**Analysis of BLADE-ON-PETIOLE interactions with auxin in control of
organ initiation and leaf patterning in *Arabidopsis thaliana***

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B.Sc., Carleton University, 2007

A thesis submitted to the Faculty of Graduate Studies and Postdoctoral Affairs in partial
fulfillment of the requirements of the degree of

Master of Science in Biology

Carleton University

Ottawa ON, Canada

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Your file *Votre référence*
ISBN: 978-0-494-81693-6
Our file *Notre référence*
ISBN: 978-0-494-81693-6

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ABSTRACT

All aerial structures in the plant are derived from the activity of the shoot apical meristem (SAM), which comprises a group of self-renewing stem cells locating at the growing tip of shoots. Accumulation of auxin and auxin-dependent signalling directs formation of organ primordia by down-regulating *KNOX* meristem genes which maintain cells in an undifferentiated state and promoting the expression of genes that promote differentiation and growth. Formation of lateral organ primordia from the SAM is associated with the formation of boundaries that separate the primordia from the meristem. The *Arabidopsis* *BLADE-ON-PETIOLE1* (*BOP1*) and *BOP2* genes are expressed in initiating lateral organ primordia where they promote differentiation and subsequently in lateral organ boundaries where they restrict growth and control the architecture of boundaries in leaves, inflorescences, and fruits. Work to date indicates that *BOP1/2* activate target genes encoding transcription factors that repress *KNOX* meristem genes and that restrict growth in organ boundaries and leaf petioles. The role of *BOP1/2* during the early stages of organ initiation and separation and leaf patterning remains unclear due to lack of phenotypes in *bop1 bop2* loss-of-function mutants during the vegetative development. The plant hormone auxin has an overlapping expression pattern with *BOP1/2* in emerging organ primordia suggesting that *BOP1/2* and auxin activities might overlap to regulate organ initiation, separation, and leaf shape. I show in my thesis that leaf initiation in *bop1 bop2* mutants is slower relative to wild-type, that there is a delayed transition to spiral phyllotaxy of leaves, and that response to auxin is enhanced in leaf petioles, consistent with the ectopic formation of leaflets and midveins in this region. Organ initiation in the meristem and dissected leaf forms depend on the localized formation of auxin maxima, which result from the polar localization of PIN-FORMED1 (*PIN1*) auxin efflux carriers, dependent in part on the kinase activity of *PINOID*

(PID). Accordingly, mutations in *pid* and *pin* cause smooth leaf margins and the inflorescence meristem cannot initiate flowers resulting in a naked “pin” inflorescence. I show that BOP1/2 and PID activities converge to control organ phyllotaxy and organ separation in vegetative seedlings. Also, phenotypic analysis of *bop1 bop2 pin1-1*, *bop1 bop2 pin1-En134*, *bop1 bop2 pid-2* and *bop1 bop2 pid-3* triple mutants shows a partial rescue of *pin1* and *pid* inflorescence phenotype by *bop1 bop2* indicating that BOP1/2 and PID/PIN function antagonistically in organ initiation from the meristem during reproductive development. Future direction will focus on understanding how BOP1/2 activity interferes with organ formation in *pid* and *pin* inflorescences. One possibility is that misexpression of BOP1/2 in *pin* and *pid* abnormally restricts growth, thereby preventing organ initiation. Alternatively, *bop1 bop2* may promote auxin biosynthesis in the *pid/pin* meristems, restoring auxin supply and organ initiation. Future experiments will examine these models.

DEDICATION

~ To my mom and dad, for their tremendous selflessness, their remarkable compassion, and their
endless encouragement and utmost support ~

ACKNOWLEDGMENTS

First, it is only appropriate to thank God to whom all thanks is due; the one and only creator, designer, and sustainer of the universe, for directing me to the righteous path. God has bestowed upon me an unconceivable amount of blessings and for that I give my deepest thanks.

I would like to give sincere gratitude to my family for their unflagging love and support throughout my life, without them this dissertation is simply impossible. I'm greatly indebted for my parents Samir Musa, and Nadia Al Loubani for all their love, care and support and everything they have done for me. I would like to give a special thanks to my exceptional siblings Mohammed, Rana, Ahmad and Binan for standing by me, and for their "kind" words of encouragement. My fiancé Majd, I am very lucky and happy to be with such an amazing and understanding person. I appreciate all my friends for being there for me with all their love, support and complete understanding when I was too busy or complained a bit too much. I have complete and utter love for all of you.

I owe a warm thank you to my supervisor Shelley Hepworth, who has provided me with continuous encouragement and support in many ways through the past two years. Her passion, perpetual energy and enthusiasm for research motivate all her advisees, including myself. In addition, she was always accessible and willing to help making research a rewarding experience. I would also like to thank members of my advisory committee Myron Smith and Brian Miki for their enthusiasm, encouragement and for their helpful suggestions. I would also like to thank the lab members of the Hepworth lab including Paul Tabb, Mike Bush, Jhadeswar Murmu and Mingli Xu, which made the lab a convivial place to work. In particular, I would like to thank Madiha Khan for her friendship, help and support in the past two years.

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List of Abbreviations

AS	ASYMMETRIC LEAVES
ANT	AINTEGUMENTA
ARF	Auxin Response Factor
ARE	Auxin Response Elements
AXR1	AUXIN RESISTANT 1
AUX1	AUXIN RESISTANT 1
AUX/IAA	ARF repressor
BOP	BLADE-ON-PETIOLE
BP	BREVIPEDICELLUS
BTB/POZ	Broad-Complex/Tramtrack/Bric-a-brac; Pox virus/Zinc finger
CL	Continuous Light photoperiod
CLV	CLAVATA
CUC	CUP-SHAPED COTYLEDON
CZ	Central Zone
dCAPS	Derived Cleaved Amplified Polymorphic Sequence
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DR5	Synthetic auxin promoter with 5 copies of an ARE
EDTA	Ethylenediaminetetraacetic acid
En	Enkheim
FMO	Flavin Monooxygenase-Like
GFP	Green Fluorescent Protein
GUS	β -Glucuronidase
IAA	Indole-3-acetic acid, auxin
IAAH	Protonated auxin
IAA ⁻	Auxin anion

IM	Inflorescence Meristem
JAG	JAGGED
JLO	JAGGED LATERAL ORGANS
KNAT	KNOTTED1-LIKE FROM ARABIDOPSIS
KNOX	KNOTTED1-LIKE HOMEODOMAIN
LAX	LIKE AUXIN-RESISTANT1
LD	Long Day photoperiod
LER	Landsberg erecta
LOB	LATERAL ORGAN BOUNDARIES
LFY	LEAFY
MP	MONOPTEROUS
NPA	1-N-naphthylphthalamic acid
NPY	NAKED PINS IN YUC MUTANTS
NOA	1-Naphthoxyacetic acid
PAT	Polar Auxin Transport
PCR	Polymerase Chain Reaction
PID	PINOID
PIN1	PIN-FORMED1
PZ	Peripheral Zone
PM	Plasma membrane
RE	Restriction Enzyme
RUB1	RELATED TO UBIQUITIN1
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RZ	Rib Zone
SAM	Shoot Apical Meristem
SCF	Skip1/Cdc53p-Cullin/F-box
SD	Short Day Photoperiod
SDS	Sodium Dodecyl Sulphate

SEM	Scanning Electron Microscopy
STM	SHOOT MERISTEMLESS
SNP	Single Nucleotide Polymorphism
T-DNA	Transposon insertion
WT	Wild type
WUS	WUSCHEL
YUC	YUCCA

Genetic Nomenclature in *Arabidopsis thaliana*

<i>BOP</i>	WT gene
BOP	WT protein or activity
<i>bop</i>	mutant gene (loss-of-function allele)
<i>bop-D</i>	mutant gene (gain-of-function allele)

CHAPTER 1 INTRODUCTION

1.1 Overview

Architectural diversity between plant species is a reflection of the central role of shape and form in optimizing plant survival and reproductive fitness. Relatively little is known about the genes that regulate plant architecture or how they interact to create structural diversity. The above-ground structures in a plant are generated post-embryonically by the shoot apical meristem (SAM), a self-renewing population of stem cells located at the apex of primary and secondary shoots. During vegetative development, the SAM generates lateral organ primordia with leaf identity during which internode elongation is repressed. Upon flowering, internode elongation begins and the SAM (now known as the inflorescence meristem) transitions to produce secondary inflorescences subtended by cauline leaves, and then flowers (reviewed in Bowman and Eshed, 2000; Barton, 2010). Lateral organs are separated from the meristem as they emerge by a distinct band of cells known as the boundary (reviewed in Aida and Tasaka, 2006ab). Thus, three distinct compartments are formed at the shoot apex: the meristem, the lateral organ primordia, and the boundary, each with a unique transcriptional profile and developmental fate (Figure 1.1; reviewed in Rast and Simon, 2008). Genetic studies show that complex interactions between genes expressed in each of these compartments as well as the plant hormone auxin play key roles in maintaining the structure and function of compartments in the shoot apex, allowing for maintenance of the meristem, the systematic production of lateral organs primordia, and their subsequent patterning (reviewed in Barton, 2010; Rast and Simon, 2008). My work seeks to understand how two boundary-associated genes, *BLADE-ON-PETIOLE1* (*BOP1*) and *BOP2*, initially expressed in emerging primordia, interact with regulators of auxin signalling and transport to control organ initiation and leaf patterning in the

model plant species, *Arabidopsis thaliana*. This work is expected to shed light on how BOP1/2 and the plant hormone auxin interact within the established genetic network that controls patterning in shoot apices.

1.2 The shoot apical meristem

Cytohistological analysis of the SAM indicate that it is divided into three cell layers, designated L1, L2, and L3, that give rise to specific cell lineages in the adult plant (Figure 1.2A). The L1 layer contributes to the epidermis of the shoots, leaves and flowers; the L2 layer produces the ground tissue and germ cells; and the L3 layer contributes to the vascular tissues of the stem and the internal tissues of leaves and flowers (reviewed in Szymkowiak, 1996; Barton, 2010). These layers can also be divided into three zones in the SAM: the central zone, which contains the core stem cells, the rib zone located beneath the central zone, which controls vasculature and interior stem structure, and the peripheral zone, located on the flanks of the SAM, which provides founder cells that contribute to initiating lateral organs (Figure 1.2A; Laufs et al., 1998; reviewed in Bowman and Eshed, 2000).

1.3 Meristem maintenance

1.3.1 WUS-CLV feedback loop

Members of the CLAVATA (CLV1, CLV2, and CLV3) family of signaling proteins interact to maintain the size and position of the stem cell population in the central zone. They do this by restricting the expression of the homeodomain transcription factor gene *WUSCHEL* (*WUS*) to small group of cells (termed the organizing centre) located in the L3 layer below the central zone (Figure 1.2B). In turn, *WUS* functions cell non-autonomously to promote the expression of *CLV3* in the overlying cells in the central zone, imparting stem cell identity (Figure

1.2B; Laux et al., 1996; Mayer et al., 1998; Brand et al., 2001). WUS-CLV interactions thus generate a stable feedback loop that balances maintenance of the stem cell population with the production of daughter cells that are incorporated into the internodes and lateral organs of the developing plant (Figure 1.2C; Fletcher et al., 1999; Schoof et al., 2000; Brand et al., 2001).

1.3.2 Class I KNOX homeodomain transcription factors

The initiation and maintenance of meristem cell fate is additionally controlled by a family of Class I KNOTTED1-like homeobox (KNOX) transcription factors. This family comprises the *SHOOTMERISTEMLESS* (*STM*), *BREVIPEDICELLUS* (*BP*), *KNOTTED-LIKE1* *FROM ARABIDOPSIS THALIANA2* (*KNAT2*) and *KNAT6* genes (reviewed in Hamant and Pautot, 2010). KNOX proteins form heterodimers with members of a related class of BELL1-LIKE homeodomain proteins to regulate transcription (e.g. Bellaoui et al., 2001; Rutjens et al., 2009; reviewed in Hamant and Pautot, 2010). Strong *stm* mutants fail to form a meristem or initiate lateral organs demonstrating a central role for *STM* in the meristem (Long et al., 1996; Clark et al., 1996; Endrizzi et al., 1996). Assistance is provided by *BP* and *KNAT6* since mutation of either gene enhances meristem defects in weak *stm* mutants (Bryne et al., 2002; Belles-Boix et al., 2006). The role of *KNAT2* in the SAM remains unclear since defects in meristem maintenance are not revealed by double mutant analysis with *stm*, *bp*, or *knat6* (Belles-Boix et al., 2006). However, misexpression of any of the class I *KNOX* genes induces ectopic meristem formation on the adaxial side of leaves, typically at the margins resulting in lobed leaves with ectopic stipules and meristems in the sinus regions located between lobes (e.g. Lincoln et al., 1994; Chuck et al., 1996; Pautot et al., 2001; Dean et al., 2004). Co-misexpression of *STM* and *WUS* together further enhances the formation of *de novo* meristems capable of supporting

organogenesis consistent with the central and complementary roles that these two genes play in meristem function (Endrizzi et al., 1996; Gallois et al., 2002; reviewed in Barton, 2010).

1.4 The meristem-organ boundary

As lateral organs emerge, a boundary develops in the form of a narrow groove that separates the primordia from the meristem, thus establishing three distinct compartments at the shoot apex: the meristem, the boundary, and the primordia (Figure 1.1; reviewed in Aida and Tasaka, 2006ab; Rast and Simon, 2008). Boundary cells are typically small and repressed in their growth with a low rate of cell division (Kwiatkowska et al., 2004; Brueil-Breyer et al., 2004; Reddy et al., 2004). Cells at the boundary become specialized for unique functions in the adult plant. For example, boundary cells in the axils of leaves have the potential to form axillary meristems (McSteen and Leyser, 2005; Aida and Tasaka, 2006ab), while boundary cells at the base of floral organs are specialized for abscission (Lewis et al., 2006). Other boundaries, such as those in the valve margin of fruits contain cells that are specialized for dehiscence (Lewis et al., 2006; Girin et al., 2009). Consistent with restricted growth in boundaries, these regions also represent auxin minima (reviewed in Rast and Simon, 2008). In the valve margin of fruits, a recent paper shows that auxin is actively transported away from this zone, which is necessary for specialization of cells for dehiscence (Sorefan et al., 2009).

Mutational analyses show that genes expressed in the boundary not only restrict growth but play important roles in SAM initiation, maintenance, and organ separation (reviewed in Rast and Simon, 2008). Members of the *CUP-SHAPED COTYLEDON* (*CUC*) gene family, which encode NAC-domain transcription factors, are thought to define boundary-cell identity (Aida et al., 1997; Hibara et al., 2006; Vroemen et al., 2003). The *cuc1 cuc2* double mutant displays

fused cotyledons (cup-shaped cotyledons) because a boundary between these organs is not established and the SAM is not maintained (Aida et al., 1997; Takada et al., 2001; Vroemen et al., 2003). *CUC1* and *CUC2* are activated in the embryo apex where they are needed to turn on *STM* to establish the SAM at heart stage. Subsequently, *STM* restricts *CUC* expression to the boundary between the meristem and the cotyledons as they emerge (Figure 1.3A; Aida et al., 1999; Takada et al., 2001; Vroeman et al., 2003). *STM* also contributes to maintenance of the boundary and *CUC* expression since loss-of-function *stm* mutants show cotyledon fusion in addition to lack of meristem (Figure 1.1; Aida et al., 1999; Takada, 1991; Long and Barton, 1998). Auxin transport genes expressed in cotyledon primordia, *PIN-FORMED1* (*PIN1*) and *PINOID* (*PID*), further restrict *CUC2* to the boundary (Furutani et al., 2004). These data demonstrate that the boundary is positioned according to positive and negative input of genes expressed in the flanking meristem and primordia compartments (Figure 1.1; reviewed in Aida and Tasaka, 2006a; Rast and Simon, 2008).

The *CUC* genes that define boundary cell identity also play an important role in establishing the unique transcriptional profile of the boundary. For example, *CUC1* and *CUC2* are required for expression of the class I *KNOX* gene *KNAT6* in the meristem-organ boundary during embryogenesis. *KNAT6* contributes to boundary maintenance, as evidenced by severe defects in cotyledon separation in the *stm-2 knat6* double mutant (Figure 1.1; Belles-Boix et al., 2006).

Members of the *LATERAL ORGAN BOUNDARIES* (*LOB*) gene family are also expressed in boundary regions (Shuai et al., 2002). Of particular importance is *JAGGED LATERAL ORGANS* (*JLO*), misexpression of which causes lobed leaves, characteristic of *KNOX* meristem

gene misexpression (Figure 1.1; Bhorgi et al., 2007; Lincoln et al., 1994). Compatible with this, inducible *JLO* activates expression of the *KNOX* genes *STM* and *BP* and down-regulates the expression of the auxin transporter-encoding *PIN1* in leaves and embryos suggesting that it serves to co-ordinate *KNOX* meristem gene expression and auxin transport from its position in the boundary (Bhorgi et al., 2007; Bureau and Simon, 2008). Another *LOB* family gene, *ASYMMETRIC LEAVES2* (*AS2*), which is expressed in emerging leaves and the boundary fulfills a complementary role. *AS2* in a complex with the MYB factor *ASYMMETRIC LEAVES1* (*AS1*) directly maintains repression of *KNOX* genes in leaves, thus guiding primordia toward differentiation (Figure 1.1; Ori et al., 2000; Semiarti et al., 2001; Bryne et al., 2002; Iwakawa et al., 2002; Lin et al., 2003; Guo et al., 2008).

AS2 is regulated in part by *BLADE-ON-PETIOLE1* (*BOP1*) and *BOP2*, a set of genes that are expressed in leaf primordia until their separation from the meristem. At this point, *BOP1/2* expression relocates to the meristem-organ boundary and the base of leaves (Ha et al., 2004; Norberg et al., 2005; Jun et al., 2010; Khan et al., 2010). *BOP1/2* contain two protein-protein interact motifs: an N-terminal BTB/POZ domain and a set of four ankyrin repeats near the C-terminus but lack a DNA-binding domain (Ha et al., 2004; Hepworth et al., 2005). Evidence suggests that they are recruited to DNA via interactions with members of the TGA family of bZIP transcription factors where they function as transcriptional co-activators (Hepworth et al., 2005; Jun et al., 2010; Xu et al., 2010). *BOP1/2* directly activates *AS2* to help maintain repression of *KNOX* genes in petioles (Ha et al., 2007; Jun et al., 2010). *BOP1/2* are also required for differentiation at the base of floral organs, the stem-pedicel boundary, and in the valve margin of fruits based on *bop1 bop2* phenotypic defects in these areas (Ha et al., 2003; Hepworth et al., 2005; McKim et al., 2008; Ha et al., 2007; Khan et al., 2010). The role of

BOP1/2 in the leaf-meristem boundary remains unclear due to lack of a phenotype in *bop1 bop2* mutant seedlings, making it likely that their function is redundant with other factors.

1.5 Organ initiation

Maintaining the structure of the SAM requires a delicate balance between stem-cell maintenance and organ initiation, which are competing processes. During vegetative development, the SAM generates leaf primordia on its flanks in a spiral phyllotactic pattern. Primordia initiation requires simultaneous directional cell division activity and outward growth from the periphery of the SAM (Donnelly et al., 1999). Organs are initiated at sites of high auxin accumulation on the flanks of the SAM called auxin maxima (Figure 1.3B; Reinhardt et al., 2000; 2003; Benková et al., 2003). As a new organ is initiated, *KNOX* meristematic and *CUC* boundary genes are switched off in an auxin-dependent manner (Figure 1.3B, Vernoux et al., 2010; Furutani et al., 2002; Aida et al., 2002; Heisler et al., 2005; Scheutz et al., 2008; reviewed in Vernoux et al., 2010). Simultaneously, genes that promote differentiation and maintain repression of *KNOX* meristem genes, including *BOP1/2* and *AS1-AS2*, are switched on, together with genes that promote cell proliferation, represented by *AINTEGUMENTA* (*ANT*) (Figures 1.1 and 1.3C; Long and Barton, 1998; Bryne et al., 2000; Norberg et al., 2005; Khan et al., 2010; Mizukami and Fischer, 2000; reviewed in Rast and Simon, 2008; Vernoux et al., 2010).

Establishing “central” and “peripheral” meristem identities in the SAM appears to distinguish the central stem cell niche from cells on the periphery that have the potential to participate in lateral organ formation. Only cells at the periphery are competent to respond to auxin and initiate organs (Reinhardt et al., 2000). *STM* only is expressed in the central zone whereas both *STM* and *BP* are expressed in the peripheral and rib zones of the meristem where

organs arise and internodes elongate (Long and Barton, 1998; Douglas et al., 2002; Venglat et al., 2002). Antagonistic interactions between *STM* and *AS1* ensure that leaf initiation is restricted to the meristem periphery: *STM* restricts the expression of *AS1* and *AS2* to sites of organ initiation, preventing ectopic leaf development at the shoot apex. Conversely, *AS1* and *AS2* maintain repression of *KNOX* genes in emerging leaves (Figure 1.3C, Byrne et al., 2002). Misexpression of *KNOX* genes in *as1* or *as2* mutants slows leaf initiation and gives rise to asymmetric lobed leaves in which ectopic meristems form along the leaf margins restricting growth (Ori et al., 2000; Bryne et al., 2002).

Presumably *BOP1/2* and *AS1-AS2* cooperate to promote organ initiation and outgrowth but their downstream targets apart from *KNOX* meristem genes have yet to be identified. Whereas *AS1-AS2* repression of *BP* is direct (Guo et al., 2008), *BOP1/2* represses *BP* indirectly by upregulating *AS2* (Ha et al., 2007; Jun et al., 2010). *BOP1/2* repression of *BP* also occurs via an *AS2*-independent mechanism as evidenced by dramatic enhancement of *KNOX* misexpression and meristem activity in *bop1 bop2 as1* and *bop1 bop2 as2* triple mutant leaves (Ha et al., 2007; Khan et al., 2010). In *bop1 bop2* mutants, *KNOX* meristem genes are co-misexpressed with *JAGGED*, which encodes a blade-promoting transcription factor, to produce the leafy petiole phenotype indicating that *BOP1/2* are important regulators of growth factors (Ha et al., 2003; 2007; Norberg et al., 2005; Khan et al., 2010).

1.6 Antagonism between auxin and *KNOX* genes regulates organ initiation and leaf shape

Studies in the embryo have shed light on how antagonism between auxin and *KNOX* meristem genes patterns the shoot apex and permits organ initiation (reviewed in Aida and Tasaka, 2006ab; Rast and Simon, 2008; Vernoux et al., 2010). Double mutants in the auxin transport facilitators *PIN1* and *PID* show defects in lateral organ formation with the most severe

defect being the complete failure to produce cotyledons in the *pin1 pid* double mutant embryo. This phenotype correlates with the expansion of *CUC* and *STM* expression throughout the dome of the embryo (Figure 1.3D; Bennett et al., 1995; Aida et al., 1997; 1999; 2002; Vernoux et al., 2000; Benjamins et al., 2001). Cotyledon formation is restored in the *cuc pin pid* triple mutants and partially restored in the *stm pin1 pid* double mutants, indicating that the inability of *pin1 pid* mutants to initiate organs (cotyledons) is caused by misexpression of *CUC1* and *STM*, which restrict growth (Aida et al., 1999; 2002; Vernoux et al., 2000; Furutani et al., 2004). Similarly, the inability of *pid* and *pin* inflorescences to produce flowers is slightly rescued by mutation of *BP* (Hay et al., 2006) consistent with the idea *KNOX* misexpression blocks organ initiation. In summary, domains of auxin maxima in the SAM are nearly complementary to the domains of *STM* and *CUC2* expression, reflecting incompatibility or antagonism between auxin and *KNOX* genes and the fact that organ initiation and meristem maintenance are two competing processes (Reinhardt et al., 2000).

1.6.1 Leaf shape

Leaf shape and complexity in many species is dependent on the spatial and temporal regulation of *KNOX* genes in the petiole and leaf margins (e.g. Barkoulas et al., 2008; Bharathan et al., 2002; reviewed in Hay and Tsiantis, 2009; Uchida et al., 2010). In *Arabidopsis*, *KNOX* gene expression is excluded from leaves resulting in a simple determinate leaf shape (Lincoln et al., 1994; Hay et al., 2006; reviewed in Hay and Tsiantis, 2009). In species with compound leaves, such as tomato and *Cardamine hirsuta*, an *Arabidopsis* relative, *KNOX* genes are reactivated in leaf petioles to generate a dissected leaf margin comprised of multiple leaflets attached to a rachis (Barkoulas et al., 2008; Hay and Tsiantis, 2006; Hareven et al., 1996; reviewed in Hay and Tsiantis, 2009). Dissected leaf shape is equally dependent on auxin

transport. For example, inhibition of auxin transport in tomato causes the conversion of compound leaves to simple leaves (Avasarala et al., 1996). In *Cardamine hirsuta*, lateral leaflet formation is stimulated by the activity of marginal cell division foci, which form in response to PIN1-dependent local auxin maxima (Barkoulas et al., 2008). Thus, compound leaf formation is analogous to how leaves initiate on the flanks of the meristem where *KNOX* expression facilitates the formation of auxin maxima, which feed back to repress *KNOX* expression, necessary for leaflet outgrowth (reviewed in Hay and Tsiantis, 2010).

1.7 Auxin - a plant morphogen

Recent studies have sought to understand how the plant hormone auxin interacts with genetic factors to regulate meristem organization, organ initiation, and leaf shape. Auxins are a group of plant hormones that exist in natural and synthetic form with indole-3-acetic acid (IAA) being the most abundant form of natural auxin (Hobbie, 1998). In conjunction with other signals, auxin regulates a myriad of developmental processes including cell division, cell expansion, cell fate determination, and phyllotaxis to name a few (reviewed in Teale et al., 2006).

Auxin displays characteristics reminiscent of animal morphogens. Morphogens are molecules expressed by a localized population of cells (a source) that diffuse to create a concentration gradient. Cells within the field of diffusion are competent to respond and differentiate according to the concentration of morphogen (Wolpert, 2007). Like conventional morphogens, auxin is distributed in a gradient in plant tissues, including embryos, roots, meristems, and leaf primordia. Auxin maxima define polarity in tissues and determine the direction of growth. Moreover, accumulation of intracellular auxin triggers changes in cell fate.

For example, auxin-dependent signalling distinguishes the developmental fate of founder cells recruited for leaf primordium initiation from surrounding peripheral meristem cells (Benková et al., 2003; Friml et al., 2003; reviewed in Benková et al., 2009). Whereas conventional morphogen gradients are driven by local production and diffusion, auxin gradients are driven by the active transport of auxin from cell-to-cell. Also, in contrast to animal morphogens where multiple developmental outcomes are achieved in a concentration-dependent manner, auxin appears to act primarily as a trigger for binary changes in cell fate. Thus, the concept of auxin being a “morphogenetic trigger” is more ideal (reviewed in Benková et al., 2009).

Auxin is synthesized in leafy organs (source tissues) and transported from cell-to-cell in a polar fashion to sites of action (sink tissues), such as initiating primordia in the meristem (reviewed in Benková et al., 2009). Polar auxin transport (PAT) is mediated by polarly localized auxin influx and auxin efflux carrier proteins, represented by AUXIN RESISTANT1/LIKE AUXIN RESISTANT1 (AUX1/LAX) and PIN1, respectively (reviewed in Kramer, 2004; Kramer and Bennett, 2006). The chemiosmotic hypothesis describes auxin movement during PAT, where auxin (IAA) exists primarily in the protonated state (IAAH) in the acidic apoplastic space. Auxin is taken up into the cell via AUX1 influx carriers or by passive diffusion. Once in the alkaline cytosol, the proton is lost to form polar IAA^- anions. IAA^- anions are unable to diffuse through the plasma membrane to the extracellular space due to their charge. As a result, IAA^- anions are exported out of the cell down their electrochemical gradient via PIN efflux carriers. In the extracellular space, auxin regains its proton and enters the next cell (Heisler and Jönsson, 2006). The polar positioning of PIN efflux carriers guides the direction of auxin transport and drives the formation of auxin concentration gradients, which are critical for auxin-mediated growth processes such as organ initiation (Gälweiler et al., 1998; Friml et al., 2003;

Michniewicz et al., 2007). PAT therefore contributes to the plasticity of plant development, since auxin mediates an array of responses through which growth is adjusted to compensate for changes in the environment (reviewed in Teale et al., 2006; Petrášek and Friml, 2009).

1.8 Polar auxin transport promotes organogenesis

The Arabidopsis genome encodes eight functionally redundant PIN transmembrane auxin efflux carriers (reviewed in Paponov et al., 2005). Live-cell imaging with PIN1:GFP shows that PIN proteins are polarly localized in cells and that their position correlates with the direction of auxin flow out of the cell. PIN1 transports auxin in the SAM epidermis towards leaf initial cells that act as auxin sinks to trigger leaf initiation. PIN1:GFP maxima on the flanks of the SAM thus predict the site of the next leaf initial (reviewed in Benjamins et al., 2005). Characteristic phenotypes in *pin1* mutants include altered leaf morphology, a slower rate of leaf initiation, altered phyllotaxis, and naked inflorescences with few or no flowers (Gälweiler et al., 1998; Okada et al., 1991). Exogenous application of auxin to *pin1* inflorescences rescues organ initiation indicating the importance of a continuous supply of auxin for proper positioning and development of organs from the inflorescence meristem (Okada et al., 1991). The expression of *PIN1* in floral organ primordia at very young stages further supports that polar auxin transport is required for organ initiation (Figure 1.1; Christensen et al., 2000; Vernoux et al., 2000).

The localization of PIN efflux carriers is regulated in part by PID, a serine-threonine kinase that is expressed in embryos, organ primordia, and vascular tissue (Benjamins et al., 2001). Mutations in *PID* cause defects in the formation of cotyledons, flowers, and floral organs similar to *pin* mutants because PID activity influences the polar localization of PIN proteins (Christensen et al., 2000). During organ initiation and outgrowth, abrupt changes are seen in the polarity of PIN proteins. Initially, organ founder cells are auxin sinks, draining auxin from the

surrounding tissues towards the organ tip to establish directional growth. Once organ initiation is established and leaf cells begin to produce their own auxin, PIN localization reverses so that auxin is drained back into the meristem and used in the initiation of new organs (e.g. Benková et al., 2003). PID plays a direct role in PIN transporter dynamics since ectopic *PID* expression causes a basal-to-apical shift in PIN1 localization leading to the loss of auxin gradients. Conversely, there is an apical-to-basal shift in PIN1 localization in loss of function *PID* mutants (Friml et al., 2004). Thus, levels of PID kinase activity act as a binary switch for PIN polar localization and the regulation of PAT during organ formation (Figure 1.4). PID regulation of PIN localization therefore plays an important role in the positioning and outgrowth of organ primordia from the meristem (Benjamins et al., 2001; Reinhardt et al., 2000; Vernoux et al., 2000).

AUX1 and the related LIKE AUX1 (LAX1, LAX2, and LAX3) proteins are putative auxin influx carriers that belong to the amino-acid permease family of proton-driven transporters, located in the plasma membrane (Bennett et al. 1996; Parry et al., 2001; Swarup et al., 2001; 2004; reviewed in Kramer, 2004). Immunolocalization studies indicate that AUX1 proteins are polarly localized on the apical side of cells, opposite to PIN1 proteins, where they mediate the rapid re-uptake of auxin, thus preventing diffusion into neighbouring cells, and thereby facilitating the formation of steep auxin maxima (Kleine-Vehn et al., 2006; Bennett et al., 1996). *aux1* mutants have defects in organ initiation and separation, leaf expansion, root elongation, growth and response to gravity, as a result of impaired local auxin redistribution between groups of cells or tissues (Parry et al., 2001; Pickett et al., 1990; Bennett et al., 1996; Reinhardt et al., 2003).

1.9 Polar auxin transport controls phyllotaxy

PAT controls the position at which organs are initiated on the flanks of the SAM. In most flowering plants, leaves are arranged in a spiral phyllotaxy with a divergence angle between successive leaves of 137.5° . Arabidopsis leaves transition from a decussate (opposite) phyllotaxy of 180° to a spiral phyllotaxy 137° marking the transition from the juvenile to the adult vegetative phase (reviewed in Reinhardt, 2005; Kuhlemeier, 2007). Using PIN1:GFP or the auxin reporter gene *DR5:GFP*, sites of auxin accumulation and organ initiation can be visualized on the meristem periphery. Chemical inhibition of auxin efflux is seen to disturb the formation of auxin maxima, causing defects in organ initiation (Benková et al., 2003).

As stem cell daughters are displaced from the central zone and move to the peripheral zone, they become competent to respond to auxin and to differentiate as organs (Reinhardt et al., 2000). As explained earlier, the most widely held model holds that leaf primordia act initially as sinks of auxin and deplete it from their surroundings, thus inhibiting the initiation of additional leaf primordia at the periphery of the SAM. Soon after their initiation, leaf primordia start to produce their own auxin, leading to repositioning of PIN transporters such that auxin is transported back through the interior of primordia into the meristem to contribute to subsequent leaf initiation events (Heisler et al., 2005; Heisler and Jonsson, 2006; reviewed in Scarpella et al., 2010). The same conserved auxin-related mechanism is thought to be involved in the formation and positioning of organs at all stages of development, including cotyledons, leaves, and floral organs (Reinhardt et al., 2000; 2003; Benková et al., 2003; reviewed in Reinhardt, 2005).

1.10 Polar auxin transport determines vein patterning

Auxin transport and its restriction or “canalization” further controls vein patterning in leaves. The “canalization of auxin flow” model integrates the biology of auxin synthesis, auxin movement, and auxin promotion of vascular differentiation, to explain vein patterning (Sachs, 1981; 1991). This model proposes that elevated auxin levels cause cells specialized for auxin polar transport to become auxin conducting such that these specialized cells elongate and position PIN1 auxin efflux carriers on their basal membrane releasing auxin at the basal end. This induces adjacent cells to also specialize in auxin transport establishing a PAT path that both delivers auxin to underlying cells and removes auxin from adjacent tissues. These canalization paths of auxin transport provide cues that define vein position (Sachs, 1981; 1991). Consistent with this model, the auxin transport mutants including *pin1* tend to have simplified vein patterns due to decreased auxin transport (Okada et al., 1991; Bennett et al., 1995; Carland and McHale, 1996; Gälweiler et al., 1998). Also consistent with the canalization model, mutants defective in auxin signalling, unable to respond to auxin, show a decrease in the size of vascular bundles in their stems (Lincoln et al., 1990). Application of chemical inhibitors of PAT tend to cause hyperaccumulation of auxin near the leaf margin and in petioles resulting in increased vein density in these locations only (Mattsson et al., 1999; Sieburth, 1999). Very high doses of chemical PAT inhibitor block canalization altogether and veins toward the base of the leaf become incomplete or disappear (Mattsson et al., 1999).

1.11 Auxin signaling

As discussed above, auxin serves as a “morphogenetic trigger” that induces changes in cell fate in response to an auxin gradient. The mechanism by which auxin regulates gene expression is well-studied (reviewed in Quint et al., 2006; Chapman and Estelle, 2009). A large

family of AUXIN RESPONSE FACTORS (ARFs) have affinity for Auxin Response Elements (AREs) in the promoters of auxin-responsive genes. ARF transcription factors are negatively regulated by a family of interacting Aux/IAA repressors. Auxin triggers the ubiquitination and proteolysis of Aux/IAA repressors. An E3 ubiquitin ligase complex known as SCF^{TIR1} (for Skp1p, Ccd53p/Cullin and F-box) has affinity for Aux/IAA repressors only when the F-box^{TIR1} subunit is bound to auxin (Gray et al., 2001; Dharmasiri et al., 2005). When auxin levels are low, Aux/IAAs are stable such that auxin-responsive gene expression remains repressed (Figure 1.5A). When auxin concentration is high and auxin is bound to the F-box^{TIR1} subunit, Aux/IAAs are ubiquitinated and degraded via the 26S proteasome (Figure 1.4B). This permits ARE-mediated gene transcription in response to auxin (reviewed in Quint et al., 2006; Chapman and Estelle, 2009).

The activity of the SCF^{TIR1} is regulated by its conjugation to the ubiquitin-like protein RELATED TO UBIQUITIN1 (RUB1) (del Pozo et al., 1998; 1999; 2002). AUXIN RESISTANT1 (AXR1) is a subunit of the RUB1 ubiquitin-activating enzyme necessary for SCF^{TIR} activity (del Pozo et al., 1998). Mutation of *AXR1* causes a reduction in conjugation of RUB1 to SCF^{TIR} thus stabilizing AUX/IAAs and blocking appropriate responses to auxin (del Pozo and Estelle, 1999; Gray et al., 2001). As a result, *axr1* mutants have a pleiotropic phenotype that includes defects in root and shoot elongation, root hair growth, root gravitropism, apical dominance, vascular development, leaf morphology, lateral branching, plant height and fertility (Lincoln et al., 1990). These pleiotropic phenotypes reflect that a vast number of processes in plant development are dependent on auxin-regulated gene expression.

1.12 Auxin biosynthesis contributes to auxin gradients

Polar auxin transport has been dominantly implicated in generating auxin gradients and auxin maxima to direct plant development. However, recent studies reinforce that localized production of auxin plays a key role in shaping auxin gradients (Cheng et al., 2006; Cheng et al., 2007). Recent studies also show that auxin biosynthesis can be mediated by tryptophan-dependent or independent pathways (Tao et al., 2008). An activation-tagging screen for *Arabidopsis* long hypocotyl mutants defective in light signaling identified the dominant *yucca1D* (*yuc1D*) mutant, with phenotypes similar to those of known auxin overproduction mutants (Zhao et al., 2001). Overproduction of auxin in *yuc1D* is caused by overexpression of a flavin monooxygenase-like enzyme that catalyzes the conversion of tryptamine to N-hydroxyl tryptamine, a rate-limiting step in auxin biosynthesis. There are ten *YUC* family members in *Arabidopsis* (Cheng et al., 2006). Similar to *YUC1*, the overexpression of other *YUC* genes leads to auxin overproduction phenotypes. Single loss-of-function *yuc* mutations do not result in obvious developmental defects due to redundancy; combinations of two or more *yuc* mutations are needed to produce dramatic developmental defects (Cheng et al., 2006; 2007a). For example, *yuc1 yuc4* double mutants have abnormal flowers lacking functional male or female floral organs and have fewer vascular strands in leaves and flowers. Expression of the auxin-responsive reporter gene *DR5:GUS* is down-regulated when *YUC* genes are inactive indicating a decrease in auxin production (Cheng et al., 2007a).

Analysis of *YUC* gene expression patterns indicates that auxin biosynthesis is regulated both temporally and spatially in small groups of discrete cell types. In developing embryos, *YUC1* is expressed in the apical region of globular embryos, and becomes gradually restricted to the SAM beginning at heart stage (Cheng et al., 2007a). The auxin reporter *DR5:GUS* is down-

regulated in *yuc1 yuc4* mutants, but only in cells expressing *YUC1* and *YUC4*, but this is sufficient to impair development, revealing the importance of locally-generated auxin peaks in root and shoot development (Cheng et al., 2006; 2007a). The *YUC* genes are collectively expressed in all organs of the plant including flowers, leaves, and roots such that each organ controls auxin gradients for development independently. For example, auxin produced in other floral organs and exported cannot compensate for the effects of inactivation of *YUC2* and *YUC6* in stamens (Cheng et al., 2006).

1.12.1 Integrating auxin transport and biosynthesis

Genetic studies are beginning to reveal the synergistic importance of both auxin biosynthesis and auxin transport in plant development (reviewed in Vernoux et al., 2010). For example, *yuc1 yuc4 pin1* triple mutants are completely defective in production of true leaves due to lack of auxin whereas single mutants are only slightly impaired in this process (Cheng et al., 2007). Similarly, the phenotype of *yuc1 yuc4* double mutants is enhanced by mutation of *NAKED PINS IN YUC MUTANTS1 (NPY1)*, which encodes a BTB/POZ domain protein that interacts with PID-like kinases to potentially control their abundance (Cheng et al., 2007b). The *yuc1 yuc4* mutant produce abnormal flowers but *pin*-like inflorescences develop in *yuc1 yuc4 npy1* triple mutants, typical of *pin1* and *pid* mutants (Cheng et al., 2007b). The isolation of such enhancers reinforces the important relationship between auxin biosynthesis and auxin transport.

1.13 Thesis rationale

The overall goal of work in the Hepworth lab is to understand mechanistically how plant architecture is governed. Current work focuses on the role the *BOP* genes whose mutation results in the abnormal patterning of lateral-organ boundaries in the plant, including those at the

base of leaves (resulting in leafy petioles) and flowers (resulting in outgrowth of subtending bracts and loss of floral-organ abscission). Compatible with these phenotypes, *BOP1/2* are expressed in initiating lateral organs (e.g. leaves and flowers) and in the boundaries that develop at the base of these organs to separate them from the meristem. However, the function of *BOP1/2* in initiating organs and the meristem-leaf organ boundary, particularly during embryogenesis and vegetative development, remains unclear due to lack of a phenotype in loss-of-function mutants. We hypothesized that *BOP1/2* function redundantly with other factors during these stages of development, making their activity best studied through double mutant interactions. In this thesis, I investigated how *BOP1/2* interacts with auxin to control organ initiation, organ separation, and leaf patterning.

Part of my rationale for focusing on *BOP1/2* and auxin interactions was provided by Hay et al. (2006) who uncovered converging roles for *ASI* and auxin in controlling the rate of leaf initiation and the shape of the Arabidopsis leaf margin. This paper showed that the activities of *ASI* and auxin converge to regulate the spatial pattern of *BP* expression pattern in the leaf margins thereby controlling the leaf margin shape. This paper also provided evidence that misexpression of *BP* in *pin1* and *pid* inflorescence meristems inhibits organ initiation (Hay et al., 2006). Given that *BOP1/2*, *ASI*, and auxin are all upregulated in incipient organ primordia and repress *KNOX* meristem genes (Hay et al., 2006; Ha et al., 2007; Khan et al., 2010), I hypothesized that *BOP1/2* and auxin may converge to regulate organ initiation, organ separation, and/or leaf patterning.

To test this hypothesis, I used phenotypic analyses to examine if loss-of-function *bop1* *bop2* mutants showed defects in leaf initiation, phyllotaxy, auxin response, vein patterning, or

showed sensitivity to chemical inhibition of auxin transport. In conjunction, I used a genetics approach to examine how BOP1/2 interacts with the auxin transport and signaling machinery to govern organ initiation, organ separation, and leaf morphology. My objectives were: 1) to determine if *bop1 bop2* plants show auxin-related defects, and 2) to analyze the genetic interactions between *bop1 bop2* mutants and mutants that are defective in auxin transport or auxin signaling. My data show that BOP1/2 activity interacts strongly with auxin transport machinery to regulate organ initiation, organ separation, and leaf shape and that the interactions differ depending on the process and phase of development.

Figure 1.1 Genetic interactions in the shoot apex maintain distinct meristem, boundary, and primordia compartments. Undifferentiated cells in the shoot apical meristem (SAM) express class I *KNOX* genes (*STM*, *BP*, and *KNAT2*). Boundary cells separate the meristem from the primordia and express boundary genes (*CUC1/2/3*, *KNAT6*, *BOP1/2*, *LOB/JLO*). Accumulation of auxin initiates organ formation by repressing meristem and boundary genes and by promoting the expression of genes required for leaf differentiation (*BOP1/2*, *ASI*, and *AS2*) and cell proliferation (*ANT*). Arrows indicate promotion of gene expression. T-bars indicate repression of gene expression. These regulatory interactions may be direct or indirect. All genes are described in the text. Figure modified from Rast and Simon (2008).

Figure 1.2 Organization and maintenance of the SAM. (A) Illustrates the position of L1, L2, and L3 cells layers in a vegetative shoot apex. Central zone, peripheral zone, and rib zones are as indicated. (B) Diagram showing the expression domain of *WUS* and the *CLV3* diffusible peptide that form a feedback loop to maintain the size of stem cell population in the central zone. (C) The *WUS*-*CLV* feed-back loop. *WUS* expressed in the organizing centre indirectly promotes *CLV3* expression in overlying cells conferring stem cell identity. *CLV3* encodes a diffusible peptide that binds to *CLV1* and *CLV2* receptors expressed in the central and peripheral zones causing activation of a signaling pathway leading to repression of *WUS*, thereby limiting its expression to the organizing center. Figure modified from Wolpert (2007).

Figure 1.3 SAM establishment and organ initiation in the embryo. (A) Expression of boundary genes *CUC1* and *CUC2* at the apex of the globular embryo causes activation of *STM* to establish the embryonic SAM. (B) Auxin accumulating on the flanks of the meristem down-regulates *KNOX* meristem and *CUC* boundary genes to permit organ specification. (C) Auxin accumulation also promotes upregulation of genes that promote leaf differentiation (AS1-AS2) and cell proliferation (ANT) to cause organ outgrowth (D) Organ initiation is blocked in *pin1 pid* double mutants. When auxin maxima cannot form, *STM* and *CUC1/2* expression expands throughout the dome of the embryo restricting growth and blocking organ initiation. Red, auxin maxima; blue, central zone of meristem; yellow, boundary region.

Figure 1.4 The polar localization of PIN efflux carriers is regulated by PID phosphorylation. Phosphorylation of PIN1 efflux carriers by PID kinase repositions them on the apical face of the cell to govern the direction of auxin flow. Unphosphorylated PIN1 carriers are localized to the basal side of the cell.

A Low auxin

B High auxin

Figure 1.5 Simplified auxin signalling pathway. (A) When auxin concentration is low, AUX/IAA repressors are stable and bind to Auxin Response Factors (ARF) to prevent their function. (B) Auxin binds directly to the F-box TIR1 subunit of the SCF^{TIR1} E3 ubiquitin ligase complex allowing recognition and binding to Aux/IAA to target it for degradation by the 26S proteasome. This allows for ARE-mediated gene transcription. U, Ubiquitin. ARE, Auxin response element.

CHAPTER 2 MATERIALS AND METHODS

2.1 Plant materials and growth conditions

Three ecotypes of wild-type *Arabidopsis thaliana* were used in this study, Columbia-0 (Col-0) in which most mutations were isolated, Landsberg *erecta* (*Ler-0*) in which the *pid-2* mutation was isolated, and Enkheim-2 (En-2) in which the *pin1-1* mutation was isolated. T-DNA mutants *bop1-2* and *bop2-1* were as described (Hepworth et al., 2005). Triple mutants were obtained by crossing *aux1-7*, *axr1-3*, *pid-2*, *pid-3*, *pin1-1*, or *pin1-En134* mutants with *bop1 bop2* homozygous double mutants. The reporter lines *DR5:GUS* and *BP:GUS* were introgressed into *bop1 bop2* double mutants by crossing. The *bop1 bop2 axr1-3 BP:GUS* line was obtained by crossing *bop1 bop2 axr1-3* homozygous triple mutant with a *bop1 bop2 BP:GUS* line 12 (Ori et al., 2000). Table 2.1 provides a complete list of plant material used in this study.

Plants were grown on plates or soil in a controlled-environment growth chamber (Conviron, Winnipeg, MB) or in an air-conditioned growth room in continuous (24 h) light at 22°C unless stated otherwise. For analysis of leaf phyllotaxy and analysis of *DR5:GUS* expression in leaves, seeds were sown directly onto fertilized soil and grown in short-day photoperiods (8h light) in a controlled-environment chamber (Percival Scientific, Perry, IA) at 22°C. For analysis of leaf initiation, plants were grown in continuous or long-day photoperiods (16h light). Seedlings were germinated on plates before transplanting to sterilized soil (Promix MPV, Premier Horticulture, Rivière-du-Loup, QC) fertilized at planting with 1g/L Plant-Pod® 20-20-20 all purpose fertilizer (Plant Product Co. Ltd, Brampton, ON). Plants were grown in 72-hole trays or in square 3.5” pots as required.

Seeds were surface-sterilized by briefly incubating in a solution of 50% (v/v) household bleach (5% sodium hypochlorite) and 0.5% (v/v) sodium dodecyl sulfate (SDS). Seeds were rinsed five times in sterile water to remove bleach and sown on agar plates containing AT minimal medium (2 mM $\text{Ca}(\text{NO}_3)_2$, 2 mM MgSO_4 , 0.02 mM $\text{Fe}(\text{EDTA})$, micronutrients [70 μM HBO_3 , 14 μM MnCl_2 , 0.5 μM CuSO_4 , 1 μM ZnSO_4 , 0.2 μM NaMoO_4 , 10 μM NaCl , 0.01 μM CoCl_2 per 1 Liter], 2.5 mM KH_2PO_4 , 5 mM KNO_3 , and 7 g/L agar) as described by Haughn and Somerville (1986). Seeds were stratified for three days at 4°C to break dormancy prior to incubation in a growth chamber. Seedlings were transplanted to soil upon emergence of true leaves, about seven days post-germination. Pots/trays were covered in plastic wrap for two days to maintain humidity during root establishment after transplantation.

2.2 Genomic DNA extraction and genotyping

For preparation of genomic DNA for PCR genotyping, leaf disks were placed in 1.5 mL microcentrifuge tubes and stored at -80 °C until processing. Frozen tissue was ground by hand for 10 seconds using a Kontes PELLET PESTLE® to break the cell wall (Kimble-Chase, Vineland, NJ). 400 μL of DNA extraction buffer (200mM Tris-HCl, 250 mM NaCl, 25 mM EDTA pH 8.0, and 0.5% SDS (v/v)) was added to the sample, which was then vortexed for 5 seconds to lyse the cells. The sample was centrifuged for 10 minutes at maximum speed (16,060 X g) in a microcentrifuge to pellet the cell debris. 350 μL of the supernatant was then transferred into a new 1.5 mL microcentrifuge tube containing 350 μL isopropanol to precipitate the DNA. The sample was mixed by inversion, incubated at room temperature for 10 minutes, and then centrifuged for 10 minutes at maximum speed to recover the DNA. The DNA pellet was washed with 800 μL of 70% ethanol (v/v) to remove impurities and dried for 20 minutes in a 37°C air incubator. The DNA was resuspended in 100 μL of purified molecular-biology-grade water

(Sigma-Aldrich, Oakville, ON) and stored at 4°C. Derived Cleaved Amplified Polymorphic Sequence (dCAPS) analysis was used to identify homozygous mutant lines containing single nucleotide polymorphisms (SNPs). In this technique, a restriction enzyme recognition site containing the SNP is introduced into the PCR product using a primer containing one or more mismatches to the genomic template DNA. The resulting PCR product is digested with an appropriate restriction enzyme and the presence or absence of the SNP is determined by the cleavage pattern (Neff et al., 1998). Table 2.2 describes the SNPs contained in the *pid-2*, *pid-3*, *aux1-7*, and *axr1-3* alleles. Table 2.3 provides a list of primers used for dCAPS genotyping. The dCaps Finder 2.0 website (<http://helix.wustl.edu/dcaps/dcaps.html>) was used for primer design (Neff et al., 2002). The *pin1-1* and *pin1-En134* mutants could not be genotyped using PCR methods and were identified by phenotype in the results.

2.3 Plant characterization

Phenotypic analysis was used to characterize homozygous triple mutant lines. Leaf initiation was scored by marking leaves at emergence. Leaves were counted daily until emergence of the inflorescence meristem (typically n=20 plants). The number of structures (flower, leaves, branches, and siliques) on the primary inflorescence and the number of petals on flowers was scored (typically n=20 plants or flowers). Whole plants, leaf series, and inflorescences were imaged using a Nikon D70s Digital SLR Camera with zoom lens (Nikon Corporation, Tokyo, Japan). Single leaves, flowers, and GUS-stained tissues were imaged using a Zeiss Discovery V12 SteREO microscope with digital camera (Carl Zeiss, Bergdorf, Germany).

2.4 Phyllotaxy measurements

For the analysis of phyllotaxy, leaves were marked with indelible ink upon emergence. Cotyledons were designated Cotyledon 1 (C1) and Cotyledon 2 (C2). As each new leaf initiated, an aerial photograph of the plant (n=2) was taken using a Zeiss Discovery V12 SterEO microscope with digital camera (Carl Zeiss, Bergdorf, Germany). The angle between consecutive leaves from C1 to Leaf 13 was measured using the “Measure Angle 3” tool in the AxioVision Software.

2.5 Chemical treatment with N-naphthylphthalamic acid (NPA)

To examine the effects of auxin transport inhibition (influx and efflux) on leaf initiation and morphology in WT and *bop1 bop2* mutants, seedlings were grown in the presence of the auxin transport inhibitor NPA. Seeds were surface sterilized and sown in magenta boxes containing AT minimal medium supplemented with 1% sucrose and NPA (Alfa Aesar, Ward Hill, MA). A 20 mM stock solution of NPA was prepared in dimethyl sulfoxide (DMSO). NPA was added to cooled molten agar to final concentrations of 0 μ M, 1 μ M, 2 μ M, 5 μ M, or 10 μ M. Ten seeds were sown per box and stratified as described above before transfer to a continuous-light growth chamber. Rate of leaf initiation was determined as described in section 2.3. Leaf 3 from a representative 21-day-old plant was photographed to document the effect of each concentration of NPA on leaf morphology.

2.6 Chemical treatment with Naphthoxyacetic acid (NOA)

To examine the effects of auxin influx inhibition on leaf vein patterning and leaf morphology in WT and *bop1 bop2* mutants, seedlings were grown in the presence of the auxin influx inhibitor NOA and leaf vein pattern was compared to that of WT control plants. Seeds

were sown in magenta boxes as described above except that medium was supplemented with various concentrations of NOA (0 μ M, 30 μ M, 50 μ M, or 100 μ M) in 95% ethanol diluted from a stock solution of 20 mM. Leaf 1 from a representative 17-day-old-plant was excised and cleared with 70% ethanol (v/v) overnight and photographed to document vein pattern. This experiment was performed by Christina Kirkham.

2.7 β -glucuronidase (GUS) staining

The promoter activities of *BP* and *DR5* (a synthetic auxin-responsive promoter) were monitored in plants by fusion to the bacterial enzyme reporter gene GUS (Jefferson et al., 1987). Excised plant material was placed in egg plates containing 90% (v/v) chilled acetone for 15 minutes to lightly fix the tissue. The acetone solution was replaced with pH 7 GUS staining buffer (4 mM 5-bromo-4-chloro-3-indoyl β -glucuronide, 0.1% Triton-X100, 0.5 mM KFe(CN), 50 mM NaPO₄). The plates were wrapped with parafilm and incubated at 37°C for colour development (~4 hours for seedlings or leaf tissue and 6 hours for the apex). Staining solution was removed and the tissue was cleared by incubating overnight in 70% ethanol (v/v) prior to imaging.

2.8 Scanning electron microscopy (SEM)

The inflorescence apices of WT and mutants (n=12 plants) were preserved for SEM in 5 ml of fixative (3% glutaraldehyde (v/v), 0.1 M NaPO₄ (pH 7.0), and 0.04% Triton X-100). Fixative was infiltrated into tissue by placing samples under a vacuum for 15 minutes prior to overnight incubation at 4°C. Afterwards, samples were rinsed with three changes of chilled 0.1 M NaPO₄ (pH 7.0) to remove the fixative. Samples were further fixed for two hours in 1% osmium tetroxide in 0.1 M NaPO₄ (pH 7.0) buffer to stain and immobilize lipid components.

Following this, samples were dehydrated with an ethanol series comprised of 30 minutes washes in 30%, 50%, 70%, and 90% ethanol (v/v). Samples were incubated overnight in anhydrous 100% (v/v) ethanol prior to critical point drying (Model K850, EMITech, Fall River, MA). Dehydrated samples were fixed to aluminum stubs such that the apex was oriented upwards. Flowers were dissected away to expose the inflorescence meristem as required. Samples were sputter-coated with gold using an Anatech Hummer VII sputter coater (Anatech, Battle Creek, MI) and imaged using a Vega-II XMU Variable Pressure SEM microscope (Tescan, Cranberry Township, PA).

Table 2.1 Plant materials

Genotype	Ecotype	Stock line	Reference
Wild type	Col-0, Ler-0		
<i>bop1 bop2</i>	Col	Homozygous double mutant	Y. Zhang (Hepworth et al., 2005)
<i>pid-2</i>	Ler	Homozygous mutant	CS8063, ABRC (Bennett et al., 1995)
<i>bop1 bop2 pid-2</i>		Homozygous triple mutant	S. Hepworth/L. Musa
<i>pid-3</i>	Col	Heterozygous	CS8064, ABRC (Bennett et al., 1995)
<i>bop1 bop2 pid-3</i>	Col	Homozygous	S. Hepworth/L. Musa
<i>pin1-1</i>	En-2	Heterozygous	CS8065, ABRC (Okada et al., 1991)
<i>bop1 bop2 pin1-1</i>		Homozygous for <i>bop1 bop2</i> , Heterozygous for <i>pin1-1</i>	M. Xu
<i>pin1- En134</i>	Col	Heterozygous	J. Friml (Gälweiler et al., 1998)
<i>bop1 bop2 pin1-En134</i>		Homozygous for <i>bop1 bop2</i> , Heterozygous for <i>pin1-1</i>	M. Xu
<i>DR5:GUS</i>	Col		E. Shultz (Ulmasov et al., 1987)
<i>bop1 bop2 DR5:GUS</i>	Col	Homozygous for <i>bop1 bop2</i> Heterozygous for transgene	M. Xu
<i>axr1-3</i>	Col	Homozygous	CS3075, ABRC (Lincoln et al., 1990)
<i>bop1 bop2 axr1-3</i>	Col	Homozygous triple mutant	M. Xu
<i>BP:GUS</i>	Col	Homozygous for transgene	N. Ori (Ori et al., 2000)
<i>bop1 bop2 BP:GUS</i>	Line 12	Homozygous for <i>bop1 bop2</i> Homozygous for transgene	M. Khan
<i>bop1 bop2 axr1-3 BP:GUS</i>		Homozygous triple mutant	L. Musa
<i>aux1-7</i>	Col	Homozygous	CS3074, ABRC (Swarup et al., 2004)
<i>bop1 bop2 aux1-7</i>	Col	Homozygous triple mutant	S. Hepworth/L. Musa

Table 2.2 Description of mutant alleles

Mutant Allele	Mutation	Mutagen ^a	Description	Amino acid change
<i>bop 1-2</i>	Insertion	T-DNA	Knock down	Insertion at nt -437 (promoter)
<i>bop2-1</i>	Insertion	T-DNA	Transcript null	Insertion at nt -154 (5'UTR)
<i>pid-2</i>	Single nucleotide substitution	EMS	Intermediate allele	R53→stop
<i>pid-3</i>	Single nucleotide substitution	EMS	Strong allele	G380→R
<i>pin1-1</i>	Unknown	Unknown	Strong allele	
<i>pin1-En134</i>	Insertion	Transposon	Transcript null	En-1 transposon in the first exon of <i>PIN1</i> at nt 134
<i>aux1-7</i>	Single nucleotide substitution	EMS	Intermediate allele	G → A in exon 9, Gly459Asp
<i>axr1-3</i>	Single nucleotide substitution	EMS	Intermediate allele	Position 461

^aEMS, ethyl methanesulphonate

Table 2.3 Primers for dCAPS genotyping and restriction enzyme used to cut mutant

Primer	Sequence 5'-3'	Tm °C	PCR product	Restriction Enzyme
dCaps <i>PID</i> -3 F1	ACGGTCACATTATGCTCTCTGACTTTGAA	57	207 bp	<i>Eco</i> RI
dCaps <i>PID</i> -3 R1	TAACCGGTTTCAGCAACAAAGAGACGGGTT			
dCaps <i>PID</i> -2 F1	TGCTTAACAAAGATCCGACTAAAAAGACT	57	202 bp	<i>Avr</i> II
dCaps <i>PID</i> -2 R1	AGTAATCGAACGCCGCTGGTTTGTTACTAC			
dCaps <i>AUX1</i> -7 F3	AACCAACTTTGTTCGTCAAGTCGACACTTTAG	57	209 bp	<i>Bgl</i> II
dCaps <i>AUX1</i> -7 R1	CTCCCTTCACGTATACGAACACAAAACAC			
dCaps <i>AXRI</i> -3 LF	AAGATTCAATGTTGAACTTGATAGAATCG	57	200 bp	<i>Cla</i> I
dCaps <i>AXRI</i> -3 RR	AGCATATATTCTCATTGGCCAATATCCTAA			

CHAPTER 3 RESULTS

3.1 Analysis of auxin-related defects in vegetative development of *bop1 bop2* mutants

3.1.1 A reduced rate of leaf initiation and delayed transition from opposite to spiral phyllotaxy in *bop1 bop2* mutants

The position of emergent leaves and the rate at which they initiate are dependent on the timing and pattern of auxin maxima formation on the periphery of the SAM (Reinhardt et al., 2000; 2003; reviewed in Reinhardt, 2005). Inhibition of auxin transport by mutation or through use of chemical inhibitors causes altered leaf phyllotaxy, altered vein patterning, and plants with fewer leaves than WT (e.g. Mattsson et al., 1999; Hay et al., 2006; Bainbridge et al., 2008). Comparison of 3-week-old WT and *bop1 bop2* plants shows differences in the appearance of the rosette that may reflect differences in the rate or pattern of leaf initiation (Figure 3.1AB). To test these possibilities, Mingli Xu in the lab examined the rate of leaf initiation in *bop1 bop2* mutants relative to WT control plants grown in continuous light (CL) or long-day (LD) photoperiods (Figure 3.1C). In both photoperiods, the rate of leaf initiation was significantly lower (Student's t-test, $P < 0.0001$) in *bop1 bop2* mutants relative to WT control plants (1 leaf/1.88 days [WT] versus 1 leaf/2.8 days [*bop1 bop2*] in LDs; 1 leaf/1.75 days [WT] versus 1 leaf/2.9 days [*bop1 bop2*] in CL). I followed this up by examining leaf phyllotaxy in WT and *bop1 bop2* mutants in short-day photoperiods in which the vegetative phase is lengthened (Figure 3.1D). In both genotypes, the cotyledons and first two true leaves formed opposite to each other with a divergence angle of 180° (decussate phyllotaxy). Following this, there was a gradual transition to spiral phyllotaxy. The divergence angle between successive leaves stabilized at 137.5° (the golden angle) at about the transition between leaves 3 and 4 in WT. In the mutant, the golden

angle was not reached until the transition between leaves 5 and 6; a slight delay in transition. Collectively, these data indicate that leaves initiate at a slower rate in *bop1 bop2* mutants, with a possible slight delay in the transition from decussate to spiral phyllotaxy. We therefore concluded that BOP1/2 activity in the vegetative shoot apex promotes leaf initiation.

3.1.2 Extra midveins in *bop1 bop2* leaf petioles

In developing leaves, auxin is transported through cells in the epidermis to a convergence point at the leaf tip. Auxin is then drained down through primordium interior, marking the position of the midvein. Higher order veins are progressively established, initially in the form of closed loops that connect to the midvein or a pre-existing vein, and subsequently in the form of open-ended veins that terminate without connection (Scarpella et al., 2006; reviewed in Scarpella et al., 2010). Analysis of PIN1:GFP shows that its expression canalizes to strands of cells in developing leaves, promoting their differentiation as vasculature and that disruption of auxin transport alters vein patterning (Scarpella et al., 2006). We therefore examined the vascular patterning of *bop1 bop2* leaves which have elongated leafy petioles (Hepworth et al., 2005; Norberg et al., 2005). No obvious change was observed in the density of veins in the leaf blade (data not shown) but there was a clear increase in the number of midveins in the petiole; these extra midveins run parallel to the main midvein without joining (Figure 3.2AB). The number of these “orphan” midveins in *bop1 bop2* leaves increased over developmental time (data not shown) indicating that new midveins are progressively added. This suggests that auxin levels in *bop1 bop2* leaf petioles are higher than in WT plants. Auxin accumulation in leaf petioles could be the result of *KNOX* misexpression which tends to restrict growth and auxin transport (Zgursky et al., 2005) or alternatively from increased auxin biosynthesis in petioles as a result of new

leaflet formation. Either of these scenarios or a combination might lead to auxin overload and increased vein density in *bop1 bop2* leaf petioles.

3.1.3 Leaf morphology of *bop1 bop2* mutants is similar to that caused by chemical inhibition of auxin influx

To further probe for auxin transport defects, Christina Kirkham in the lab monitored the effects of 1-naphthoxyacetic acid (NOA) inhibition of auxin influx (uptake) on leaf shape and vein patterning in WT and *bop1 bop2* plants (Figure 3.3). Studies in tobacco cells show that NOA interferes with membrane vesicle transport causing defects in the polar localization of AUX1:YFP in the plasma membrane (Laňková et al., 2010). If auxin uptake were impaired in *bop1 bop2* mutants, we reasoned that NOA-induced changes in leaf morphology might be obvious at a lower concentration than in WT leaves. For this experiment, WT and *bop1 bop2* mutants were grown on agar medium in the presence of 0 μ M, 30 μ M, 50 μ M, or 100 μ M NOA. As expected, treatment of WT leaves with 30 μ M NOA caused broadening of the leaf petiole and formation of extra midveins (Sieburth, 1999; Mattsson et al., 1999), similar to the *bop1 bop2* mutant phenotype. Treatment of *bop1 bop2* leaves with NOA further enhanced petiole broadening and midvein complexity but the effects were less pronounced than for WT. At 50 μ M of NOA, the midvein became indistinct in both genotypes and was abolished at 100 μ M NOA in *bop1 bop2* mutants only. The leaf blade became progressively smaller in both genotypes with increasing NOA concentration. We therefore concluded that the elongated leaf shape and extra midveins in *bop1 bop2* leaves are consistent with auxin overload in the petiole.

To further examine how auxin transport inhibition affects leaf shape and leaf initiation in WT and *bop1 bop2* mutants, I treated plants with the chemical auxin efflux inhibitor N-

napthylphthalamic acid (NPA) (Sieburth, 1999; Mattsson et al., 1999). Dynamic localization of PIN1 results from rapid actin-dependent cycling of transporters between the plasma membrane and endosomal compartments and is disrupted by treatment with NPA (Geldner et al., 2001). Treatment of WT plants with NPA reduces the rate of leaf initiation and impairs leaf growth such that petioles are tubular (no longer flat and planar) and blades do not expand. The density of veins and their diameter increases at the leaf margin and in the petiole (Mattsson et al., 1999). I therefore monitored the rate of leaf initiation in WT and *bop1 bop2* mutants and the morphology of 21-day-old leaves. For this experiment, plants were grown on agar medium supplemented with 0 μ M, 1 μ M, 2 μ M, 5 μ M, or 10 μ M NPA (Figure 3.4AB). These low concentrations were chosen based on previous data from Mingli Xu in the lab suggesting that *bop1 bop2* may be more sensitive than WT to concentrations of NPA below 5 μ m (data not shown). Figure 3.4A shows the representative morphology of leaf 3 for WT and *bop1 bop2* mutants. In both genotypes, NPA treatment resulted in narrower leaf blades with smoother margins and elongated petioles. This analysis confirmed that *bop1 bop2* mutants have a lower rate of leaf initiation than WT (1 leaf/2.78 days [WT] versus 1 leaf/4.65 days [*bop1 bop2*] in 0 μ m NPA; Student's t-test, $P < 0.0001$) but treatment with low concentrations of NPA (up to 5 μ m) did not significantly impair the rate of leaf initiation in either WT or *bop1 bop2* mutant plants in comparison to control plants grown without NPA (Figure 3.4B, Student's t-test, $P = 1$ for all). These data indicate that both WT and *bop1 bop2* mutants are insensitive to low levels of auxin efflux inhibitor.

3.1.4 Increased auxin reporter activity in *bop1 bop2* leaf petioles

The presence of extra midveins in *bop1 bop2* leaf petioles suggests that more auxin may be present relative to WT petioles (Figure 3.2). In WT leaves, the blade produces more auxin than the petiole, where growth is inhibited. Auxin maxima occur at the tips of leaf primordia and in the hydathodes (serration tips) along the leaf margin (e.g. Scarpella et al., 2006). Auxin response maxima are easily visualized in leaves using a *DR5:GUS* reporter gene that contains five copies of an auxin response element driving expression of β -glucuronidase (Ulmasov et al., 2007). I therefore monitored the pattern of *DR5:GUS* expression in WT and *bop1 bop2* rosette leaves over a 3-week time-course of development. In both genotypes, *DR5:GUS* expression was observed at the leaf tip and in hydathodes along the leaf margin. However, only in the *bop1 bop2* mutant was *DR5:GUS* expression observed at the base of mature leaves (Figure 3.5A). This increased expression correlates with the ectopic development of blade-on-petiole and is associated with a significant increase in the total number of auxin maxima (localized auxin accumulation at the serration tips) in *bop1 bop2* leaves relative to WT leaves (Figure 3.5B; Student t-test, $P < 0.0001$). We therefore concluded that auxin-responsive reporter gene activity is markedly higher in the elongated leafy petioles of *bop1 bop2* mutants, consistent with extra auxin in this region, relative to WT leaves with smooth petioles.

3.2 Genetic interactions between *bop1 bop2* mutants and mutants defective in auxin transport, auxin signalling, or auxin-response

3.2.1 BOP1/2 and AS1 interact differently with the auxin signalling factor AXR1 to pattern leaves

Auxin regulates gene expression through a well-characterized auxin signalling pathway. AXR1 encodes a subunit of the RUB1 ubiquitin-activating enzyme whose activity promotes SCF^{TIR} E3 ubiquitin ligase activity and auxin-dependent gene expression (Figure 1.5; reviewed in Chapman and Estelle, 2009). Hay et al. (2006) showed that AXR1 and AS1 activities converge to negatively regulate *BP* expression in the leaf margins so as to determine blade shape. AS1 excludes *BP* from the midvein, petiole, and serration tips of leaves, whereas AXR1 excludes *BP* from leaf serration tips. Double *as1 axr1* mutants have deeply lobed leaves with *BP* misexpression sharply localized to margin cells in the sinus of the lobes, reminiscent of compound leaves (Hay et al., 2006). Given that AS1 and BOP1/2 activities converge to maintain repression of *BP* in leaf petioles (Ori et al., 2000; Ha et al., 2003; 2007; Khan et al., 2010), I wanted to determine if BOP1/2 and AXR1 also converge to regulate *BP* expression and leaf shape. To address this, I analyzed leaf morphology in *bop1 bop2 axr1-3* triple mutants relative to WT and parental control plants (Figure 3.6A,B,D). No dramatic changes in leaf morphology were observed in the triple mutant; leaves resembled the *bop1 bop2* parent with leafy petioles. However, a subtle difference was observed in the morphology of leaf 4 in *bop1 bop2 axr1* triple mutants. Triple mutants more often developed leaflets in comparison to the continuous blade-on-petiole typical of *bop1 bop2* leaves 3 and 4 (Figure 3.6D), suggesting that auxin signalling promotes to some extent blade outgrowth on the petiole margin. Collectively, these data indicate differences in the way that BOP1/2 and AS1 interact with AXR1 to control leaf shape. Our data

show that BOP1/2 and AXR1 activities do not converge to cause changes in leaf shape nor is meristematic activity enhanced in *bop1 bop2 axr1* triple mutant leaves. To correlate these finding with *BP:GUS* expression patterns, I attempted to monitor the spatial expression of *BP:GUS* in *bop1 bop2 axr1-3* triple mutants versus *bop1 bop2* and *axr1-3* control plants but was unable to isolate a suitable line due to silencing of the *BP:GUS* transgene (data not shown).

3.2.2 BOP1/2 and the auxin influx carrier AUX1 function independently to regulate leaf initiation and leaf shape

AUX1 encodes a membrane-associated permease that promotes the uptake of extracellular auxin into the cell (Swarup et al., 2004; Bennett et al., 1996; reviewed in Kramer, 2004). The chemiosmotic model of auxin transport suggests that auxin influx carriers are required for the rapid uptake of auxin in order to maintain auxin gradients, which in turn regulates organ initiation and phyllotaxis (Swarup et al., 2001; Kramer and Bennett, 2006; Bainbridge et al., 2008). The *aux1* mutant displays no phenotypes in the aerial part of the plant due to redundancy with LAX transporters (Parry et al., 2001) but a role for AUX1 in controlling primordia initiation is suggested by formation of abnormally wide primordia in response to local application of auxin to the inflorescence meristem of *pin1 aux1* double mutants (Reinhardt et al., 2003). Similar to the *bop1 bop2* mutant phenotype, chemical inhibition of auxin influx by NOA reduces the rate of leaf initiation and leads to elongated leaves with broadened petioles and ectopic midveins (Figures 3.1 and 3.2). To further examine how disrupting auxin influx in a *bop1 bop2* mutant background affects leaf initiation and leaf shape, I constructed *bop1 bop2 aux1-7* triple mutants and analyzed the phenotype of these plants relative to parental control plants. This analysis showed that the phenotype of *bop1 bop2 aux1-7* triple mutants was indistinguishable from that of the *bop1 bop2* parent (Figure 3.7AC). The number of leaves

initiated in *bop1 bop2* versus triple mutants was also similar (Figure 3.7B; Student's t-test, $P=0.337$). These data are consistent with the model BOP1/2 and AUX1 work independently to regulate leaf initiation and leaf morphology. To further examine this, we can make quadruple mutants with the LAX family of genes which function redundantly with AUX1 (Parry et al., 2001). It would also be interesting to look at the vein pattern of the *bop1 bop2 aux1-7* triple mutant leaves, to see if this is further disrupted relative to parental control leaves.

3.2.3 BOP1/2 and the auxin efflux carrier PIN1 interact to regulate organ initiation and leaf shape

The PIN family of auxin efflux carriers is essential for polar auxin transport in plant tissues. PIN1 plays a central role in PAT and its role in development has been extensively studied (reviewed in Papanov et al., 2005). Loss-of-function *pin1* causes a reduced rate of leaf initiation and a smooth leaf blade margin because organ outgrowth is restricted (Okada et al., 1991; Hay et al., 2006). Initiation of lateral branches and flowers is particularly dependent on auxin transport such that *pin1* inflorescences are naked and devoid of flowers (Okada et al., 1991; Gälweiler et al., 1998). Studies in embryos indicate that organ initiation in *pin1* mutants is hampered by failure to generate auxin maxima resulting in misexpression of *CUC* boundary and *STM* meristem genes throughout the embryo apex, thereby restricting growth and preventing cotyledon outgrowth (Furutani et al., 2004; reviewed in Aida and Takada, 2006ab). Both *BOP1/2* and *PIN1* are co-expressed in emerging leaf and floral primordia suggesting potential functional interactions (Benková et al., 2003; Heisler et al., 2005; Xu et al., 2010; Khan et al., 2010). To further investigate the genetic interaction between *BOP1/2* and *PIN1*, we therefore crossed *bop1 bop2* to *pin1-1* (ecotype Enkheim-2) and *pin1-En134* (ecotype Col-0) mutants and characterized the resulting *bop1 bop2 pin* triple mutant phenotypes by examining the rate of leaf initiation, leaf

morphology, inflorescence architecture, and floral morphology (Figures 3.8-3.11). In both *bop1 bop2* triple mutants with *pin1*, the rate of leaf initiation was additively reduced with respect to parental control lines (Figures 3.8C and 3.9C; Student's t-tests, $P < 0.0001$). There was a similar additive reduction in leaf initiation in *asl pin1* double mutants (Hay et al., 2006). These data suggest that BOP1/2 and PIN1 promote leaf initiation by non-interacting mechanisms.

Leaves in the *bop1 bop2 pin1-1* mutant closely resembled those in *bop1 bop2 asl* plants, which have trumpet-shaped blades and tubular (radial) petioles (Figure 3.8D). On the other hand, leaves in the *bop1 bop2 pin1-En134* mutant resembled *asl* mutant leaves, which lack a petiole and have short broad dissected blades (Figure 3.10D). Either leaf phenotype can be produced by treatment with high concentrations of the auxin transport inhibitor NPA (data not shown; Mattsson et al., 1999) or by co-misexpression of *KNOX* meristem and *YABBY* abaxial polarity genes in leaves (Bryne et al., 2000; Ori et al., 2000; Xu et al., 2003; Ha et al., 2004; 2007; 2010). These phenotypes indicate that BOP1/2 and PIN1 activities converge to regulate petiole morphology and may function synergistically to maintain repression of *KNOX* meristem or *YABBY* abaxial polarity genes in leaves, but this remains to be tested. It is interesting that the same synergistic interactions are not observed in *bop1 bop2 aux1* and *bop1 bop2 axr1* triple mutants. This suggests that leaf patterning changes in *bop1 bop2 pin* triple mutants are not strongly dependent on AUX1-mediated auxin influx or AXR1-mediated auxin signaling.

Inhibition of auxin transport has a dramatic effect on the initiation of reproductive organs (lateral branches and flowers) such that *pin1* inflorescences are naked, bearing few or no flowers (Okada et al., 1991). We found that mutation of *bop1 bop2* partially rescues the *pin1* naked inflorescence phenotype (Figures 3.9AB and 3.11AB). A significantly greater number of

structures, including leaves, branches, and flowers, were produced in *bop1 bop2 pin1-1* triple mutants relative to *pin1* control inflorescences (Figures 3.9D and 3.11D; Student's t-tests, $P < 0.0001$). Secondly, flowers in the triple mutant contained numerous randomly arranged petals relative to the *bop1 bop2* parental control (Figure 3.9CE; 8.45 ± 0.6 versus $5.85.8 \pm 0.3$ petals respectively). An identical inflorescence rescue phenotype was seen in *bop1 bop2 pin1-En134* triple mutants (Figure 3.11). Rescue of *pin1* by *bop1 bop2* indicates that BOP1/2 and PIN1 function antagonistically in the inflorescence. This means that in *pin1* mutants, BOP1/2 activity inhibits the initiation and outgrowth of lateral shoots (branches and flowers) from the inflorescence meristem, consistent with its role as a growth restrictor. In contrast to leaves, lateral branches and flowers are products of the axillary meristem, originating from the boundary that separates the leaf from the meristem. During reproductive development, the axillary meristem proliferates at the expense of the leaf that subtends it. We therefore concluded that PIN1 is essential for outgrowth of lateral shoots from the inflorescence meristem and that mutation of *BOP1/2* partially compensates for *pin1* loss-of-function.

3.2.4 BOP1/2 and PID kinase interact to regulate organ initiation and phyllotaxy

The polar localization of PIN1 auxin transporters is regulated in part by PID-mediated phosphorylation (Figure 1.4; Benjamins et al., 2001; Friml et al., 2004). Thus, *pid* loss-of-function phenotypes are similar to *pin1* loss-of-function phenotypes (Bennett et al., 1995; Friml et al., 2004; Vernoux et al., 2000, Christensen et al., 2000; Gälweiler et al., 1998, Benjamins et al., 2001). We therefore crossed *bop1 bop2* to *pid-2* (ecotype Ler-0, weak allele) and *pid-3* (ecotype Col-0, strong allele) mutants and analyzed the resulting *bop1 bop2 pid* triple mutant phenotypes by examining the rate of leaf initiation, leaf morphology, inflorescence architecture, and floral morphology (Figures 3.12 to 3.15). No change in leaf initiation was observed in *bop1*

bop2 pid-2 or *bop1 bop2 pid-3* triple mutants relative to *bop1 bop2* control lines (Figures 3.12AC and 3.14 AC). The total number of leaves produced in the *bop1 bop2 pid-2* triple mutant was similar to the *bop1 bop2* parental line (Figure 3.12C; P=0.145). A similar result was obtained for the *bop1 bop2 pid-3* triple mutant (Figure 3.14C). No enhancement of the *bop1 bop2* leafy petiole phenotype was seen in either triple mutant (Figures 3.12BD and 3.14B). If anything, leaf 3 petioles were smoother in *bop1 bop2 pid-2* triple mutant leaves than in *bop1 bop2* control plants (Figure 3.12BD) again suggesting that auxin may in part promote *bop1 bop2* leafy outgrowths. Previous analysis has shown that strong *pid-3* mutants have rare defects in phyllotaxy resulting in fused or extra cotyledons and true leaves (Bennett et al., 1995). The frequency of such phenotypes was dramatically enhanced in *bop1 bop2 pid-3* triple mutants as evidenced by an increase in the number of seedlings with fused cotyledons (organs initiated too closely with no boundary to separate them) or extra first true leaves (three instead of two). In the triple mutant, 13 out of 20 plants displayed defects while none of the 20 characterized *pid-3* seedlings had defects (Figure 3.14A). These data reveal a redundant role for BOP1/2 in controlling the position and separation of leaf primordia.

Like *pin* mutants, strong *pid-3* mutants show severe defects in inflorescence architecture such that few or no flowers are initiated by the inflorescence meristem (Benjamins et al., 2001; Figure 3.15ABCE). As seen for *pin1*, loss-of-function *bop1 bop2* partially rescued the naked “pin” phenotype of *pid-3* mutants; triple mutants initiated significantly more structures (leaves, branches, and flowers) relative to *pid-3* control plants (Figure 3.15E; Student’s t-test, $p < 0.0001$). The inflorescence meristem of weak *pid-2* mutants terminates in a mass of flowers with an excess of petals. Mutation of *bop1 bop2* caused a dramatic rescue of this phenotype resulting in plants with inflorescences similar to WT (Figure 3.13ABD). Whereas *pid-2* flowers initiate an

excess of randomly arranged petals, triple mutant flowers were WT in architecture with an average of four petals (Figure 3.12E). Triple *bop1 bop2 pid-3* mutant flowers initiated an excess of randomly arranged petals but unlike *pid-3* single mutants, triple mutant flowers were fertile (Figures 3.15DE; Figure 3.15B; Students t-test, $P < 0.0001$). These data provide further support that BOP1/2 and PIN/PID activities in the inflorescence meristem are antagonistic with respect to initiation and outgrowth of lateral shoots and flowers.

3.3 SEM microscopy of *bop1 bop2 pin* and *bop1 bop2 pid* apices confirms rescue of inflorescence meristem activity.

To confirm the rescue of organ initiation in *pid* and *pin* inflorescence meristems by loss-of-function *bop1 bop2*, I used SEM to examine the detailed morphology of WT, *bop1 bop2*, *pid-2*, *bop1 bop2 pid2*, *pid-3*, *bop1 bop2 pid-3*, *pin1-1* and *bop1 bop2 pin1-1* inflorescence apices (Figure 3.16). This analysis showed that mutation of *bop1 bop2* restores organization in *pin* and *pid* shoot apices, permitting primordia initiation and organ outgrowth. In the strong *pin1* and *pid-3* mutants, organ initiation is only partly restored but in the weak *pid-2* allele organization of the inflorescence and floral meristem is similar to WT. These data confirm that BOP1/2 activity interferes with organ initiation in *pin* and *pid* inflorescence defects, reminiscent of how CUC activity interferes with cotyledon initiation in *pin pid* mutant embryos (Figure 1.3D; Furutani et al., 2004).

Figure 3.1 Analysis of leaf initiation rate and phyllotaxy in WT and *bop1 bop2* mutants. 3.3-week-old plants are shown for: (A) WT. (B) *bop1 bop2* mutant. (C) Graph depicting the average rate of leaf initiation for WT and *bop1 bop2* plants grown in long-day (LD) or continuous light (CL) photoperiods (n=20). (D) Graph depicting the angle of divergence between successive leaves of WT and *bop1 bop2* plants grown in short-day photoperiods (n=2). Red line, Golden Angle (137.5°). Arrow, point where *bop1 bop2* leaves show spiral phyllotaxy. C, cotyledon. L, true leaf. Error bars, s.e.m. Asterisks in (C), significantly different than WT control (Student's t-test, $P < 0.0001$). Scale bars, 2cm. Panels A-C were generated by Mingli Xu.

Figure 3.2 Ectopic midveins in *bop1 bop2* mutants. (A) Representative petioles are shown for 22-day-old WT and *bop1 bop2* leaf 3 cleared with 70% ethanol. Arrows denote midveins. Scale bars, 1mm. (B) Graph depicting the average number of midveins in 17-day-old WT and *bop1 bop2* mutant leaves ($n \geq 13$). Error bars, s.e.m. Data in this figure was generated by Christina Kirkham.

Figure 3.3 Treatment of WT and *bop1 bop2* mutants with the chemical auxin influx inhibitor naphthoxyacetic acid (NOA). Plants were grown on medium containing NOA at the indicated concentrations. Leaf 1 was harvested at day 17 and cleared to reveal the vein pattern (n=20). Representative leaves are shown. Arrow, midvein is abolished in *bop1 bop2* mutant at 100 μ m NOA. Scale bars, 1mm. Data in this figure was generated by Christina Kirkham.

Figure 3.4 Treatment of WT and *bop1 bop2* mutants with the chemical auxin efflux inhibitor N-Naphthylphthalamic acid (NPA). Plants were grown on medium containing NPA at the concentrations indicated. (A) Morphology of a representative leaf 3 at day 21 is shown (n=20). Scale bars, 1mm. (B) Graph depicting the average rate of leaf initiation per day in WT and *bop1 bop2* mutants (n=10). Error bars, s.e.m.

Figure 3.5 Analysis of *DR5:GUS* expression in WT and *bop1 bop2* leaves. (A) *DR5:GUS* expression in leaf 3 of 18- or 28-day-old WT and *bop1 bop2* plants. Representative leaves are shown (n=20). Arrows, region of elevated *DR5:GUS* expression in *bop1 bop2* leaves. Scale bars, 1 mm. **(B)** Graph depicting the average number of auxin maxima in leaf 3 of 28-day-old WT and *bop1 bop2* mutant plants (n=10). Error bars, s.e.m.

Figure 3.6 Analysis of genetic interactions between *bop1 bop2* and *axr1-3* in control of leaf shape. Representative 28-day-old plants or leaves are shown. Genotypes are as indicated. (A) Whole plants. (B) Leaf series; C1-L(n) from left to right. (C) Graph depicting that triple mutant leaf 4 petioles are more likely to have a smooth petiole with leaflet versus continuous leafy petiole in *bop1 bop2* control leaves (n=20). Error bars, s.e.m. (D) Leaf 4; arrow denotes smooth petiole with leaflet in the triple mutant. Scale bars, 1cm (A-B); 0.5 cm (D).

Figure 3.7 Analysis of genetic interactions between *bop1 bop2* and *aux1-7* in control of leaf initiation and leaf shape. Representative 28-day-old plants or leaves are shown. Genotypes are as indicated. (A) Whole plants. (B) Graph showing the average number of leaves initiated by WT and mutants (n=20). Error bars, s.e.m. (C) Leaf series. C1-L (n) form left to right, Scale bars, 1cm.

Figure 3.8 Analysis of genetic interactions between *bop1 bop2* and *pin1-1* in control of leaf initiation and leaf shape. Representative 18-day-old plants or leaves are shown. Genotypes are as indicated. (A) Whole plants. (B) Leaf series. C1-L (n) form left to right. (C) Graph depicting the average rate of leaf initiation for WT and mutants; triple mutants initiate leaves more slowly than parental controls (n=20). Error bars, s.e.m. (D) Morphology of single leaf 4; tubular petiole similar to *bop1 bop2 as1* mutants that misexpress *KNOX* meristem genes. Scale bars, 1cm (A-B); 0.5 cm (D).

Figure 3.9 Analysis of genetic interactions between *bop1 bop2* and *pin1-1* in inflorescence and floral development. Representative 7-week-old plants, apices, and flowers are shown. Genotypes are as indicated. **(A)** Whole plants; partial rescue of “pin” phenotype in *bop1 bop2 pin1-1* triple mutants. **(B)** Inflorescence apices; floral initiation is partially rescued in triple mutants. **(C)** Flowers; triple mutant flowers initiate a random excess of petals. **(D)** Graph depicting the average number of structures (leaves, branches, and flowers) generated on WT and mutant primary inflorescences (n=20). **(E)** Graph depicting the average number of petals per flower for WT and mutants (n=15). Error bars, s.e.m. Scale bars, 1 cm (A); 0.5 cm (B); 1 mm (C).

Figure 3.10 Analysis of genetic interactions between *bop1 bop2* and *pin1-En134* in control of leaf initiation and leaf shape. Representative plants and leaves of 21-day-old plants are shown. Genotypes are as indicated. (A) Whole plants; fewer leaves are initiated in *bop1 bop2* and *pin1-En134* mutant. (B) Leaf series. C1-L (n) form left to right. (C) Graph showing the total number of leaves initiated in WT and mutants; an additive decrease in leaf initiation occurs in triple mutants (n=20). Error bars, s.e.m. (D) Single leaf 3; triple mutant leaves have wide short petioles. Scale bars, 1cm (A-B); 0.5 cm (D).

Figure 3.11 Analysis of genetic interactions between *bop1 bop2* and *pin1-En134* in inflorescence and floral development. Representative 7-week-old plants, apices, or flowers are shown. Genotypes are as indicated. **(A)** Whole plants; partial rescue of “pin” phenotype in *bop1 bop2 pin1-En134* triple mutants. **(B)** Inflorescence apices; floral initiation is partially rescued in triple mutants. **(C)** Flowers; triple mutant flowers initiate a random excess of petals. **(D)** Graph depicting the average number of structures (leaves, branches, and flowers) generated on WT and mutant primary inflorescences (n≥8). Error bars, s.e.m. **(E)** Graph depicting the average number of petals per flower in WT and mutants (n≥14). Error bars, s.e.m. Scale bars, 1 cm (A-B); 1 mm (C).

Figure 3.12 Analysis of genetic interactions between *bop1 bop2* and *pid-2* in control of leaf initiation and leaf shape. Representative 28-day-old plants and leaves are shown. Genotypes are as indicated. (A) Whole plants. (B) Leaf series; C1-L (n) form left to right. (C) Graph depicting the total number of leaves initiated in WT and mutants (n=20). Error bars, s.e.m. (D) Single leaf 3. Scale bars, 1cm (A-B); 0.5 cm (D).

Figure 3.13 Analysis of genetic interactions between *bop1 bop2* and *pid-2* in inflorescence and floral development. Representative 7-week-old plants, apices, and flowers are shown. Genotypes are as indicated. WT and *pid-2* are Ler ecotype. (A) Whole plants. (B) Inflorescence apices; *pid-2* apical phenotype rescued by loss-of-function *bop1 bop2*. (C) Flowers; *pid-2* floral patterning rescued by loss-of-function *bop1 bop2*. (D) Graph depicting the average number of structures (leaves, branches, and flowers) on the primary inflorescence in WT and mutants (n≥14). (E) Graph depicting the average number of petals per flower in WT and mutants (n≥14); ectopic petal initiation in *pid-2* is rescued by loss-of-function *bop1 bop2*. Error bars, s.e.m. Scale bars, 1cm (A); 0.5 cm (B); 1mm (C).

Figure 3.14 Analysis of genetic interactions between *bop1 bop2* and *pid-3* in control of leaf initiation and leaf shape. Representative plants and leaves of 28-day-old plants or 14-day-old seedlings are shown. Genotypes are as indicated. (A) Whole plants. (B) Leaf series. (C) Graph depicting the average number of leaves initiated per day in WT and mutants (n=20). Error bars, s.e.m. (D) Seedlings; triple mutants show an increase in the frequency of fused cotyledons and true leaves and/or irregular phyllotaxy (arrows). Scale bars, 1cm (A-B); 0.5 cm (D).

Figure 3.15 Analysis of genetic interactions between *bop1 bop2* and *pid-3* in inflorescence and floral development. Representative 6-week-old plants, apices, and flowers are shown. Genotypes are as indicated. Loss-of-function *bop1 bop2* partially rescues organ initiation and fertility in *pid-3* mutants. (A) Whole plants. (B) Graph depicting the average number of siliques per plant in WT and mutants ($8 \leq n \leq 13$). Triple mutant siliques are fertile. (C) Inflorescences apices. (D) Flowers. (E) Graph depicting the average number of structures (leaves, branches, and flowers) on the primary inflorescence of WT and mutants ($8 \leq n \leq 13$). (F) Graph depicting the average number of petals per flower in WT and mutants ($n=20$). Error bars, s.e.m. Scale bars, 1 cm (A); 0.5 cm (C); 1 mm (D).

Figure 3.16 Scanning electron micrographs of inflorescence apices in WT and mutants. Genotypes are indicated in panels. Organ initiation is partially restored in *bop1 bop2* triple mutant apices relative to *pin1-1*, *pid-3*, and *pid-2* single mutant apices. Scale bars, 20 μ m.

CHAPTER 4 DISCUSSION

Plant development is modular with organs successively produced by the shoot apical and inflorescence meristems. Each module has three components: a leaf primordium, a boundary with the potential to form an axillary meristem, and an internode. During *Arabidopsis* vegetative development, both axillary meristem development and internode elongation are repressed giving rise to compact rosette of leaves. During *Arabidopsis* reproductive development, internode elongation occurs between lateral organs and axillary meristems proliferate to generate secondary inflorescences and then flowers (reviewed in Barton, 2010).

BOP1/2 are expressed in initiating leaf primordia but localize to the boundary at about the time that the primordia becomes distinct from the meristem (Norberg et al., 2005; Khan et al., 2010) suggesting that they may play a role in organ initiation (similar to auxin) and/or organ separation (similar to other boundary genes). Previous work has shown that *BOP1/2* activity regulates plant architecture in several ways. First, their expression in young organ primordia promotes differentiation. In emerging leaves and petioles, this entails repression of *KNOX* meristem genes and in the floral meristem this entails the activation of genes that confer floral fate (*APETALA1*) and the repression of genes that confer inflorescence identity (e.g. *AGAMOUS-LIKE24*) (Ha et al., 2003; 2004; Xu et al., 2010). Second, *BOP1/2* in boundaries promotes differentiation of specialized cells types. For example, at the stem-pedicel junction in inflorescences and at the base of floral organs, *BOP1/2* promotes the differentiation of cell-types specialized for abscission (McKim et al., 2008). Third, *BOP1/2* restricts growth. In petioles and floral bracts, *BOP1/2* restricts growth by excluding expression of *JAGGED*, which encodes a zinc-finger transcription factors that stimulates cell division and leafy outgrowth (Norberg et al., 2005). Misexpression of *BOP1/2* in stems likewise restricts growth leading to compact

inflorescences (Khan et al., 2010). Lack of organ initiation and separation defects in *bop1 bop2* mutants at the embryo and seedling stage suggests that they probably function redundantly with other factors in vegetative development.

To better understand the role of BOP1/2 during the initial stages of organ initiation and leaf patterning, my work focused on genetic interactions between BOP1/2 and the plant hormone auxin whose transport and accumulation selects sites of organ initiation and is required for leaf outgrowth and patterning. Here, I show that *bop1 bop2* mutants have several auxin-related defects not previously described. First, *bop1 bop2* mutants initiate leaves more slowly than WT and there is a slight delay in the transition from opposite to spiral phyllotaxy. Second, *bop1 bop2* mutants have a wider petiole with extra midveins, reminiscent of phenotypes caused by chemical inhibition of auxin transport (Mattsson et al., 1999; Sieburth, 1999). Third, auxin response in *bop1 bop2* leaf petioles is higher than in WT correlating with ectopic leaflet outgrowth and a wider petiole with extra midveins. To follow this up, I used double mutant analysis to examine in detail *BOP1/2* interactions with genes controlling polar auxin transport and auxin signaling. Interactions with PIN1 and PID were the most informative and showed that BOP1/2 interact differentially with auxin transport machinery to promote leaf initiation, phyllotaxy, organ separation, and floral initiation. Strikingly, mutation of *bop1 bop2* partially restored floral initiation in *pid* and *pin* shoot apices but additively slowed leaf initiation in vegetative apices highlighting differences in the way that BOP1/2 promote initiation of leaves versus flowers. I also showed genetically that BOP1/2 acts redundantly with PID to control phyllotaxy and organ separation in vegetative development and that BOP1/2 and PIN1 activities converge to regulate petiole morphology. These data show convergent roles for BOP1/2 and

auxin in regulating organ initiation, phyllotaxy, and organ patterning and highlight differences in the way that auxin interacts with BOP1/2 during vegetative and reproductive development.

4.1 *bop1 bop2* mutants exhibits auxin related defects

Several lines of phenotypic evidence support that patterns of auxin transport are disrupted in *bop1 bop2* mutants. First, the *bop1 bop2* mutants display a slower rate of leaf initiation and a possible delay in transition to spiral leaf phyllotaxy relative to WT control plants (Figure 3.1). Similar phenotypes arise in auxin transport mutants such as *pin1* which have a slow rate of leaf initiation, a decrease in the number of leaves initiated, and a disruption in phyllotaxy (Okada et al., 1991; Gälweiler et al., 1998). Second, *bop1 bop2* mutant leaves have broad petioles containing multiple ectopic midveins (Figure 3.2). This phenotype is symptomatic of auxin overload, for example when plants are grown in the presence of a PAT inhibitor. At very high doses of PAT inhibitor (e.g. NPA or NOA) no auxin gradient can be formed and the midvein becomes incomplete or disappears (Figure 3.3; Mattsson et al., 1999; Sieburth, 1999). Monitoring the expression of an auxin responsive *DR5:GUS* reporter gene in *bop1 bop2* leaves confirmed ectopic reporter gene activity in petiole (Figure 3.5). In simple WT leaves, auxin response is normally higher in the blade than in the petiole but in *bop1 bop2* mutants the gradient was shifted with higher expression in the petiole. These data provide evidence to support that BOP1/2 activity influences auxin distribution in the shoot apex and leaves, contributing in part to the rate and position at which leaves initiate and their patterning. The mechanism of disruption in auxin transport however remains unclear.

The LOB-domain gene *JLO* is also expressed in lateral organ boundaries and interacts with auxin to regulate organ initiation, organ position, and organ patterning (Borghi et al., 2007;

Bureau and Simon, 2008). Like *BOP1/2*, the *JLO* boundary gene is initially expressed in the adaxial half of young organ primordia and is subsequently restricted to the boundary between initiating organs and the meristem (Borghi et al., 2007). Phenotypes in the *jlo-ID* gain-of-function mutant include dwarfed form, serrated rosette leaves with short petioles, and shoots with a *pin*-like structure (Borghi et al., 2007; Bureau and Simon et al., 2008). As illustrated in Figure 1.1, JLO in the boundary promotes *STM* expression in the meristem and restricts *PIN1* expression to the initiating organ primordia, necessary for lateral organ outgrowth (Heisler et al., 2005; Borghi et al., 2007). *BOP1/2* may play a similar role in co-ordinating PIN1 and KNOX activities in emerging lateral organs or in the boundary but this must be established. *BOP1/2* functions as a direct activator of the LOB-domain gene *AS2* in leaf petioles, thereby maintaining repression of *KNOX* genes in the leaf (Ha et al., 2007; Jun et al., 2010). Interactions between *BOP1/2* and *JLO* have yet to be investigated.

4.2 BOP1/2, AS1, and PIN1 additively promote leaf initiation

Convergence points of auxin flow in epidermal cells of the meristem generate auxin maxima that direct the formation of leaf primordia at precise locations at the periphery of the meristem by down-regulating KNOX meristem genes (Long et al., 1996; Reinhardt et al., 2000; Benková et al., 2003; Heisler et al., 2005; Reinhardt et al., 2003; reviewed in Vernoux et al., 2010). Auxin-dependent signaling is required for the initial down-regulation of *KNOX* meristem genes since mutation of the ARF encoded by *MONOPTEROUS* (*MP*) blocks the ability of *pin1* meristems to initiate organs in response to exogenous application of auxin (Reinhardt et al., 2003). Experiments in maize confirm that treatment of apices with NPA leads to the ectopic accumulation of KNOX proteins at the shoot apex, blocking leaf initiation (Scanlon, 2003). Leaf initiation is mildly reduced in *pin1* mutants but severely restricted in *pin1 pid* double mutants

presumably because a steep auxin gradient cannot be formed (Bennett et al., 1995; Hay et al., 2006; Furutani et al., 2004; reviewed in Vernoux et al., 2010). The result is delocalized expression of *STM* meristem and *CUC* boundary genes throughout the peripheral zone (Figure 1.3; Furutani et al., 2004; Vernoux et al., 2000; Reinhardt et al., 2003; Schuetz et al., 2008). A number of *pin* enhancers that block organ initiation have been identified. These include mutations in *YUC1* and *YUC4* which encode auxin biosynthetic genes (Cheng et al., 2007a), mutations in *MP*, which encodes an ARF protein required for auxin-dependent gene expression (Schuetz et al., 2008), and mutations in *NPY1* (for *NAKED PINS IN YUC MUTANTS1*) which probably regulates PID protein abundance (Cheng et al., 2007b). In single or double mutants of *BOP1/2*, *AS1*, and *PIN1*, leaf initiation is slower but not prevented (Hay et al., 2006; Khan et al., 2010; Figure 3.8,3.10). Thus, mutation of *BOP1/2* and *AS1* are insufficient to block formation of auxin maxima in *pin1* vegetative meristems. Nevertheless, it would be interesting to examine the rate of leaf initiation in *bop1 bop2 as1* triple mutants and in *bop1 bop2 as1 pin1* quadruple mutants and to examine the expression patterns of *KNOX* meristem (*STM*, *BP*) and *CUC* boundary genes using *in situ* hybridization in each of these genotypes and in *pin pid* double mutants to gain more insight into BOP1/2-AS1 function. A reduced rate of leaf initiation in *as1* and *bop1 bop2* mutants may simply reflect the role of these genes in maintaining repression of *KNOX* gene in leaf primordia after their emergence from the meristem (Bryne et al., 2002; Guo et al., 2008) or they may promote the expression of growth regulators that speed organ outgrowth and feed a supply of auxin to the rest of the meristem.

4.3 BOP1/2 and auxin regulate phyllotaxy and promote organ separation

Phyllotaxy and organ fusion defects occur at a low frequency in *pin1-1* and *pid-3* seedlings (Bennett et al., 1995). These phenotypes have been attributed to auxin overload which reduces separation between successively initiated leaf primordia by altering auxin distribution and transport dynamics (Okada et al., 1991; Bennett et al., 1995; Reinhardt et al., 2000; reviewed in Scarpella et al., 2010). *PID* encodes a kinase that regulates the polar localization of PIN1 transporters; mutations in *pid* therefore impair polar auxin transport (Benjamins et al., 2001; Friml et al., 2004). My data show that *bop1 bop2* mutations dramatically enhance phyllotaxy and organ fusion defects in *pid-3* mutants, suggesting that auxin overload may be significantly enhanced in the triple mutant (Figure 3.14). This interpretation is supported by phenotypic analysis of *bop1 bop2* petioles showing extra midveins and increased auxin response (Figures 3.2 and 3.3). *BOP1/2* are initially co-expressed with *PIN/PID* in organ primordia but become restricted to the boundary as primordia separate from the meristem, similar to *JLO* (Khan et al., 2010; Benjamins et al., 2001; Bhorgi et al., 2007). Leaf primordia act initially as sinks of auxin and deplete it from their surroundings, thus inhibiting the initiation of additional leaf primordia at the periphery of the SAM. However, soon after their initiation, leaf primordia start to produce their own auxin, leading to a reversal in PIN polarity such that auxin is transported back into the meristem to contribute to subsequent leaf initiation events (Heisler et al., 2005; reviewed in Scarpella et al., 2010). Relocation of *BOP1/2* and *JLO* expression to the boundary correlates closely with the switch to auxin synthesis and redirection of auxin flow from the primordia, an event that requires PID activity. If this process was synergistically disrupted in *bop1 bop2 pid-3* triple mutants, defects in phyllotaxy leading to organ separation problems might be the expected result.

Organ fusion can also be viewed as ectopic growth in the boundaries that separate cotyledons or leaves. Interestingly, *cuc* cotyledon fusion defects are enhanced by *pin* or *pid* mutations but not by *bop1 bop2* mutations (Furutani et al., 2004; data not shown). This may reflect the fact that BOP1/2 do not play a strong role in embryo development or that CUC and BOP activities interact differentially with the auxin transport machinery. BOP1/2 are likely to act downstream of the *CUC* genes with respect to their boundary function because *CUC* expression precedes *BOP* expression in boundaries and mutation of *bop1 bop2* does not alter *CUC* expression pattern, at least in shoot boundaries of the inflorescence (Aida et al., 1999; Ha et al., 2004; Khan et al., 2010). It remains to be seen if localization of *BOP1/2* expression to the boundary is CUC-dependent. Both BOP and CUC proteins function as growth restrictors but their loss- and gain-of-function phenotypes are quite different indicating that they may use different transcriptional targets to regulate growth (Hepworth et al., 2005; Xu et al., 2010; Khan et al., 2010; Norberg et al., 2005; Ha et al., 2007; Furutani et al., 2004; Peaucelle et al., 2007; Seiber et al., 2007). For example, loss-of-function *bop1 bop2* prominently results in leafy petioles and flowers with bracts whereas loss-of-function *cuc1 cuc2* mutants results in cup-shaped cotyledons (e.g. Hepworth et al., 2005; Aida et al., 1997).

4.4 BOP1/2, AS1, and auxin regulate leaf shape

Leaves can assume a variety of shapes, sizes, and arrangements in nature but are broadly classified as simple or compound. Simple leaves such as those in *Arabidopsis* have a single blade and petiole. By contrast, compound leaves possess multiple blade units (leaflets) attached to a rachis (reviewed in Uchida et al., 2010; Hay and Tsiantis, 2010). Class I *KNOX* genes and auxin are the two main regulators of leaf shape. In species with simple leaves, *KNOX* meristem genes are excluded from leaf primordia so that leaves are paddle-shaped with a distinct blade and

petiole. The margin of simple leaves may be smooth, lobed, or serrated, distinctions that are controlled by the pattern of *KNOX* gene expression and PIN1-mediated production of auxin maxima at the leaf margin. In compound leaf species such as tomato, *KNOX* genes are reactivated in leaves so as to allow the production of multiple leaflets on the rachis. Available data suggests the following as the sequence of events in the formation of serrations, lobes, and leaflets: *KNOX* expression facilitates the formation of auxin maxima, which feed back to repress *KNOX* expression, necessary for leaflet outgrowth. This pattern is analogous to how leaves initiate on the flanks of the meristem (reviewed in Hay and Tsiantis, 2010).

Mutation of *BOP1/2* or *AS1* both cause reactivation of *KNOX* meristem genes in leaves, but in different places, leading to distinct leaf shapes. In *as1* mutants, *KNOX* genes are misexpressed both in leaf margins and petioles causing lobed blade margins and broad and short or tubular petioles, respectively (Bryne et al., 2000; Ori et al., 2000; Xu et al., 2003). In *bop1 bop2* mutants, *KNOX* misexpression is confined to the petioles, resulting in leaflets or blade-on-petiole, similar to compound leaves (Ha et al., 2003; Khan et al., 2010). In *bop1 bop2 as1* triple mutants, *BP* is further misexpressed in petioles but no further misexpression occurs in the blade (Jun et al., 2010; Khan et al., 2010).

Hay et al. (2006) showed that AS1 and auxin signaling activities converge to regulate the pattern of *BP* expression in leaf margins leading us to examine if BOP1/2 and auxin activities likewise converge to regulate petiole structure. Although *axr1* mutations did not enhance *bop1 bop2* leaf defects as seen in figure 3.6, my data showed that mutations in *pin1* mutations enhance *bop1 bop2* petiole defects. In *bop1 bop2 pin1-1* triple mutants, petioles were radialized (tubular) similar to those in *bop1 bop2 as1* triple, indicating loss of abaxial-adaxial polarity (Figure 3.8).

Tubular petioles are also seen in leaves treated with NPA (Ni et al., 1999; Scanlon, 2003). In *bop1 bop2 pin1-En134* triple mutants, petioles were absent or very short and wide, similar to *asl* mutants (Figure 3.10; Bryne et al., 2000). These data indicate that BOP1/2 and auxin converge to regulate petiole morphology. The next step would be to examine patterns of *KNOX* meristem and *YABBY* abaxial polarity genes in the triple mutant petioles. Both classes of genes are misexpressed in *bop1 bop2* leaves (Ha et al., 2007) and may be synergistically upregulated in *bop1 bop2 pin1* triple mutant leaves. It is also possible that auxin biosynthetic genes are upregulated in *bop1 bop2* petioles contributing to enhanced auxin overload in *bop1 bop2 pin1* triple mutant leaves.

4.5 BOP1/2 activity antagonizes floral initiation in auxin transport mutants

During reproductive development, the apical meristem stops making rosette leaves and produces secondary inflorescences subtended by cauline leaves and subsequently flowers. Secondary inflorescences and flowers are the products of axillary shoot meristems derived from the boundary between the leaf and the inflorescence meristem. Shoot meristem proliferation blocks outgrowth of the subtending leaf primordia so that branches are subtended by small cauline leaves and flowers lack bracts altogether (e.g. Nilsson et al., 1998; Hepworth et al., 2006; Xu et al., 2010). For reasons that are unclear, initiation and proliferation of axillary shoots is particularly sensitive to defects in auxin transport such that auxin maxima cannot be generated in *pin1* or *pid* single mutants and organ initiation from the inflorescence meristem is blocked (e.g. Okada et al., 1991; Mattsson et al., 1999; Bennett et al., 1995). This could reflect that the inflorescence meristem is more dependent on auxin supply because organs are produced at a much faster rate than the vegetative meristem (three flowers per day versus one leaf per day) (Schuetz et al., 2008) or could reflect differences in the way that organs are initiated during

reproductive versus vegetative development. In double *mp pin1* double mutants, a leafless dome from the SAM is formed and organs fail to initiate completely, a phenotype not present in either parent line (Schuetz et al., 2008). Sensitivity of organ initiation to defects in auxin transport is a good example of the competitive relationship between meristem maintenance and organ initiation. My data show that mutation of *bop1 bop2* rescue in part the naked “pin” phenotype of strong *pid* and *pin* mutants (Figures 3.9 and 3.11). Mutation of *bp* causes a similar partial rescue of *pin* and *pid* mutants (Hay et al., 2006).

The interaction between auxin, meristem, and boundary genes has been studied in developing embryos and helps to explain why *bop1 bop2* mutations might partially suppress *pin* and *pid* naked inflorescences (Figure 4.1). In *pin1 pid* double mutants, *STM* meristem and *CUC2* boundary genes are misexpressed throughout the entire apical dome of the embryo, restricting organ outgrowth. In this context, cotyledon formation is restored by inactivating *CUC* or *STM* (Figure 4.1A-C; Furutani et al., 2004). Thus, despite the fact that auxin gradients are not properly formed in *pin pid* apices, organs initiate when unopposed by growth restrictors. In *pin1* inflorescence apices, the *CUC2* boundary gene is similarly misexpressed in a continuous ring that encircles the apex (Vernoux et al., 2000). Two other early markers of floral organ initiation, *LEAFY* (*LFY*) and *ANT* are misexpressed in an identical pattern (Long and Barton, 2000; Vernoux et al., 2000). These data led to the proposal that in the absence of threshold levels of auxin, a hybrid of compartment identities occurs in the peripheral zone of the *pin* meristem, preventing organ initiation and outgrowth (Vernoux et al., 2000). Loss-of-function in either *bp* or *bop1 bop2* partly alleviates the block (Hay et al., 2006; this study) suggesting that *BP* and *BOP1/2* are probably also misexpressed (Figure 4.1D-F). To test this, *in situ* hybridization or GUS reporter genes can be used to monitor the expression patterns of these genes in *pin1*

inflorescence apices. To test for misexpression of *KNOX* meristem genes in *pin1* apices, I looked for up-regulation of transcript using RT-PCR (Appendix A). Unfortunately, quantification was not accurate and differences could not be accurately documented. This method is not sensitive enough to detect small spatial differences in transcript abundance. It would also be interesting to examine the pattern of *CUC2*, *LFY*, and *ANT* expression in *bop1 bop2 pin1* apices, where organ initiation is partially restored. These experiments would help to distinguish between mechanisms for rescue. Inactivation of *BOP1/2* or *BP* may simply alleviate growth restriction, tipping the balance in favor of canalization and organ outgrowth. Alternatively, *bop1 bop2* may rescue *pin1* inflorescence defects by promoting auxin biosynthesis, thereby increasing the auxin supply such that auxin maxima are generated and organization is restored. The *YUCCA* genes are a family of auxin biosynthetic genes and *yuc1 yuc4 yuc2 yuc6* quadruple mutants display a “pin” inflorescence phenotype illustrating the important role that auxin biosynthesis plays in sustaining organ initiation (Cheng et al., 2007a). To test whether rescue is due to upregulation of auxin biosynthetic genes, we can look at expression of *YUC1* and *YUC4* in the inflorescence apices of WT, *pin1*, and *bop1 bop2 pin1* triple mutants using *in situ* hybridization. In the *bop1 bop2 pin1* triple we would expect to see up-regulation of *YUC1* and *YUC4* transcript. I looked for up-regulation of *YUC* transcripts using RT-PCR but unfortunately, quantification was not accurate and reproducible differences were not observed (Appendix A). Finally, it would be useful to examine the pattern of PIN1:GFP expression in the rescued *bop1 bop2 pin1* and *bop1 bop2 pid* apices to determine if auxin maxima are reestablished as predicted.

4.6 Conclusion

My experiments show that BOP1/2 and polar auxin transport converge to regulate organ initiation, phyllotaxy, organ separation, and leaf patterning. PIN1 convergence points in the meristem periphery create auxin maxima that stimulate organ outgrowth by creating a specialized niche of cells in which *KNOX* meristem and *CUC* boundary genes are repressed and primordium markers such as *BOP1/2* and *ASI* are activated. If polar auxin transport is blocked and auxin maxima cannot be created, compartmentalization cannot occur and expression of meristem, primordia, and boundary genes are intermingled such that regional differences in growth cannot be generated (Vernoux et al., 2000; Furutani et al., 2004; Schuetz et al., 2008). During vegetative development, mutation of *bop1 bop2* predominantly enhances *pin* and *pid* phenotypes related to auxin overload and *KNOX* misexpression (slower leaf initiation, phyllotaxy and organ fusion defects). Conversely, during inflorescence development, mutation of *bop1 bop2* has the opposite effect, promoting outgrowth of lateral branches and flowers. This likely represents *BOP1/2* misexpression in *pin1* or *pid-3* meristems, revealing the importance of auxin transport in generating gradients to achieve compartmentalization within the shoot apex, necessary for organ initiation and outgrowth. Future studies will focus on parallels between the production of embryonic leaves and the production of reproductive organs to identify what factors controls the spatial expression *BOP1/2* during organogenesis and to identify molecular targets of BOP1/2 that promote organ initiation and meristem compartmentalization.

Figure 4.1 Model showing parallels between the mechanism of organ outgrowth in the embryo and in the inflorescence meristem. (A) In WT embryos, formation of auxin maxima via PIN1/PID directs repression of *KNOX* meristem genes (*STM*) and boundary genes (*CUC1/2*). Leaf differentiation genes (*ASI*) and genes that promote cell proliferation (*ANT*) are upregulated at auxin maxima to stimulate cotyledon specification and outgrowth. (B) In *pin1 pid* mutants, no auxin maxima are generated and the expression domains of *STM* and *CUC2* expand to block primordia formation. (C) In *pin1 pid cuc2* triple mutants, cotyledon outgrowth is restored, but the meristem cannot be established. (D) In WT inflorescence meristems, formation of auxin maxima via PIN/PID down-regulate *KNOX* meristem genes (*STM* and presumably *BP*) at peripheral sites and promote the activation of floral differentiation genes (*LFY*) and genes that promote cell proliferation (*ANT*). (E) In *pin1* inflorescence apices, no auxin maxima are generated and the expression of meristem, boundary, and floral differentiation genes is delocalized throughout meristem periphery. (F) In *pin1 bop1 bop2* mutants, initiation of floral primordia is restored, presumably because *BOP1/2* misexpression in the *pin1* meristem antagonizes growth. When *BOP1/2* are inactivated, compartmentalization is restored.

Formation of cotyledons in the embryo

Formation of flowers at the inflorescence apex

CHAPTER 5 REFERENCES

- Aida M., Ishida, T., and Tasaka, M.** (1999). Shoot apical meristem and cotyledon formation during Arabidopsis embryogenesis: interactions among the CUP-SHAPED COTYLEDON and SHOOT MERISTEMLESS genes. *Development* **126**, 1563-1570.
- 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. *Plant Cell* **9**, 841-857.
- Aida, M., and Tasaka, M.** (2006a). Genetic control of shoot organ boundaries. *Curr. Opin. Plant Biol.* **9**, 72-77.
- Aida, M., and Tasaka, M.** (2006b). Morphogenesis and patterning at the organ boundaries in the higher plant shoot apex. *Plant Mol. Biol.* **60**, 915-928.
- Aida, M., Vernoux, T., Furutani, M., Traas, J., and Tasaka, M.** (2002). Roles of PIN-FORMED1 and MONOPTEROS in pattern formation of the apical region of the Arabidopsis embryo. *Development* **129**, 3965-3974.
- Avasarala, S., Wang, J., and Caruso, J.L.** (1996). Production of phenocopies of the lanceolate mutant in tomato using polar auxin transport inhibitors. *J. Exp. Bot.* **47**, 709-712.
- Bainbridge, K., Guyomarch, S., Bayer, E., Swarup, J., Bennett, M., Mandel, T., and Kuhlemeier, C.** (2008). Auxin influx carriers stabilize phyllotactic patterning. *Genes Dev.* **22**, 810-823.

- Barkoulas, M., Hay, A., Kougiousmoutzi, E., and Tsiantis, M.** (2008). A developmental framework for dissected leaf formation in the Arabidopsis relative Cardamine hirsuta. *Nat. Genet.* **40**, 1136-1141.
- Barton, M.K.** (2010). Twenty years on: the inner workings of the shoot apical meristem: a developmental dynamo. *Dev. Biol.* **341**, 95-113.
- 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. *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. *Plant Cell* **18**, 1900-1907.
- Benjamins, R., Malenica, N., and Luschnig C.** (2005). Regulating the regulator: the control of auxin transport. *Bioessays* **27**, 1246-1255.
- 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.
- Benková, E., Ivanchenko, M.G., Friml, J., Shishkova, S., and Dubrovsky, J.G.** (2009). A morphogenetic trigger: is there an emerging concept in plant developmental biology? *Trends Plant Sci.* **14**, 189-193.

- Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G., and Friml, J.** (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. *Cell* **115**, 591-602.
- Bennett, M.J., Marchant, A., Green, H.G., May, S.T., Ward, S.P., Millner, P.A., Walker, A.R., Schulz, B., and Feldmann, K.A.** (1996). Arabidopsis AUX1 gene: a permease-like regulator of root gravitropism. *Science* **273**, 5277: 948-50.
- Bennett, S.R.M., Alvarez, J., Bossinger, G., and Smyth, D.R.** (1995). Morphogenesis in pinoid mutants of *Arabidopsis thaliana*. *Plant J.* **8**, 505-520.
- Bharathan, G.T.E., Goliber, C., Moore, S., Kessler, T., Pham, N.R.** (2002). Homologies in leaf form inferred from KNOXI gene expression during development. *Science* **296**, 1858-1860.
- Borghi, L., Bureau M., and Simon R.** (2007). Arabidopsis JAGGED LATERAL ORGANS is expressed in boundaries and co-ordinates KNOX and PIN activities. *Plant Cell* **19**, 1795-1808.
- Bowman, J.L., and Eshed, Y.** (2000). Formation and maintenance of the shoot apical meristem. *Trends Plant Sci.* **5**, 110-115.
- Brand, U., Fletcher, J.C., Hobe, M., Meyerowitz, E.M., and Simon, R.** (2001). Dependence of stem cell fate in *Arabidopsis* on a feedback loop regulated by CLV3 activity. *Science* **289**, 617-619.

- Breuil-Broyer, S., Morel, P., de Almeida-Engler, J., Coustham, V., Negrutiu, I., and Trehin, C.** (2004). High-resolution boundary analysis during *Arabidopsis thaliana* flower development. *Plant J.* **38**, 182-192.
- Bryne, M.E., Simorowski, J., and Martienssen, R.A.** (2002). ASYMMETRIC LEAVES1 reveals knox gene redundancy in *Arabidopsis*. *Development* **129**, 1957-1965.
- Bureau, M., and Simon, R.** (2008). JLO regulates embryo patterning and organ initiation by controlling auxin transport. *Plant Signal. Behav.* **3**, 145-147.
- 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.
- Carland, F.M., and McHale, N.A.** (1996). LOP1: a gene involved in auxin transport and vascular patterning in *Arabidopsis*. *Development* **122**, 1811-1819.
- Chapman, E.J., and Estelle, M.** (2009). Mechanism of auxin-regulated gene expression in plants. *Annu. Rev. Genet.* **43**, 265-285.
- Cheng, Y., Dai, X., and Zhao, Y.** (2006). Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in *Arabidopsis*. *Genes Dev.* **20**, 1790-1799.
- Cheng, Y., Dai, X., and Zhao, Y.** (2007a). Auxin synthesized by the YUCCA flavin monooxygenases is essential for embryogenesis and leaf formation in *Arabidopsis*. *Plant Cell* **19**, 2430-2439.

- Cheng, Y., Qin, G., Dai, X., and Zhao, Y.** (2007b). NPY1, a BTB-NPH3-like protein, plays a critical role in auxin-regulated organogenesis in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **104**, 18825-18829.
- Christensen, S.K., Dagenais, N., Chory, J., and Weigel, D.** (2000). Regulation of auxin response by the protein kinase PID. *Cell* **100**, 469-478.
- Chuck, G., Lincoln, S., and Hake, S.** (1996). KNAT1 induced lobed leaves with ectopic meristems when overexpressed in Arabidopsis. *Plant Cell* **8**, 1277-1289.
- Clark, S.E., Jacobsen, S. E., Levin, J.Z., and Meyerowitz, E.M.** (1996). The CLAVATA and SHOOT MERISTEMLESS loci competitively regulate meristem activity in Arabidopsis. *Development* **122**, 1567-1575.
- Dean, G., Casson, S, and Lindsey, K.** (2004). KNAT6 gene of Arabidopsis is expressed in roots and is required for correct lateral root formation. *Plant Mol. Biol.* **54**, 71-84.
- del Pozo, J.C., and Estelle M.** (1999). The Arabidopsis cullin AtCUL1 is modified by the ubiquitin-related protein RUB1. *Proc Natl. Acad. Sci. USA* **96**, 15342-15347.
- del Pozo, J.C., Dharmasir, S., Hellmann H., Walker, L., Gray, W.M., and Estelle, M.** (2002). AXR1-ECR1-dependent conjugation of RUB1 to the Arabidopsis cullin AtCUL1 is required for auxin response. *Plant Cell* **14**, 421-433.
- 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.

- Dharmasiri, N., Dharmasiri, S., and Estelle, M.** (2005). The F-box protein TIR1 is an auxin receptor. *Nature* **435**, 441-445.
- 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. *Dev. Biol.* **215**, 407-419.
- Douglas, S.J., Chuck, G., Dengler, R.E., Pelecanda, L., and Riggs, D.C.** (2002). KNAT1 and ERECTA regulate inflorescence architecture in Arabidopsis. *Plant Cell* **14**, 547-558.
- Endrizzi, K., Moussian, B., Haecker, A., Levin, J.Z., and Laux, T.** (1996). The SHOOT MERISTEMLESS gene is required for maintenance of undifferentiated cells in Arabidopsis shoot and floral meristems and acts at a different regulatory level than the meristem genes WUSCHEL and ZWILLE. *Plant J.* **10**, 967-979.
- 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.
- Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R., and Jürgens, G.** (2003). Efflux-dependent auxin gradients establish the apical-basal axis of Arabidopsis. *Nature* **426**, 147-153.
- Friml, J., Yang, X., Michneiwick, M., Weijers, D., Quint, A., Tietz, O., Benjamins, R., Ouwerkerk, P.B.F., Ljung, K., Sandberg, G., Hooykaas, P.J.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.

- Furutani, M., Vernoux, T., Traas, J., Kato T., Tasaka, M., and Aida M.** (2004). PIN-FORMED1 and PINOID regulate boundary formation and cotyledon development in *Arabidopsis* embryogenesis. *Development* **131**, 5021-5030.
- Gallois J.L., Woodward, C., Reddy, G.V., and Sablowski, R.** (2002). Combined SHOOT MERISTEMLESS and WUSCHEL trigger ectopic organogenesis in *Arabidopsis*. *Development* **129**, 3207–3217.
- Gälweiler, L., Guan, C., Müller, A., Wisman, E., Mendgen, K., Yephremov, A., Palme, K.** (1998). Regulation of polar auxin transport by AtPIN1 in *Arabidopsis* vascular tissue. *Science* **282**, 2226–2230.
- Geldner, N., Friml, J., Stierhof, Y-D, Jürgens, G., and Palme, K.** (2001). Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature* **413**, 425-428.
- Girin, T., Sorefan, K., and Østergaard, L.** (2009). Meristematic sculpting in fruit development. *J. Exp. Bot.* **60**, 1493-1502.
- Gray, W.M., Kepinsky, S., Rouse, D., Leyser, O., and Estelle, M.** (2001). Auxin regulates SCF^{TIR1}-dependent degradation of AUX/IAA proteins. *Nature* **414**, 271–276.
- Guo, M., Thomas, J., Collins, G., and Timmermans, M.C.** (2008). Direct repression of KNOX loci by the ASYMMETRIC LEAVES1 complex of *Arabidopsis*. *Plant Cell* **20**, 48-58.

- Ha, C.M., Jun, J.H., and Fletcher, J.C.** (2010) Control of Arabidopsis leaf morphogenesis through regulation of the YABBY and KNOX families of transcription factors. *Genetics* (e-pub ahead of print)
- Ha, C.M., Jun, J.H., Nam, H.G., and Fletcher, J.C.** (2004). BLADE-ON-PETIOLE1 encodes BTB/POZ domain protein required for leaf morphogenesis in Arabidopsis thaliana. *Plant Cell Physiol.* **45**, 1361-1370.
- Ha, C.M., Jun, J.H., Nam, H.G., and Fletcher, J.C.** (2007). BLADE-ON-PETIOLE1 and 2 control Arabidopsis lateral organ fate through regulation of LOB domain and adaxial–abaxial polarity genes. *Plant Cell* **19**, 1809-1825.
- Ha, C.M., Kim, G.-T., Kim, B.C., Jun, J.H., Soh, M.S., Ueno, Y., Machida, Y., Tsukaya, H., and Nam, H.G.** (2003). The BLADE-ON-PETIOLE1 gene controls leaf pattern formation through the modulation of meristematic activity in Arabidopsis. *Development* **130**, 161-172.
- Hamant, O., and Pautot, V.** (2010). Plant development: a TALE story. *C.R. Biol.* **333**, 371-381.
- Hamant, O., Nogue, F., Belles-Boix, E., Jublot, D., Grandjean, O., Traas, J., and Pautot, V.** (2002). The KNAT2 homeodomain protein interacts with ethylene and cytokinin signaling. *Plant Physiol.* **130**, 657-665.
- 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**, 735-74.
- Haughn, G.W., and Somerville, C.** (1986). Sulfonyleurea-resistant mutants of Arabidopsis thaliana. *Mol. Gen. Genet.* **204**, 430-434.

- Hay, A., and Tsiantis, M.** (2006). The genetic basis for differences in leaf form between *Arabidopsis thaliana* and its wild relative *Cardamine hirsuta*. *Nat. Genet.* **38**, 942-947.
- 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., and Tsiantis, M.** (2009). A KNOX family TALE. *Curr. Opin. Plant Biol.* **12**, 593-598.
- 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. *Curr. Biol.* **15**, 1899-1911.
- Heisler, M., and Jönsson, H.** (2006). Modeling auxin transport and plant development. *J. Plant Growth Regul.* **25**, 302-312.
- Hepworth, S.R., Klenz, J.E., and Haughn, G.W.** (2006). UFO in the *Arabidopsis* inflorescence apex is required for floral-meristem identity and bract suppression. *Planta* **223**, 769-778.
- 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*. *Plant Cell* **17**, 1434-1448.
- Hibara, K., Karim, M.R.K., Takada, S., Taoka, K., Furutani, M., Aida, M., and Tasaka M.** (2006). *Arabidopsis* CUP-SHAPED COTYLEDON3 regulates postembryonic shoot meristem and organ boundary formation. *Plant Cell* **18**, 1946-2957.

- Hobbie, L.J.** (1998). Auxin: molecular genetic approaches in *Arabidopsis*. Plant Physiol. Biochem. **36**, 91-102.
- Iwakawa, H., Ueno, Y., Semiarti, E., Onouchi, H., Kojima, S., Tsukaya, H., Hasebe, M., Soma, T., Ikezaki, M., Machida, C., Machida, Y.** (2002). The ASYMMETRIC LEAVES2 gene of *Arabidopsis thaliana*, required for formation of a symmetric flat leaf lamina, encodes a member of a novel family of proteins characterized by cysteine repeats and a leucine zipper. Plant Cell Physiol. **43**, 467-78.
- Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W.** (1987). GUS fusions: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6**, 3901-3907.
- 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. Plant Cell **22**, 62-76.
- Khan, M., Xu, M., Hu, T., Tabb, P., Murmu, J., McKim, S.M., Musa, L., Storey, K., Mercado, J., and Hepworth, S.R.** (2010). Antagonistic interaction of *BLADE-ON-PETIOLE1* and 2 with *BREVIPEDICELLUS* and *PENNYWISE* regulates *Arabidopsis* inflorescence architecture. Plant Cell (submitted Oct 30 2010)
- Kleine-Vehn, J., Dhonukshe, P., Swarup, R., Bennett, M., and Friml, J.** (2006). Subcellular trafficking of the *Arabidopsis* auxin influx carrier AUX1 uses a novel pathway distinct from PIN1. Plant Cell **18**, 3171-3181.

- Kramer, E.M.** (2004). PIN and AUX/LAX proteins: their role in auxin accumulation. *Trends Plant Sci.* **9**, 578-582.
- Kramer, E.M., and Bennett, M.J.** (2006). Auxin transport: a field in flux. *Trends Plant Sci.* **11**, 382-386.
- Kuhlemeier, C.** (2007). Phyllotaxis. *Trends Plant Sci.* **12**, 143-150.
- Kuhlemeier, C., and Reinhardt, D.** (2001). Auxin and phyllotaxis. *Trends Plant Sci.* **6**, 187–189.
- Kwiatkowska, D.** (2004). Structural integration at the shoot apical meristem: models, measurements, and experiments. *Am. J. Bot.* **91**, 1277-1293.
- Laňková, M., Smith, R.S., Pešek, B., Kubeš, M., Zažímalová, E., Petrášek, J., and Hoyerová, K.** (2010). Auxin influx inhibitors 1-NOA, 2-NOA, and CHPAA interfere with membrane dynamics in tobacco cells. *J. Exp. Bot.* **61**, 3589-3598.
- Laufs, P., Grandjean, O., Jonak, C., Keiü, K., and Traas, J.** (1998). Cellular parameters of the shoot apical meristem in Arabidopsis. *Plant Cell* **10**, 1375-1390.
- Laux T., Mayer, K.F., Berger, J., and Jürgens, G.** (1996). The WUSHEL gene is required for shoot and floral meristem integrity in Arabidopsis. *Development* **122**, 87-96.
- Lewis, M.W., Leslie, M.E., and Liljegren, S.J.** (2006). Plant separation: 50 ways to leave your mother. *Curr. Opin. Plant Biol.* **9**, 59-65.

- Lin, W., 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. *Plant Cell* **15**, 2241-2252.
- Lincoln, C., Britton, J.H., and Estelle, M.** (1990). Growth and development of the *axr1* mutants of Arabidopsis. *Plant Cell* **2**, 1071-1080.
- Lincoln, C., Long, J., Yamaguchi, J., Serikawa, H., and Hake, S.** (1994). A knotted1-like homeobox gene in Arabidopsis is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. *Plant Cell* **6**, 1859-1876.
- Long, J., and Barton, M.K.** (2000). Initiation of axillary and floral meristems in Arabidopsis. *Dev. Biol.* **218**, 341-353.
- Long, J.A., and Barton M.K.** (1998). The development of apical embryonic pattern in Arabidopsis. *Development* **125**, 3027-3035.
- 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.
- Mattsson J., Sung, Z.R., and Berleth, T.** (1999). Responses of plant vascular systems to auxin transport inhibition. *Development* **126**, 2979-2991.
- Mayer, K.F.X., Schoof, H., Haecker, A., Lenhard, M., Jürgens, G., and Laux T.** (1998). Role of WUSCHEL in regulating stem cell fate in the Arabidopsis shoot meristem. *Cell* **95**, 805-815.

- McKim, S.M., Stenvik, G-E., Butenko, M.A., Kristiansen, W., Cho, S.K., Hepworth, S.R., Aalen, R.B., and Haughn, G.W.** (2008). The BLADE-ON-PETIOLE genes are essential for abscission zone formation in Arabidopsis. *Development* **135**, 1537-1546.
- McSteen, P., and Leyser, O.** (2005). Shoot branching. *Annu. Rev. Plant Biol.* **56**, 353-374.
- Michniewicz, M., Zago, M.K., Abas, L., Weijers, D., Schweighofer, A., Meskiene, I., Heisler, M.G., 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.
- Mitzukami, Y., and Fischer, R.L.** (2000). Plant organ size control: AINTEGUMENTA regulates growth and cell numbers during organogenesis. *Proc. Natl. Acad. Sci. USA* **97**, 942-947.
- Neff, M.M., Neff, J.D., Chory, J., and Pepper, A.E.** (1998). dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in *Arabidopsis thaliana* genetics. *Plant J.* **14**, 387-392.
- Neff, M.M., Turk, E., Kalishman, M.** (2002). Web-based primer design for single nucleotide polymorphism analysis. *Trends Genet.* **18**, 613-615.
- Ni, D.A., Wang, L.J., Xu Z.H., and Xia, Z.A.** (1999). Foliar modifications induced by inhibition of polar transport of auxin. *Cell Res.* **9**, 27-35.
- Nilsson, O., Wu, E., Wolfe, D.S., and Weigel, D.** (1998). Genetic ablation of flowers in transgenic *Arabidopsis*. *Plant J.* **15**, 799-804.

- Norberg, M., Holmlund, M., and Nilsson, O.** (2005). The BLADE ON PETIOLE genes act redundantly to control the growth and development of lateral organs. *Development* **132**, 2203-2213.
- 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. *Plant Cell* **3**, 677-684.
- 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.
- Papanov, I.A., Teale, W.D., Trebar, M., Blilou, I., and Palme, K.** (2005). The PIN auxin efflux facilitators: evolutionary and functional perspectives. *Trends Plant Sci.* **10**, 170-177.
- Parry, G., Delbarre, A., Marchant, A., Swarup, R., Napier, R., Perrot-Rechenmann, C., Bennett, M.J.** (2001). Novel auxin transport inhibitors phenocopy the auxin influx carrier mutation *aux1*. *Plant J.* **25**, 399–406.
- Pautot, V., Dockx, J., Hamant, O., Kronenberger, J., Grandjean, O., Jublot, D., and Traas, J.** (2001). KNAT2: evidence for a link between knotted-like genes and carpel development. *Plant Cell* **13**, 1719-1734.
- Peaucelle, A., Morin, H., Traas, J., and Laufs, P.** (2007). Plants expressing a miR164-resistant CUC2 gene reveal the importance of post-meristematic maintenance of phyllotaxy in Arabidopsis. *Development* **134**, 1045-50.
- Petrášek, J., and Friml, J.** (2009). Auxin transport routes in plant development. *Development* **136**, 2675-2688.

- Pickett, F.B. and Wilson, A.K., and Estelle, M.** (1990). The aux1 mutation of Arabidopsis confers both auxin and ethylene resistance. *Plant Physiol.* **94**, 1462-1486.
- Quint, M., and Gray, W.M.** (2006). Auxin signaling. *Curr. Opin. Plant Biol.* **9**, 448-453.
- Rast, M.I., and Simon, R.** (2008). The meristem-to-organ boundary: more than an extremity of anything. *Curr. Opin. Genet. Dev.* **18**, 287-294.
- 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.
- Reinhardt, D.** (2005) Regulation of phyllotaxis. *Int. J. Dev. Biol.* **46**, 539-546.
- Reinhardt, D., Mandel, T., and Kuhlemeier, C.** (2000). Auxin regulates the initiation and radial position of plant lateral organs. *Plant Cell* **12**, 507-518.
- Reinhardt, D., Pescel, 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.
- 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. *Plant J.* **58**, 641-654.
- Sachs, T.** (1981). The control of the patterned differentiation of vascular tissues. *Adv. Bot. Res.* **9**, 151-262.
- Sachs, T.** (1991). Cell polarity and tissue patterning in plants. *Development Suppl.* **1**, 83-93.

- Scanlon, M.J.** (2003). The polar auxin transport inhibitor N-1-naphthylphthalamic acid disrupts leaf initiation, KNOX protein regulation, and formation of leaf margins in maize. *Plant Physiol.* **133**, 597–605.
- Scarpella, E., Barkoulas, M., and Tsiantis, M.** (2010). Control of leaf and vein development by auxin. *Cold Spring Harb. Perspect. Biol.* **2**, a001511.
- Scarpella, E., Marcos, D., Friml J., and Berleth, T.** (2006). Control of leaf patterning by polar auxin transport. *Genes Dev.* **20**, 1015-1027.
- Scheutz, M., Berleth, T., and Mattsson, J.** (2008). Multiple MONOPTEROUS-dependent pathways are involved in leaf initiation. *Plant Physiol.* **148**, 870-880.
- Schoof, H., Lenhard, M., Haecker, A., Mayer, K.F. X., Jürgens, G., and Laux, T.** (2000). The stem cell population of Arabidopsis shoot meristems is maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. *Cell* **100**, 635-644.
- Semiarti, E., Ueno, Y., Tsukaya, H., Iwakawa, H., Machida, C., and Machida, Y.** (2001). The *ASYMMETRIC LEAVES2* gene of *Arabidopsis thaliana* regulates lamina formation, establishment of venation and repression of meristem-related homeobox genes in the leaf. *Development* **128**, 1771–1783.
- Shuai, B., Reynaga-Peña, C.G., and Springer, P.S.** (2002). The *LATERAL ORGAN BOUNDARIES* gene defines a novel, plant-specific gene family. *Plant Physiol.* **129**, 1–15.

- 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.
- Sieburth, L.E. (1999).** Auxin is required for leaf vein pattern in *Arabidopsis*. *Plant Physiol.* **121**, 1179-1190.
- Sorefan, K., Girin, T., Liljegren, S.J., Ljung, K., Robles, P., Galván-Ampudia, C.S., Offringa, R., Friml, J., Yanofsky, M.F., and Østergaard, L. (2009).** A regulated auxin minimum is required for seed dispersal in *Arabidopsis*. *Nature* **459**, 583-587.
- Swarup, R., Friml, J., Marchant, A., Ljung, K., Sandberg, G., Palme, G., and Bennett, M.J. (2001).** Localisation of the auxin permease AUX1 in the *Arabidopsis* root apex reveals two novel functionally distinct hormone transport pathways. *Genes Dev.* **15**, 2648–2653.
- Swarup, R., Kargul, J., Marchant, A., Zadik, D., Rahman, A., Mills, R., Yemm, A., May, S., Williams, L., Millner, P., Tsurumi, S., Moore, I., Napier, R., Kerr, I.D., and Bennett, M.J. (2004).** Structure-function analysis of the presumptive *Arabidopsis* auxin permease AUX1. *Plant Cell* **16**, 3069-3083.
- Szymkowiak, E.J. (1996).** Is the extent of the proliferation of component cell lineages critical during organ morphogenesis? *Semin. Cell Dev. Biol.* **7**, 849-856.
- Takada, S., Hibara, K-I., Ishida, T., and Tasaka, M. (2001).** The CUP-SHAPED COTYLEDON1 gene of *Arabidopsis* regulates shoot apical meristem formation. *Development* **128**, 1127-1135.

- Tao, Y., Ferrer, J.-L., Ljung, K., Pojer, F., Hong, F., Long, J.A., Li, L., Moreno, J.E., Bowman, M.E., and Ivans, L.J.** (2008). Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. *Cell* **133**, 164–176.
- Teale, W.D., Paponov, I.A., and Palme, K.** (2006). Auxin in action: signaling, transport, and the control of plant growth and development. *Nat. Rev. Mol. Cell. Biol.* **7**, 847-859.
- Treml, B.S., Winderl, S., Radykewicz, R., Herz, M., Schweizer, G., Hutzler, P., Glawischnig, E., and Ruiz, R.A.** (2005). The gene *ENHANCER OF PINOID* controls cotyledon development in the *Arabidopsis* embryo. *Development* **132**, 4063-4074.
- 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. *Plant Cell* **9**, 1963-1971.
- Venglat, S.P., Dumonceaux, T., Rozwadowski, K., Parnell, L., Babic, V., Keller, W., Martienssen, R., Selvaraj, G., and Datla, R.** (2002). The homeobox gene *BREVIPEDICELLUS* is a key regulator of inflorescence architecture in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **99**, 4730-4735.
- Vernoux, T., and Besnard, F., and Traas, J.** (2010). Auxin at the shoot apical meristem. *Cold Spring Harb. Perspect. Biol.* **2**, a001487.
- Vernoux, T., Kronenberger, J., Grandjean, O., Laufs, P., and Traas, J.** (2000). *PIN-FORMED1* regulates cell fate at the periphery of the shoot apical meristem. *Development* **127**, 5157-5165.

- Vroemen, C. W., Mordhorst, A.P., Albrecht, C., Kwaaitaal, M.A.C.J., and de Vries, S.C.** (2003). The CUP-SHAPED COTYLEDON3 gene is required for boundary and shoot meristem formation in Arabidopsis. *Plant Cell* **15**, 1563-1577.
- Western, T.L., Young, D.S., Dean, G.H., Tan, W.L., Samuels, A.L., and Haughn, G.W.** (2004). MUCILAGE-MODIFIED4 encodes a putative pectin biosynthetic enzyme developmentally regulated by APETALA2, TRANSPARENT TESTA GLABRA1, and GLABRA2 in the Arabidopsis seed coat. *Plant Physiol.* **134**, 296-306.
- Wolpert, L.** (2007). Principles of Development. 3rd edition. Oxford University Press Inc. NY.
- Xu, L., Xu, Y., Dong, A., Sun, Y., Pi, L., 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, M., Hu, T., McKim, S., J. Murmu, Haughn, G.W., and Hepworth, S.R.** (2010). Arabidopsis BLADE-ON-PETIOLE1 and 2 promote floral meristem fate and determinacy in a previously undefined pathway targeting APETALA1 and AGAMOUS-LIKE24. *Plant J.* **63**, 974-989.
- Zgurski, J.M., Sharma, R., Bolokoski, D.A., and Schultz, E.A.** (2005). Asymmetric auxin response precedes asymmetric growth and differentiation of asymmetric leaf1 and asymmetric leaf2 Arabidopsis leaves. *Plant Cell* **17**, 77-91.
- Zhao, Y., Christensen, S.K., Fankhauser, C., Cashman, J.R., Cohen, J.D., Weigel, D., and Chory, J.** (2001). A role for flavin monooxygenase-like enzymes in auxin biosynthesis. *Science* **291**, 306-309.

APPENDIX A Semi-Quantitative Reverse-Transcriptase PCR (RT-PCR)

Semi-quantitative RT-PCR was used to monitor the relative transcript levels of *STM*, *KNAT2*, *KNAT6*, *YUC1*, and *YUC4* in the inflorescence apices of WT and mutants. Total RNA was extracted from the apices of ~12 plants per genotype. Apices were harvested from plants with 5-7 cm bolts and open flowers were removed. Tissues were flash-frozen in liquid nitrogen and stored at -80° C until processing. Frozen tissue was ground to a powder using a mortar and pestle cooled with liquid nitrogen. Powder was placed in a sterile 1.5 mL microcentrifuge tube and 1mL of TRIzol® reagent (Invitrogen, Carlsbad, CA) was added to the sample. Samples were vortexed and stored on ice until all samples likewise processed. Samples were then incubated at room temperature for 8 minutes prior to the addition of 200 µL of chloroform. Samples were mixed vigorously for 15 seconds and incubated at room temperature for 3 minutes before centrifugation for 10 minutes at 4°C in a refrigerated microcentrifuge at 15,142.4 g. Three 170 µL aliquots of the upper aqueous layer were transferred to a new tube containing 510 µL isopropanol to precipitate the RNA, which was harvested by microcentrifugation as above. The resulting RNA pellet was washed with 75% ethanol (v/v) prepared with DEPC-treated water. Excess ethanol was removed from the pellet, which was then dried in a sterile laminar flow hood for about 10 minutes. The pellet was resuspended in 50 µl of DEPC-treated water and diluted 10-fold. 1 µL of diluted RNA was resolved on a 0.8% agarose gel to assess integrity. The concentration of RNA was determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Ottawa, Ontario). One µg of total RNA in a volume of 10 µL was then used as a template for cDNA synthesis. The product was denatured by heating at 70°C for 5 minutes and then quick-chilled on ice. Each 10 µL reaction contained 1.2 µL 50µM Oligo-dT , 4

μL 5X first strand buffer, 2 μL 0.1M DTT, 1 μL 10 mM dNTPs, 0.5 μL RNase inhibitor (40U per μl), and 0.25 μL Superscript III RT (Invitrogen, Carlsbad, CA) and 1.05 μL dH₂O. The reaction was performed at 50°C for 60 minutes followed by incubation at 70°C for 15 minutes to heat-inactivate the enzyme. The cDNA was diluted 10-fold with sterile water and 2 μl used as template for the amplification of *STM*, *KNAT2*, *KNAT6*, *YUC1* and *YUC4* transcripts by conventional PCR using the gene-specific primers listed in Table A.1. The primers were designed to anneal to exon junctions to prevent the amplification of cDNA products derived from contaminating genomic DNA. *GAPC* (cytosolic glyceraldehyde-3-phosphate dehydrogenase) served as a control transcript (Western et al., 2004). Forty cycles of amplification were used for transcript detection. For *GAPC*, 26 cycles of amplification were used. PCR amplification was performed a minimum of three times for each set of primers. The PCR products were resolved on a 0.8% (w/v) agarose gel by electrophoresis with ethidium bromide dye. The Alphamager 2200 gel-imager system (Alpha Innotech, San Leandro, CA) was used to photograph the gel and to quantify the bands.

Table A.1 Primers for RT-PCR

Primer name	Sequence 5' - 3'	Tm °C	PCR Product
TW GAPC P1	TCAGACTCGAGAAAGCTGCTA	57	300 bp
TW GAPC P2	GATCAAGTCGACCACACGG		
KNAT2-F	ACCACCGGAGACAATCAAAG	57	540 bp
KNAT2-R	TCCGCTGCTATGTCATCATC		
KNAT6--pU161	ATGTACAATTTCCATTTCGGCCGGTG	57	552 bp
KNAT6--pU162	TCATTCCTCGGTAAAGAAGAATGATCCACTG		
STM-RT F1	GGTTCCAACAGCACTTCTTGTCCAA	57	682 bp
STM-RT R1	TCTCTCCATAACCGGAGAAAGAGG		
YUC1-RT-F1	TTTCTGGAGAGTAAAGACTCATGAT	57	535 bp
YUC1-RT-R1	TTACACCTTCCATTATCTGAATCAT		
YUC4-RT-F1	ACACATCCACGTGGCTGGTGGTTGCC	57	495 bp
YUC4-RT-R1	ACGGCTTGCGTCACTTTAATTTGTC

Figure A.1 RT-PCR analysis of Class I *KNOX* genes (*STM*, *KNAT2*, *KNAT6*) and auxin biosynthetic genes (*YUC1* and *YUC4*) in the inflorescence apices of WT and mutants. Representative data are shown. Gene transcripts, 40 cycles. *GAPC* control transcript, 26 cycles.

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