ROLE OF *BLADE-ON-PETIOLE1* AND *2* IN PATTERNING THE *ARABIDOPSIS THALIANA* LEAF AND INFLORESCENCE

by

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ABSTRACT

The architectural design of plants is selected by nature for optimal survival and reproductive fitness. A major goal of plant developmental biologists is to identify genes that control plant shape and form and to understand how these genes interact to create structural diversity. BLADE-ON-PETIOLE1 (BOP1) and BOP2 are an ancient and conserved set of plant-specific transcriptional co-regulators that play a key role in determining the architecture of leaves, inflorescences, and fruits in the model plant species, *Arabidopsis thaliana*. Phenotypes in *bop1 bop2* loss-of-function mutants include leafy petioles, asymmetric flowers subtended by a bract, lack of floral-organ abscission, and the partial conversion of flowers to shoots. Conversely, *BOP1/2* gain-of-function mutants have severe defects in internode elongation resulting in short, compacted inflorescences. However, the mechanisms behind these architectural variations have yet to be determined. In my thesis, I show that *BOP1/2* are expressed in initiating lateral organ primordia during both the vegetative and reproductive phases of *Arabidopsis* development. As these primordia separate from the meristem, *BOP* expression stably shifts to the proximal boundary region that separates these primordia from the meristem or from adjacent structures. In floral meristems, I show that BOP1 and BOP2 function redundantly with LEAFY, a master regulator of floral-meristem identity, to promote the expression of *APETALA1*, a key marker of commitment to floral fate. All three activities then converge in the floral meristem during stages 2-3 to down-regulate the expression of inflorescence identity genes including *AGAMOUS-LIKE24* to promote the formation of a determinate floral shoot. In leaves, I show that BOP1 and BOP2 in the petiole function redundantly with leaf patterning factors ASYMMETRIC LEAVES1 (AS1) and AS2 to

maintain the stable repression of Class I *KNOTTED1-LIKE HOMEOBOX* (*KNOX1*) meristematic genes to facilitate simple leaf formation. In inflorescences, I show that the expression of *BOP* genes and the *KNOX1* family member *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA6* (*KNAT6*) are confined to pedicel axils (a boundary that separates the pedicel from the stem) by the combined activities of the *KNOX1* family member *BREVIPEDILLUS* (*BP*) and its interacting partner *PENNYWISE* (*PNY*). My data show that *BOP2* and *KNAT6* expression domains are differentially enlarged in *bp* and *pny* mutants, corresponding to the distinctive patterns of short internodes, clustered or downward-oriented siliques, and defects in epidermal cell differentiation characteristic of these mutants. These data indicate that BP-PNY and BOP1/2-KNAT6 have reciprocal functions in the inflorescence. Further evidence shows that BOP1/2 are positive regulators of *KNAT6* expression and that this regulation may be direct. Collectively, my work shows that BOP1/2 are key regulators of determinacy in lateral shoot and organ primordia and illustrate that changes in determinacy lead to dramatic architectural variation in these structures. My data also indicate that BOP1/2-KNAT6 (boundary factors) and BP-PNY (meristematic factors) have antagonistic functions in the inflorescence stem: the combined activities of BP-PNY restrict BOP1/2-KNAT6 to pedicel axils to facilitate the production of an inflorescence in which lateral branches and flowers are equally spaced along elongated internodes. Future studies will determine if differences in BOP1/2 activity or expression pattern form a common module for creating architectural diversity in plant species.

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Preface

The purpose of this thesis is to dissect the role of BLADE-ON-PETIOLE1 and 2 in patterning the *Arabidopsis thaliana* leaf and inflorescence at molecular level. I have carried out most of the experiments to elucidate how BOP1/2 interact with other growth regulators to sculpt the *Arabidopsis thaliana* leaf and inflorescence. However, some parts were carried out in collaboration with other graduate students or technicians; some Figures in Chapter1 are adapted from published sources with permissions from the publishers for better understanding of the knowledge. Detailed information concerning these contributions to my thesis are discussed below.

Papers published or submitted at the time of thesis submission

1. **Xu, M.**, Hu, T., McKim, S., Murmu, J., 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 Journal* **63**, 974-989. This paper was published in July, 2010 and was reprinted in my thesis as Chapter 2 with permission of the publisher (John Wiley and Sons).

2. Khan, M., **Xu, M.**, Hu, T., Tabb, Murmu, J., McKim, S.M., Story, K., Mercado, J., and Hepworth, S.R. Antagonistic interaction of BLADE-ON-PETIOLE1 and 2 with BREVIPEDICELLUS and PENNYWISE regulates Arabidopsis inflorescence architecture. This manuscript was submitted to the Plant Cell on October 30th, 2010 and the submission number is: PLANTCELL/2010/080747. I am a co-first author of this

manuscript and we are revising it according to the reviewers' comments. In my thesis I only presented the work that I did or work done by technicians or students that I co-supervised. The manuscript is about the role of BOP1/2 in *Arabidopsis* leaf and inflorescence patterning. However, I divide it into two chapters in my thesis: Chapter 3 is about the role of BOP1/2 in leaf patterning and Chapter 4 is about the role of BOP1/2 in inflorescence patterning. Detailed information about the contributions to this thesis is presented below.

Statement of contributions

I have contributed to the research described in this thesis as followers:

1. In Chapter 2, Dr. Hepworth and I formulated the hypothesis and I carried out all of the experiments described in this Chapter with the following exceptions: Sarah McKim provided the scanning electron micrographs and BiFC data, Tieqiang Hu provided the qRT-PCR data for *AP1*, *AGL24*, *SOC1*, and *FUL* transcripts, and Jhadeswar Murmu made the pBOP1::BOP1-GFP plants used for ChIP assays. Dr. Hepworth and I analyzed the data and prepared the manuscript.

2. In Chapter3, Dr. Hepworth and I formulated the hypothesis. I co-supervised Tieqiang Hu (Technician) and Jethro Mercado (undergraduate) with Dr. Hepworth for the following experiments: Jethro Mercado helped with the isolation of *bop1 bop2 as1* and *bop1 bop2 as2* triple mutants and I analyzed the plants and collect the data; Tieqiang Hu performed the qPCR experiment on *STM*, *BP*, *KNAT2*, *KNAT6* and *PNY* and Dr. Hepworth analyzed the data; Madiha Khan made the *bop1 bop2*

KNAT2::GUS and *bop1 bop2 KNAT6::GUS* lines and I used them for GUS staining and sectioning.

3. In Chapter4, Dr. Hepworth and I formulated the hypothesis and I carried out all of the experiments described in this Chapter except that Madiha Khan isolated the *BOP2::GUS bp-2* and *BOP2::GUS pny* lines and I used them for whole mount GUS staining and sectioning. I initiated the project for this chapter with the crosses *bop1 bop2* x *bp-1*; and *bop1 bop2* x *pny*. I co-supervised Madiha Khan with Dr. Hepworth to isolate *bop1 bop2 bp* and *bop1 bop2 pny* triple mutants. She found that the defects in *bp* and *pny* were rescued by *bop1 bop2*. We collaborated on this project and I did the qPCR of *KNAT6* and *BP* in various tissues and I did the expression analysis of *BOP*, *KNAT6*, *BP* and *CUC3* in different genotypes.

4. With the help of Dr. Hepworth, I prepared the manuscript of this thesis.

The acknowledgement of the use of Copyright material

As the first author of Xu *et al.* Plant Journal (2010), I reprinted it and enclosed it as Chapter 2 in this thesis with the permission from the publisher John Wiley and Sons. I also adopted some figures from published papers in Chapter 1 with permission from the publishers. I redrew Figure 1.3 from Barton (2010) with permission from the publisher Elsevier; I reprinted Figure 1.4 from Rast and Simon (2008) with permission from the publisher Elsevier; I reprinted Figure 1.5 from Byrne (2006) with permission from the publisher PLoS Genetics.

List of Abbreviations

ABRC	Arabidopsis Biological Resource Center
AG	AGAMOUS
AGL24	AGAMOUS-LIKE24
ANK	Ankyrin
AP1	APETALA1
AP2	APETALA2
AP3	APETALA3
AS1	ASYMMETRIC LEAVES1
AXR1	AUXIN RESISTANT1
ATH1	ARABIDOPSIS THALIANA HOMEOBOX1
BEL1	BELL1
BiFC	Biomolecular Fluorescence Complementation
BOP1	BLADE-ON-PETIOLE1
BP	BREVIPEDICELLUS
BTB/POZ	Broad-Complex, Tramtrack, and Bric-a-Brac/POX virus and Zinc finger
bZIP	basic leucine zipper
CAL	CAULIFLOWER
CHX	Cycloheximide
ChIP	Chromatin Immunoprecipitation
CK	Cytokinin
CL	Continuous Light

CLV	CLAVATA
CO	CONSTANS
Col	Columbia
CUC	CUP-SHAPED COTYLEDONS
Cys	Cysteine
CZ	Central Zone
EREBP	Ethylene response element binding protein
EYFP	Enhanced Yellow Florescent Protein
FD	FLOWERING LOCUS D
FIL	FILAMENTOUS FLOWER
FLC	FLOWERING LOCUS C
FMI	Floral Meristem Identity
FOI	Floral Organ Identity
FT	FLOWERING LOCUS T
FUL	FRUITFULL
GFP	Green Florescent Protein
GA	Gibberellic Acid
GAPC	Glyceraldehyde-3-phosphate dehydrogenase
GUS	β-Glucuronidase
h	hours
HD-ZIP	homeodomain leucine-zipper
I	Initiation
IM	Inflorescence Meristem

PM	Primary Morphogenesis
SM	Secondary Morphogenesis
JAG	JAGGED
KAN	KANADI
KNAT	KNOTTED-LIKE FROM ARABIDOPSIS THALIANA
KNOX	KNOTTED-LIKE HOMEOBOX
KNOXI	Class I KNOTTED-LIKE HOMEOBOX
Ler	Landsberg *erecta*
LFY	LEAFY
LOB	LATERAL ORGAN BOUNDARIES
LBD	LATERAL ORGAN BOUNDARIES DOMAIN
LOF	LATERAL ORGAN FUSION
LD	Long Day
LMI	LATE MERISTEM IDENTITY1
MADS-box	MCM1-AGAMOUS-DEFICIENS-SERUM RESPONSE FACTOR conserved sequence motif
NPA	N-napthylphthalamic acid
NPR1	NON-EXPRESSOR OF PATHOGENESIS RELATED GENES1
OC	Organizing Centre
PAN	PERIANTHIA
PCR	polymerase chain reaction
PHB	PHABULOSA
PHV	PHAVOLUTA

PI	PISTILATTA
PIN1	PIN-FORMED1
PNF	POUNDFOOLISH
PNY	PENNYWISE
PR1	PATHOGENESIS-RELATED1
PTL	PETAL LOSS
PZ	Peripheral Zone
qPCR	Quantitative RT-PCR
RT-PCR	Reverse transcriptase polymerase chain reaction
RZ	Rib Zone
SA	Salicylic Acid
SAM	Shoot Apical Meristem
SAR	Systemic Acquired Resistance
SD	Short Day
SEM	Scanning Electron Microscopy
s.e.m.	standard error of mean
SEP	SEPALLATA
SOC1	SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1
STM	SHOOT MERISTEMLESS
STP	STAMINA PISTILLOIDA
SPL	SQUAMOSA PROMOTER BINDING PROTEIN-LIKE
SVP	SHORT VEGETATIVE PHASE
TGA	TGACG-BOX BINDING

TALE	Three-Amino Acid-Loop-Extension
UFO	UNUSUAL FLORAL ORGANS
UNI	UNIFOLIATA
UTR	Untranslated region
YAB	YABBY
Y2H	Yeast-Two-Hybrid
WT	Wild-Type
WUS	WUSCHEL

Genetic Nomenclature in *Arabidopsis thaliana*

BOP1	wild-type gene
BOP1	gene product or activity
bop1	mutant gene allele (loss-of-function)
bop1-6D	mutant gene allele (gain-of-function)

Chapter 1

General introduction

1.1 Overview of thesis

Plants of a species show a characteristic patterning for leaves, inflorescences, and flowers that is selected for in nature to optimize reproductive success. Forward and reverse genetics approaches in the model plant species, *Arabidopsis thaliana*, have identified many genes that control plant architecture. However, it remains to be seen how many of these genes function at the molecular level and how their activities interact to generate structural diversity.

The aerial parts of a plant are generated through the activity of the shoot apical meristem (SAM), a self-renewing population of stem cells located at the growing shoot tip. During *Arabidopsis* vegetative development, the SAM generates leaves without internode elongation to produce a compact rosette. Upon floral induction, internode elongation is allowed and the SAM (now known as the inflorescence meristem, IM) transitions to produce lateral branches and then flowers (reviewed in Bowman and Eshed, 2000; Barton, 2010). However, the patterning of these organs and shoots varies between plant species.

The main objective of this thesis is to investigate the role of two conserved plant-specific transcriptional co-regulators, BLADE-ON-PETIOLE1 (BOP1) and BOP2, which control the architecture of leaves, inflorescences, and flowers in plants. *BOP1* and *BOP2* (referred to as *BOP1/2*) are expressed in emerging leaf and floral primordia. As these primordia partition from the SAM, *BOP1/2* expression shifts to the boundary at the base of the organ where expression is maintained thereafter. This pattern of expression suggests a role for these genes in organ initiation and boundary patterning. Loss-of-function *bop1 bop2* mutations cause a range of patterning defects including leafy petioles, asymmetric flowers subtended by a bract, and the partial conversion of flowers to shoots. Conversely, gain-of-function *BOP1/2* plants are

restricted in stem growth and have compact internodes. While it is clear that these genes control plant architecture, how they function at the molecular level is largely unknown.

To address this, I used a combination of molecular, genetic and biochemical approaches to elucidate how BOP1/2 interact with developmental-specific regulators to influence (a) floral meristem identity, (b) leaf patterning, and (c) internode elongation. As a background to understand the rationale and significance of these studies, I will first provide an overview of *Arabidopsis* development with an emphasis on plant architecture. I will then explain how BOP1/2 interact with other regional and developmental-specific factors to promote floral-meristem identity, simple leaf shape, and elongation of internodes in inflorescences.

1.2 Study species

In the late 1970's, *Arabidopsis thaliana* was selected as a model species to study plant development for four main reasons:

a) It is a diploid organism with a small compact genome compared to rice or wheat.

b) It is easy to obtain *Arabidopsis* mutants and to make transgenic plants.

c) It is easy to grow in a lab because its size is small (30-40 cm), its life-time is short (6-8 weeks), and it has high seed set.

d) It can be self- or cross-fertilized at will (Meyerowitz, 1987).

Since then, the *Arabidopsis* genome has been completely sequenced and key resources such as a library of T-DNA insertion mutants are readily available from the Arabidopsis Biological Resource Center (ABRC). Although *Arabidopsis* has no economic value, it is in the same family as cabbage, broccoli, and horseradish. Information discovered about this plant can be applied to modify plants or accelerate breeding for human benefit (Jack, 2004).

1.3 The life cycle of *Arabidopsis thaliana*

The life cycle of the flowering plant *Arabidopsis* can be broadly divided into three developmental phases: an embryogenesis phase, a vegetative phase and a reproductive phase. After germination, *Arabidopsis* undergoes vegetative development and a compact rosette is formed from the activities of the meristem established from embryogenesis (Fletcher, 2002; Figure 1a). Upon perceiving endogenous and environmental cues, the plants stops making leaves and transits to generate flowers, initiating the reproductive phase of development. Reproductive development itself has two distinct stages (Ratcliffe *et al.*, 1998): the first-inflorescence (FI) stage and the second-inflorescence (SI) stage. During the FI stage, several cauline leaves are produced, each in association with a secondary inflorescence (Ratcliffe *et al.*, 1998; Figure 1b). During the SI stage, leaf development is fully repressed and flowers are produced without leaves (Figure 1b). Some biologists consider the FI stage as part of the vegetative phase, since cauline leaves are still produced (Liljegren *et al.*, 1999). In this study, however, we followed the convention of Ratcliffe *et al.* (1998) who defined the period of cauline leaf growth as part of the reproductive phase because mutations in some genes specifically affect the transition from indeterminate secondary inflorescences to determinate flowers.

As explained above, the above-ground parts of a plant are generated post-embryonically by the apical meristem. Each set of structures produced by this meristem can be viewed as a standard module comprised of three parts: a leaf, an axillary meristem, and an internode. How the parts of each module are elaborated however, depends on the plant species and the phase of development (Sussex, 1989). For example, during the vegetative phase of *Arabidopsis* development, a leaf with the potential to form an axillary meristem is developed but internode elongation is repressed. During the first-inflorescence stage of reproductive development, the

module is fully developed and consists of a cauline leaf, an axillary meristem that develops into a secondary inflorescence, and an elongated internode. During the second-inflorescence stage of reproductive development, leaf development is fully suppressed, while the axillary meristem develops as a flower, and the internode is elongated. The position of each module and its subsequent development is strongly influenced by the environment and by genetic cues, allowing the overall architecture of a plant to unfold over developmental time (Sussex, 1989).

1.4 Establishment and maintenance of the shoot apical meristem

Unlike animals, where the body plan is completed during embryogenesis, organs and tissues are continuously produced over the life cycle of a plant. New organs or tissues are initiated from groups of stem cells located at the shoot tip or root tip of a plant (Fletcher, 2002). As explained earlier, the above-ground parts of the plant are built from a series of repeating modules that are produced one-at-a-time by the SAM. This strategy works well for plants because they are immobile and must continually respond to the changing environment to thrive. Their modular make-up gives them the flexibility to adapt by changing their patterns of growth and development (Fletcher, 2002).

1.4.1 Organization of the shoot apical meristem

Two apical meristems, the shoot apical meristem (SAM) and the root apical meristem (RAM), are established during embryogenesis and determine the architecture of aerial and underground parts of a plant, respectively. The RAM will not be considered further. The SAM is organized into three distinct zones on the basis of morphological differences and cell division rates: the central zone (CZ), the peripheral zone (PZ), and the rib zone (RZ) (Figure 1.2). The CZ cells are large and highly vacuolated, and they lie at the very centre of the SAM. These cells divide

infrequently relative to other cells in the SAM. Surrounding the CZ is the PZ. The PZ cells are smaller and divide more frequently than the CZ cells. Importantly, the PZ cells give rise to lateral organs that build up the aerial body of a plant; thus this zone represents a transitional region of the SAM. Subtending the CZ is the RZ and its cells are large and vacuolated. Cell division in this zone is suppressed during vegetative development and activated during reproductive development giving rise to internodes. Plants can maintain a reservoir of stem cells and continuously produce lateral organs on the flanks of the meristem by coordinating cell fate at different zones: the progeny of CZ cells either stay in the centre of the SAM and maintain their stem cell activity, or they enter the PZ or RZ and provide founder cells for the formation of lateral organs or internodes (reviewed in Liljegren and Yanofsky, 1996; Bowman and Eshed, 2000; Fletcher, 2002; Barton, 2010).

The SAM can also be subdivided into distinct L1, L2 and L3 cell layers (Figure 1.2). The outmost cell layer in *Arabidopsis* is termed the L1 layer. The L2 cell layer is the sub-epidermal layer of cells underlying the L1 layer. Underneath the L2 layer is the L3 layer, which is comprised of multiple cell layers. The L1 and L2 layers constitute the tunica of the SAM, whereas the L3 layers constitute the corpus of the SAM. Cells from the L1, L2, and L3 layers encompass the CZ and PZ, while the RZ consists of cells from the L3 layer (Fletcher, 2002; William and Fletcher, 2005; Barton, 2010).

1.4.2 Establishment and maintenance of the shoot apical meristem

Because of the significance and complexity of the SAM in plant development, biologists are very interested in knowing how the SAM is established and maintained throughout the plant life cycle. Two transcriptional regulators, WUSCHEL (WUS) and SHOOT MERISTEMLESS (STM) from

the Class 1 KNOTTED1-LIKE HOMEOBOX (KNOX1) family are key factors in the establishment and maintenance of the SAM.

1.4.2.1 WUSCHEL functions through CLAVATA signaling in the establishment and maintenance of the SAM

Molecular and genetic data support the idea that WUS is a master regulator for maintenance of the SAM. *WUS* encodes a homeodomain (a highly conserved protein motif that binds to DNA and regulates gene expression subsequently) transcription factor, and loss of *WUS* function results in failure to maintain the CZ, indicating a central role for WUS in maintaining the reservoir of stem cells (Mayer *et al.*, 1998). *WUS* is not expressed in CZ, but rather in a small group of cells underneath termed the Organizing Centre (OC) (Laux *et al.*, 1996).

Maintenance of the stem cell population relies on a *WUS*-mediated inductive signal, which must be targeted to the overlying apical cells. Members of the CLAVATA (CLV1, CLV2, and CLV3) family of signaling proteins interact to maintain the size and position of the stem cell population. *CLV1* and *CLV2* encode receptor-like proteins that are expressed primarily in the L3 corpus layers. The *CLV3* gene encodes a small, diffusible secreted ligand that expresses in the L1 and L2 tunica cells of the SAM, corresponding to the CZ (Clark *et al.,* 1993; 1996; Kayes *et al.*, 1998). CLV3 interacts with the CLV1-CLV2 receptor complex in the underlying L3 layer cells. The CLV proteins negatively regulate *WUS* expression, thereby limiting the size and position of the OC (Williams and Fletcher, 2005).

There is antagonistic activity between WUS and CLV regulators in the establishment and maintenance of stem cells (Fletcher, 2002; William and Fletcher, 2005). Loss-of-function in *CLV1*, *CLV2,* or *CLV3* genes causes enlarged shoot and floral meristems throughout development, opposite to loss-of-function in *WUS*. In addition, *WUS* expression is not restricted

to the OC and expands laterally in *clv3* mutants, while there is no *WUS* expression in *CaMV35S::CLV3* transgenic plants (Fletcher *et al.*, 1999; Brand *et al.*, 2000; Schoof *et al.*, 2000), indicating that CLV3 represses *WUS*.

Taken together, a WUS-CLV feed-back loop is present in the SAM (Figure 1.3): WUS activity in the OC specifies the overlying cells as stem cells thereby inducing *CLV3* expression. Consequently, activation of the CLV signaling complex feeds back to limit the domain of *WUS* expression. Any change in *CLV3* or *WUS* expression levels cause subsequent adjustments in other gene expression levels and in SAM activity.

1.4.2.2 Class I KNOX proteins function with BEL1-like homeodomain proteins in the establishment and maintenance of the SAM

Class 1 *KNOX* genes (*KNOX1* genes) are another class of positive regulators of SAM formation and maintenance. *KNOX1* genes encode the homeodomain-containing proteins SHOOT MERISTEMLESS (STM), BREVIPEDICELLUS (BP) (also known as KNOTTED-LIKE FROM ARABIDOPSIS THALIANA1, KNAT1), KNAT2 and KNAT6. *STM* is expressed throughout the SAM and has a central role in meristem maintenance since strong alleles of *stm* are unable to establish and maintain a functional SAM (Long *et al.*, 1996). *BP* is expressed in the PZ of the SAM and at the boundaries of leaf primordia (Lincoln *et al.*, 1994; Ori *et al.*, 2000) while *KNAT6* is expressed in the embryonic SAM and in the adaxial part of the boundary that separates leaf primordia from the SAM (Belles-Boix *et al.*, 2006). Neither *bp* nor *knat6* mutants display any SAM defects, but their mutations enhance the phenotype of plants with weak *stm* alleles to give a strong *stm* mutant phenotype (Byrne *et al.*, 2002; Belles-Boix *et al.*, 2006). These findings reveal that BP and KNAT6 have roles in maintaining the SAM in addition to STM. A third gene, *KNAT2*, is expressed at the base of the SAM but its role in the SAM is

unclear since its mutation does not enhance the phenotype of weak *stm* mutants (Byrne *et al.,* 2002; Belles-Boix *et al.,* 2006).

Gain-of-function studies further support the idea that *KNOX1* genes promote meristem formation. Misexpression of any KNOX1 family member induces ectopic meristem formation (no organogenesis) on the adaxial surface or margins of leaves (Lincoln *et al.,* 1994; Chuck *et al.,* 1996, Pautot *et al.,* 2001; Gallois *et al.,* 2002; Dean *et al.,* 2004). However, combined misexpression *STM* and *WUS* induces ectopic organogenesis on the hypocotyl and cotyledons, demonstrating that the KNOX1 and WUS-CLV systems have complementary roles in meristem formation (Gallois *et al.,* 2002; Barton, 2010).

Biochemical studies show that KNOX1 proteins interact with members of the related BELL1-like class of homeodomain proteins to perform their function in the SAM (Belloui *et al.,* 2001; Byrne *et al.*, 2003; Smith and Hake 2003; Kanrar *et al.*, 2006; Rutgens *et al.* 2009). Three BELL1-like members are expressed in the SAM: PENNYWISE (PNY), POUNDFOOLISH (PNF) and ARABIDOPSIS THALIANA HOMEOBOX1 (ATH1) (Kanrar *et al.*, 2006; Rutjens *et al.*, 2009). Loss-of function *pny,* but not *pnf,* enhances the phenotype of plants with a weak *stm* allele and when all three loci are mutated, the meristem is not maintained (Byrne *et al.*, 2003; Smith and Hake 2003; Kanrar *et al.*, 2006; Rutgens *et al.* 2009). These data indicate that SAM establishment and maintenance requires the activity of KNOX1-BELL heterodimers.

A few of the KNOX1 transcriptional targets have been uncovered recently, revealing how KNOX1 proteins function for the establishment and maintenance of the SAM. KNOX1 proteins directly activate cytokinin (CK) biosynthesis genes or gibberellin (GA) deactivation genes to modulate the abundance of the plant hormones CK and GA. Activation of STM can rapidly increase the transcription level of *ISOPENTENYL TRANSFEREASE7* (*IPT7*), a CK biosynthesis

gene (Yanai *et al.*, 2005). As a result, CK accumulation levels are elevated (Yanai *et al.*, 2005). Similarly, GA deactivation genes *AtGA2ox2* and *AtGA2ox4* are activated in response to STM induction, resulting in decreased GA accumulation (Jasinski *et al.*, 2005). Therefore, a high CK to GA ratio in the SAM is yielded by activation of CK biosynthesis and GA deactivation genes. Elevating CK biosynthesis can partially rescue *stm* mutant phenotypes, and reducing CK levels or elevating GA levels can enhance *stm* mutant phenotypes, again suggesting that STM exerts its effect by regulating CK and GA abundance (Jasinski *et al.*, 2005; Yanai *et al.*, 2005). Interestingly, plants overexpressing CK display high levels of *STM* and *BP* transcripts and phenocopy *35S::STM* or *35S::BP* plants (Chuck *et al.,* 1996; Rupp *et al.*, 1999; Gallois *et al.* 2002) suggesting that CK acts in a feed-forward loop targeting *STM* and *BP* for establishment and maintenance of the SAM.

In summary, the SAM is central to plant development because it gives rise to all aerial parts of the plant. WUS and KNOX1 proteins are the major regulators of SAM establishment and maintenance and they work co-operatively to perform this task. WUS function is through a WUS-CLV feed-back loop, whereas KNOX1 proteins form heterodimers with BELL homeodomain proteins and function in part by elevating CK levels and lowering GA levels.

1.5 Boundary establishment

When lateral organ primordia are partitioned from the SAM, a boundary develops in the form a narrow groove that separates the primordia from the meristem (Figure 1.4, Aida and Tasaka 2006ab; Rast and Simon, 2008). Boundaries are first established between the embryonic SAM and the cotyledons, and the pattern is repeated for all subsequent lateral organs as they emerge from the SAM. Cells in the boundary zone divide at a slower rate than cells in adjacent domains indicating that growth is restricted in this zone (Breuil-Broyer *et al.*, 2004; Aida and Tasaka,

2006ab). Members of the *CUP-SHAPED COTYLEDON* (*CUC*), *LATERAL ORGAN BOUNDARIES DOMAIN* (*LBD*) (defined by an expression domain at lateral organ boundaries), *LATERAL ORGAN FUSION* (*LOF*), *BOP*, and *KNOX1* gene families (e.g. *KNAT6*) all display boundary-localized expression patterns and their combined activities are required for: a) organ separation, b) meristem maintenance, and c) boundary cell specialization (reviewed in Aida and Tasaka, 2006ab; Rast and Simon, 2008). Boundaries often contain specialized cell types that are important later in plant development. For example, cells in the axils of leaves have the potential to form axillary meristems (McSteen and Leyser, 2005) whereas cells at the base of floral organs are specialized for abscission (Lewis *et al.*, 2006; McKim *et al.*, 2008). Boundary genes are therefore important regulators of plant architecture.

The NAC (for NAM, ATAF1, and CUC2) domain transcription factors encoded by *CUC1*, *CUC2*, and *CUC3* restrict growth and are viewed as the central regulators of boundary-cell identity. Genetic data show that the *CUC* genes are redundantly required for organ separation and meristem initiation (Aida *et al.,* 1997; Vroemen *et al.,* 2003; Aida and Tasaka, 2006ab; Hibara *et al.,* 2006). *CUC1* and *CUC2* are activated early in embryogenesis and promote meristem initiation by activating *STM.* Once activated, *STM* feeds back to restrict *CUC2* to the boundary, thus separating meristem and organ domains in the embryo (Aida *et al.*, 1997; 1999; Takada *et al.,* 2001). Accumulating auxin in the developing cotyledon tips additionally restricts *CUC2* expression to the boundary (Aida *et al.,* 2002; Furutani *et al.,* 2004). Inactivation of *CUC* genes in *Arabidopsis* results in fused cotyledons (cup-shaped cotyledons) and a blunted stem apex lacking a SAM (Aida *et al.*, 1999; Hibara *et al.*, 2006). *CUC* genes are expressed earlier than *KNAT6* and correct *KNAT6* expression requires CUC activity (Belles-Boix *et al.*, 2006) suggesting that *CUC* genes play a role in establishing the unique transcriptional profile of the

boundary, but direct transcriptional targets are still unknown. *KNAT6* contributes to boundary maintenance, as evidenced by severe defects in cotyledon separation in the *stm-2 knat6* double mutant (Belles-Boix *et al.,* 2006).

During reproductive development, the *CUC* genes promote the formation of axillary meristems since some *cuc* mutants sometimes lack secondary inflorescences in the axil of cauline leaves (Hibara *et al.,* 2006). *LOF1* and *LOF2* encode MYB transcription factors (for Myeloblast, an oncogene first identified in avian myloblastosis virus) that are additionally required for separation of the cauline leaf and secondary inflorescence: these structures are fused together in the double mutant (Lee *et al.,* 2009). Combined loss-of-function *lof* and *cuc* mutants display enhanced organ fusion defects relative to single mutants and are more often unable to form secondary shoots in the axils of cauline leaves (Lee *et al.*, 2009). Organ fusions are also frequent in the *bop1 bop2* mutant during reproductive development (data not shown) and the cellular structure of pedicel-stem junction is disrupted (McKim *et al.,* 2008) indicating that BOP1/2 contribute to organ separation and boundary patterning in reproductive development. However, defects in organ separation and meristem maintenance are not seen in *bop1 bop2* mutant seedlings suggesting that BOP1/2 activity is more important during reproductive development.

A common theme among genes expressed in the boundary is their modulation of *KNOX1* expression. As explained above, *CUC1* and *CUC2* genes promote *STM* expression in the apex of globular stage embryos to establish the embryonic meristem (Aida *et al.,* 1997; 1999; Tasaka *et al.,* 2001). In addition, misexpression of *CUC1* and the LBD family member *JAGGED LATERAL ORGANS* (*JLO*) direct the ectopic expression of *BP* when misexpressed in leaves (Takada *et al.,* 2001; Borghi *et al.,* 2007) and CUC and LOF activities are required to reactivate

STM expression for the formation of axillary meristems in cauline leaf axils (Lee *et al.,* 2009). These data define *CUC*, *LOF*, and *JLO* as positive regulators of meristem formation. Conversely, BOP1/2 and ASYMMETRIC LEAVES2 (AS2; another LBD family member) negatively regulate *KNOX1* expression in leaves (Ori *et al.,* 2000; Ha *et al.,* 2003; 2007; 2010; Jun *et al.* 2010;) defining these boundary genes as negative regulators of meristem activity. These data suggest that interplay between boundary genes maintains an appropriate balance between meristem activity and cell differentiation. This balance is important as meristem activity and organ differentiation are two competing processes that must be compartmentalized in the shoot apex for the continuous production of modules by the SAM.

In summary, boundary cells have a unique transcriptional profile and provide a physical barrier between meristematic and non-meristematic cells in the plant. The combined activities of CUC, LOF, LBD, BOP1/2, and KNAT6 proteins are required to restrict growth, regulate meristematic potential, achieve organ separation, and promote the differentiation of boundary cells for functions such as axillary meristem formation or abscission. It remains unclear how genes in the boundary coordinate the competing processes of meristem maintenance and organ production.

1.6 Leaf initiation and architecture

Leaf development can be divided into the three stages termed leaf initiation (I), primary morphogenesis (PM), and secondary morphogenesis (SM). During stage I, organ phyllotaxy and growth rate are established, and during the PM and SM stages, the architecture of the leaves is established (Hay and Tsiantis, 2010). Leaf primordia may also be described according to morphological stages in their development, defined as P0, P1, P2, P3, and P4 (Long and Barton, 2000). These stages are defined as follows: P0, when an *STM*-negative region first appears on

the flanks of the SAM; P1, when a lateral bulge is seen on the flanks of the SAM; P2, when a visible cleft is seen between the primordia and the SAM; P3, when primordia extend farther apically and a deep cleft separates the primordia and the SAM; and P4, when the primordia has extended and curves over to about the midpoint of the SAM. A summary of the regulators for leaf initiation and leaf patterning (leaf shape and form) is provided next.

1.6.1 Leaf initiation

In *Arabidopsis*, leaf primordia are initiated on the flanks of the SAM in a spiral phyllotactic pattern (Reinhardt *et al.* 2000; Byrne *et al.*, 2003). Organs are initiated at sites of high auxin response in the PZ called auxin maxima (Reinhardt *et al.*, 2000; 2003; Benkova *et al.*, 2003). The orientation of PIN-FORMED (PIN) auxin efflux facilitators, particularly PIN1, play a crucial role in draining auxin from the SAM epidermis and transporting it towards maxima that predict the site of new organ initiation (Gälweiler *et al.,* 1998; Benková *et al.,* 2003; reviewed in Vernoux *et al.,* 2010). To initiate a new organ, *KNOX1* meristematic and *CUC* boundary genes are switched off in an auxin-dependent manner (Vernoux *et al.*, 2000; Furutani *et al.,* 2004; Aida *et al.,* 2002; Vernoux *et al.,* 2010). At the same time, genes that promote differentiation and maintain repression of *KNOX1* meristem genes, including *BOP1/2*, *AS1*, and *AS2* are switched on (Byrne *et al.*, 2000; Ori *et al.*, 2000; Semiarti *et al.*, 2001; Iwakawa *et al.*, 2002; Ha *et al.*, 2003; 2007; Jun *et al.*, 2010), together with genes that promote cell division, such as *AINTEGUMENTA* (*ANT*) (Long and Barton, 1998; 2000; reviewed in Rast and Simon, 2008).

Leaf initiation and stem-cell maintenance are two competing processes that are compartmentalized in the shoot apex. The SAM is marked by *KNOX1* gene expression (Lincoln *et al.*, 1994; Long *et al.*, 1996; Long and Barton, 2000). By contrast, *AS1*, *AS2*, and *BOP1/2* are expressed in P0 and P1 leaf primordia where their activities maintain the repression *KNOX1* in

the leaf primordia (Byrne *et al.*, 2000; Ori *et al.*, 2000; Semiarti *et al.*, 2001; Iwakawa *et al.*, 2002; Lin *et al.*, 2003; Norberg *et al.*, 2005; Ha *et al.*, 2007; 2010; Jun *et al.*, 2010).

AS1 encodes a MYB transcription factor that is expressed on both abaxial and adaxial sides of the leaf primordia (Byrne *et al.*, 2000) whereas *AS2* is a member of the LBD family and is expressed on the adaxial side of the leaf primordia (Semiarti *et al.*, 2001; Iwakawa *et al.*, 2002; Jun *et al.* 2010). AS1 and AS2 bind to separate promoter sites in *BP* but interact in a complex that directs stable changes in the chromatin structure by recruiting the chromatin remodeling factor HIRA (Xu *et al.*, 2003; Phelphs-Durr *et al.*, 2005; Guo *et al.*, 2008). *BOP1* and *BOP2* encode BTB-ankyrin domain-containing transcriptional co-regulators that are expressed in a partly overlapping domain with *AS1* and *AS2* (Ha *et al.*, 2004; Hepworth *et al.*, 2005; Norberg *et al.*, 2005; see also Chapter 3). Jun *et al.* (2010) showed that BOP1 directly promotes *AS2* expression to maintain *BP* repression in leaves.

1.6.2 Leaf patterning

After stage I, leaf primordia proceed to the PM and SM stages for maturation. At these stages, leaf primordia are manipulated by various regulators that establish the adaxial-abaxial and proximal distal axes and determine leaf shape and form,

The adaxial-abaxial axis is established early in the development of leaf primordia. The dorsal or adaxial side of the leaf is the side that is next to the SAM whereas the ventral or abaxial side is the side faces away from the SAM (Figure 1.5a). Tricomes are a useful marker for identifying the adaxial side of the leaves since they are more abundant on the adaxial side (Byrne, 2006; Figure 1.5b). Adaxial identity is primarily specified by Class III HD-ZIP transcription factors, which share a common homeodomain DNA-binding motif (HD) and a leucine zipper dimerization motif (ZIP) (Figure 1.3). There are five class III HD-ZIP

transcription factors in *Arabidopsis*, including PHABULOSA (PHB), PHAVOLUTA (PHV), ATHB8, ATHB15, and REVOLUTA (REV) (Byrne, 2006). The LBD transcription factor encoded by *AS2* and BTB-ankyrin transcriptional co-activator encoded by *BOP1/2*, expressed at the adaxial base of leaf primordia (Norberg *et al.*, 2005; Jun *et al.*, 2010) also help specify adaxial leaf identity, mainly through regulating Class III HD-ZIP transcription factors (Xu *et al.*, 2003; Ha *et al.*, 2007). Abaxial identity is mainly specified by two families of transcription factors, the YABBY (YAB) family that includes FILAMENTOUS FLOWER (FIL) and the KANADI (KAN) family (Figure 1.3) (Kerstetter *et al.*, 2001; Emery *et al.*, 2003; Eshed *et al.*, 2004).

In addition to adaxial-abaxial polarity, patterning along the proximal-distal axis further defines the architecture of leaves and primarily contributes to the diversity of leaf architectures. There are two major types of leaves, simple or compound, defined based on their degree of complexity along the proximal-distal axis. A leaf with several leaflets (each leaflet may or may not have a short petiolude) on a petiole is termed a compound leaf whereas a leaf with a single undivided flat blade (no leaflet) is termed a simple leaf. Accordingly, meristematic activity that causes growth in the petiole is allowed to different degrees in the compound leaves, whereas it is prohibited in the simple leaves (reviewed in Hasson *et al.*, 2010; Uchida *et al.*, 2010).

There are at least three interconnected pathways that control the simple-compound leaf deviation in plants. The first pathway involves the *KNOX1* genes. In simple leaf species such as *Arabidopsis*, *STM* and *BP* are expressed in the SAM but repressed in leaf primordia (Ori *et al.* 2000; Hay *et al.*, 2006ab). However, in compound leaf species such as tomato and *Cardamine*, *STM, KNAT2*, and *BP* are at first repressed but reactivated at later stages in the leaf primordia (Hay and Tsiantis, 2006b; Shani *et al.*, 2010). Remarkably, inhibition of *KNOX1* genes in tomato

and *Cardamine* leaves results in less complicated compound leaves, whereas induction of *KNOX1* genes in *Cardamine* leaves results in more complicated compound leaves (Hay and Tsiantis, 2006b; Shani *et al.*, 2010). In *Arabidopsis*, *KNOX1* genes are repressed by the overlapping activities of AS1, AS2, and BOP1/2 so that smooth petioles are formed (Byrne *et al.*, 2000; Ori *et al.*, 2000; Hay *et al.*, 2006a; Ha *et al.*, 2007; 2010). As stated above, AS1 and AS2 form a complex that recruits the chromatin-remodeling protein HIRA, leading to stable repression of *BP* and *KNAT2* in developing leaves (Xu *et al.,* 2003; Phelps-Durr *et al.,* 2005; Guo *et al.*, 2008). BOP1/2 is proposed to repress *KNOX1* genes indirectly based on the fact that BOP1 directly activates *AS2* and AS2 directly represses *BP* (Guo *et al.*, 2008; Jun *et al.*, 2010).

The second pathway involves the plant hormones auxin and CK. Local auxin response has been shown to trigger organ initiation at discrete sites in the SAM peripheral zone (e.g. Okada *et al.,* 2001; Benková *et al.*, 2003). Consistent with this, auxin response maxima and the polar auxin transport facilitator PIN1 are present in the *Cardamine* rachis directly corresponding to the sites of leaflet outgrowth. By contrast, these features are not present in the petiole of *Arabidopsis*, thereby indicating the role of auxin in leaflet promotion. Consistent with this, loss-of-function *pin1* in *Cardamine* or applying the auxin transport inhibitor 1-N- naphthylphthalamic acid (NPA) to wild type *Cardamine* leaves results in partial or complete transformation of compound leaves to simple leaves (Barkoulas *et al.*, 2008). Auxin-dependent signaling may also regulate leaflet formation since the combined loss of AUXIN-RESISTANT1 (AXR1) and AS1 activities leads to the formation of leaflets at the junction between the blade and petiole, mimicking the compound leaf form (Hay *et al.*, 2006b). *AXR1* encodes a subunit of the RUB1 ubiquitin-activating enzyme that promotes SCF^{TIR} E3 ubiquitin ligase activity and auxin-dependent gene expression (Leyser *et al.*, 1993). AXR1 and AS1 activities converge to repress

BP expression in leaves, suggesting that they facilitate simple leaf formation by regulating *KNOX1* meristematic genes in leaves (Hay *et al.*, 2006b). Although BOP1/2 function redundantly with AS1 and AS2 in leaf patterning (Ha *et al.*, 2003; 2007; Chapter 3) the combined mutation of *bop1 bop2* and *axr1* does not lead to ectopic leaflet formation (Musa, 2010). Therefore, it remains to be seen if BOP1/2 negatively regulates auxin accumulation to promote simple leaf formation. CK is also involved in simple-compound leaf patterning. Up or down regulation of CK biosynthesis results in more or less complicated compound leaves, respectively, in tomato plants (Shani *et al.*, 2010). As auxin and *KNOX1* genes are involved in compound leaf formation, how does CK interact with them in compound leaf formation? Shani and co-workers (2010) showed that the effect of CK on leaf complexity correlates with auxin accumulation and that KNOX1 acts upstream of CK in leaf patterning.

A third pathway involves the *Arabidopsis* organ boundary *CUC* genes. Hasson *et al.* (2011) showed that ectopic expression of *CUC1* triggers ectopic leaflet formation on the petiole, transforming simple leaves to compound leaves. They also showed that the ectopic expression of *CUC1* results in ectopic expression of *STM* and *BP* but not *KNAT2* at the blade-petiole junction.

In summary, variation in leaf architecture contributes to the diversity of plants. Leaf initiation is a coordinated process that involves the down regulation of *KNOX1* genes and up regulation of *PIN1*, *AS1/2*, and *BOP1/2* in leaf primordia. Two aspects of leaf patterning occur after leaf initiation: adaxial-abaxial patterning and proximal-distal patterning. Adaxial identity is specified by Class III HD-ZIP transcription factors, as well as BOP1/2 and AS2, whereas abaxial identity is specified by YAB and KAN transcription factors. Proximal-distal patterning is controlled by KNOX1 homeodomain proteins, the plant hormones auxin and CK, and the organ boundary gene *CUC1*. In *Arabidopsis*, the combined activities of BOP1/2, AS1, AS2, and AXR1

maintain repression of *KNOX1* genes in leaves to control leaf shape. Studies in pea show that STAMINA PISTILLOIDA (STP), a homolog of *Arabidopsis* UNUSUAL FLORAL ORGANS (UFO), and UNIFOLIATA (UNI), the homolog of *Arabidopsis* LEAFY (LFY), are required for pea compound leaf development (Hofer *et al.*, 1997; Taylor *et al.*, 2001). UFO and LFY promote floral meristem identity and floral organ identity in *Arabidopsis*, however, a role for UFO and LFY in *Arabidopsis* simple leaf development has not been found.

1.7 Reproductive plant architecture

In response to appropriate endogenous and environmental cues, *Arabidopsis* plants transition to reproductive development during which time the architecture of the plant changes dramatically. Modules produced during by the apical meristem are elaborated differently during reproductive development. In *Arabidopsis*, internodes are not elongated until floral induction. In the first-inflorescence stage, cauline leaves with axillary meristems that differentiate as secondary inflorescences (an indeterminate shoot-type) are produced. In the second-inflorescence stage, leaf development is fully repressed and axillary meristems differentiate as flowers (a determinate shoot-type). Variables that contribute to the diversity of inflorescence architecture include the fate and determinacy of the axillary meristem, internode length, the phyllotaxy and the orientation of the lateral structures (inflorescences and flowers). These various architectural traits are controlled by the interacting activities of several classes of regulators in *Arabidopsis* that are explained in the sections that follow.

1.7.1 The decision to flower

The transition from vegetative to reproductive development requires the SAM to adopt an IM fate, such that inflorescences and flowers are produced at the expense of leaves. Therefore, acquiring an IM fate is the first step of reproductive development. The transition from SAM to

IM identity is tightly controlled by several pathways: the long-day photoperiod pathway, the vernalization pathway, the gibberellin (GA) pathway, the thermosensory pathway, and the autonomous pathway (reviewed in Jack, 2004; Liu *et al.*, 2009a; Figure 1.6). In *Arabidopsis*, the long-day photoperiod pathway promotes flowering whereas GA accelerates flowering especially under short-day conditions. The vernalization pathway facilitates the response to cold temperature induction whereas the autonomous pathway regulates flowering by perceiving endogenous signals related to the age of the plant. Input from these pathways is integrated by a small set of genes (termed floral integrators) that include *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) and *LEAFY* (*LFY*), which are expressed at low levels during vegetative development but strongly up regulated in the SAM upon floral induction (Parcy, 2005). The up regulation of these genes in shoot apices directs the formation of an inflorescence and causes the activation of floral-meristem identity (FMI) genes in lateral organ primordia so as to confer floral fate. In *Arabidopsis*, the two key FMI regulators are *LFY* and *APETALA1* (*AP1*). These genes have several important functions: a) to promote the expression of genes that specify floral meristem fate, b) to repress the continued expression of genes that confer inflorescence meristem identity, and c) to activate genes that specify floral-organ identity (FOI). These activities are required to ensure the specification of floral meristems that are determinate and terminate after the production of four whorls of floral organs (sepals, petals, stamens, and carpels).

1.7.2 Internode elongation and pedicel orientation

In *Arabidopsis*, the three-amino-acid loop-extension (TALE) super family of homeodomain proteins consists of two structurally and functionally related subclasses: KNOX homeodomain proteins and BEL1-like (BELL) homeodomain proteins (Hamant and Pautot, 2010). Members

from these two subclasses such as BP from the KNOX subclass and PENNYWISE (PNY) (also called BELLRINGER, REPLUMLESS and VAAMANA) from the BELL subclass control internode length and pedicel orientation (Douglas *et al.*, 2002; Venglat *et al.*, 2002; Byrne *et al.*, 2003; Smith and Hake, 2003). Molecular and genetic analyses have revealed both redundant and antagonistic interactions among them. During *Arabidopsis* vegetative development, STM, BP, KNAT6, PNY, PNF, and ATH1 function redundantly to establish and maintain the SAM (Byrne *et al.*, 2000; 2002; 2003; Ori *et al.*, 2000; Belles-Boix *et al.*, 2006). However, during reproductive development, KNAT2, KNAT6, and ATH1 function antagonistically to BP and PNY in regulating internode elongation and pedicel orientation (Ragni *et al.*, 2008).

1.7.2.1 BP and PNY-PNF promote internode formation

In addition to their roles in establishing and maintaining the SAM, BP and PNY-PNF homeodomain proteins regulate inflorescence architecture. Loss-of-function *bp* mutants display reduced internode length and horizontal or downward-pointing siliques (Douglas *et al.*, 2002; Venglat *et al.*, 2002). In contrast, loss-of-function *pny* mutants display internodes of irregular length and clusters of siliques (Byrne *et al.*, 2003; Smith and Hake, 2003). Strikingly, the inflorescence of *bp pny* double mutants is extremely short compared to its parents, indicating redundant roles for BP and PNY in promoting internode elongation (Smith and Hake, 2003; Ragni *et al.*, 2008). Mutations in *PNF*, a gene closely related to *PNY*, do not obviously affect internode elongation but the *pny pnf* double mutant fails to produce an inflorescence and continues to produce leaves indefinitely (Smith *et al.*, 2004). This suggests a redundant role for PNY and PNF in co-coordinating internode elongation and the decision to flower.

Since there are synergistic interactions between BP, PNY, and PNF, what are the mechanisms that explain the functions of these proteins in internode patterning? This question is

only partly answered. Consistent with the overlapping activities of these genes, *BP* and *PNY* are expressed in the cortex of internodes and pedicels whereas *PNF* is only expressed in the IM (Lincoln *et al.,* 1994; Byrne *et al.,* 2003; Smith *et al.,* 2004). Yeast-two-hybrid assays have revealed heterodimers of BP/PNY and BP/PNF providing a partial explanation for redundancy in their activities (Kanrar *et al.*, 2006).

In addition to promoting internode elongation, BP and PNY affect cell differentiation in the internodes. Histological analyses of *pny* and *bp* stem cross-sections reveal an abnormal pattern of cell differentiation and lignin accumulation, albeit in slightly different patterns for the two mutants (Venglat *et al.*, 2002; Mele *et al.*, 2003; Smith and Hake, 2003). In *bp* mutants, lignin is ectopically deposited in discrete regions of the stem epidermis and cortex. In the epidermis, lignin is ectopically deposited in stripes of abnormally-differentiated epidermal tissue that extend below nodes, whereas in the cortex, lignin is prematurely deposited in intrafascicular bundles indicating the accelerated differentiation of secondary xylem (Douglas *et al.,* 2002; Mele *et al.,* 2003; Venglat *et al.,* 2002). In *pny* stems, there are a greater number of vascular bundles resulting in a continuous ring of lignified stem cortex (Smith and Hake, 2003). Further, DNA microarray and *in vitro* binding studies performed by Mele *et al.* (2003) indicate that *BP* is a direct negative regulator of lignin biosynthetic genes. Similar studies have not been done with *PNY*. One interpretation of these data is that *BP* and *PNY* in the stem cortex serve to coordinate internode elongation with the developmental timing of xylem differentiation.

1.7.2.2 KNAT2-KNAT6 and ATH1 inhibit internode elongation

In the vegetative SAM, STM, BP, KNAT6, PNY, PNF, and ATH1 function redundantly to maintain the SAM. Surprisingly, KNAT2, KNAT6, and ATH1 function antagonistically to BP and PNY in the inflorescence. In WT inflorescences, reporter gene studies show that *KNAT2* and

KNAT6 expression domains are restricted to the pedicel axil. However, in *bp* and *pny* mutants, the expression domains of both genes expand to stems and pedicels (Ragni *et al.*, 2008). Not surprisingly, *knat2 knat6* double mutations rescue internode length and pedicel orientation in *bp* and *pny* single mutants and in *bp pny* double mutants (Ragni *et al.*, 2008). Mutations in *KNAT6* were more effective than mutations in *KNAT2* in rescue of *bp* and *pny* defects indicating unequal requirements for these genes (Ragni *et al.,* 2008). These data indicate that BP-PNY and KNAT2-KNAT6 function antagonistically to regulate growth patterns in the inflorescence stem. It is not yet known if *knat2 knat6* can rescue the lignification pattern of *bp* and *pny* or if *knat2 knat6* mutations rescue the *pny pnf* block in inflorescence formation.

Mutation of the BELL homeodomain protein encoded by *ATH1* also fully rescues *pny* inflorescence defects and at least partially restores the *pny pnf* bolting defect when mutated (Rutjens *et al.*, 2009) indicating antagonistic functions for ATH1 and PNY/PNF in the inflorescence. It is not known if *ATH1* mutations also rescue *bp* inflorescence defects. How these factors function antagonistically is not clear but earlier characterization of *ATH1* has shown that it promotes the expression of *FLOWERING LOCUS C* (*FLC*), a potent repressor of flowering (Proveniers *et al.*, 2007; Liu *et al.,* 2009a). Thus, regulation of ATH1 may provide an important link between coordination of internode elongation and flowering that has not yet been investigated. This idea is discussed further below.

1.7.2.3 A role for AS2 in inflorescence patterning

AS1 and AS2 have been shown to repress *BP* expression during leaf development (Byrne *et al.*, 2000; Ori *et al.*, 2000; Semiarti *et al.*, 2001; Iwakawa *et al.*, 2002). Consistent with this, ectopic expression of *AS1* and *AS2* results in reduced internode length and downward pointing siliques, resembling loss-of-function *bp* mutants (Lin *et al.*, 2003; Xu *et al.*, 2003). Remarkably, over-

expression of *BOP1* or *BOP2* also causes internode length and pedicel orientation defects (Norberg *et al.*, 2005; Ha *et al.*, 2007) similar to loss-of-function *bp* mutants or overexpression of AS1/2. However, inactivation of *AS2* in *35S::BOP1* plants only slightly rescues its inflorescence architecture defects (Ha *et al.*, 2007) indicating that the effect of BOP1/2 on inflorescence architecture is largely independent of AS2.

Taken together, the length of internodes and orientation of pedicels in inflorescences is regulated by the activities of KNOX and BELL homeodomain proteins. BP, PNY and PNF function redundantly to promote internode elongation and proper pedicel orientation, whereas KNAT2, KNAT6, and ATH1 have opposing functions. How these groups of regulators exert their antagonistic functions remain to be elucidated. Gain-of-function AS1/2 and BOP1/2 also perturb inflorescence architecture. However, in the inflorescence, BOP1/2 does not primarily exert its effect through AS2.

1.7.3 Coordination of internode elongation and flowering

In many plant species, including *Arabidopsis*, the switch to flowering and elongation of the first internode are tightly linked. Several lines of data suggest that coordination of these two events requires PNY and PNF activities (Smith *et al.*, 2004; Kanrar *et al.*, 2008). As explained above, *pny pnf* double mutants do not flower in response to appropriate inductive signals (Smith *et al.*, 2004). While the transcripts of several floral integrators accumulate normally in *pny pnf* apices, *LFY* and *AP1* transcripts (FMI genes) do not accumulate. While overexpression of *LFY* rescues *pny pnf* flowering, overexpression of the floral integrator *FT* does not, suggesting that FT requires the activities of PNY and PNF to be functional (Kanrar *et al.*, 2008). Mutation of the BELL-like gene *ATH1* partially rescues the *pny pnf* non-flowering phenotype (Rutjens *et al.*, 2009) suggesting that its misexpression in *pny pnf* mutants may interfere with floral evocation,

but this remains to be tested. Plants overexpressing *ATH1* are very late flowering due to up regulation of the floral repressor gene *FLOWERING LOCUS C* (*FLC*) (Proveniers *et al.*, 2007; Liu *et al.,* 2009a), suggesting a link between flowering and internode elongation. PNY and PNF also regulate flower identity together with LFY, UFO, and WUS (Yu *et al.*, 2009). Identification of direct targets of the TALE proteins, as with the case of *AGAMOUS* (*AG*) for PNY (Bao *et al.*, 2004), would help us to understand the mechanism that coordinates the internode elongation and flowering.

1.7.4 Specification of floral meristem identity

Upon floral induction, the IM produces secondary inflorescences and then flowers (Figure 1.1). In *Arabidopsis*, the architecture of a secondary inflorescence differs from a flower in three aspects. First, a secondary inflorescence has an indeterminate meristem with the potential to produce numerous flowers, whereas flower meristems (FMs) are determinate and produce a set number of floral organs before the stem-cell population is terminated. Second, inflorescence meristems produce flowers arranged in a spiral pattern with internode elongation between organs, whereas floral meristems produce floral organs arranged in a whorled pattern without internode elongation between organs. Third, a secondary inflorescence has a cauline leaf at its base, whereas an *Arabidopsis* flower is bractless (Huala and Sussex, 1992; Weigel *et al.*, 1992; Levin *et al.*, 1995).

The morphological changes that occur during *Arabidopsis* floral development can be divided into 12 stages according to a series of landmark events (Smyth *et al.,* 1990). Floral primordia are generally considered as floral meristems (FM) as they produce floral organs. Stage 0 FMs are morphologically invisible, but can be distinguished from other cells in the IM by the expression of marker genes such as *LFY* (Hempel *et al.,* 1997). Stage 1 FMs as seen as lateral

bulges on the flanks of the IM. Stage 2 FMs are separated from the IM by a groove and are enlarged into rounded structures. In Stage 3 FMs, the pedicel begins to elongate and sepal primordia develop on the meristem periphery. They develop into stage 4 FMs when the sepal primordia grow to cover the FM. After stage 4, stamen and petal primordia develop, while the inner most cells in the FM gives rise to the carpel primordia.

As explained in the introduction to this section, the expression of FMI markers such as *LFY* and *AP1* in lateral organ primordia confers floral fate and they activate a chain of events resulting in the production of a flower (Irish and Sussex, 1991; Weigel *et al.,* 1992; Bowman *et al.,* 1993; reviewed in Krizek and Fletcher, 2005). Mutations or combinations of mutations in FMI genes cause a transition to indeterminacy, flowers with bracts or internode elongation between successive floral organs, which are all characteristics of secondary inflorescences (Parcy, 2005, Liu *et al.*, 2009a).

1.7.4.1 LFY

LFY is viewed as the central regulator of FMI. However, its expression is dependent on input from several flowering time pathways, so it also behaves as a floral integrator (Weigel *et al.*, 1992; Parcy 2005, Liu *et al.,* 2009a). Thus far, SOC1 is the only transcriptional regulator that is found to bind to the *LFY* promoter directly (Lee *et al.*, 2008). SOC1 is directed into the nucleus by AGL24, another MADS-box (for MCM1-AGAMOUS-DEFICIENS-SERUM RESPONSE FACTOR) protein with a domain of expression overlapping with *SOC1*. Based on these observations, Lee *et al.* (2008) suggested that SOC1-AGL24 heterodimers directly activate *LFY* expression *in vivo*. The floral integrator FT and its co-factor FLOWERING LOCUS D (FD) also activate *LFY* expression, but this activation could be indirect since FT/FD activates *SOC1* (Abe *et al.*, 2005) and SOC1 directly promotes *LFY* expression (Lee *et al.*, 2008).

LFY transcripts are very low in vegetative leaf primordia but accumulate to high levels in floral primordia from the earliest stages (Blazquez *et al.,* 1997; Hempel *et al.*, 1997). *LFY* expression first occurs in P0 primordia. At stage 3, *LFY* expression concentrates in the dome of the FM where its activity turns on genes that confer floral organ identity (Weigel *et al.*, 1992; Hempel *et al.,* 1997; Liu *et al.,* 2009a).

Consistent with its role as a master regulator of FMI, mutations in *LFY* cause the complete conversion of early-arising flowers into inflorescences. In strong *lfy* mutants, more flowers are transformed into secondary inflorescences than in weak alleles, and late-arising flowers retain partial characteristics of secondary inflorescences (e.g. secondary flowers arise in the axils of the outer floral organs, floral organs are separated by internodes, and flowers are subtended by bracts) (Schultz and Haughn, 1991; Huala and Sussex, 1992; Weigel *et al.*, 1992). Once *LFY* is activated in floral primordia, it directs the activation of additional FMI genes including the MADS-domain transcription factors encoded by *AP1* and *CAULIFLOWER* (*CAL*) and the homeodomain-leucine zipper protein encoded by *LMI1*. These factors reinforce *LFY* expression in a feed-forward loop that makes the switch from inflorescence to flower production unidirectional (Parcy *et al.,* 1998; Wagner *et al.,* 1999; Ferrandiz *et al.,* 2000; Williams *et al.,* 2004; Saddic *et al.,* 2006).

1.7.4.2 AP1

AP1 is first activated in stage 1 FMs and is the earliest known marker of commitment to floral fate (Hempel *et al.,* 1997). LFY is a direct regulator of *AP1* (Parcy *et al.*, 1998; Wagner *et al.,* 1999) but its expression is also directed by a complex of FT/FD (Abe *et al.*, 2005; Wigge *et al.*, 2005) and by SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) proteins that control age-related flowering time (Wang *et al.,* 2009; Yamaguchi *et al.,* 2009).

Unlike *LFY*, *AP1* is not expressed in vegetative tissues, nor does it appear in the floral meristem until stage 1 (Mandel *et al.*, 1992; Bowman *et al.*, 1993; Hempel *et al.*, 1997). In *ap1-1* mutants, secondary FMs arise in the axils of the first-whorl floral organs and this pattern is repeated several times, resulting in highly branched floral structures (Irish and Sussex, 1990; Bowman *et al.*, 1993). These structures represent the partial conversion of flowers to inflorescences (indeterminate shoots because the FMs that used to give rise to a single flower now produce several flowers). When *ap1-1* is added to strong or weak alleles of *lfy*, the difference between strong and weak alleles of *lfy* largely disappears and the later-arising flowers are more shoot-like than in single mutants, indicating redundant roles for *LFY* and *AP1* in promoting FMI (Huala and Sussex, 1992; Weigel *et al.*, 1992).

1.7.4.3 Inflorescence meristem identity genes

LFY and AP1 activities are required to block the continued expression of inflorescence meristem-identity genes in the floral meristem so that shoots become determinate and develop as flowers (Yu *et al.*, 2004; Liu *et al.*, 2007; 2009b). There are three main IM identity genes in *Arabidopsis* that encode MADS-domain transcription factors: *SOC1*, *AGL24*, and *SHORT VEGETATIVE PHASE* (SVP). All three genes are expressed in the IM and early FMs (stages 0-2) but the continued expression of any one of these genes in FMs partially transforms flowers into shoots with inflorescence-like characteristics, similar to mutation of *lfy* or *ap1-1* (Irish and Sussex, 1991; Weigel *et al.*, 1992; Yu *et al.*, 2004; Liu *et al.*, 2007; 2009a). Consistent with this, loss-of-function *agl24* is sufficient to rescue floral branching in *ap1-1* mutants. Chromatin immunoprecipitation experiments suggest that *AGL24*, *SOC1*, and *SVP* are directly repressed by AP1 whereas repression by LFY is probably indirect (Yu *et al.*, 2004; Liu *et al.* 2007; Gregis *et al.*, 2008). Paradoxically, in the early stages of FM development (stages 1 and 2) *SOC1*, *SVP*,

and *AGL24* expression in the FM promotes FMI (Samach *et al.,* 2000; Yu *et al.,* 2002; Michaels *et al.,* 2003; Gregis *et al.,* 2008). Several lines of evidence support this idea. First, SOC1 and AGL24 contribute to *LFY* up regulation (Lee *et al.,* 2008), Second, *ap1 agl24 svp* triple mutants develop a cauliflower-like apex that produces shoots in place of flowers, indicating that mutations in *agl24* and *svp* enhance *ap1-1* FMI defects (Gregis *et al.*, 2008), similar to *ap1 cal* mutants that are also compromised for FMI (Ferrándiz *et al.*, 2000).

In summary, LFY and AP1 are the master regulators of FMI. This task requires both the activation of secondary FMI genes and the repression of IM identity genes. Both activities are essential for the maintenance of FM fate so that flowers have a determinate structure.

1.7.5 Floral patterning

Floral organs begin to differentiate at floral stage 3. Sepals are seen to emerge first on the edges of the FM, followed by petal and stamen primordia. The FM is consumed by the formation of two central carpels that fuse to form the gynecium (Smyth *et al.* 1990). In contrast to floral primordia that arise from the IM in a spiral pattern, floral organ primordia are generated in concentric whorls (Smyth *et al.*, 1990). The establishment of FMI and promotion of FOI are consecutive events, and some genes, such as *LFY* and *AP1*, play roles in both processes (Irish and Sussex, 1990; Schultz and Haughn, 1991; 1993; Weigel, *et al.*, 1992).

Our detailed understanding of how floral organs are patterned started with the characterization of mutants in which floral organs developed normally, but in inappropriate whorls of the flower (Bowman *et al.,* 1991; Coen and Meyerowitz, 1991). It is now known that floral organ identity is determined by the overlapping activities of four classes of homeotic genes termed A, B, C, and E (Figure 1.7). With one exception, all of these genes encode MADS domain transcription factors (Krizek and Fletcher, 2005). According the ABCE model, A-class

genes (e.g. *AP1* and *AP2*) are expressed in the first whorl and specify sepals. In the second whorl, A-class and B-class genes (e.g. *PISTILLATA* (*PI*) and *AP3*) are co-expressed and their products combine to specify petals. In the third whorl, B-class and C-class genes (e.g. *AGAMOUS* (*AG*)) are co-expressed and their products combine to specify stamens. In the fourth whorl, only C-class genes are expressed and carpels are specified. The Class E genes (*SEPALLATA1-4*) were discovered later as a result of the *Arabidopsis* genome sequencing project. These genes function redundantly, are expressed in all whorls, and are required for the specification of all four organ types (Pelaz *et al.*, 2000; Honma *et al.*, 2001; Ditta *et al.*, 2004). To account for the mutant phenotypes observed, the model also stipulates that A-class and C-class activities are mutually exclusive and repress each other. Consistent with this, in A-class mutants, C-class activity expands into all whorls, such that sepals are replaced by carpels and petals by stamens. Similarly, in C-class mutants, A-class activity expands into all whorls, such that carpels are replaced by sepals and stamens by petals. In C-class mutants, the flowers also become indeterminate because there is no mechanism to extinguish the stem-cell population and the production of sepals and petals continues indefinitely (Lenhard *et al.*, 2001; Lohmann *et al.*, 2001; Lohmann and Weigel, 2002).

LFY plays a central role in promoting FOI in all four whorls because its activity turns on the expression of key A-Class, B-Class, and C-Class genes in the FM. The A-class gene *AP1* is expressed in the perianth (sepal and petal) whorls of the flower and is a direct target of LFY activation (Parcy *et al.*, 1998; Wagner *et al.*, 1999). The A-class gene *AP2* is also expressed in the outer floral whorls but its spatial domain of expression is determined post-transcriptionally by miR172 (Wollmann *et al.*, 2010; Chen, 2004).

The B-class gene *AP3* is directly activated by LFY in the petal and stamen whorls but in

this case, LFY requires the activity of an F-box co-factor called UNUSUAL FLORAL ORGANS (UFO). Most F-box proteins form the substrate adaptor component of an SCF complex which acts an E3 ubiquitin ligase to mark targets with polyubiquitin so that they are recognized the 26S proteosome and degraded (Deshaies, 1999). The requirement for UFO is surprising since the LFY protein has an activation domain and does not require a co-factor to turn on *AP1* (Parcy *et al.,* 1998; Wagner et al., 1999). Nevertheless, several lines of evidence suggest that LFY and UFO activities are both needed to activate *AP3* expression. First, flowers of strong *lfy* and *ufo* mutants do not have petals and stamens (Weigel *et al.*, 1992; Wilkinson and Haughn, 1995), similar to that of *ap3* mutant. Consistent with this, *AP3* expression is significantly down-regulated in these mutants (Weigel and Meyerowitz, 1993; Levin and Meyerowitz, 1995). Second, over expression of *AP3* and *PI* can partially restore the petals and stamens in *lfy* and *ufo* mutants and Lamb *et al.* (2002) demonstrated that LFY binds directly to the *AP3* promoter. Work by Chae *et al.* (2008) found that the LFY-UFO complex directly binds to the *AP3* promoter, suggesting that UFO is a co-factor for LFY in activating *AP3*. The other B-class gene *PI* is expressed at the same domain as *AP3* and functions as a heterodimer with AP3 in floral patterning (Riechmann *et al.*, 1996; Sundstrom *et al.*, 2006).

The C-class gene *AG* is expressed in the central dome of the FM beginning at stage 3 and specifies stamen and carpel identity. LFY, together with the meristem factor WUS, are the primary activators of *AG*. Both LFY and WUS have binding sites in the *AG* second intron, which is the main regulatory region for *AG* (Busch *et al.,* 1999). *WUS* is co-expressed with AG from floral stage 3 to stage 5 for this function but it is turned off from floral stage 6 by AG to ensure that the stem-cell population in the FM terminates (Lenhard *et al.*, 2001; Lohmann *et al.*, 2001).

The bZIP transcription factor PERIANTHIA (PAN) expressed in IMs and FMs also activates *AG* expression directly (Das *et al.*, 2009; Meier *et al.*, 2009).

The *SEP1-4* genes are expressed in overlapping domains that encompass all four whorls of the flowers. They function in association with Class A, B, and C genes to specify FOI (Krizek and Fletcher, 2005). Several lines of evidence support this model. First, *sep1 sep2 sep3 sep4* quadruple mutants produce flowers with four whorls of leaf-like organs, resembling the phenotype of *abc* mutants (Pelaz *et al.*, 2000; Ditta *et al.*, 2004). Second, the ectopic expression of B-class and *SEP* genes together is required for the successful conversion of leaves into petals (Honma and Goto, 2001; Pelaz *et al.*, 2001b).

All of the floral homeotic genes with the exception of *AP2* encode MADS domain transcription factors, a type DNA-binding domain that is conserved among eukaryotes. How do these factors work in combination to specify FOI? Biochemical experiments show that MADS domain proteins form dimers *in vitro* and interact with a conserved DNA target sequence called a 'CArG box'. The B-class proteins AP3 and PI only bind to DNA as heterodimers, indicating that different complexes of MADS box proteins can bind selectively to sites in the promoters of their target genes (Reichmann *et al.*, 1996; Honma and Goto, 2000). Large-scale interaction studies in yeast have led to the model that the assembly of MADS box proteins into higher order complexes containing four members might be the principal way in which A, B, C, and E-class genes function combinatorially (Hongma and Goto, 2001; reviewed in Theissen, 2001). For example, AP1 interacts with SEP3, and AG interacts with SEP1, SEP2, and SEP3 (Pelaz *et al.*, 2001a). Moreover, a large complex of AP3/PI/SEP3/AG was also discovered (Honma and Goto, 2001). SEP3 is proposed to act as a scaffold for the formation of quartet complexes of MADS box proteins (Immink *et al.*, 2009).

In summary, the overlapping activities of four classes of floral homeotic genes, termed A, B, C, and E specify *Arabidopsis* floral organ identity by forming multimeric complexes of four proteins that activate genes required for sepal, petal, stamen, and carpel differentiation (Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005). The expression of several key A, B, and C class genes (*AP1*, *AP3*, and *AG*) are directly dependent on LFY for their activation in specific domains of the FM (Busch *et al.*, 1999; Wagner *et al.*, 1999; Lamb *et al.*, 2002) whereas the *AP2* expression domain is determined by miR172 (Chen, 2004; Wollman *et al.*, 2010). The E-class *SEP* genes are activated independently of LFY and play an important role as the "glue" that mediates the formation of MADS-domain protein "quartets" for the specification of FOI (Theissen, 2001; Pelaz *et al.*, 2000; 2001b; Ditta *et al.*, 2004; Immink *et al.*, 2009).

1.8 BOP signaling mechanism

BOP1 and BOP2 belong to the NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) family of proteins, all of which contain an N-terminal BTB/POZ (for Broad-Complex, Tramtrack, and Bric-a-Brac/POX virus and Zinc finger) domain and a C-terminal set of four ankyrin repeats (Cao *et al.*, 1997; see Hepworth *et al.*, 2005 for a phylogenetic tree of the six NPR1 family members in *Arabidopsis*). The BTB/POZ domain is found to interact with Cullin 3 proteins that target substrates for ubiquitin-mediated degradation whereas the ankyrin domain interacts with TGA (TGACG-motif binding) bZIP (basic leucine zipper) transcription factors (Hepworth *et al.*, 2005). NPR1 is the founding member of the gene family, and it was identified by mutation as a central regulator of systemic acquired resistance (SAR) in plants (Cao *et al.*, 1997). SAR is an induced immune response triggered by local infection that protects the entire plant against subsequent bacterial, fungal, and viral infection (Dong, 2004). BOP1/2 have no apparent function in plant disease resistance and instead regulate the architecture of leaves,

inflorescence, fruits, and flowers (Hepworth *et al.*, 2005; Norberg, *et al.*, 2005; Ha *et al.*, 2003; 2007; McKim *et al.*, 2008). Genes or transcripts with homology to *BOP1* and *BOP2* are found in various plant species including moss, trees, dicots and monocots (McKim, 2009). In moss, BOP proteins promote cell differentiation (Saleh *et al.*, 2011). These findings suggest that BOP proteins are ancient and conserved and play a fundamental role in controlling plant architecture and development. Because of the conserved domain structure of BOP1/2 and NPR1, studies pertaining to NPR1 serve as a paradigm for understanding how BOP1/2 might be regulated and how they might control transcription at the biochemical level.

Briefly, NPR1 promotion of SAR in plants is regulated primarily at the post-transcriptional level (Cao *et al.* 1998; Spoel *et al.* 2010). In response to challenge with pathogen, the plant hormone salicylic acid (SA) accumulates systemically in plants leading to changes in the intracellular redox potential of cells that convert NPR1 into an active monomer that is allocated to the nucleus. In the nucleus, NPR1 is recruited to the promoter of its target genes primarily through association with members of the TGA subclass of bZIP transcription factors thus activating plant defense (Zhang *et al.,* 1999; Despres *et al.,* 2000; Mou *et al.,* 2003; reviewed in Dong, 2004). Evidence suggests that this basic mechanism may apply in part to BOP1 and BOP2. First, BOP1/2 also forms complexes in yeast with TGA bZIP family members, including TGA8/PAN that functions in the same pathway as BOP1/2 to control sepal number in flowers (Hepworth *et al.,* 2005). Second, BOP1-GFP fusion proteins localize to both the cytoplasm and the nucleus of cells (Hepworth *et al.* 2005; Jun *et al.* 2010). Third, BOP1/2 function as transcriptional activators (Jun *et al.* 2010; Xu *et al.* 2010). However, it remains unclear if BOP1/2 nuclear localization, stability, transcriptional activation, and interactions with TGA factors are regulated by redox-dependent modification of Cys residues since only two of

several Cys regulatory residues in NPR1 are conserved in BOP1 and BOP2 (Hepworth *et al.*, 2005; reviewed in Spoel *et al.*, 2010).

Analysis of *NPR1* expression before and after SA treatment suggests that NPR1 activity is primarily posttranscriptionally regulated, as *NPR1* is constitutively expressed and its transcripts are increased only two-fold after SA treatment (Cao *et al.*, 1998). Kinkema and co-workers (2000) first found that nuclear localization of NPR1 is essential for its activity during SAR. Later on, Mou and co-workers (2003) found that NPR1 normally resides in the cytoplasm in oligomeric form, holding together by intermolecular disulfide bonds. Upon pathogen challenge or SA treatment, the cellular redox state changes, leading to the reduction of these disulfide bonds, and resulting in nuclear localization of monomeric NPR1 and activation of its target genes. These authors also showed that mutations of cysteine at Cys82 and Cys216 to Alanine in NPR1 lead to increased monomer accumulation, constitutive nuclear localization and expression of *PR1* (a target gene) without induction, thereby confirming that monomerization of NPR1 mediates its nuclear localization and activity. However, the fact that plants treated with SA not only release NPR1 monomer but also accumulate NPR1 oligomer, and that removing reducing agent from NPR1 protein solutions results in the oligomer reformation, indicated that NPR1 undergoes conformational changes under different cellular environment (Tada *et al.*, 2008).

Further molecular, genetic and biochemical analysis demonstrated that reversion of NPR1 monomers to oligomers is carried out through S-nitrosylation, probably on Cys156 (Tada *et al.*, 2008). As such, S-nitrosoglutathione, a natural NO donor, can effectively reduce monomer NPR1 levels without changing the total NPR1. Moreover, the *atgnsor1-3* mutant, which displays increased S-nitrosylation activity, cannot effectively induce nuclear localization of NPR1 and

PR1 expression upon SA treatment, indicating that there is little or no NPR1 monomer available due to the oligomerization of NPR1 by S-nitrosylation (Tada *et al.*, 2008). These findings confirm that NPR1 is oligomerized by S-nitrosylation.

Localization of NPR1 monomer in the nucleus is necessary but not sufficient for its transcriptional activity. Spoel and co-workers (2009) demonstrated that under non-inducing conditions the nuclear localized monomer NPR1 is degraded by the CUL3-mediated activity, preventing the costly activation of SAR. By contrast, during SAR, NPR1 is phosphorylated at Ser11 and Ser15 in the nucleus, and the phosphorylation of NPR1 stimulates its transcriptional activity. NPR1 has no DNA binding domain but has ankyrin repeats that interact with TGA transcription factors (Zhang *et al.*, 1999; Despres *et al.*, 2000). After SA treatment, NPR1 binds to TGA transcription factors (such as TGA2), forming a enhanceosome on the *PR1* promoter to activate the transcription of *PR1* (Zhang *et al.*, 1999; Despres *et al.*, 2000; Rochon *et al.*, 2006; Pape *et al.*, 2010).

In moss, *BOP* expression is regulated post-transcriptionally by miRNA534, which controls the timing of juvenile-to-adult phase change (Saleh *et al.*, 2011). It is possible that *BOP* is targeted by miRNAs in *Arabidopsis* since it has various effects on plant development, resembling the mutation of the miRNA targets *AP2* (Chen *et al.*, 2004) and *SPL3/9* (Wang *et al.*, 2009; Wu *et al.*, 2009; Yamaguchi *et al.*, 2009) but no such miRNAs have been identified for *BOP* in *Arabidopsis* (Saleh *et al.,* 2010). Future work will need to address whether Cys residues in the BOP1 and BOP2 post-transcriptionally control BOP activity through oxidation-reduction, nitrosylation and/or phosphorylation to regulate association with DNA-binding partners or transcriptional activation potential.

1.9 Thesis rationale

The overall goal of our work is to understand the mechanisms that control plant architecture. Our lab is interested in how two conserved BTB-ankyrin domain-containing transcriptional co-regulators, BOP1 and BOP2, control the architecture of *Arabidopsis* leaves, inflorescences and flowers (Ha *et al.,* 2003; Hepworth *et al.,* 2005; Norberg *et al.,* 2005; Ha *et al.,* 2007; McKim *et al.,* 2008). These genes are expressed in initiating lateral organ primordia produced during both vegetative and reproductive phases of development and then partition to the boundaries that separate these organs from the meristem. In support of a central role for these genes in regulation of plant architecture, homologs are found in a wide variety of species (McKim, 2009) and they promote phase change in moss which is a lower plant (Saleh *et al.,* 2010). Both loss and gain of BOP function in *Arabidopsis* cause dramatic changes in the patterning of leaves, inflorescences, and flowers (Ha *et al.*, 2003; Hepworth *et al.,* 2005; Norberg *et al.,* 2005; Ha *et al.,* 2007; McKim *et al.,* 2008) suggesting that the temporal and spatial pattern of *BOP* expression is an important determinant of plant architecture. While it is clear that these genes control plant architecture, how they function at the molecular level is poorly understood.

To address this gap in knowledge, I used a molecular genetics approach to address the role and mechanism of BOP1/2 activity in:

(1) floral meristems;

(2) leaves; and

(3) the node/internode junction of inflorescences

The results of these investigations form the basis of Chapters 2, 3, and 4 in my thesis. Chapter 5 provides a summary of these finding and future direction. Appendix A contains a glossary of the regulatory genes discussed in the thesis. Appendix B explains how double mutant analysis is

used to infer genetic interactions between genes, a method that forms the basis of many of the experiments in this thesis.

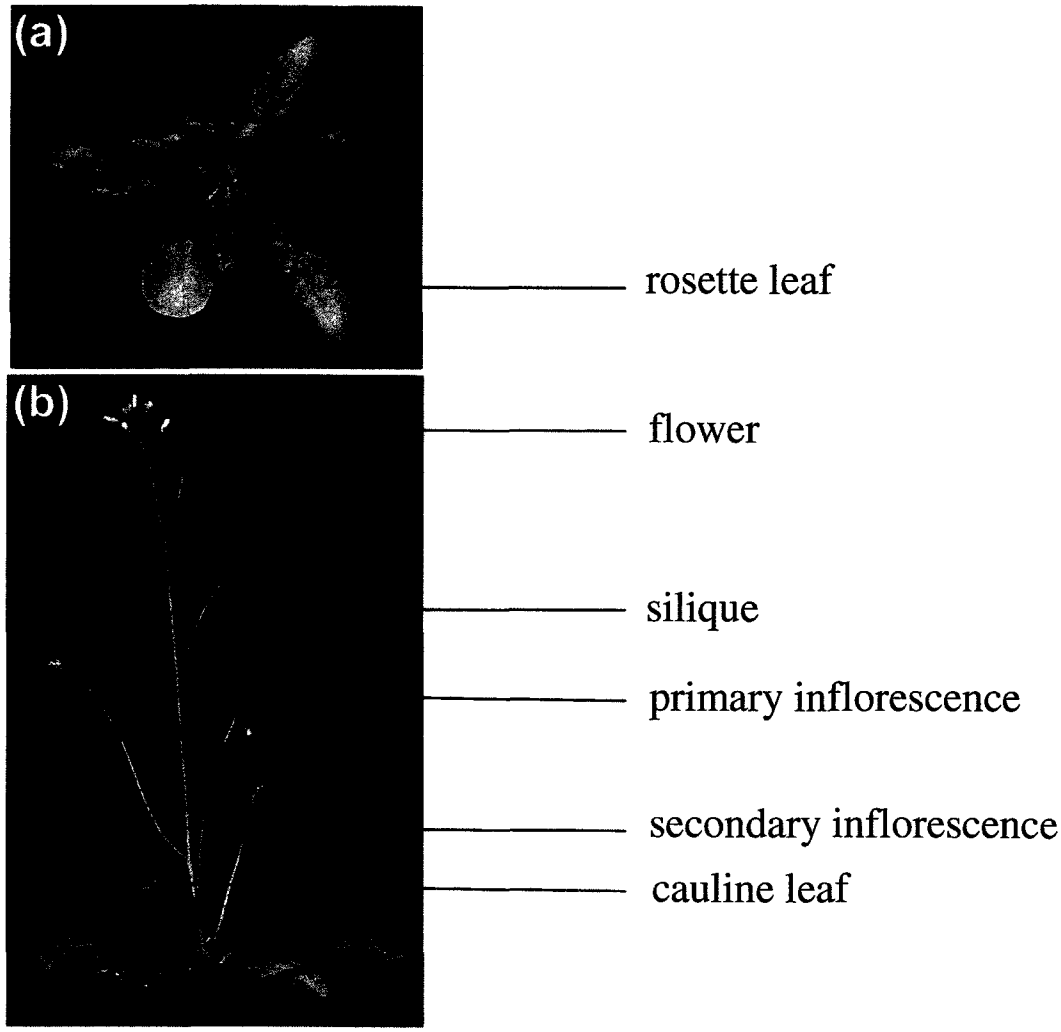

Figure 1.1 ***Arabidopsis thaliana*** **developmental stages.**

(a) Vegetative stage. Rosette leaves are produced without internode elongation. Axillary meristems may later develop in the axils of these leaves to produce a paraclade.

(b) Reproductive stage. In the first stage of reproductive development, cauline leaves form with secondary inflorescences in their axils. In the second stage of reproductive development, leaf development is repressed and axillary meristems develop as single flowers that mature into fruits (siliques). Internode elongation occurs during reproductive development so that lateral branches and flowers are evenly distributed along the primary inflorescence.

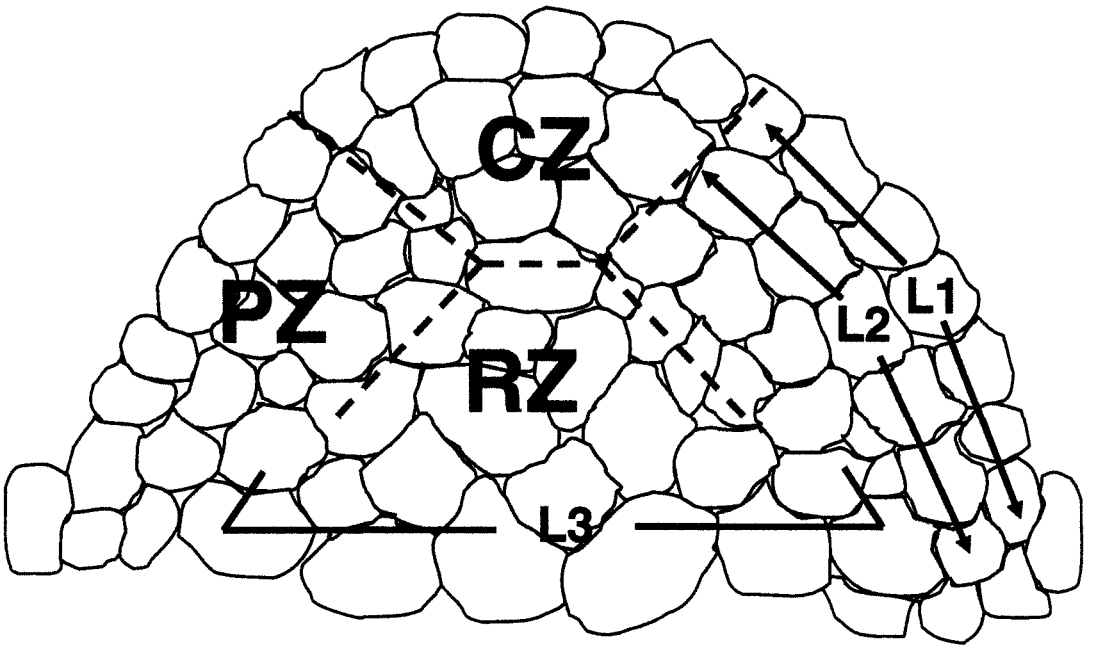

Figure 1.2 Organization of shoot apical meristem.

At the apex of the meristem, cells in the central zone (CZ) divide slowly to replenish the fund of pluripotent stem cells. The central zone is flanked by the peripheral zone (PZ), where organ primordia are initiated. The rib zone (RZ) lies beneath the central zone. The dashed lines indicate the boundaries between the different zones. It is not clear precisely how many cells constitute each region. Nevertheless, the outer L1 cell layer and the sub-epidermal L2 cell layer are a single cell thick, and the internal L3 cells lie beneath them.

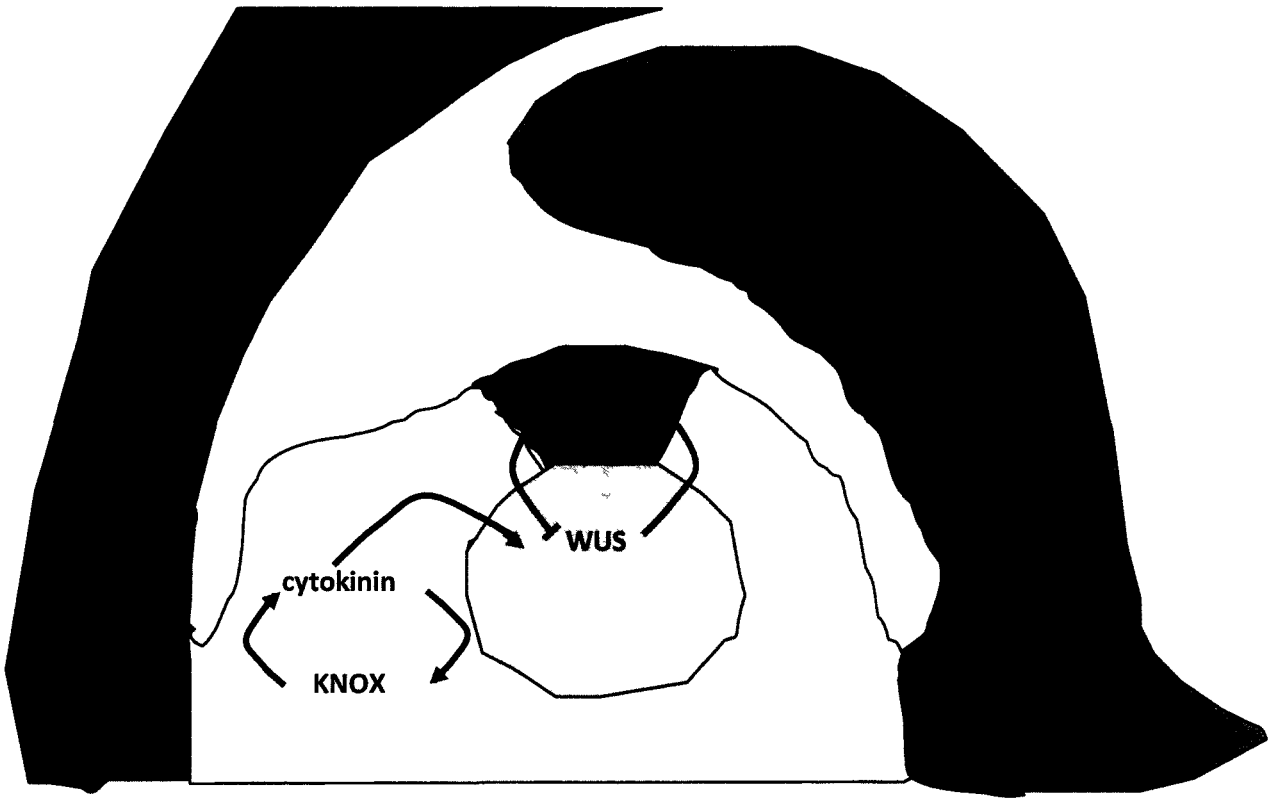

Figure 1.3 The shoot apical meristem is established and maintained by WUS-CLV3 signaling and KNOX-auxin/cytokinin signaling.

Diagram showing the expression domains of transcription factors involved in maintenance of the SAM and abaxial-adaxial leaf patterning. The transcription factor WUS is expressed in the organizing center induces the expression of a diffusible peptide CLV3 in the overlying central zone cells. CLV3 activates a signaling pathway that represses *WUS* expression, thereby creating a feed-back loop that maintains the size of the stem-cell population. Class I KNOX proteins modulate cytokinin levels which in turn activate *KNOX* and *WUS* expression. KAN and YAB activities promote abaxial leaf identity while HD-ZIPIII proteins in association with BOP1/2 and AS1-AS2 promote adaxial leaf identity. This figure is redrawn from Barton (2010) with permission from the publisher (Elsevier).

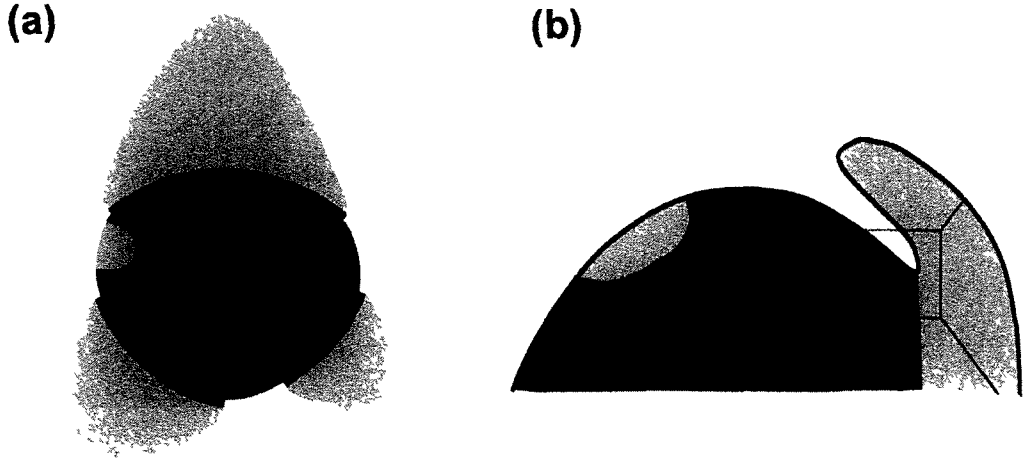

Figure 1.4 Schematic representations of the SAM and boundaries.

(a) Top view of the SAM. Red: meristematic region; blue: boundary domain; green: primordia. CZ: Central Zone. Dotted lines indicate the stem cell domain.

(b) Side view of the SAM.

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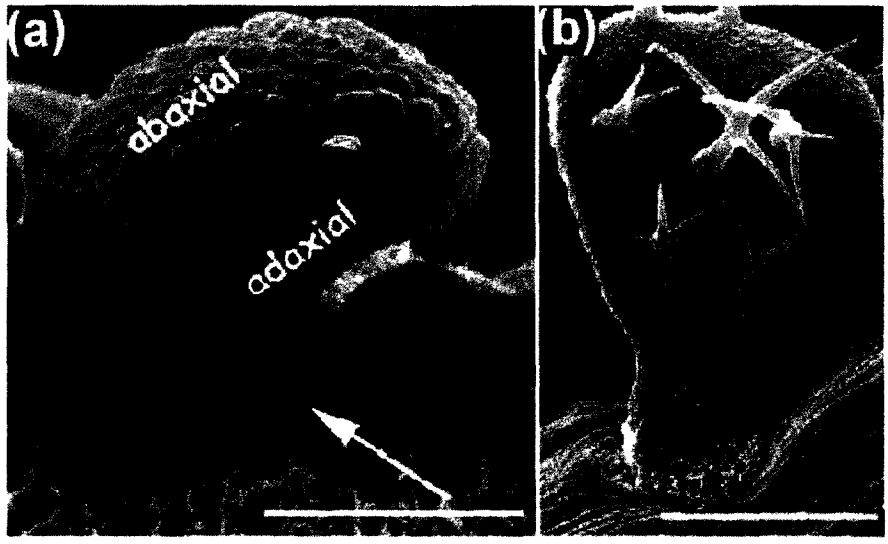

Figure 1.5 Adaxial-abaxial polarity in leaves.

SEM micrographs depicting:

(a) The adaxial and abaxial sides of an young *Arabidopsis* leaf. The adaxial side of the leaf is adjacent to the central SAM (arrow) whereas the opposite, abaxial, side of the leaf is farther from the SAM. Scale bar, 50 μm.

(b) A developing vegetative leaf. Trichomes are marker of adaxial leaf identity in early leaves. Scale bar, 250 μm.

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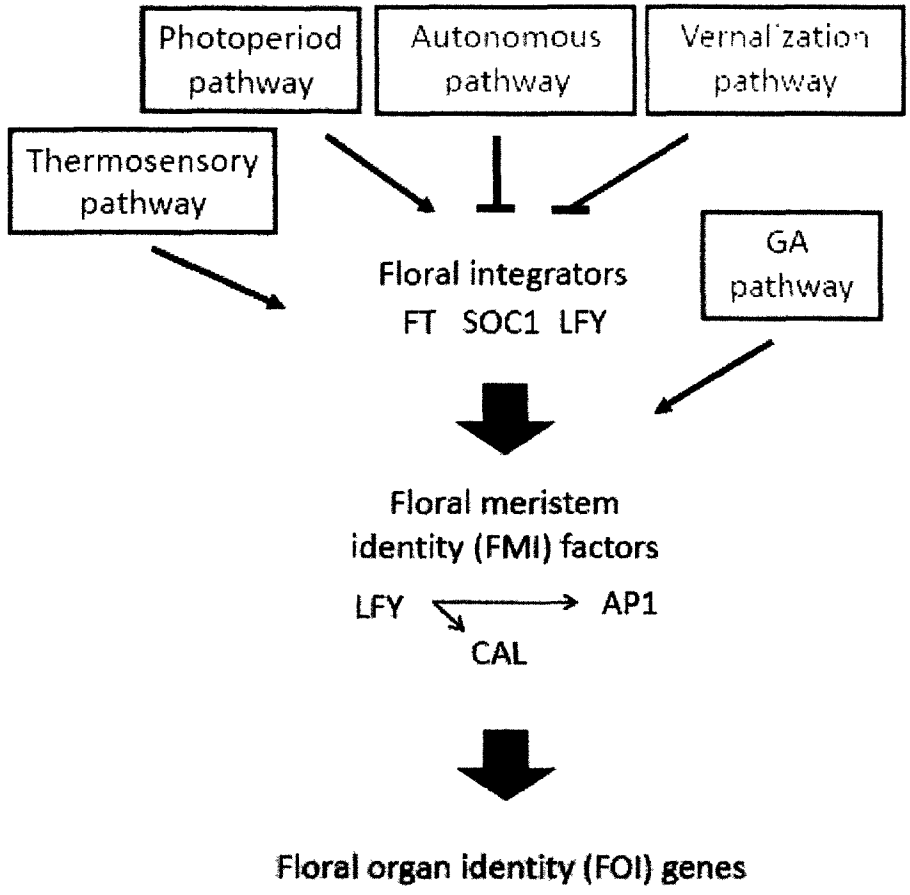

Figure 1.6 Simplified diagram showing the pathways that control FMI.

Positive and negative inputs from five different flowering-time pathways converge to regulate the expression of a small number of genes with floral integrator activity. Upregulation of these genes in shoot apices promotes inflorescence meristem (IM) identity and the activation of floral-meristem identity (FMI) genes in lateral organ primordia. FMI genes in turn promote floral fate by turning on the expression of three overlapping sets of genes that combinatorially specify floral-organ identity (FOI) leading to the formation of flowers.

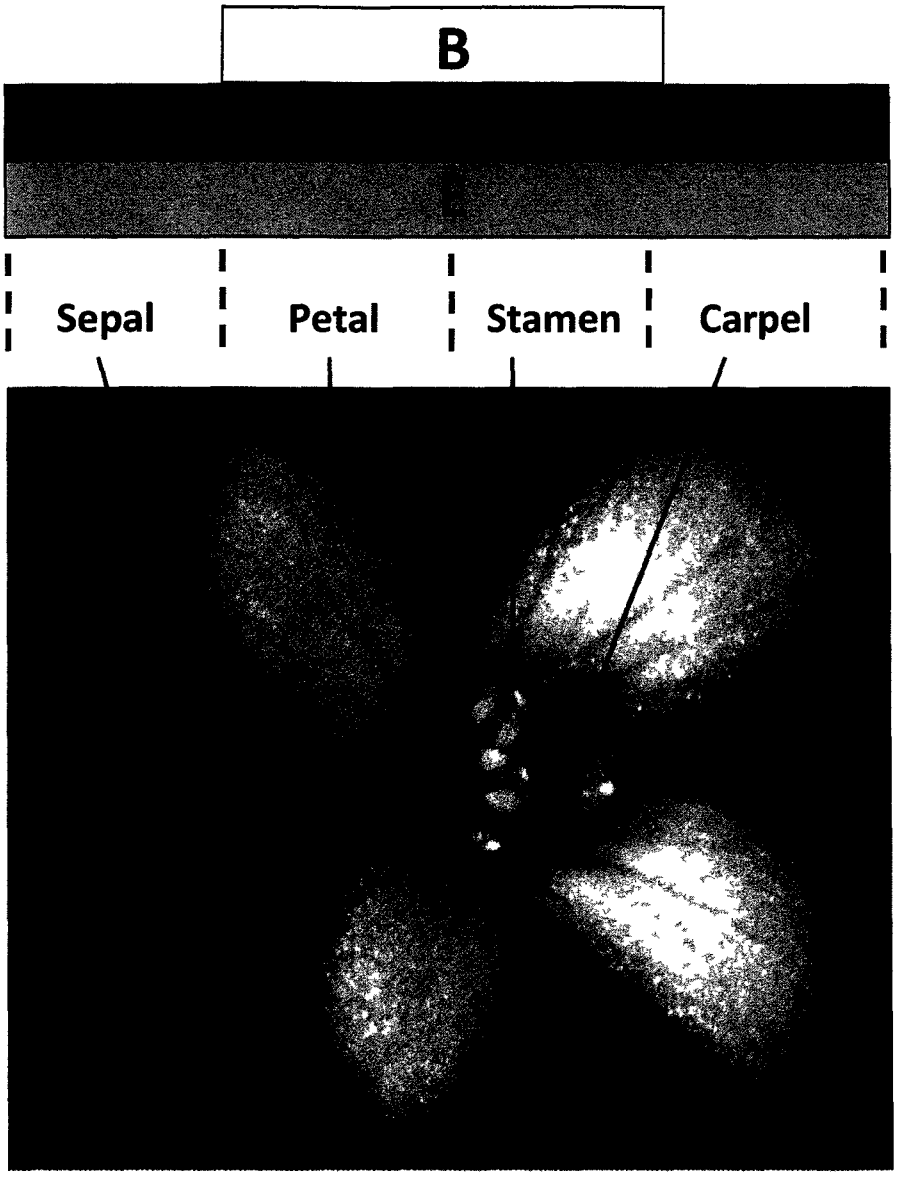

Figure 1.7 The ABCE model of floral development in *Arabidopsis*.

Floral-organ identity is defined by the overlapping expression domains of three sets of genes, termed Class A, B, and C that function in association with Class E genes that are expressed in all four whorls. Class A genes alone specify sepal identity; Class A+B genes specify petal identity; Class B+C genes specify stamen identity; and Class C genes alone specify carpel identity. Class E genes are required in all four whorls of the flower.

Chapter 2

Arabidopsis BLADE-ON-PETIOLE1 and 2 promote floral meristem fate and determinacy in a previously undefined pathway targeting *APETALA1* and *AGAMOUS-LIKE24*

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I carried out all of the experiments described in this Chapter with the following exceptions: Sarah McKim provided the scanning electron micrographs and BiFC data, Tieqiang Hu provided the qRT-PCR data for *AP1*, *AGL24*, *SOC1*, and *FUL* transcripts, and Jhadeswar Murmu made the *pBOP1::BOP1-GFP* plants used for ChIP assays. This article is reprinted with permission of the publisher (John Wiley and Sons).

Abstract

The transition to flowering is a tightly controlled developmental decision in plants. In *Arabidopsis*, *LEAFY* (*LFY*) and *APETALA1* (*AP1*) are key regulators of this transition and expression of these genes in primordia produced by the inflorescence meristem confers floral fate. Here, we examine the role of architectural regulators *BLADE-ON-PETIOLE1* (*BOP1*) and *BOP2* in promotion of floral meristem identity. Loss-of-function *bop1 bop2* mutants show subtle defects in inflorescence and floral architecture but in combination with *lfy* or *ap1*, synergistic defects in floral meristem fate and determinacy are revealed. The most dramatic changes occur in *bop1 bop2 ap1-1* triple mutants where flowers are converted into highly branched inflorescence-like shoots. Our data show that BOP1/2 function distinctly from LFY to upregulate *AP1* in floral primordia and that all three activities converge to down-regulate flowering-time regulators including *AGAMOUS-LIKE24* in stage 2 floral meristems. Subsequently, BOP1/2 promote A-class floral-organ patterning in parallel with LFY and AP1. Genetic and biochemical evidence support the model that BOP1/2 are recruited to the promoter of *AP1* through direct interactions with TGA bZIP transcription factors, including PERIANTHIA. These data reveal an important supporting role for BOP1/2 in remodeling shoot architecture during the floral transition.

2.1 Introduction

The switch from vegetative to reproductive development in *Arabidopsis* is a tightly controlled process mediated by multiple genetic pathways in response to developmental cues and environmental signals (Kobayashi and Weigel, 2007; Turck *et al.*, 2008). Inputs from flowering-time pathways converge to regulate the expression of a small number of genes with floral integrator activity including *LEAFY* (*LFY*), *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) whose up-regulation in shoot apices promotes the production of an inflorescence (Parcy, 2005). The MADS-box flowering-time genes *AGAMOUS-LIKE24* (*AGL24*), *SOC1*, and *SHORT VEGETATIVE PHASE* (*SVP*) encode key determinants of inflorescence meristem identity in *Arabidopsis* (Hartmann *et al.*, 2000; Michaels *et al.*, 2003; Yu *et al.*, 2004; Liu *et al.*, 2007). Early in the transition to flowering, the inflorescence meristem produces cauline leaves, which generate secondary inflorescences in their axils. Subsequently, leaf development is fully repressed and lateral shoots acquire floral fate (Sablowski, 2007).

Two key regulators of floral meristem identity in *Arabidopsis* are *LFY* and *APETALA1* (*AP1*). Expression of these genes in lateral organ primordia confers floral fate (Blázquez *et al.*, 2006). The initial up-regulation of *LFY* specifies floral meristems by activating floral meristem identity genes including the MADS-box transcription factors encoded by *AP1* and *CAULIFLOWER* (*CAL*) and the homeodomain leucine-zipper (HD-ZIP) transcription factor encoded by *LATE MERISTEM-IDENTITY1* (*LMI1*) (Kempin *et al.*, 1995; Liljegren *et al.*, 1999; Saddic *et al.*, 2006). LFY is a direct regulator of *AP1* (Parcy *et al.*, 1998; Wagner *et al.*, 1999) but activation is also directed by a complex of FT/FD (Abe *et al.*, 2005; Wigge *et al.*, 2005) and by SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) proteins that control age related flowering time (Wang *et al.*, 2009; Yamaguchi *et al.*, 2009).

Prior to their specification of floral organ identity, AP1 and LFY activities block the continued expression of inflorescence meristem identity genes in the floral meristem so that these shoots become determinate and develop as flowers (Yu *et al.*, 2004; Liu *et al.*, 2007; Liu *et al.*, 2009a). Over-expression *AGL24*, *SOC1*, or *SVP* partially transforms floral meristems into inflorescence meristems leading to branched flowers and floral bracts. These phenotypes are thereby suppressed in *lfy* and *ap1* by loss-of-function mutations in *agl24*, *soc1*, or *svp* (Yu *et al.*, 2004; Liu *et al.*, 2007). After commitment to flowering, activation of *AGAMOUS* (*AG*) in the dome of the floral meristem leads to repression of the stem-cell organizer *WUSCHEL* (*WUS*) ensuring that shoot determinacy is complete (Lenhard *et al.*, 2001; Lohmann *et al.*, 2001).

BLADE-ON-PETIOLE1 (*BOP1*) and *BOP2* encode BTB-ankyrin transcriptional co-regulators that are expressed in lateral organ boundaries and that control the architecture of leaves, fruits, and flowers. *bop1 bop2* mutants develop leafy petioles and receptacle defects in flowers. Flowers have bracts and two petalloid structures typically replace the abaxial sepal (Hepworth *et al.*, 2005; Norberg *et al.*, 2005; McKim *et al.*, 2008). The role of BOP1/2 is best understood in leaves where they are direct activators of the lateral organ boundary marker *ASYMMETRIC LEAVES2* (Jun *et al.*, 2010). Co-misexpression of meristematic genes such as *BREVIPEDICELLUS* (*BP*) and the blade-promoting transcription factor encoding *JAGGED* (*JAG*) in *bop1 bop2* mutants promotes indeterminacy in leaf petioles leading to ectopic leaflet formation (Ha *et al.*, 2003, 2004, 2007; Norberg *et al.*, 2005).

Two previous reports have provided preliminary evidence that the *BOP* genes also promote floral meristem identity. *In situ* experiments by Karim *et al.* (2009) have suggested that the redundant activities of BOP1/2 and PUCHI, an EREBP transcription factor, promote *LFY* expression in lateral meristems. In *bop1 bop2 puchi* triple mutants, extra inflorescence like

shoots arise with the eventual production of *bop1 bop2*-like flowers. Norberg *et al.* (2005) also showed that *bop1 bop2* enhances *lfy-26* in bract formation and floral meristem identity defects but the mechanism was not addressed.

Here, we use a genetics approach to examine the role of BOP1/2 in the floral transition. Loss-of-function *bop1 bop2* mutants show minor defects in inflorescence and floral architecture but in combination with *lfy* or *ap1*, synergistic defects in floral fate and shoot architecture are revealed. We show that BOP1/2 function in parallel with LFY to control determinacy in floral shoots through activation of *AP1* and repression of *AGL24* in developing flowers. To establish mechanism, we show that BOP1/2 are recruited to the promoter of *AP1* in part through direct interactions with the TGA bZIP factor PERIANTHIA (PAN). These data reveal an important supporting role for BOP1/2 in remodeling shoot architecture during the floral transition.

2.2 Materials and methods

2.2.1 Plant material and growth conditions

Plants were grown on agar plates or in soil at 21°C in long-day (16 h) or continuous (24 h) light. Wild-type was the Columbia-0 (Col-0) ecotype of *Arabidopsis thaliana*. Mutant alleles were obtained from the Arabidopsis Biological Resource Center unless otherwise stated. *bop1-3 bop2-1* and *pan-1* mutants were previously described (Hepworth *et al.*, 2005). The *ap1-1* allele was introgressed into Col-0. The *agl24–3* allele (SALK_095007) was provided by Richard Amasino and *ft-1* (introgressed into Col-0), *fd-2*, and *fd-2 lfy-12* were gifts from Hao Yu and Philip Wigge. Mitsuhiro Aida provided the *bop1 bop2 puchi* mutant and Detlef Weigel provided *spl9* and *spl9 pSPL9:GFPSPL9* lines. All mutant combinations were constructed by crossing and confirmed by genotyping. For genotyping *lfy-2*, a 168-bp product was amplified by PCR; *Bam*HI cleaves only the wild-type product. For genotyping *ft-1*, a 183-bp product was amplified by PCR; *Sac*II

cleaves only the wild-type product. Floral stages were determined according to Smyth *et al.* (1990). Primers are listed in Table 2.1.

2.2.2 Scanning electron microscopy (SEM)

Samples were prepared for SEM as described (Hepworth *et al.*, 2005). Images were acquired on Hitachi VP-6400 (http://www.hitachi-hitec.com) or Tescan VegaII XMU VPSEM (http://www.tescan-usa.com) microscopes.

2.2.3 *BOP2::GUS* reporter lines, GUS staining and *in situ* hybridization

pBOP2::GUS containing the BOP2 5'-UTR (nt -4015 to +16) fused in-frame with the GUS gene *uidA* was created using the strategy described (Hepworth *et al.*, 2002). Wild-type plants were transformed by floral dipping (Clough and Bent, 1998). Tissues were stained for GUS activity, fixed, embedded, and sectioned as described in Sieburth and Meyerowitz (1997). Sections were adhered to glass slides and de-waxed with tert-butanol prior to imaging. *In situ* hybridization and probe synthesis was performed essentially as described (Hepworth *et al.*, 2005). The *AG* probe was based on Drews *et al.* (1991).

2.2.4 RT-PCR and quantitative RT-PCR (qPCR)

Total RNA was isolated from inflorescence apices using Trizol reagent (Invitrogen, http://www.invitrogen.com). Total cDNA was synthesized from 1 μg of RNA template using Superscript III reverse transcriptase (Invitrogen) followed by RT-PCR using Taq polymerase (Invitrogen). *GAPC* served as a control transcript (Hepworth *et al.*, 2005). Band intensities were quantified with AlphaImager software (Cell Biosciences, http://www.cellbiosciences.com). qPCR was performed in triplicate with SYBR Green (Sigma, http:// www.sigmaaldrich.com) and IQ Supermix (BioRad, http://www.bio-rad.com) using a Rotor-Gene 6000 (Qiagen,

http://www.qiagen.com) thermocycler. Conditions were optimized for each primer pair and data quality was verified by melting curve analysis. qPCR was performed with triplicates and relative transcript levels were calculated from mean of threshold cycle values and standard curves. Values were normalized to *GAPC* and then to the wild-type control. Experiments were repeated twice with independently-isolated RNA to ensure reproducibility.

2.2.5 ChIP assay

1 g of 25-day-old *bop1 bop2 BOP1::BOP1–GFP* apices were used for ChIP as described in Saleh *et al.* (2008) omitting the nuclear isolation step. Anti-GFP antibodies were used for immunoprecipitation (ab290; Abcam, http://www.abcam.com). Fold-enrichment of DNA sequences was determined by qPCR in triplicate with *ACTIN2/7* as a control according to Liu *et al.* (2008). Primers are listed in Table 2.2. Assays were repeated twice to ensure reproducibility.

2.2.6 Yeast 2-hybrid analysis

We used the GAL4-based yeast 2-hybrid system described in Kohalmi et al. (1998). For all pair-wise interactions, the bait was BOP2 coding region fused in-frame with the GAL4 DNA-binding domain and the preys were PAN, FD, LFY, AGL24, SPL3, SPL9, SEP1, SEP2, SEP3, or SEP4 coding regions fused in-frame to GAL4 transcriptional activation domain. The BOP2 bait and PAN prey constructs were as described previously (Hepworth et al., 2005). All other prey constructs were constructed likewise. The coding sequences of *FD*, *LFY*, *AGL24*, *SPL3*, *SPL9*, *SEP1*, *SEP2*, *SEP3*, and *SEP4* were amplified by PCR using cDNA derived from inflorescences apices as the template and iProof as the polymerase (Biorad, www.bio-rad.com). PCR products were subcloned into pCR-BluntII-TOPO (Invitrogen, www.invitrogen.com) and sequenced to ensure fidelity. Recognition sites for restriction enzymes were incorporated at the 5' ends of the primers used for amplification to facilitate their subsequent directional cloning into the prey

plasmid pBI-881 (Kohalmi et al., 1998). Co-transformation of yeast with bait and prey plasmids and quantitative determination of β-galactosidase activity were as described previously (Hepworth et al., 2005).

2.2.7 Bimolecular fluorescence complementation (BiFC)

Constructs were based on the pSAT modular vectors modified for BiFC, gifts from Dr. Stanton Gelvin (Citovsky et al. 2006). The pSAT6A vector contained full-length EYFP (Invitrogen, www.invitrogen.com), pSAT1A contained the C-terminal half of EYFP (residues 1-174) and pSAT4A contained the N-terminal end of EYFP (residues 175-stop). The coding sequences of *BOP2* and *PAN* were amplified by PCR from cloned cDNA template using primers incorporating *Kpn*1 sites at the 5' end. Products were digested with *Kpn*I and ligated into the corresponding site of each pSAT vector. All constructs were sequenced to confirm fidelity and translational fusion. Protoplasts were transformed according to Yoo et al. (2007) using 20 μg of plasmid DNA for single vector controls and 10 μg of each plasmid for bimolecular fluorescent complementation co-transformation. Protoplasts were allowed to rest for 12-20 hours before visualization by epifluorescent microscopy.

2.3 Results

2.3.1 Dynamic pattern of *BOP* expression in the inflorescence apex

To clarify the dynamics of *BOP* expression during floral development, we examined the expression pattern of a *GUS* reporter gene driven by the *BOP2* promoter. *BOP2* expression quickly cleared from lateral inflorescence meristems and localized to the axil of cauline leaves (Figure 2.1a). Expression was detected in floral anlagen (stage 0) and in the adaxial portion of stage 1 floral primordia (Figure 2.1c, d). At late stage 2, expression shifted to the boundary

between the floral primordium and the cryptic bract (Figure 2.1a, b, d, e, asterisks). *BOP2* expression was focused in the dome of the floral meristem at early stage 3 but shifted to the sepal axils and was maintained (Figure 2.1c, f, g, arrows). *BOP1::GUS* was expressed in a similar pattern (not shown). These data are in agreement with previous *in situ* data (Hepworth *et al.*, 2005; Norberg *et al.*, 2005; Karim *et al.*, 2009) and are consistent with a role for BOP1/2 in promotion of floral meristem identity.

2.3.2 Weak floral meristem identity defects in *bop1 bop2* mutants

Close inspection of *bop1 bop2* plants grown in inductive photoperiods revealed phenotypes consistent with mild defects in floral meristem identity. Some of these defects were reported previously but here they are considered collectively. First, visible bracts subtended about 20% of flowers grown in continuous light (Table 2.3 and Figure 2.2a). Hepworth *et al.* (2005) showed that bracts are initiated on most flowers at stage 1–2 but that their further development is variable. Second, *bop1 bop2* mutants displayed a small but reproducible increase in secondary inflorescences compared with wild-type (Figure 2.2b; Norberg *et al.*, 2005). Cauline leaves were sometimes absent from the base of shoots preceding the node of first flower (Figure 2.2c). Sometimes this was due to ectopic pedicel elongation causing displacement of the cauline leaf but other times the cauline leaf was lacking indicating that its development was repressed. Third, branched flowers arose at a low frequency (Figure 2.2d; Table 2.3; Ha *et al.*, 2007). This phenotype is characteristic of *ap1* mutants, caused by the ectopic initiation of floral meristems in the axils of sepal-whorl organs, but occurs less commonly in *lfy* mutants (Irish and Sussex, 1990; Schultz and Haughn, 1991, 1993; Weigel *et al.*, 1992). Overall, the range of floral meristem identity defects in *bop1 bop2* closely resembles those in *lfy* mutants consistent with the notion that like LFY, BOP1/2 promote floral fate.

2.3.3 Interactions with LEAFY and LATE-MERISTEM-IDENTITY1

LFY is the central floral meristem identity regulator in *Arabidopsis* and its loss-of-function generates a large increase in secondary inflorescences, floral bracts, and some branched flowers (Schultz and Haughn, 1991; Weigel *et al.*, 1992). Enlarged floral bracts and nodes without flowers were reported for *bop1 bop2* crossed to the strong *lfy-26* mutant suggesting that BOP1/2 and LFY contribute independently to floral meristem identity (Norberg et al., 2005). To examine this further, we crossed *bop1 bop2* mutants to strong (*lfy-1*) and weak (*lfy-2*) alleles and assayed the triple mutants for defects in floral meristem identity (Tables 2.3 and 2.5; Figure 2.3). In continuous light, *lfy-2* plants generated about 3.8 more secondary inflorescences than wild-type but no further increase occurred in triple mutants with *bop1 bop2* suggesting that BOP1/2 do not function redundantly with LFY to control the number of secondary inflorescences (Table 2.3). Comparison of *lfy-2* mutants to *bop1 bop2 lfy-2* triple mutants showed a significant increase in plants with branched flowers (54.4% versus 100%) and floral bracts (4.2% versus 100%). Branching patterns in *bop1 bop2 lfy-2* flowers were more complex than in *lfy-2* with enhanced internode elongation between successive floral organs (Figure 2.3a–c). Bracts in *bop1 bop2 lfy-2* were enlarged and late in the primary inflorescence, nodes containing a bract but no flower developed (Figure 2.3d, g). Bracts and floral branching were also elaborated in *bop1 bop2 lfy-1* mutants (Figure 2.3e, f) confirming separate roles for BOP1/2 and LFY in promotion or maintenance of floral meristem identity.

LFY promotes flowering by activating a suite of downstream floral meristem identity regulators. *LMI1*, which encodes an HD-ZIP transcription factor, is upregulated by LFY in stage 1 flowers before localizing to the cryptic bract (Saddic *et al.*, 2006). Loss-of-function *lmi1* enhances the number of secondary inflorescences in weak *lfy* mutants, showing that LMI1

functions in part as a meristem-identity factor downstream of LFY. LMI1 has a second LFY-independent role in leaf and bract repression: in short-day photoperiods *lmi1* mutants develop petiole leaflets similar to *bop1 bop2* mutants (Hepworth *et al.*, 2005; Saddic *et al.*, 2006). No increase in the number of secondary inflorescences or floral branching occurred in *bop1 bop2 lmi1* triple mutants (Table 2.3) nor were petiole leaflets in *bop1 bop2* mutants enhanced (data not shown). However, the frequency of flowers with bracts was much higher in the triple mutant relative to *bop1 bop2* (53.7% versus 11.2%; Figure 2.3o–r) indicating that BOP1/2 and LMI1 contribute separately to bract repression and have little impact on the number of secondary inflorescences when LFY is functional.

2.3.4 Interactions with APETALA1

The primary target of LFY in promotion of floral fate is *AP1*. Activation of *AP1* together with *CAL* and *LMI1* feed-forward to reinforce *LFY* expression so that floral induction is sharp and unidirectional (Saddic *et al.*, 2006). The strong *ap1-1* (Col) mutant shows elaborate floral branching only in the first few nodes after the switch to flowering (Table 2.3; Irish and Sussex, 1990; Schultz and Haughn, 1993). In *bop1 bop2 ap1-1* triple mutants, floral meristems were dramatically converted to partial inflorescences, with curd-like apices similar to *ap1 cal* mutants (Figure 2.4a–e; Bowman *et al.*, 1993). Highly branched shoots, often indeterminate, developed in place of all floral nodes. Single peduncles showed enhanced floral branching and internode elongation between successive floral organs (Figure 2.4f–j; Tables 2.3 and 2.5). Floral bracts in stage 1–2 flowers of the triple mutant were highly developed relative to parental controls and first whorl organs showed spiral instead of whorled phyllotaxy (Figure 2.5). Branching was enhanced in *bop1 bop2 ap1-12* mutants but all shoots remained determinate, likely due to residual AP1 function in *ap1-12* (Table 2.3 and Figure 2.4l–m). These interactions reveal that

BOP1/2 and AP1 redundantly suppress inflorescence characteristics in floral shoots.

2.3.5 BOP activity has A-class function in flowers

After flowers are initiated, LFY and AP1 promote floral patterning. Floral organ identity is determined by the overlapping activities of three classes of homeotic genes termed A, B, and C that specify sepals, petals, stamens and carpels according to the ABC model (Haughn and Somerville, 1988; Coen and Meyerowitz, 1991). Strong *lfy* mutants lack petals and stamens due lack of B-class gene activation and sepal whorl organs become carpelloid as a result of *AG* misexpression (Drews *et al.*, 1991; Weigel and Meyerowitz, 1993; Liu and Meyerowitz, 1995). Comparison of *lfy-2* and *bop1 bop2 lfy-2* triple mutants showed enhancement of *lfy* floral organ identity defects (Table 2.4 and Figure 2.3c, j–m). Whereas weak *lfy-2* mutants develop some petals and stamens, sepal-like or mosaic sepal/carpel organs formed in all whorls of *bop1 bop2 lfy-2* flowers, with *AG* misexpression in the outer whorls (Figure 2.3i). Mutation of *bop1 bop2* similarly enhanced the carpelloid character of outer-whorl organs in strong *lfy-1* mutants (Table 2.4 and Figure 2.3h, n) similar to *lfy ap1* mutants (Schultz and Haughn, 1993). AP1 also contributes to A-function by specifying sepal and petal identity (Irish and Sussex, 1990; Schultz and Haughn, 1991; Mandel *et al.*, 1992). Flowers in *bop1 bop2 ap1-12* triple mutants closely resembled those in the strong *ap1-1* mutant, lacking petals (Figure 2.4g, l, m; Table 2.4). In *bop1 bop2 ap1-1* triple mutants, sepal-whorl organs gained carpelloid features (Figure 2.4k and Table 2.4) consistent with misexpression of *AG* in sepal margins (data not shown). Overall, these data reveal that BOP1/2 promote A-class floral patterning.

2.3.6 BOP1/2 and LFY are distinct regulators of *AP1* in floral meristems

Given that BOP activity promotes floral meristem identity, we reasoned that *LFY* and/or *AP1* might be targets of regulation. *In situ* hybridization by Karim *et al.* (2009) showed a dramatic

lack of *LFY* expression in *bop1 bop2 puchi* apices, providing one explanation for the severe flower-to-shoot reversions observed in *bop1 bop2 ap1-1* triple mutants. We therefore monitored *LFY* expression in the inflorescence apices of wild-type, *bop1 bop2*, *ap1-1*, and *bop1 bop2 ap1-1* plants grown in continuous light, using *bop1 bop2 puchi* triple mutants as a control. Apices were examined at three time-points: 14-day-old seedlings committed to flowering, 1-cm bolts, and 5-cm bolts. However, *LFY* transcript accumulation in both triple mutants was similar to *ap1-1* control apices in which *LFY* accumulates to WT levels (Figure 2.6; Weigel *et al.*, 1992) indicating that the dramatic flower to shoot phenotypes in *bop1 bop2 ap1-1* mutants cannot easily be attributed to lack of *LFY* expression.

Rather, *in situ* hybridization revealed that in combination with all mutant genotypes examined, loss of BOP activity had a significant impact on *AP1* transcript levels. *AP1* is independently activated by LFY and FD/FT in stage 1 flowers and represents the earliest known marker of commitment to floral fate (Hempel *et al.*, 1997; Wagner *et al.*, 1999; Abe *et al.*, 2005; Wigge *et al.*, 2005). As seen previously, *AP1* transcript in *lfy-1* and *lfy-2* apices was reduced (Figure 2.7; Mandel and Yanofsky, 1995a; Ruiz-Garcia *et al.*, 1997; Liljegren *et al.*, 1999). In *lfy-1* apices, *AP1* first accumulated in the sepal whorl of stage 3 flowers and slightly earlier in *lfy-2* apices (Figure 2.7g–i, m–o). In contrast, little or no *AP1* accumulated in *bop1 bop2 lfy-1* or *bop1 bop2 lfy-2* flowers at stage 1–2, nor was there expression at stage 3 in *bop1 bop2 lfy-1* flowers (Figure 2.7j–l, p–r). Reduced *AP1* expression was also apparent in *bop1 bop2 puchi* control apices (Figure 2.7s–j). These data identify *AP1* as a major target of BOP regulation.

To examine this further, we used qPCR to monitor the expression of *LFY* direct targets *AP1* and *CAL* in the inflorescence apices of *lfy-1* versus *bop1 bop2 lfy-1* triple mutants with 5-cm bolts. Both genes are expressed specifically in floral primordia (Wagner *et al.*, 1999; Ferrandiz *et*

al., 2000; William *et al.*, 2004). A dramatic reduction in *AP1* transcript was observed in *bop1 bop2 lfy-1* triple mutants; *CAL* levels were also slightly reduced (Figure 2.8a). We further monitored *AP1*, *CAL*, and *LMI1* transcript levels: (i) in apices of *lfy-1* versus *bop1 bop2 lfy-1*; and (ii) in *fd-2* versus *fd-2 lfy-12* control apices; using the experimental design of Abe *et al.* (2005) (1-cm bolts; Figure 2.8b). Dramatically lower levels of *AP1* were observed in *bop1 bop2 lfy-1* mutants relative to *lfy-1* mutants [comparable with *fd-2* versus *fd-2 lfy-12* apices (Abe *et al.*, 2005; Wigge *et al.*, 2005)] and in *bop1 bop2 puchi* control apices. *CAL* and *LMI1* transcripts were also slightly reduced in *bop1 bop2 lfy-1* triple mutants compared with *lfy-1*, suggesting that BOP1/2 also have some effect on genes other than *AP1*. This finding promoted us to test if BOP activity resides in the FT pathway. However, levels of *AP1* and *CAL* in *bop1 bop2 ft-1* apices were much lower than in *ft-1* single mutants (Figure 2.8b) and bolting was greatly delayed in *bop1 bop2 ft-1* triple mutants (apex first visible at 54.2 ± 0.66 days, n = 33) compared with *ft-1* mutants (44.4 ± 0.55 days, n = 34) and *ft-10* null mutants (47.8 ± 0.55 days; n = 24). These genetic data indicate that BOP1/2 promote floral meristem identity independently of LFY and FT/FD.

2.3.7 BOP activity contributes to down-regulation of inflorescence identity genes

During the transition to flowering, *AGL24*, *SOC1*, and *FUL* are up-regulated in shoot apices to drive the production of primary and secondary inflorescences (Mandel and Yanofsky, 1995; Hempel *et al.*, 1997; Ferrandiz *et al.*, 2000; Yu *et al.*, 2002; Michaels *et al.*, 2003). Together with AP1, these factors initially promote floral meristem fate but are subsequently down-regulated to permit development of the determinate floral shoot (Yu *et al.*, 2004; Liu *et al.*, 2007, 2009b; Gregis *et al.*, 2008). The architecture of floral nodes in *bop1 bop2 ap1* and *bop1 bop2 lfy-2* triple mutants suggests that BOP activity contributes to this down-regulation. Supporting this, qPCR

analysis showed elevated *AGL24*, *SOC1*, and *FUL* transcript in triple mutant apices relative to control apices (Figure 2.8c). *In situ* hybridization was used to monitor the expression patterns of these genes in more detail.

In wild-type and *bop1 bop2* apices, *AGL24* was expressed in the inflorescence meristem and floral primordia until late stage 2 when expression became restricted to the cryptic bract (Figure 2.9a, b; Michaels *et al.*, 2003). At stage 3, when floral organs begin to differentiate, AGL24 expression sometimes occurred in the dome of *bop1 bop2* flowers (Figure 2.9d). In *ap1-1* and *lfy-2* control apices, ectopic expression of *AGL24* was consistently detected in the dome and/or sepal whorl of stage 2 and 3 flowers (Figure 2.9f, g, h, i). Misexpression was dramatically enhanced in *bop1 bop2 ap1-1* and *bop1 bop2 lfy-2* triple mutants, detected throughout stage 1–3 floral primordia, consistent with their partial conversion into inflorescences (Figure 2.9j, k). Misexpression of *SOC1* and *FUL* in triple mutant apices was similar to *AGL24* (Figures 2.10 and 2.11). These data indicate that BOP1/2, AP1, and LFY activities converge at stage 2 to down-regulate genes that confer inflorescence identity.

2.3.8 Rescue of floral branching but not bract formation by loss-of-function *agl24*

Loss-of-function *agl24* rescues floral branching in *ap1-1* and bract formation in *lfy-6* (Yu *et al.*, 2004; Liu *et al.*, 2007; Ler ecotype) identifying misexpression of *AGL24* as a leading cause flower-to-shoot reversion in these mutants. Whilst loss-of-function *agl24* did not significantly reduce floral branching in *bop1 bop2*, *lfy-2*, or *ap1-1* plants (Col ecotype), branching in the triple mutants *bop1 bop2 ap1-1* and *bop1 bop2 lfy-2* was dramatically rescued as evidenced by steep reductions in the average number of flowers per peduncle and reestablishment of determinacy at all floral nodes in *bop1 bop2 ap1-1* plants (Table 2.5; Figures 2.12 and 2.13). These data confirm that shoot architectural defects in *bop1 bop2 lfy-2* and *bop1 bop2 ap1-1* triple mutants are due in

part to continued expression of *AGL24* in floral meristems.

2.3.9 Evidence that BOP1/2 are recruited to the *AP1* promoter by direct interaction with PAN

BOP1/2 are transcriptional regulators of *AP1* and *AGL24* in floral meristems raising the possibility that this regulation is direct. BOP1/2 are BTB-ankyrin proteins similar to the pathogen defense regulator NPR1, a transcriptional co-activator that exerts most or all of its function via TGA bZIP transcription factors (Zhang *et al.*, 1999; Despres *et al.*, 2000; Rochon *et al.*, 2006; Boyle *et al.*, 2009). BOP similarly interacts with a subset of TGA factors including PAN and BOP1/2-PAN function in the same genetic pathway to control perianth floral organ number (Hepworth *et al.*, 2005). Several other TGA factors are broadly expressed in inflorescence apices (e.g. Li *et al.*, 2009; Maier *et al.*, 2009) suggesting that BOP1/2 may function through one or more of these factors to promote floral meristem identity. Supporting this, *pan-1* mutants display floral meristem identity defects similar to *bop1 bop2* mutants, albeit at a lower frequency: floral bracts (2.8% of plants, n = 71), cauline leaves absent from the base of shoots preceding node of first flower (5.6% of plants, n = 71), and branched flowers (2.68% of node 1–5 flowers, n = 149) (Figure 2.14a–c). Using bimolecular fluorescence complementation (BiFC) assays, BOP–PAN interaction was confirmed in the nucleus of *Arabidopsis* mesophyll cells (Figure 2.15b–j). Conversely, no interaction in yeast was detected between BOP proteins paired with LFY, FD, AGL24, SPL3, SPL9, or SEPALATTA1-4 (Figure 2.15a; data not shown) representing other direct regulators of *AP1* and *AGL24* (Wagner *et al.*, 1999; Wigge *et al.*, 2005; Liu *et al.*, 2007, 2008; Gregis *et al.*, 2008; Wang *et al.*, 2009; Yamaguchi *et al.*, 2009). Using the AthaMap tool (Galuschka *et al.*, 2007) we identified potential TGA binding sites in the promoter of *AP1* (Figure 2.14d) and tested for BOP1 occupancy at these sites using chromatin

immunoprecipitation (ChIP) assays. These assays used *bop1 bop2* plants complemented by a pBOP1:BOP1–GFP fusion protein. Strong and selective occupancy of BOP1–GFP was reproducibly detected at sites 1 and 3 in the *AP1* promoter (Figure 2.14f). Site 3 maps close to binding sites for FD, LFY, and SPL3/9 in the main control region for *AP1* (Parcy *et al.*, 1998; Wigge *et al.*, 2005; Wang *et al.*, 2009; Yamaguchi *et al.*, 2009). Enrichment of GFP–SPL9 at site 1 in the *AP1* promoter served as a positive control (Figure 2.14f; Wang *et al.*, 2009). These results collectively provide strong evidence that BOP1/2 are recruited to sites in the *AP1* promoter via TGA binding factors where they function as transcriptional co-regulators thereby expanding the role of TGA transcription factors in development and revealing a previously undefined pathway for promotion of floral fate.

2.4 Discussion

In this study, we use a genetics approach to examine the role of architectural regulators BOP1 and BOP2 in promotion of floral fate. These genes are expressed in lateral organ primordia, including floral meristems, together with regulators of floral meristem identity including LFY, LMI1, and AP1. Loss-of-function *bop1 bop2* show only subtle defects in floral fate making their function more easily analyzed in sensitized genetic backgrounds. Double mutants with *lfy*, *ap1*, and *lmi1* mutants define in detail how BOP activity promotes floral meristem identity (Figure 2.16). We show that BOP1/2 promote flowering distinctly from LFY, contributing to activation of *AP1* and repression of inflorescence identity genes including *AGL24*, *SOC1*, and *FUL*, crucial for generating a determinate floral shoot. At stage 3, BOP1/2 exerts A-class floral patterning activity in parallel with LFY and AP1. Several lines of evidence support the model that BOP1/2 bind in vivo to regulatory sequences in the *AP1* promoter through direct interaction with TGA transcription factors, including PAN.

2.4.1 BOP activity is required for maintaining floral fate

Mutations that impair floral meristem identity cause the full or partial reversion of flowers into lateral branches. Delayed commitment to floral fate is associated with extra secondary inflorescences and floral bract outgrowth corresponding to defects in LFY expression or activity (Liu *et al.*, 2009a). In *ap1-1* mutants, *LFY* transcript accumulation is normal but maintenance of floral fate is compromised leading to branched flowers (Weigel *et al.*, 1992). The combination of *bop1 bop2* with *ap1-1* also shows robust expression of *LFY* throughout the floral transition and normal levels of *CAL* (data not shown) but extreme floral branching and indeterminate shoot growth, indicating that BOP1/2 plays a strong role in commitment to floral fate. The situation is similar in *bop1 bop2 puchi* plants (see also Karim *et al.*, 2009). Moreover, *bop1 bop2* mutations fail to increase the formation of secondary inflorescences in weak *lfy* and *lmi1* mutants but rather increase bract formation and floral branching. These data indicate that BOP1/2 function in parallel with LFY and that the redundant activities of BOP1/2 and AP1 enforce floral fate.

2.4.2 Down-regulation of flowering-time genes

In stage 2 floral meristems, down-regulation of inflorescence identity genes prevents continuation of the shoot developmental program and permits LFY and AP1 to initiate differentiation of floral organs (Liu *et al.*, 2009a). *AGL24*, *SOC1*, and *SVP* are directly repressed by AP1, whereas repression by LFY is indirect (Yu *et al.*, 2004; Liu *et al.*, 2007; Gregis *et al.*, 2008). BOP1/2 contributes to this down-regulation as evidenced by dramatic misexpression of *AGL24*, *FUL*, and *SOC1* in *bop1 bop2 ap1-1* and *bop1 bop2 lfy-2* apices. *SVP* was not a target of BOP repression (data not shown). Loss-of-function *agl24* strongly suppressed floral branching in triple mutants but rescue was incomplete, likely due to continued misexpression of *SOC1* and *FUL*. It remains unclear if BOP1/2 directly repress *AGL24*, but the promoter contains two TGA

binding motifs (not shown). In petioles, BOP1/2 promotes determinacy by repression of *BP* (Ha *et al.*, 2003, 2007; Chapter 3) but the mechanism appears to differ in flowers as *BP* is not misexpressed in triple mutant apices nor do *bp* mutations rescue floral branching (data not shown).

2.4.3 Bract formation

Arabidopsis flowers develop in the absence of a visible bract, which is specified but repressed in its development (Hepworth *et al.*, 2006). The number and/or size of bracts was enhanced in all double mutant combinations tested: *lfy lmi1*, *bop1 bop2 lfy*, *bop1 bop2 lmi1*, *bop1 bop2 ap1*, and *bop1 bop2 agl24–3*, indicating that several inter-related pathways contribute to bract repression. Of these, *LMI1* and *AGL24* expression localizes to the cryptic bract in late stage 2 whereas *BOP1/2* expression localizes to the boundary between the floral meristem and the cryptic bract (this study; Karim *et al.*, 2009; Saddic *et al.*, 2006). *LFY* and *AP1* are expressed in the floral meristem contributing non-cell autonomously to bract repression (Sessions *et al.*, 2000; Hepworth *et al.*, 2006). One mutual target of repression is *JAG*, whose mutation alleviates bract formation in *lfy* and strong *ap1* mutants (Dinneny *et al.*, 2004; Ohno *et al.*, 2004). BOP1/2 also represses *JAG* in the cryptic bract but *jag* mutations fail to rescue bract formation in *bop1 bop2* mutants supporting the involvement of additional factors (Norberg *et al.*, 2005).

2.4.4 BOP1/2 and LFY are independent regulators of *AP1*

BOP1/2 and LFY are co-expressed in floral anlagen and activate *AP1*, a key marker of commitment to floral fate. Apical expression of *AP1* is strongly delayed in *lfy* mutants and essentially abolished in *lfy ft* and *lfy fd* double mutants indicating that FT/FD and LFY are the major direct regulators of *AP1* expression (Parcy *et al.*, 1998; Abe *et al.*, 2005; Wigge *et al.*, 2005). SPL transcription factors provide additional positive input (Wang *et al.*, 2009; Yamaguchi

et al., 2009). In *lfy ft* double mutants, leaves are generated in place of flowers (e.g. Ruiz-Garcia *et al.*, 1997). Similar nodes containing only a bract occurred late in the primary inflorescence of *bop1 bop2 lfy* plants providing evidence that BOP and LFY are independent regulators of floral meristem fate. ChIP analysis showed enrichment of BOP1–GFP at two potential TGA binding sites in the *AP1* promoter close to binding sites for LFY, FD, and SPL3/9 (Parcy *et al.*, 1998; Wigge *et al.*, 2005; Wang *et al.*, 2009; Yamaguchi *et al.*, 2009). Consistent with this, we found weak floral meristem identity defects in *pan-1* mutants similar to *bop1 bop2* and show that BOP2 interacts selectively with PAN over known direct regulators of AP1 expression. BOP1/2 interact in yeast with several TGAs, including PAN, which functions in the same genetic pathway as BOP1/2 to control sepal number in flowers (Hepworth *et al.*, 2005). Several other TGAs are expressed in inflorescence apices including TGA2, TGA3, and TGA7, which interact with the floral glutaredoxin ROXY1 (Xing *et al.*, 2005; Li *et al.*, 2009) and TGA4, which binds *in vitro* to the *FT* promoter and with CONSTANS, a direct regulator of *FT* (Samach *et al.*, 2000; Song *et al.*, 2008). One of these may preferentially contribute to repression of *AGL24* since no enhancement of floral branching occurs in *pan-1 ap1-1* mutants (data not shown). Thus far, genetic redundancy has hampered our attempts to identify which of these might function with PAN in the floral transition.

2.4.5 BOP activity regulates shoot architecture by controlling determinacy

The transition to flowering involves dramatic changes in shoot architecture, beginning with the formation of primary and secondary inflorescences and ending with the production of flowers. How changes in shoot architecture are coordinated with the decision to flower remains unclear. In leaves, BOP1/2 control the determinacy and hence architecture of petioles through co-repression of *KNOXI* homeobox genes and blade-growth regulators such as *JAG* (Ha *et al.*, 2004,

2007; Jun *et al.*, 2010). This study shows that BOP1/2 likewise control determinacy and architecture of floral shoots through activation of *AP1* and repression of *AGL24*. Shoot determinacy further depends on the activation of *AG* at the dome of the floral meristem causing termination of the stem cell population. LFY and WUS are the main activators of *AG* (Busch *et al.*, 1999; Lenhard *et al.*, 2001; Lohmann *et al.*, 2001) but positive input is also provided by PAN (Das *et al.*, 2009; Maier *et al.*, 2009). Similar to *pan-2 lfy* mutants, unfused carpels occur in *bop1 bop2 lfy-1* flowers (Das *et al.*, 2009; Figure 2.3h) indicating that BOP1/2 may likewise fine-tune *AG* expression in developing flowers, but this remains to be tested. Unlike flowering-time regulators, BOP1/2 are broadly expressed in initiating lateral organs and their loss-of-function affects leaf, fruit, and floral architecture (e.g. Ha *et al.*, 2003; Hepworth *et al.*, 2005; McKim *et al.*, 2008). Plants over-expressing BOP1 or 2 are reduced in stature with clustered or downward-pointing siliques, similar to mutation of *BP* or *PENNYWISE* (*PNY*) (Norberg *et al.*, 2005; Ha *et al.*, 2007). Interestingly, double mutants of *pny* and the related gene *poundfoolish* (*pnf*) cannot complete floral evocation: flowering signals direct the up-regulation of floral integrators *FT*, *SOC1*, and *FUL* but *LFY* and *AP1* are not activated nor is an inflorescence generated (Smith *et al.*, 2004; Kanrar *et al.*, 2008) indicating that PNY/PNF coordinate shoot architecture and flowering. Our work indicates that BOP1/2 are likely also involved in this co-ordination and illustrates how modulation of determinacy contributes to shoot architectural diversity.

Table 2.1 Primers used for genotyping, making *BOP2::GUS* construct, qPCR analysis, and making *in situ* hybridization probes.

Primer	Sequence 5' – 3'
Genotyping	
lfy-2 dCAPs F	GTTTGGGGACAGAGAGACAGAGGGAGGATC
lfy-2 dCAPs R	CGCCACGGTCTTTAGCAATTGTCTGG
agl24-3 F	GAATGAGAGACATATTGGGAAGGTA
agl24-3 R	AAGTGTCGGAGTCATCCTCAAG
4H Salk RP	CGTACCCTTTGATTTTAGTATGCTG
4H Salk LP	GCACAATCTTTCGACTTCATCACC
5H Salk RP	CCCTTTTTATAATCAGCATCAAGA
5H Salk LP	TCGACGCCGAAGTAACGAGAG
ft-1-F	TACAATTGTCAGAGGGAGAGTGGCCGCG
ft-1-R	CACTTATATATTGAACTACTATAGGCATCAT
GUS reporter	
BOP2-4kb-Bam-F1	ATAGGATCCGAGAAAGGTGAAGAAAAGGGA
BOP2-4kb-Nco-R1	TCACCATGGCTTCAAGATTGCTCATCTT
qPCR	
LFY qPCR-F1	TCTAGACGCCGTCATTTGCTA
LFY qPCR-R1	CCTCAGATAACCCTTCTTGGG
AP1 qPCR-LF	TCCACTGATTCTTGTATGGAGAAG
AP1 qPCR-RP2	TCTTCCCCAAGATAATGCCTCTGGT
agl24 P15	GAGGCTTTGGAGACAGAGTCGGTGA
agl24 P16	AGATGGAAGCCCAAGCTTCAGGGAA
SOC1 qPCR-F2	GCTCCAATATGCAAGATACCATAG
SOC1 qPCR-R2	CCCAAGAGTTTACGTTTAGAAGC
FUL qPCR-F1	CTCCAGAAGAAGGATAAAGCCT
FUL qPCR-R1	ACAAAGCCATCTCTGGAGGAG
CAL qPCR-F1	GCTTACCAAACAGATAAAGGAGAG
CAL qPCR-R1	GGTACAAACCACCCATATTTAGG
***In situ* probes**	
AP1-LF	TCCACTGATTCTTGTATGGAGAAG
AP1-T7-RR	CATAATACGACTCACTATAGGTATGATGATATAAGAACATCGAACATTTG
LFY-LF	GATCCTGAAGGTTTCACGAGTGGC
LFY-T7-RR	CATAATACGACTCACTATAGGCAGTGGAGAGCGTAACAGTGAACG
AGL24 RT-F	ATGGCGAGAGAGAAGATAAGGATAAAG
AGL24 T7-RP2	CATAATACGACTCACTATAGGGACCAATAACACGTACAATATCTGAAAC
SOC1-LF	ATGGTGAGGGGCAAAACTCAGAT
SOC1-T7-RR	CATAATACGACTCACTATAGGGAGGGAAGAAAGCTAAATTGATACATG
FUL-RT-F1	TCATTTCAGGGTTGTCGTTTCT
FUL-T7-R1	CATAATACGACTCACTATAGGGGACAACGGAGTTCCATCATACAT

Table 2.2 Primers for qPCR of ChIP products

Primer	Sequence 5' – 3'
AP-1F	CAATATATATGGATATAACGTACAAAC
AP-1R	GATGATCAGGACAAAAATCAGTTG
AP-2F	TATTTTGGTTGGTTCAGATTTTGTT
AP-2R	ACTGGTCCTTCCCCAAGTGT
AP-3F	CACGAGACGTCGATAATCAAATTG
AP-3R	GGTTTCTTTAGGATTTGCGTGTCG
AP-4F	AAAAGGATCAAAAATGGGAAGGGG
AP-4R	CCCTTATGGGAGAAGACAACAAGAGC
ACTIN-F	CGTTTCGCTTTCCTTAGTGTTAGCT
ACTIN-R	AGCGAACGGATCTAGAGACTCACCTTG

Table 2.3 Quantitative analysis of floral meristem identity phenotypes in wild-type and mutants

Genotype	No. secondary inflorescences	Flowers with bracts	Plants with branched flowers	Branched flowers
WT (Col)	1.94±0.32 (n=36)	0.0% (n=360)	0.0% (n=24)	0.0%
bop1 bop2	2.83±0.14* (n=36)	22.3% (n=235)	14.9% (n=22)	0.3%
lfy-2	5.74±0.23 (n=35)	4.2% (n=407)	54.5% (n=22)	5.6%
bop1 bop2 lfy-2	5.51±0.28 (n=29)	100% (n=281)	100% (n=32)	60.1%
WT (Col)	n.d.	0.0% (n=333)	0.0% (n=12)	0.0%
bop1 bop2	n.d.	28.6% (n=297)	25.0% (n=12)	1.0%
lfy-1	n.d.	51.5% (n=94)	100% (n=12)	18.1%
bop1 bop2 lfy-1	n.d.	100% (n=89)	100% (n=6)	29.2%
WT (Col)	1.69±0.11 (n=36)	0.0% (n=360)	0.0% (n=24)	n.d.
bop1 bop2	3.17±0.12* (n=36)	11.2% (n=420)	0.0% (n=24)	n.d.
lmi1-1	1.78±0.11 (n=36)	0.0% (n=360)	0.0% (n=24)	n.d.
bop1 bop2 lmi1-1	2.54±0.11 (n=36)	53.7% (n=808)	0.0% (n=24)	n.d.
WT (Col)	1.65±0.11 (n=35)	0.0% (n=525)	0.0% (n=35)	0%
bop1 bop2	2.67±0.14* (n=36)	21.6% (n=540)	0.0% (n=36)	0%
ap1-12	2.04±0.16 (n=26)	0.0% (n=390)	100% (n=26)	27.7%
ap1-1	2.7±0.11 (n=27)	0.0% (n=525)	100% (n=35)	35.8%
bop1 bop2 ap1-12	2.94±0.11 (n=32)	31.7% (n=480)	100% (n=32)	81.0%
bop1 bop2 ap1-1	5.4±0.34 (n=17)	69.2% (n=240)	100% (n=19)	89.5%
WT (Col)	1.83±0.17 (n=12)	0% (n=180)	0% (n=12)	0%
agl24-3	2.33±0.26 (n=12)	0% (n=180)	0% (n=12)	0%
lfy-2	9.71±0.60 (n=17)	3.9% (n=255)	70.6% (n=6)	14.9%
lfy-1	11.50±0.50 (n=6)	32.8% (n=171)	83.0% (n=6)	9.9%
agl24-3 lfy-2	11.30±0.50 (n=20)	75.4 (n=285)	80.0% (n=20)	15.4%
agl24-3 lfy-1	12.90±0.76 (n=13)	95.3% (n=358)	53.9% (n=13)	3.6%

*Significantly different from WT as determined by a Student's t-test, $p<0.05$.

Table 2.4 Quantitative analysis of floral-organ identity phenotypes in wild-type and mutants

Genotype	No[a] of flowers scored	Sepal whorl		Petal whorl			Stamen whorl		Carpel whorl	
		sepal	carpelloid attributes	petal	sepalloid attributes	carpelloid attributes	stamen	sepalloid or petalloid attributes	fused	unfused
WT (Col)	20	4 00±0 00		4 00±0 00			5 90±0 07		2 00±0 00	
bop1 bop2	23	4 78±0 09		4 04±0 00			6 74±0 09			
lfy-2	90	3 97±0 02		3 11±0 09	0 10±0 05			2 04±0 09	1 78±0 08	0 34±0 09
bop1 bop2 lfy-2	34	1 03±0 23	1 97±0 67*	0 03±0 03	0 53±0 19	0 56±0 18*	0 68±0 16	0 32±0 12	0 85±0 18	1 21±0 18*
lfy-1	51	3 90±0 04	0 04±0 03	0 02±0 02	2 57±0 19	0 78±0 14	0 08±0 04	0 25±0 09	0 68±0 15	1 56±0 16
bop1 bop2 lfy-1	21	1 76±0 30	2 05±0 33*	0 00±0 00	0 10±0 10	3 05±0 30*	0 00±0 00	0 00±0 00	0 10±0 10	2 67±0 22*
WT (Col)	25	4 00±0 00		4 00±0 00			5 75±0 09		2 00±0 00	
bop1 bop2	25	4 88±0 07		4 42±0 07			6 70±0 09		2 00±0 00	
ap1-12	25	3 24±0 16		1 36±0 27			5 68±0 10		2 00±0 00	
bop1 bop2 ap1-12	25	4 20±0 12	0 04±0 04	0 44±0 16	0 08±0 05		5 80±0 18	0 52±0 15	2 00±0 00	
ap1-1	25	3 16±0 19		0 08±0 05			5 68±0 13	0 44±0 17	2 00±0 00	
bop1 bop2 ap1-1	29	3 38±0 17	0 72±0 16*	0 38±0 14			6 00±0 11	0 10±0 06	2 00±0 00	

[a]Flowers from nodes 1-15 were scored

* Significantly different from the *lfy* parental control as determined using a Student's t-test, $p<0.05$.

Table 2.5 Quantitative analysis of floral branching and bract suppression by loss-of-function *agl24*

Genotype	No. of secondary inflorescences	No. of flowers per pedicel/peduncle			No. of plants scored	Flowers with bracts (%)
		Floral node 1-5	6-10	11-15		
WT (Col)	n.d.	1.00±0.00	1.00±0.00	1.00±0.00	24	0.0 (n=360)
bop1 bop2	n.d.	1.04±0.02	1.05±0.03	1.00±0.00	22	26.5 (n=287)
ap1-1	n.d.	3.84±0.23	1.50±0.14	1.03±0.01	25	0.0 (n=375)
agl24-3	n.d.	1.00±0.00	1.00±0.00	1.00±0.00	24	0.0 (n=360)
ap1-1 agl24-3	n.d.	2.58±0.20	1.20±0.04	1.01±0.01	30	0.0 (n=360)
bop1 bop2 agl24-3	n.d.	1.01±0.01	1.00±0.00	1.00±0.00	24	56.8 (n=572)
bop1 bop2 ap1-1	n.d.	9.95±0.87	6.42±0.42	3.56±0.33	22	73.6 (n=336)
bop1 bop2 ap1-1 agl24-3	n.d.	3.96±0.30*	2.72±0.28*	1.45±0.15*	20	79.0 (n=238)
WT (Col)	2.28±0.18	1.00±0.00	1.00±0.00	1.00±0.00	18	0.0 (n=270)
bop1 bop2	3.43±0.25	1.01±0.01	1.00±0.00	1.00±0.00	23	10.1 (n=345)
lfy-2	9.71±0.60	1.23±0.07	1.08±0.04	1.20±0.06	17	3.9 (n=255)
agl24-3	4.90±0.35	1.00±0.00	1.00±0.00	1.00±0.00	24	0.0 (n=360)
lfy-2 agl24-3	11.2±0.52	1.33±0.04	1.18±0.05	1.24±0.07	19	75.4 (n=285)
bop1 bop2 agl24-3	4.45±0.29	1.01±0.01	1.00±0.00	1.00±0.00	22	51.2 (n=330)
bop1 bop2 lfy-2	9.18±0.83	2.33±0.17	n.d.[a]	n.d.[a]	11	100 (n=165)
bop1 bop2 lfy-2 agl24-3	9.35±0.35	1.03±0.06*	n.d.[a]	n.d.[a]	23	100 (n=345)

*Significantly different from the triple mutant control as determined using a Student's t-test, $p<0.05$.

[a]Floral branching could not be accurately scored due to presence of nodes without a flower (bract only): *bop1 bop2 lfy-2* (21.4%, n=121), *bop1 bop2 lfy-2 agl24-3* (49.36%, n=235)

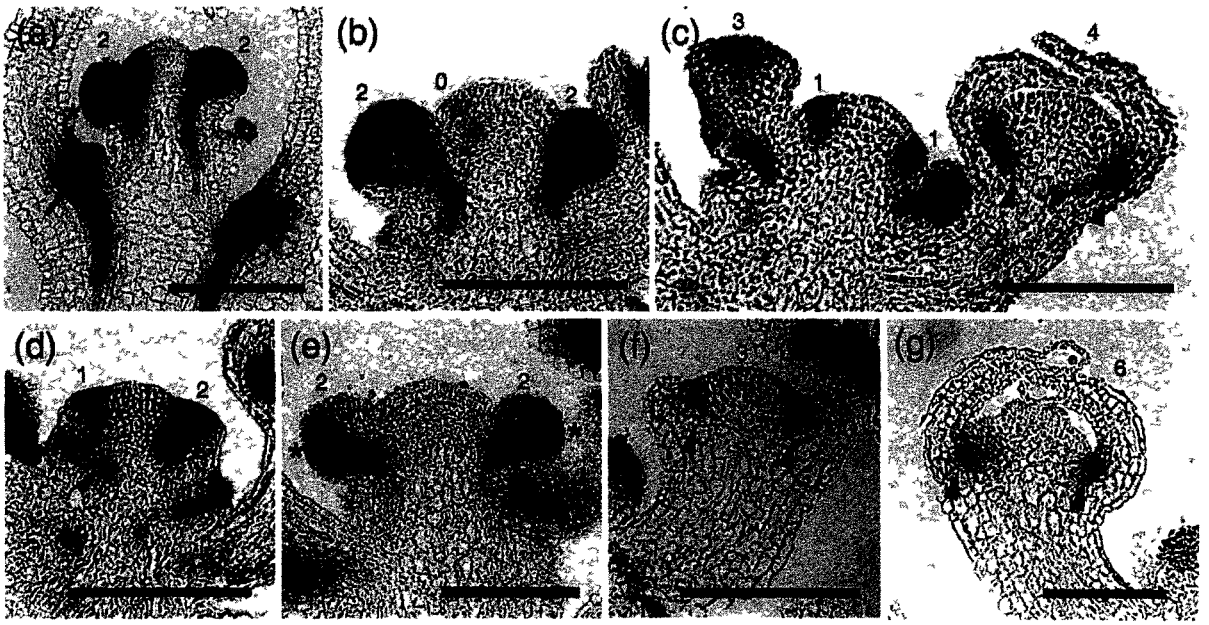


Figure 2.1 *BOP2::GUS* expression in inflorescence apices.

(a) 14-day-old seedling; expression at the boundary of a lateral shoot meristem (arrow).

(b-e) Expression begins in floral anlagen (0) and associates with the adaxial part of floral meristems until late stage 2 when expression shifts to the floral meristem/bract boundary (asterisks).

(c, f, g) Expression is in the dome of early stage 3 flowers but shifts to sepal axils (arrowheads). Numbers in panels indicate floral stage. Scale bars, 100 μm

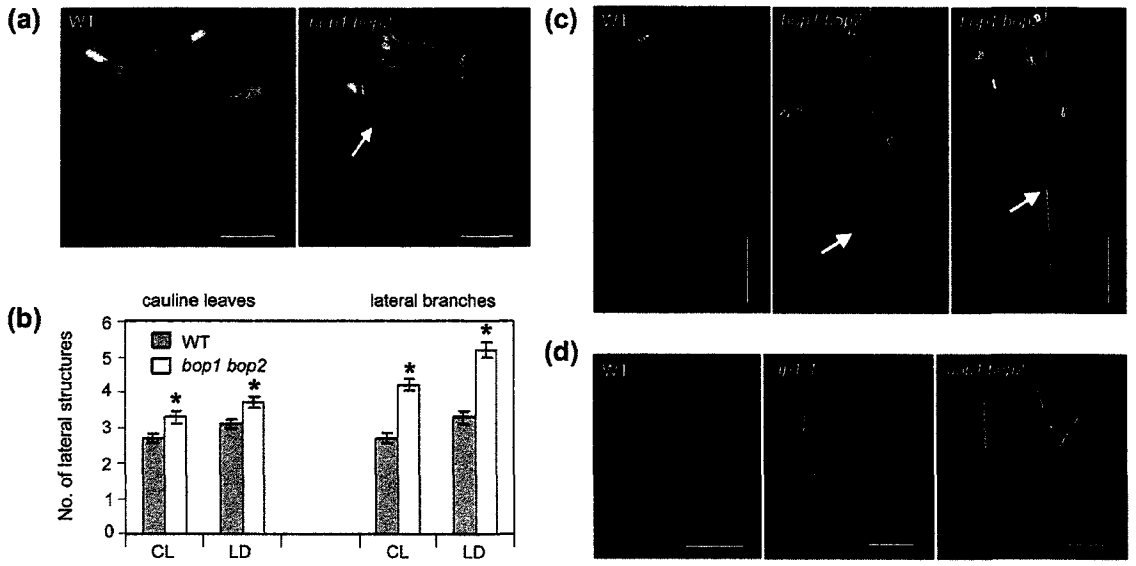


Figure 2.2 Analysis of floral meristem identity defects in *bop1 bop2* mutants.

(a) Flowers in *bop1 bop2* plants often have a bract (arrow). Scale bars, 2 cm.

(b) Graph showing that lateral branches are more numerous in *bop1 bop2* mutants relative to WT in continuous light (CL) or long-day (LD) photoperiods and that not all branches have a cauline leaf. Asterisks indicate a significant difference from WT (Student's t-test, $p<0.05$). Error bars indicate s.e.m.

(c) Cauline leaves may be absent or displaced at transitional nodes in *bop1 bop2* plants (arrows). Scale bars, 0.5 cm.

(d) Siliques of WT, *ap1-1*, and *bop1 bop2* mutants. Scale bars, 1 cm.

Figure 2.3 Mutation of *bop1 bop2* enhances *lfy* and *lmi1-1* floral defects.

(a) *lfy-2*.

(b) *bop1 bop2 lfy-2*; enhanced branching complexity (arrow).

(c) SEM of *bop1 bop2 lfy-2* flower; carpelloid sepals and internode elongation between floral organs (arrow).

(d) Comparison of floral bracts in *bop1 bop2*, *lfy-2*, and *bop1 bop2 lfy-2* mutants.

(e-f) Comparison of *lfy-1* and *bop1 bop2 lfy-1* inflorescences; bracts are larger and more numerous (arrows).

(g) *bop1 bop2 lfy-2* bract-only nodes.

(h, j-n) Representative flowers of the indicated genotypes. Ectopic stigmatic papillae and ovules and unfused carpels are more prevalent in *bop1 bop2 lfy-2* and *bop1 bop2 lfy-1* flowers compared with *lfy-2* or *lfy-1* respectively.

(i) Misexpression of *AG* in the perianth whorls of *bop1 bop2 lfy-2* triple mutant flowers.

(o-r) Representative inflorescences of the indicated genotypes; bract formation in *bop1 bop2* is enhanced by *lmi1* (arrows). Scale bars: 1 mm except a-b, d, 5 mm; c, 0.5 mm; i, 100 μm; o-r, 2 cm.

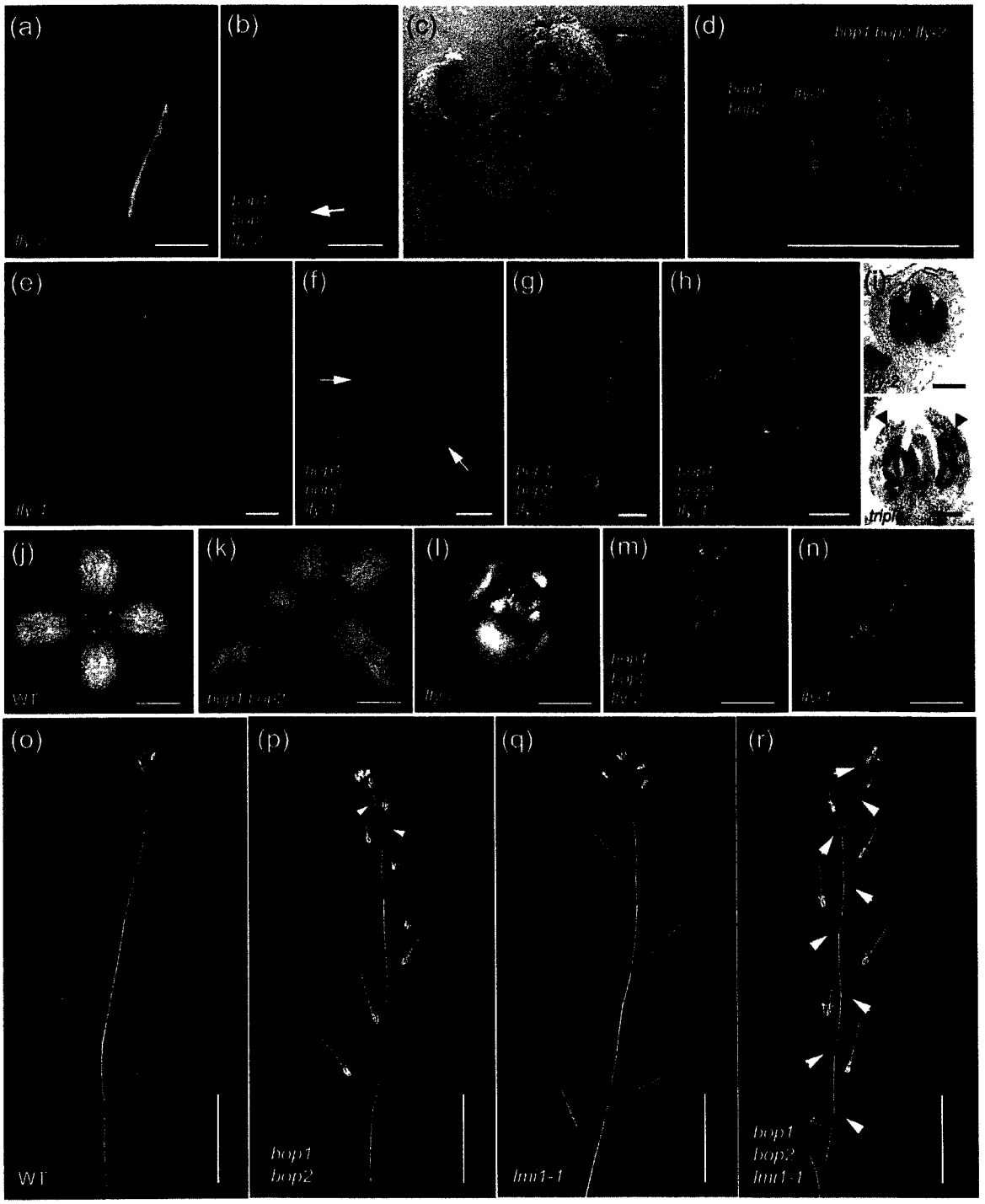

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
(m)
(n)
(o)
(p)
(q)
(r)
WT
bop1
bop2
lmi1-1
bop1
bop2
lmi1-1

Figure 2.4 Mutation of *bop1 bop2* enhances *ap1* floral defects.

(a-d) Representative inflorescences for the genotypes indicated. Arrows denote node of first flower. Floral nodes in *bop1 bop2 ap1-1* are highly branched and often indeterminate.

(e) Curd-like *bop1 bop2 ap1-1* inflorescence apex.

Comparison of floral architectures:

(f) *bop1 bop2;* most flowers are unbranched, abaxial sepals are petalloid.

(g) *ap1-1*; secondary flowers in the axils of first-whorl floral organs.

(h) *bop1 bop2 ap1-1* floral shoot with determinate architecture. Large leafy sepal/bracts subtend flowers (arrows).

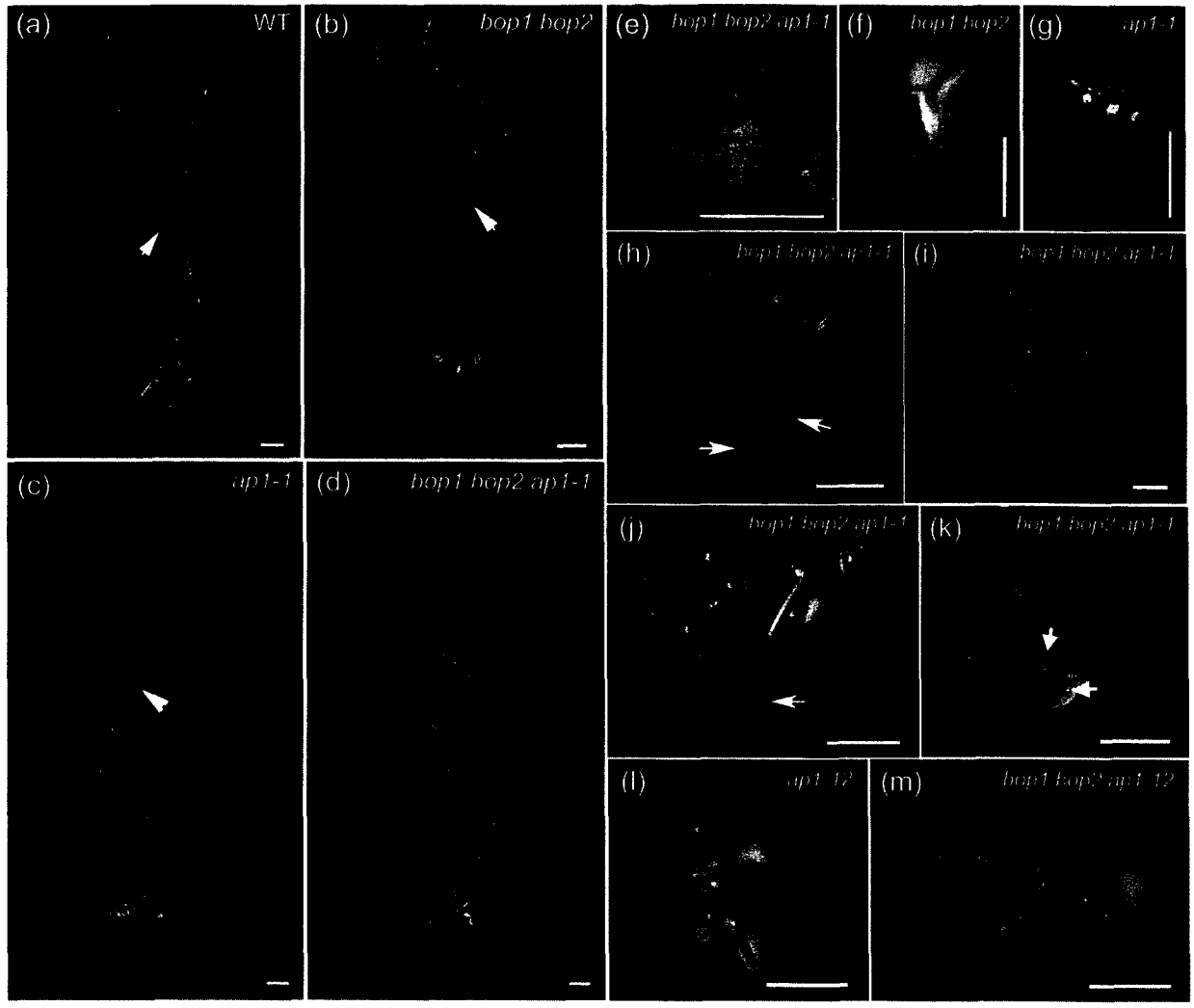
(i) Late stage *bop1 bop2 ap1-1* floral node showing complex branching architecture.

(j) Early stage *bop1 bop2 ap1-1* floral node; internode elongation between first-whorl organs and floral bracts (arrow).

(k) *bop1 bop2 ap1-1* flower; arrows indicate ovules on the margin of carpelloid sepals.

(l) *ap1-12* flower; weak allele.

(m) *bop1 bop2 ap1-12* flower; similar to *ap1-1*. Scale bars: a-d, 2.5 cm; e-g, 2 mm; h, 0.5 cm; i-m, 1 mm.


(a) WT
(b) bop1 bop2
(c) ap1-1
(d) bop1 bop2 ap1-1
(e) bop1 bop2 ap1-1
(f) bop1 bop2
(g) ap1-1
(h) bop1 bop2 ap1-1
(i) bop1 bop2 ap1-1
(j) bop1 bop2 ap1-1
(k) bop1 bop2 ap1-1
(l) ap1-12
(m) bop1 bop2 ap1-12

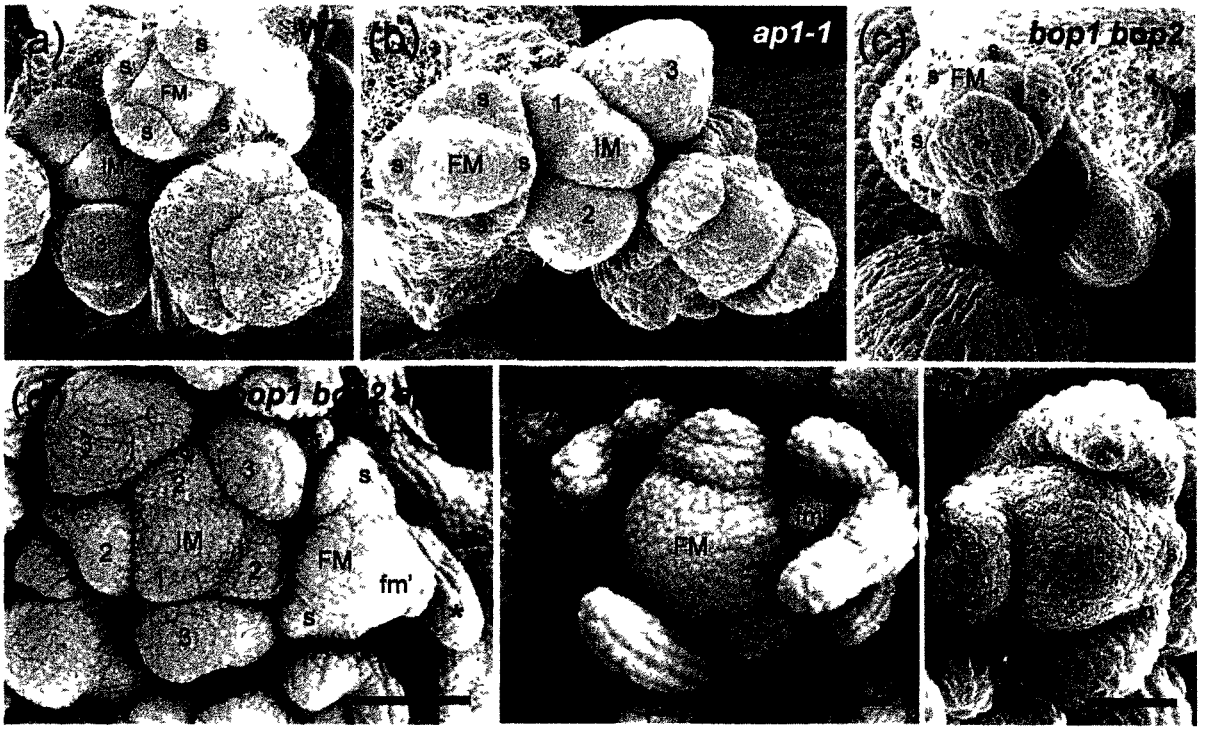


Figure 2.5 Scanning electron micrographs of WT and mutant inflorescence apices.

(a) WT control.

(b) *ap1-1*.

(c) *bop1 bop2*; asterisk denotes floral bract.

(d) *bop1 bop2 ap1-1*; asterisks denote floral bracts. Note the aberrant morphology of stage 3 primordia lacking clearly defined sepals. Ectopic axillary flowers (fm') are first evident in late stage 3 structures.

(e) *bop1 bop2 ap1-1*; late stage 3 flower showing large leafy bracts and a secondary floral meristem (fm') developing in the axil of a first whorl bract/sepal.

(f) *bop1 bop2 ap1-1;* stage 3 structure showing spiral initiation of first whorl organs. Numbers in panels indicate floral stages. Scale bars, 100 μm except 50 μm in (f). IM, inflorescence meristem. FM, floral meristem. s, sepal.

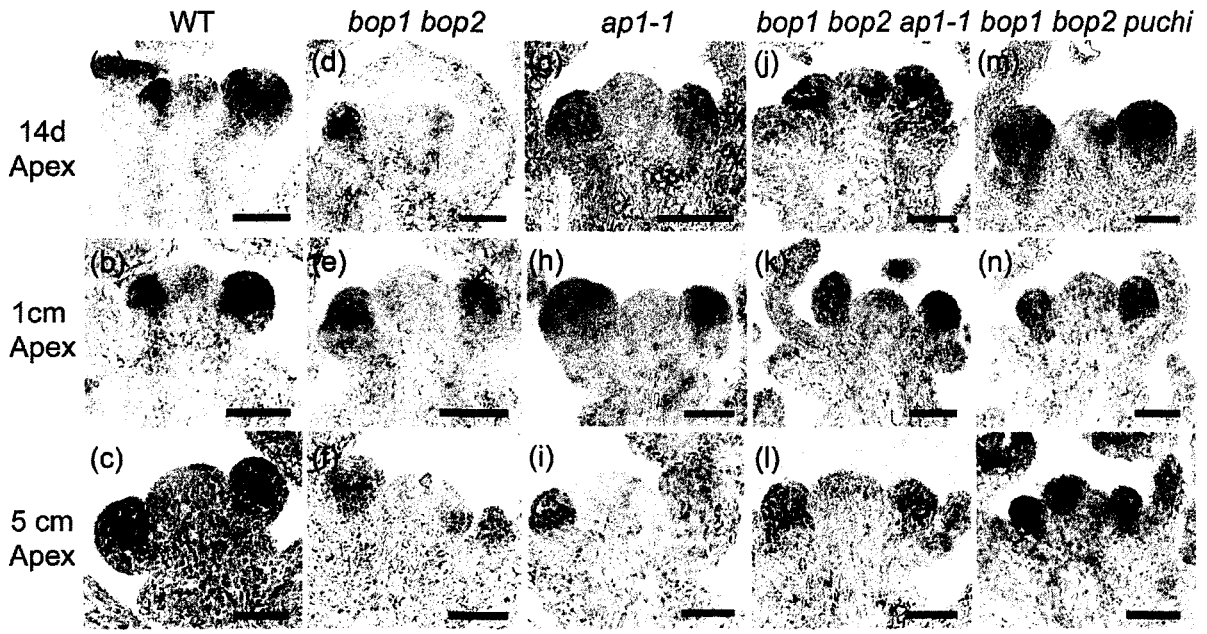


Figure 2.6 Expression of *LFY* in WT and mutant inflorescence apices.

Inflorescence apices sectioned from 14-day old seedlings or plants with 1-cm and 5-cm bolts were hybridized with a *LFY* anti-sense probe.

(a-c) WT control.

(d-f) *bop1 bop2* apices.

(g-i) *ap1-1*.

(j-l) *bop1 bop2 ap1-1*.

(m-o) *bop1 bop2 puchi* control. *LFY* expression in all genotypes is similar to that in *ap1-1* mutants. Scale bars, 100 μm.

Figure 2.7 In situ analysis of *AP1* expression in WT and mutant apices.

Inflorescence apices from 1-cm or 5-cm bolts were examined.

(a-c) WT.

(d-f) *bop1 bop2*.

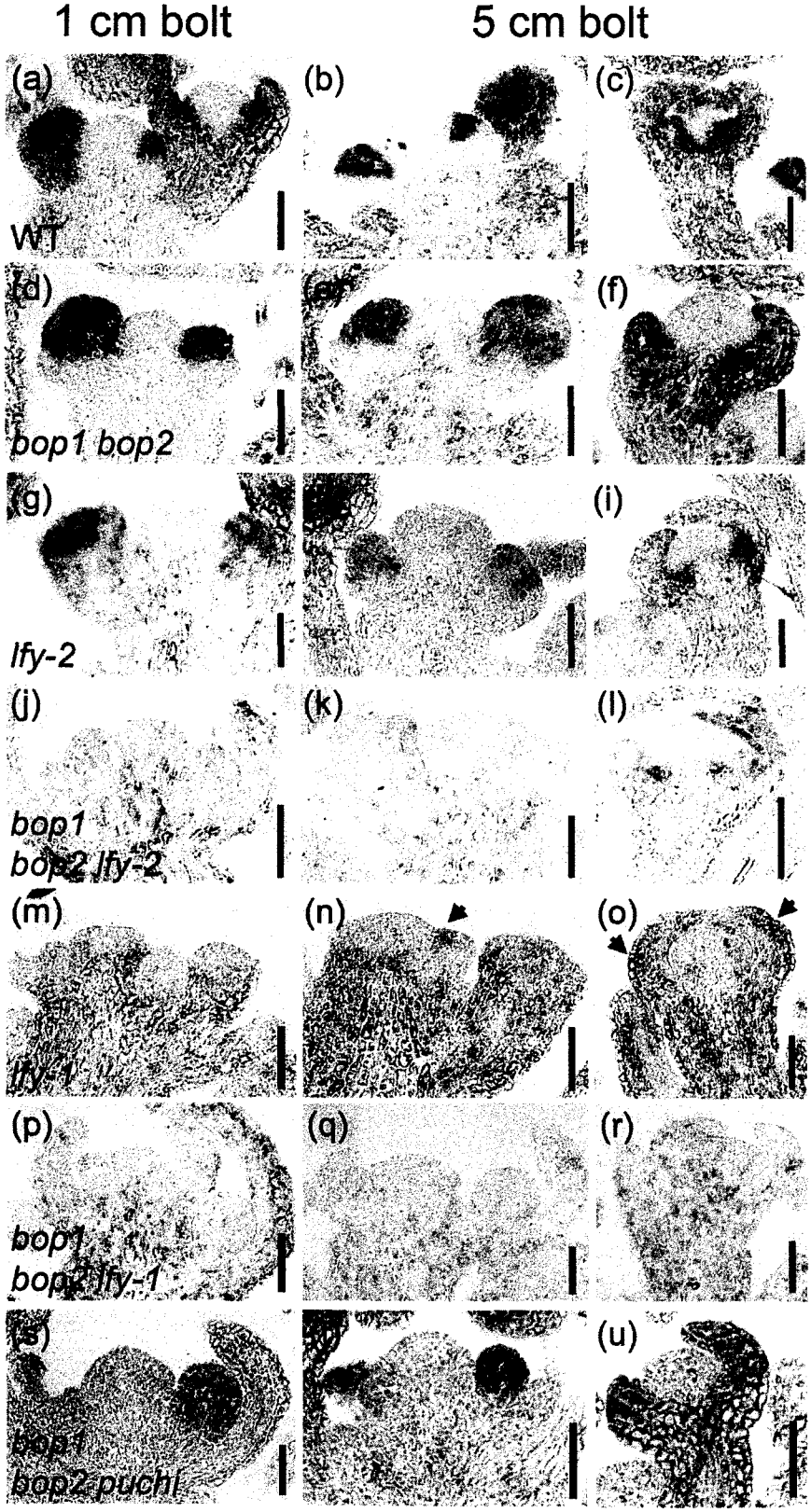
(g-i) *lfy-2*.

(j-l) *bop1 bop2 lfy-2*; *AP1* transcript reduced relative to *lfy-2*.

(m-o) *lfy-1*. Arrows indicate expression in stage 2-3 flowers.

(p-r) *bop1 bop2 lfy-1*; no detectable *AP1* transcript.

(s-u) *bop1 bop2 puchi* control. Scale bars, 50 μm.


1 cm bolt
5 cm bolt
(a)
(b)
(c)
WT
(d)
(e)
(f)
bop1 bop2
(g)
(i)
lfy-2
(j)
(k)
(l)
bop1
bop2 lfy-2
(m)
(n)
(o)
lfy-1
(p)
(q)
(r)
bop1
bop2 lfy-1
(s)
(u)
bop1
bop2 puchi

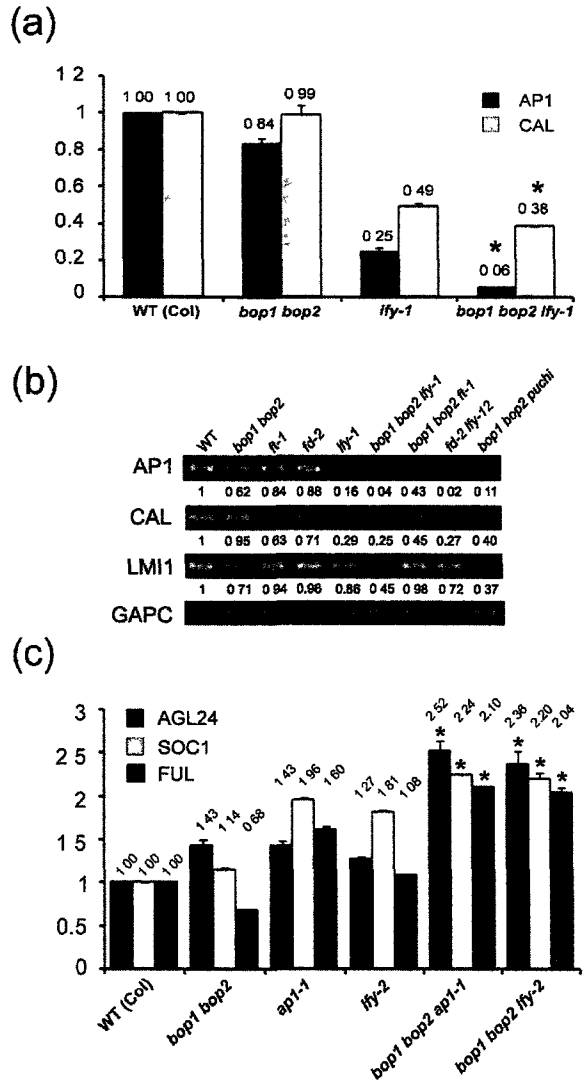


Figure 2.8 Quantitative analysis of *AP1*, *CAL*, and flowering-time gene expression in WT and mutant apices.

(a) Relative *AP1* and *CAL* transcript levels in apices of 5-cm bolts. *AP1* transcript is significantly lower in plants lacking both BOP and LFY activities.

(b) *AP1* transcript levels in the apices of 1-cm bolts for genotypes as indicated. Loss-of-function *bop1 bop2* lowers *AP1* transcript accumulation in both *lfy* and *ft* mutants.

(c) Relative *AGL24, SOC1,* and *FUL* transcript levels in apices of 5-cm bolts for the indicated genotypes. Transcript levels are highest in triple mutant apices. Asterisks in (a) and (c) indicate that values are significantly different from parental controls (Student's t-test, $p<0.05$). Values represent mean. Error bars indicate s.e.m.

Figure 2.9 *In situ* analysis of *AGL24* expression in WT and mutant inflorescence apices.

(a-c) WT control; transcript localizes to the cryptic bract (arrows) at late stage 2.

(d-e) *bop1 bop2*; enlarged bracts (arrows) and misexpression in the dome of stage 3 flowers (asterisk).

(f-g) *ap1-1*; ectopic expression in the dome of stage 2 flowers and in stage 3 sepals (arrows).

(h-i) *lfy-2*; ectopic expression in the dome of stage 3 flowers.

(j-k) *bop1 bop2 ap1-1*; enhanced misexpression.

(l-m) *bop1 bop2 lfy-2*; enhanced misexpression. Numbers in panels indicate floral stage. Scale bars, 50 μm.

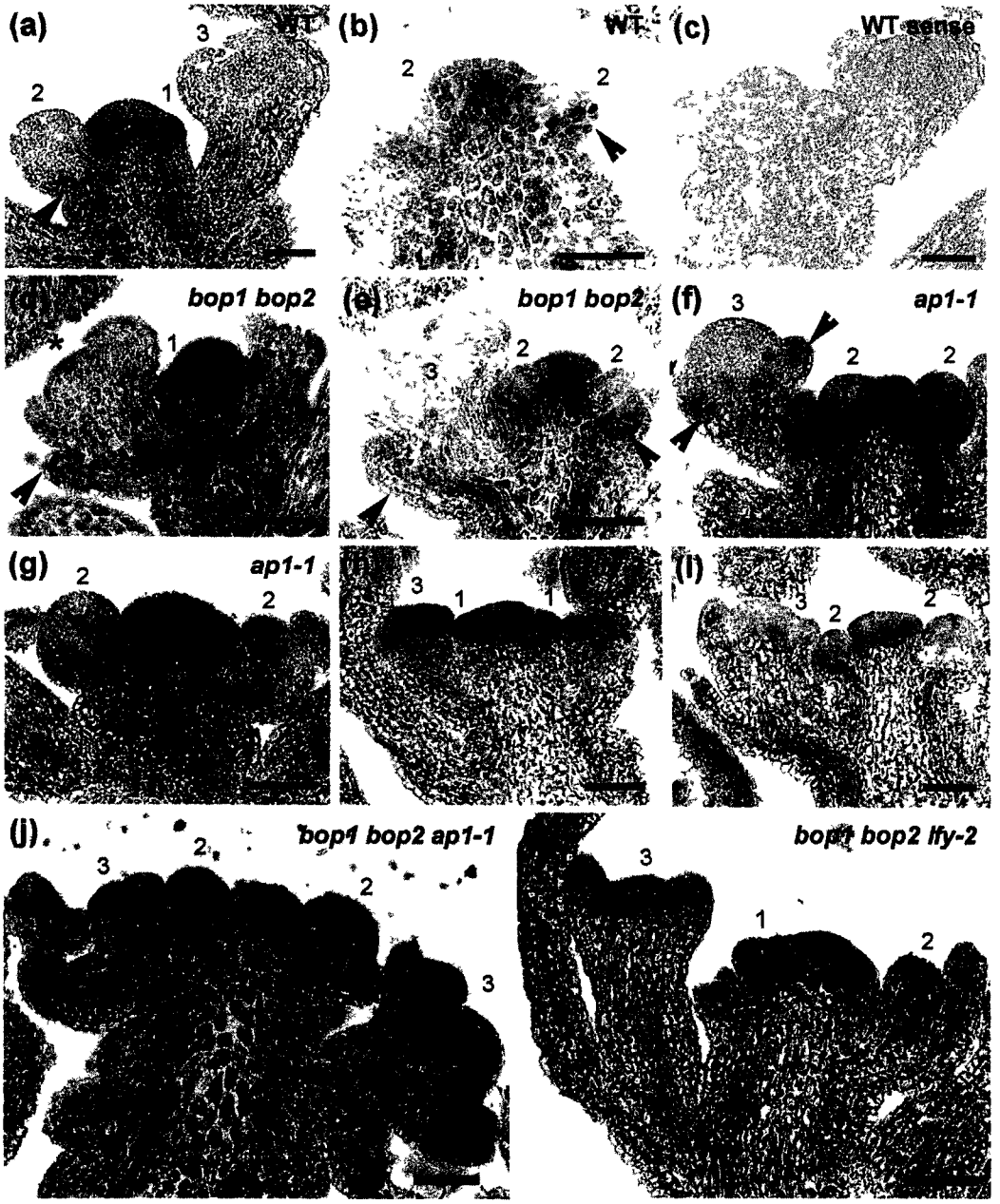

(a)
WT
3
2
1
(b)
WT
2
2
(c)
WT sense
(d)
bop1 bop2
*
1
(e)
bop1 bop2
3
2
2
(f)
3
ap1-1
2
2
(g)
2
ap1-1
2
(h)
3
1
1
(i)
3
2
2
(j)
bop1 bop2 ap1-1
3
2
2
3
bop1 bop2 lfy-2
3
1
2

Figure 2.10 Expression of *SOC1* in WT and mutant inflorescence apices.

Inflorescence apices sectioned from plants with 5-cm bolts were hybridized with a *SOC1* anti-sense probe.

(a-b) WT control apices; *SOC1* is expressed throughout the inflorescence meristem and stage 1 flowers but clears from the dome of the primordia in late stage 2 and is restricted to the cryptic bract. Expression resumes in early stage 3 in the center of the floral meristem and expands in the interior of the flower but is excluded from the developing sepals.

(c-d) *bop1 bop2* apices; resembles wild-type.

(e-f) *ap1-1* apices; *SOC1* expression clears from the dome of the primordia in late stage 2 but is ectopically expressed in the sepal whorl of stage 3 flowers (arrow).

(g-h) *lfy-2* apices; resembles wild-type.

(i-j) *bop1 bop2 lfy-2* apices; expression of *SOC1* fails to clear from the dome of stage 2 primordia and is upregulated throughout stage 3 flowers.

(k-l) *bop1 bop2 ap1-1*apices; expression of *SOC1* fails to clear from the dome of stage 2 primordia and is upregulated throughout stage 3 flowers. Numbers in panels indicate floral stage. Scale bars, 100 μm.

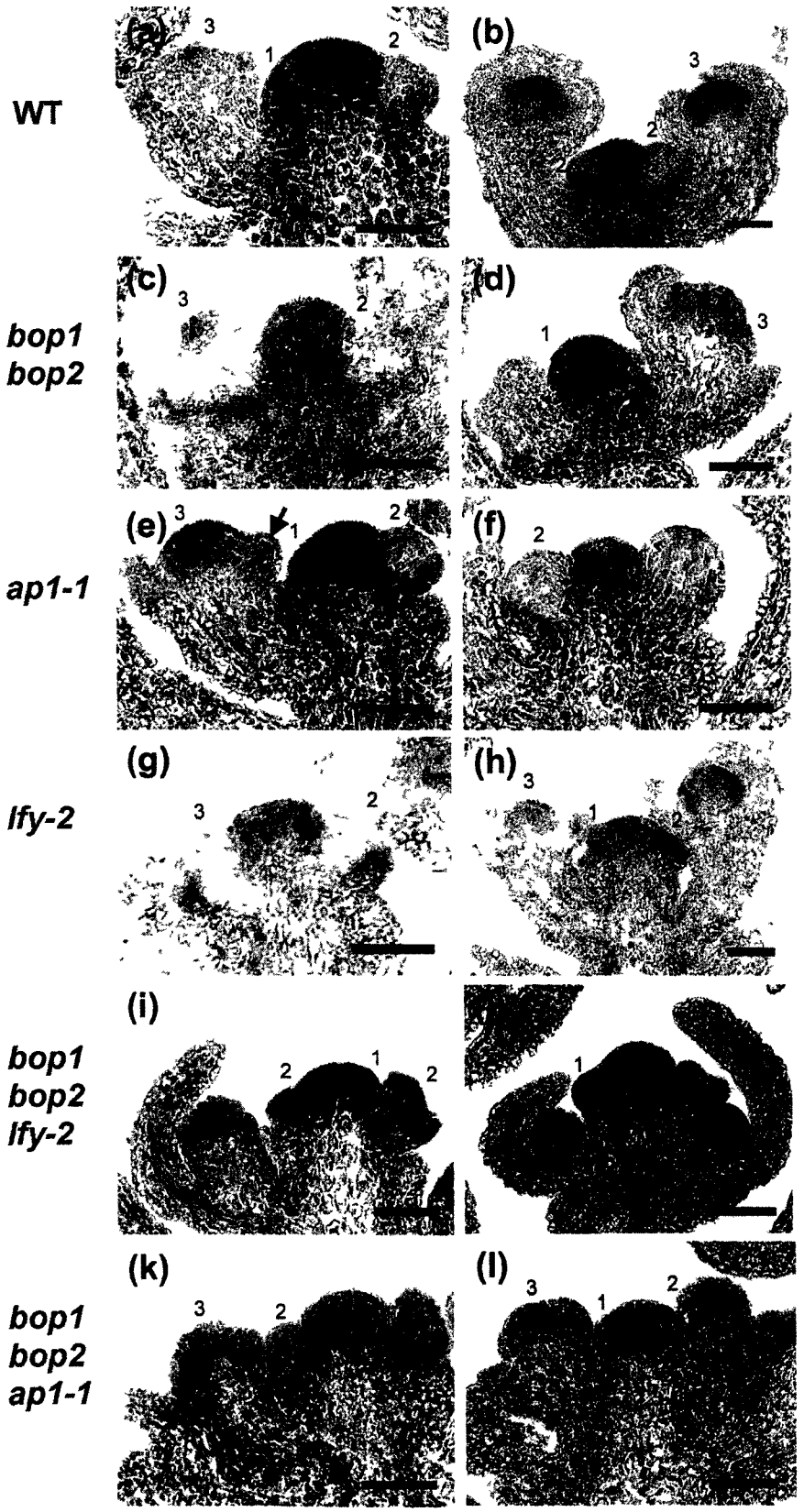

WT
bop1
bop2
ap1-1
lfy-2
bop1
bop2
lfy-2
bop1
bop2
ap1-1
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(k)
(l)

Figure 2.11 Expression of *FUL* in WT and mutant inflorescence apices.

(a-c) WT control apices; *FUL* is expressed throughout the inflorescence meristem and stage 1 flowers but clears from the dome of the primordia in late stage 2 and is restricted to the cryptic bract (arrow). Expression resumes in the center of the floral meristem in stage 3.

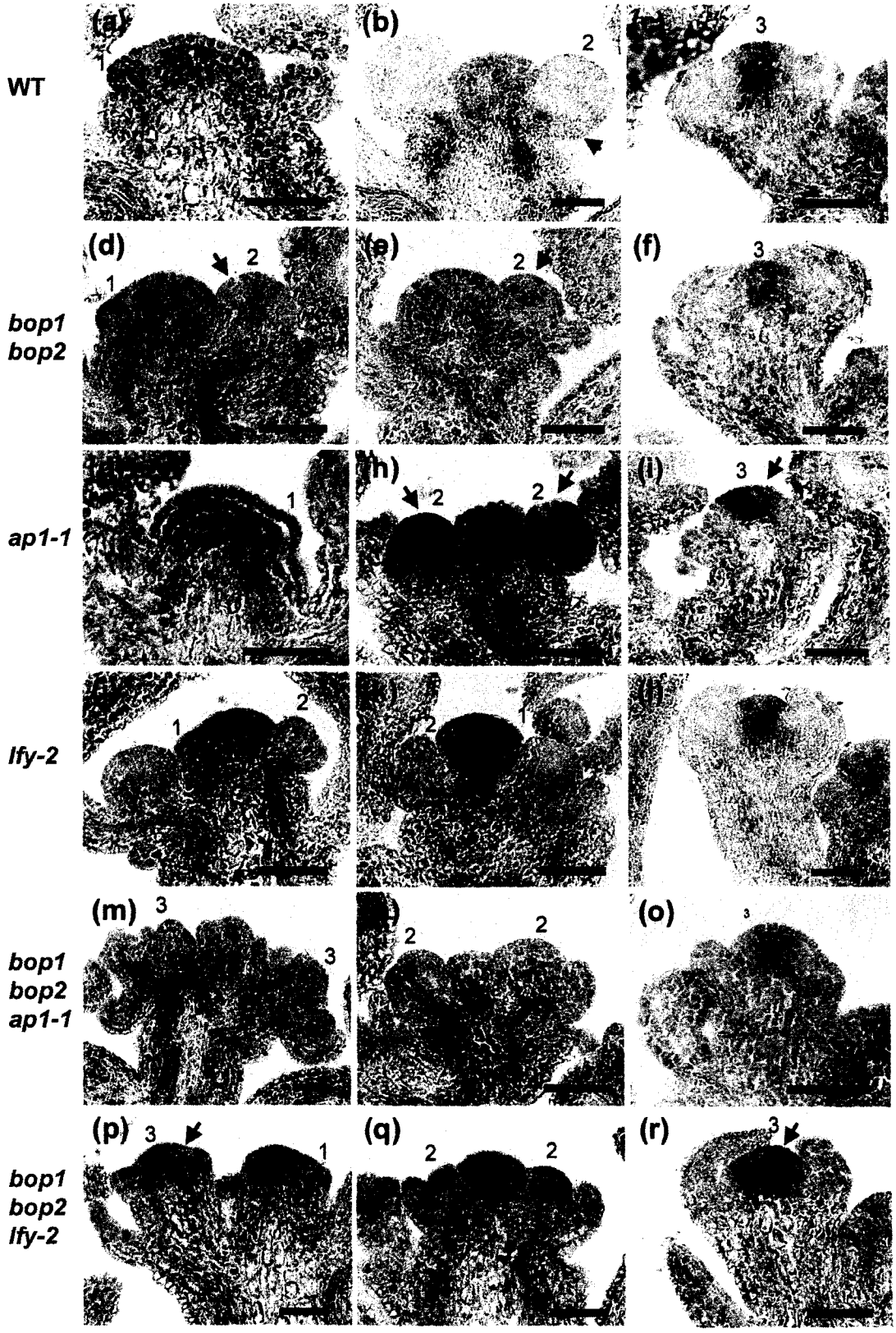
(d-f) *bop1 bop2* apices; *FUL* expression persists in the dome of primordia at late stage 2 (arrows) but expression at stage 3 appears normal.

(g-i) *ap1-1* apices; *FUL* expression fails to clear from the dome of stage 2 primordia and is expanded in the central part of stage 3 flowers (arrows).

(j-l) *lfy-2* apices; *FUL* expression persists in the dome of stage 2 flowers.

(m-o) *bop1 bop2 ap1-1* apices. *FUL* is expressed throughout stage 2 and early stage 3 structures and its domain of expression is expanded in stage 3 flowers.

(p-r) *bop1 bop2 lfy-2* apices; *FUL* is expressed throughout stage 2 and early stage 3 structures and its domain of expression is expanded in stage 3 flowers. Numbers in panels indicate floral stage. Scale bars, 100 μm


WT
bop1
bop2
ap1-1
lfy-2
bop1
bop2
ap1-1
bop1
bop2
lfy-2
(a)
(b)
(d)
(e)
(f)
(h)
(i)
(m)
(o)
(p)
(q)
(r)

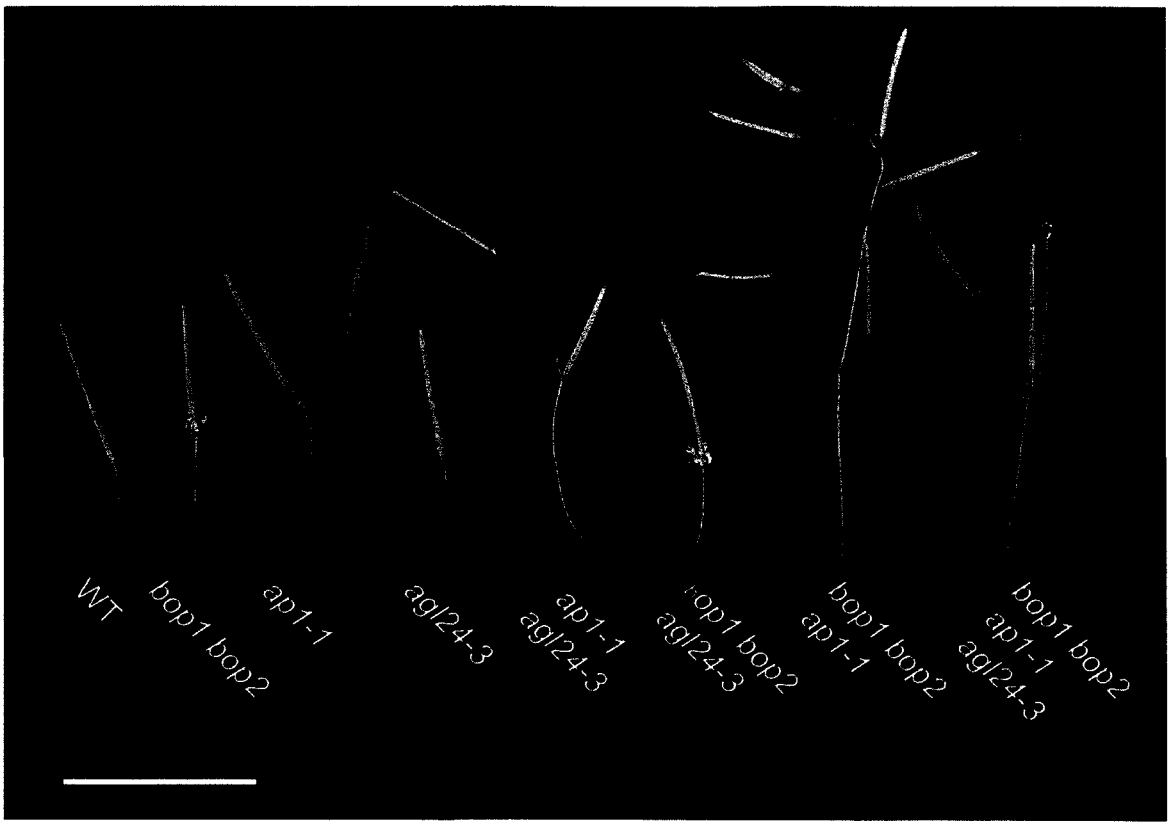


Figure 2.12 Floral branching in *bop1 bop2 ap1-1* triple mutants is suppressed by *agl24*.
Representative shoots (nodes 6 to 10) are shown for the indicated genotypes. Scale bar, 2 cm.

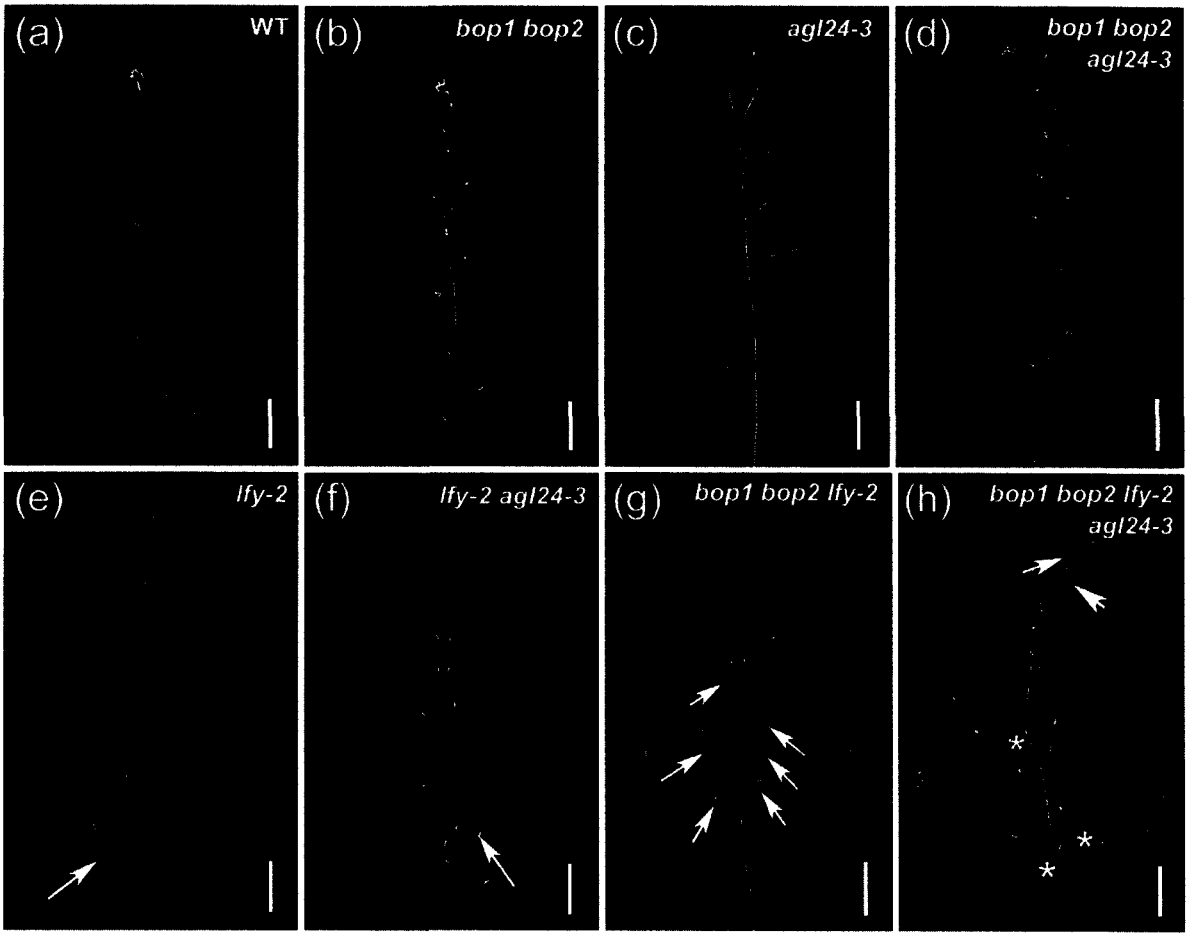


Figure 2.13 Floral branching in *bop1 bop2 lfy-2* triple mutants is suppressed by loss-of-function *agl24*.

Inflorescence apices from:

(a) WT control plant.

(b) *bop1 bop2* mutant.

(c) *agl24-3* mutant.

(d) *bop1 bop2 agl24-3* mutant.

(e) *lfy-2* mutant, arrow indicates a branched flower.

(f) *lfy-2 agl24-3* mutant, arrow indicates a branched flower.

(g) *bop1 bop2 lfy-2* mutant, arrows indicate branched flowers.

(h) *bop1 bop2 lfy-2 agl24-3* mutant, asterisks indicate bracts and arrows indicate nodes lacking a flower (bract only). Scale bars, 2 cm.

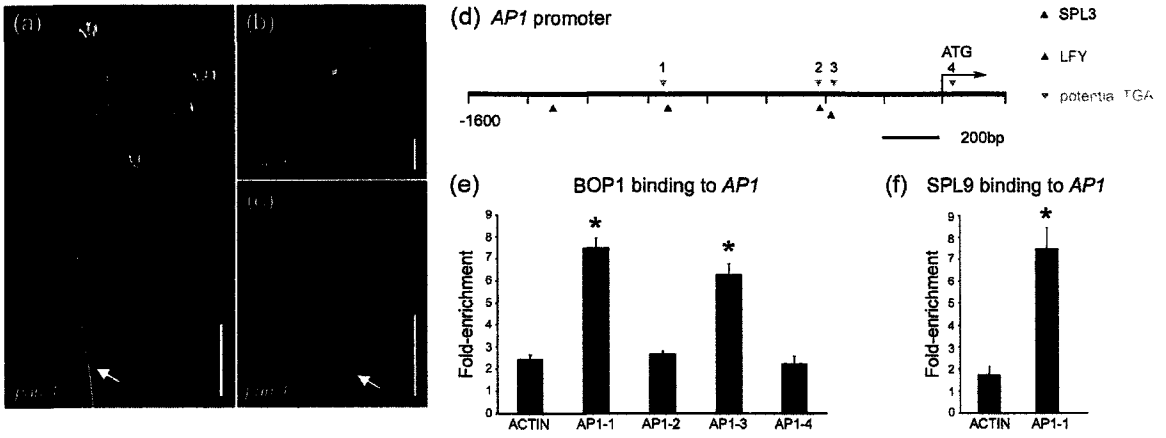


Figure 2.14 BOP1 binds *in vivo* to sites containing TGA binding motifs in the *AP1* promoter.

Floral meristem identity defects in *pan-1* mutants:

(a) Cauline leaf absent at transitional node (arrow). Scale bar, 2 cm.

(b) Branched flowers.

(c) Flower with bract. Scale bars, 5 mm.

(d) Scale diagram of the *AP1* promoter indicating the binding sites of direct regulators and potential TGA binding motifs. Numbers indicate regions tested for BOP1-GFP occupancy in ChIP assays (e-f). Binding site locations derived from: Wang et al., 2009; Wigge et al., 2005; Yamaguchi et al., 2009; Wang et al., 2009. See text for identification of potential TGA binding sites.

(e) BOP1-GFP occupancy at *AP1* promoter sites.

(f) GFP-SPL9 occupancy at *AP1* promoter site 1. Asterisks in e and f indicate significantly different from *ACTIN* control (Student's t-test, $p<0.05$). Error bars indicate s.e.m.

Figure 2.15 Yeast 2-hybrid and BiFC analysis of BOP protein-protein interactions.

(a) Pair-wise interactions of BOP2 with transcription factors directly regulating *AP1* and/or *AGL24*. Only PAN interacts with BOP2.

(b-j) BiFC analysis of interactions and localization of BOP2 and PAN YFP-tagged fusion proteins. Mesophyll protoplasts were monitored for YFP fluorescence 12-20 h after transfection. Inset panels show the corresponding DIC image under visible light.

(b) No vector background fluorescence control.

(c) Free EYFP localizes to the nucleus and the cytoplasm.

(d) PAN-EYFP fusion protein localizes to the nucleus.

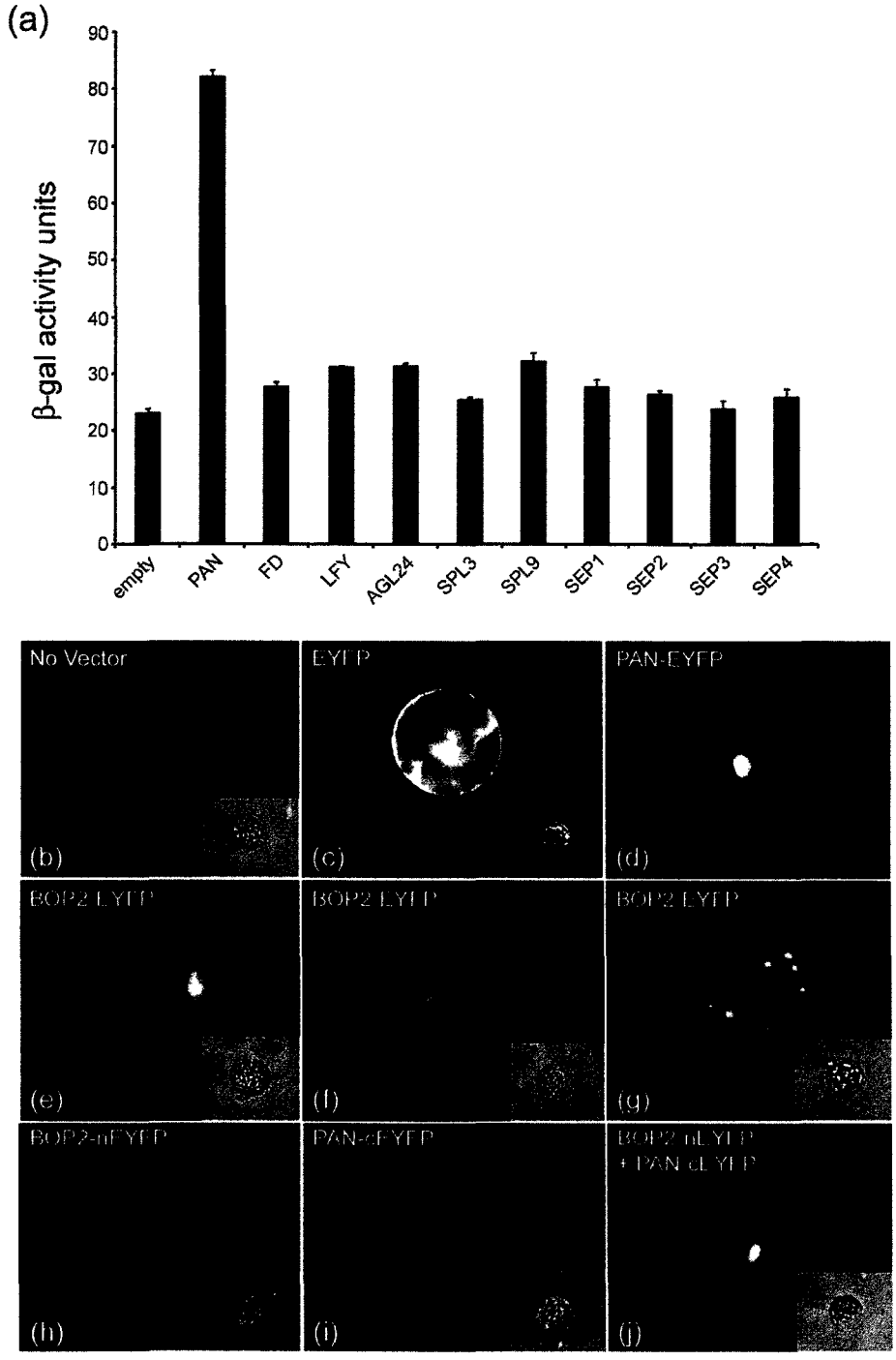
(e-f) BOP2-EYFP fusion protein localizes to the cytoplasm and the nucleus.

(g) Infrequently, the fluorescent signal occurs in punctuate spots throughout the cytoplasm.

(h) BOP2-nEYFP fusion protein shows no signal.

(i) PAN-cEYFP fusion protein shows no signal.

(j) Protoplasts expressing both BOP2-nEYFP and PAN-cEYFP show nuclear localized fluorescence.


(a)
β-gal activity units
90
80
70
60
50
40
30
20
10
0
empty
PAN
FD
LFY
AGL24
SPL3
SPL9
SEP1
SEP2
SEP3
SEP4
No Vector
(b)
EYFP
(c)
PAN-EYFP
(d)
BOP2-EYFP
(e)
BOP2-EYFP
(f)
BOP2-EYFP
(g)
BOP2-nEYFP
(h)
PAN-cEYFP
(i)
BOP2-nEYFP
+ PAN-cEYFP
(j)

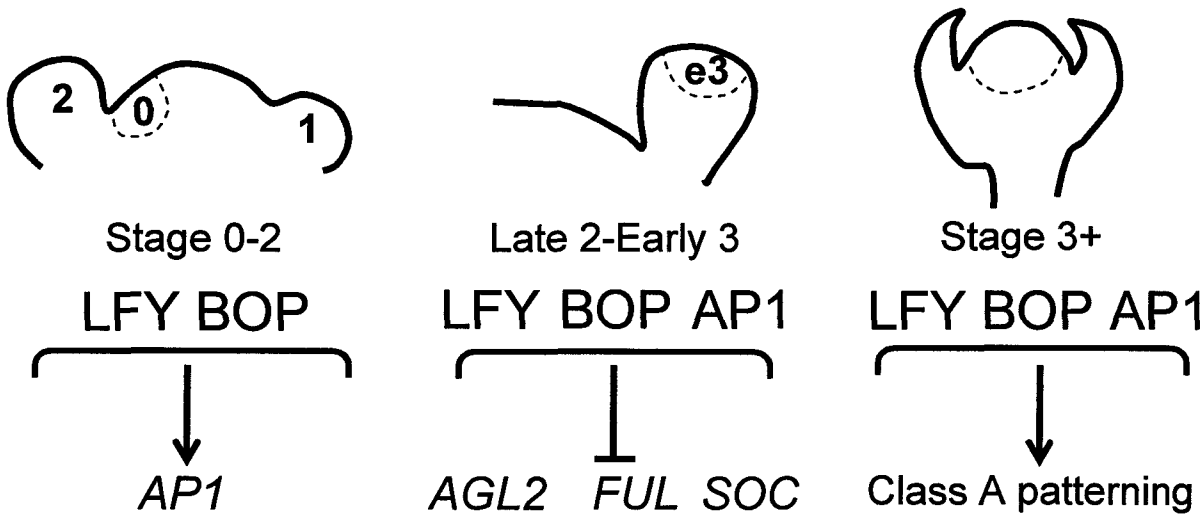


Figure 2.16 Model for BOP promotion of floral meristem identity.

The *BOP* genes are co-expressed with *LFY* in floral anlagen and function distinctly from LFY to upregulate *AP1* in stage 1 flowers. During stage 2, the overlapping activities of BOP1/2, LFY, and AP1 down-regulate *AGL24*, *SOC1*, and *FUL* in floral meristems to maintain floral fate. After stage 3, the continued activities of BOP1/2, LFY, and AP1 promote A-class floral patterning.

Chapter 3

BLADE-ON-PETIOLE1 and 2 function redundantly with ASYMMETRIC LEAVES1 and 2 to promote simple leaf shape by preventing reactivation of *KNOTTED1-LIKE HOMEOBOX* meristem genes in the petiole

I provided all of the data in this Chapter with the following exception: Tieqiang Hu performed the qPCR experiment shown in Figure 3.3.

Abstract

Leaves are flat structures specialized for photosynthesis. Leaf shapes are classified into simple and compound forms according to their degree of complexity on the petiole. Three main factors have been shown to promote the transformation of simple leaves to compound leaves: 1) the expression of Class I *KNOTTED1-LIKE HOMEOBOX* (*KNOX1*) meristem genes, 2) the expression of *CUP-SHAPED COTYLEDON* (*CUC*) boundary genes; and 3) the formation of auxin maxima. A number of factors expressed in leaves including ASYMMETRIC LEAVES1 (AS1), AS2, BLADE-ON-PETIOLE1 (BOP1) and BOP2 have been identified as negative regulators of *KNOX1* gene expression, but how these genes interact to control the level and spatial pattern of *KNOX1* misexpression in leaves has not been investigated. Here, I use a combination of genetic and expression studies to show that BOP1/2 differentially repress *KNAT2* and *KNAT6* expression in vegetative apices and that BOP1/2 and AS1-AS2 function synergistically to maintain *KNOX1* gene repression in leaf petioles though AS2-dependent and AS2-independent pathways. My data show that the major target of repression in leaves is *BREVIPEDICELLUS* (*BP*). Consistent with this, I show that *bp* mutations partially rescue petiole defects in *bop1 bop2 as1* and *bop1 bop2 as2* triple mutants. Collectively, my data support the model that BOP1/2 function redundantly with AS1-AS2 to promote simple leaf shape by maintaining repression of *KNOX1* meristematic genes in the petiole.

3.1 Introduction

Land plants display a variety of leaf architectures designed to optimize the capture of light and defend against biotic and abiotic stresses. Although leaves vary dramatically in size and shape, they are classified into two major groups according to their degree of complexity: simple and compound. The major difference between simple and compound leaves is that simple leaves have one undivided blade on the main axis (petiole) whereas compound leaves have several leaves arranged bisymmetrically on the petiole. Though very different in shape, simple and compound leaves can be found in related species of the same genus (reviewed in Hasson *et al.*, 2010; Uchida *et al.*, 2010).

Both simple and compound leaves are initiated from stem cell located in the SAM. *SHOOT MERISTEMLESS* (*STM*) from *KNOX1* family is expressed in the SAM except at the incipient leaf primordia and is one of the master regulators for the establishment and maintenance of SAM (Long *et al.*, 1996). Other KNOX1 members, such as *BREVIPEDICELLUS* (*BP*) (also known as *KNOTTED*-LIKE from ARABIDOPSIS THALIANA or *KNAT1*) and *KNAT6*, which are expressed in the PZ of SAM or the primordia-SAM boundary, respectively, also contribute to the maintenance of SAM since loss-of-function mutants *bp* and *knat6* enhance to strong the phenotype of weak *stm-2* alleles (Byrne *et al.*, 2002; 2003; Belles-Boix *et al.*, 2006). Strikingly, *KNAT2* is also expressed in the SAM but its inactivation does not enhance *stm-2*, *knat6 stm-2*, or *bp knat6 stm-2* phenotypes, making the role of KNAT2 in the SAM unclear (Belles-Boix *et al.*, 2006). KNOX1 homeodomain proteins form hereodimers with BELL homeodomain proteins in vivo (Kanrar *et al.*, 2006) and the BELL protein PENNYWISE (PNY), POUNDFULLISH (PNF), and ARABIDOPSIS THALIANA HOMEOBOX1 (ATH1) also contribute to SAM establishment and maintenance since the *pny pnf ath1* triple mutant also

lacks of SAM (Rutjens *et al.*, 2009). *KNOX1* homeodomain proteins function in part via balancing cytokinin and gibberellin levels in plants to establish and maintain the SAM (Rupp *et al.*, 1999; Jasinski *et al.* 2005; Yanai *et al.*, 2005; reviewed in Barton, 2010; Hay and Tsiantis, 2010). Though each of the *KNOX1* genes has a unique expression profile in the SAM, they share a common repression zone--the incipient leaf primordia (Lincoln *et al.*, 1994; Long *et al.*, 1996). This repression zone represents a transition of cell fate (from indeterminate to determinate) and is maintained differently in simple and compound leaf primordia.

In simple leaf species such as *Arabidopsis*, *KNOX1* genes are repressed throughout leaf development. If they are overexpressed, lobed leaves and ectopic meristems in the sinus of these lobes resembling compound leaves are developed (Lincoln *et al.*, 1994; Chuck *et al.*, 1996; Gallois *et al.*, 2002), In the compound leaves of plants such as tomato and *Cardamine, KNOX1* genes are transiently repressed in the early stages of leaf development but reactivated in later stages of leaf primordia development (Hareven *et al.*, 1996; Hay and Tsiantis, 2006b). Genetic studies show that higher and prolonged expression of *KNOX1* genes in tomato and *Cardamine hirsuta* leaves results in a higher order of leaflets (Hareven *et al.*, 1996; Hay and Tsiantis, 2006b) indicating that the temporal and spatial regulation of *KNOX1* genes is important for the simple-compound leaf deviation.

A number of genes in *Arabidopsis* contribute to maintenance of *KNOX1* repression during leaf development. These include *AS1* that encodes a MYB transcription factor, *AS2* that belongs to the LBD family (Byrne *et al.*, 2000; Ori *et al.*, 2000; Semiarti *et al.*, 2001; Iwakawa *et al.*, 2002; Lin *et al.*, 2003), and *BOP1/2* that encode BTB-ankryin transcriptional co-activators (Ha *et al.*, 2003; 2007; Hepworth *et al.*, 2005). Both *AS1* and *BOP1/2* are expressed in stage 1 leaf primordia and are excluded from the SAM by STM (Byrne *et al.*, 2000; Norberg *et al.*,

2005; Jun *et al.*, 2010). *AS2* is expressed on the adaxial side of leaves, overlapping with *BOP1/2* expression at the adaxial base of leaves and in the petiole (Norberg *et al.*, 2005; Jun *et al.*, 2010). In loss-of-function *as1*, *as2*, and *bop1 bop2* mutants, leaflets or ectopic blade growth occurs to different degrees on the petiole and *BP* transcripts are up-regulated. Reciprocally, overexpression of *AS1*, *AS2,* or *BOP1/2* phenocopies loss-of-function *bp* phenotypes, indicating that AS1, AS2 and BOP1/2 are negative regulators of *BP* (Byrne *et al.*, 2000; Ori *et al.*, 2000; Semiarti *et al.*, 2001; Iwakawa *et al.*, 2002; Ha *et al.*, 2003; 2007; 2010; Lin *et al.*, 2003).

The mechanism for repression of *BP* and *KNAT2* by AS1-AS2 was recently elucidated. AS1 and AS2 bind to the *BP* and *KNAT2* promoters directly, forming a repressor complex that contains the chromatin-remodeling protein HIRA (Xu *et al.,* 2003; Phelps-Durr *et al.,* 2005; Guo *et al.*, 2008). BOP1 activates AS2 directly (Jun *et al.*, 2010) suggesting that *BOP*1/2 repression of *BP* is indirect. Independent experiments showed that AS1, AS2 and BOP1/2 repress *BP, KNAT2* and *KNAT6* expression in leaves (Byrne *et al.*, 2000; Ori *et al.*, 2000; Semiarti *et al.*, 2001; Iwakawa *et al.*, 2002; Ha *et al.*, 2003; 2007; Lin *et al.*, 2003) and that leaf patterning defects in *bop1 bop2 as1* and *bop1 bop2 as2* triple mutants are more severe than in *bop1 bop2*, *as1*, or *as2* (Ha *et al.*, 2003; 2007). However, how this phenotypic synergism correlates spatially and quantitatively with *KNOX1* gene misexpression in the SAM and in leaves was not known. My work was designed to address these questions.

3.2 Materials and methods

3.2.1 Plant material and growth conditions

Plant growth conditions were identical to those described in Chapter 2. Mutant alleles of *as1-1* (CS3374), *as2-1* (CS3117) and *axr1-3* (CS3075) were obtained from the ABRC and have been described previously (Byrne *et al.*, 2000; Iwakawa *et al.*, 2002; Smith and Hake, 2003; Belles-

Boix *et al.*, 2006; Hay *et al.*, 2006a). Mutant alleles of *bp-1* and *bp-2* (introgressed into Col-0) were provided by Raju Datla (Venglat *et al.*, 2002). The reporter lines *BOP1::GUS* and *BOP2::GUS* were previously described (McKim *et al.*, 2008; Xu *et al.*, 2010). *KNAT2::GUS* (C24 ectotype) and *KNAT6::GUS* (WS ectotype) were gifts from Veronique Pautot (Dockx *et al.*, 1995; Belles-Boix *et al.*, 2006). All mutant combinations were constructed by crossing, and where possible they were confirmed by PCR genotyping.

3.2.2 Primers and genotyping

Primers used for genotyping and transcript analysis are listed in Table 3.1. The strategy for genotyping *bop1-3* and *bop2-1* alleles is described in Chapter 2. For genotyping *bp-2*, primers bp-2dCAPs-F1 and bp-2dCAPs-F2 were used to amplify a 240-bp product from genomic DNA. Only the *bp-2* product is cleaved by *Mun*I to yield a 211-bp fragment.

3.2.3 Analysis of leaf initiation

For analysis of leaf initiation, plants were grown in continuous (24h light) or long-day (16h light) photoperiods. Leaf initiation was scored by marking leaves at emergence. Leaves were counted daily until emergence of the inflorescence meristem (n=20 plants).

3.2.4 GUS staining

Tissues were stained, embedded, and sectioned as described in Chapter 2.

3.2.5 Quantitative RT-PCR (qPCR)

RNA isolation, reverse transcription and qPCR were performed essentially as described in Chapter 2. Tissues were harvested from 21-day-old leaves grown in continuous light. Total cDNA was synthesized from 1 μg of RNA template using Superscript III reverse transcriptase (www.invitrogen.com). qPCR was performed in triplicate using 5 μl of diluted cDNA as the

template in a reaction containing SYBR® Green I (www.sigma-aldrich.com) and IQ Supermix (www.biorad.com) using a Rotor-Gene 6000 (www.qiagen.com) thermocycler. Conditions were optimized for each primer pair and data quality was verified by melting curve analysis. Primers for qPCR analysis were as listed in Table 3.1. qPCR was performed with triplicates and relative transcript levels were calculated from mean of threshold cycle values Pfaffl (2001). Expression levels were normalized with values obtained using the reference gene *GAPC* (Hepworth *et al.,* 2005). Experiments were repeated at least twice using independently-isolated RNA. Representative data from one biological replicate is shown.

3.3 Results

3.3.1 Expression pattern of *BOP1* and *BOP2* in vegetative apices

The double mutant *bop1 bop2* has broad, leafy petioles that are similar to *as1* mutants, which have short and broad petioles, and to *as2* mutants, which form isolated leaflets near the junction of the blade and the petiole. This suggests that their functions in leaves might be similar. *In situ* hybridization and GUS reporter gene analysis indicate that *AS1* is expressed in emerging leaf primordia and that *AS2* is expressed at the adaxial side of leaf primordia (Byrne *et al.*, 2000; Jun *et al.*, 2010). To confirm that the expression domain of *BOP1* and *BOP2* overlap with *AS1* and *AS2* in shoot apices, I examined the expression patterns of *BOP1::GUS* and *BOP2::GUS* reporter genes in short-day grown seedlings. *BOP2* was not detected in the SAM or P0 primordia but readily detected in stage 1 leaf primordia, similar to *AS1* (Figure 3.1a; Byrne *et al.,* 2000). During stage 2 of leaf development, *BOP2* expression was restricted to the boundary between the leaf primordia and the SAM (Figure 3.1b). At stages 3 and 4 of leaf development, *BOP2* expression was localized to the adaxial base of leaf primordia (Figure 3.1a, b) in an overlapping pattern with *AS2* (Ha *et al.*, 2003; 2004; 2007; Hepworth *et al.*, 2005; Norberg *et al.*, 2005). In

expanded leaves, *BOP2* expression was detected in the petiole and mid-vein (Figure 3.1d). *BOP1::GUS* was expressed in a similar pattern (not shown). These data confirm the overlapping expression domains for *BOP1/2*, *AS1*, and *AS2* in developing leaves.

To confirm whether BOP1/2 has a defect in leaf initiation similar to *as1* (Hay *et al.*, 2006a), I compared the leaf initiation rate of wild-type and *bop1 bop2* mutant plants in long-day and continuous light photoperiods. My data showed that the leaf initiation rate in *bop1 bop2* plants was consistently slower than in wild-type control plants (Figure 3.1c). These data confirm a probable role for BOP1/2 in leaf initiation, similar to AS1 (Hay *et al.*, 2006a).

3.3.2 BOP1/2 function redundantly with AS1/2 in promoting simple leaf form

Similar expression patterns and loss-of-function mutant phenotypes for BOP1/2 and AS1-AS2 led me to hypothesize that BOP1/2 function redundantly with AS1/2 or that they function in a linear pathway. While Ha *et al* (2003) reported that leaf defects in *bop1-1 as1* and *bop1-1 as2* double mutants were more severe than in single mutants, this data was considered problematic since *bop1-1* is a dominant-negative allele in the Landsberg *erecta* (L*er*) ecotype that causes a leaf phenotype but does not cause the floral defects seen in loss-of-function *bop1 bop2* mutants (Hepworth *et al.*, 2005). We therefore constructed the *bop1 bop2 as1* and *bop1 bop2 as2* triple mutants in a Col background and analyzed the resulting phenotypes. Leaves of *bop1 bop2*, *as1*, and *as2* have growths on their petioles to different degrees (Figure 3.2b, f, g; Byrne *et al.*, 2000; Semiarti *et al.*, 2001; Hepworth *et al.*, 2005; Norberg *et al.*, 2005; Ha *et al.*, 2007). At an early developmental stage, the leaves of *bop1 bop2 as1* and of *bop1 bop2 as2* triple mutants displayed ectopic growth of tissures on the adaxial side of leaf petioles (Figure 3.2c, h). In old leaves (>5 weeks old) this tissue developed into leaflets, stipules, inflorescences, or flowers (Figure 3.2d, i), resembling the form of compound leaves. The growth of leaflets, inflorescence and flowers is

never observed in *bop1 bop2*, *as1*, or *as2* mutants, therefore, the genetic interaction between BOP1/2 and AS1/2 is synergistic. No synergistic defects in floral development were observed (data not shown).

Collectively these data confirm that BOP1/2 activity overlaps AS1 and AS2 in leaves of by repressing meristematic activity in the petiole region, necessary for the formation of simple leaves. These data are in agreement with Ha *et al.* (2007) who published a study of triple mutant phenotypes during the early stages of my project.

3.3.3 BOP1/2 act alongside AS1 and AS2 to exclude *KNOX1* gene expression from leaves

Arabidopsis thaliana produces simple leaves, whereas its close relative *Cardamine hirsuta* produces dissected leaves with individual leaflets. These two leaf forms are the result of differential expression of *KNOX1* genes during leaf development. Studies have shown that *KNOX1* genes are repressed throughout *Arabidopsis* leaf development (Byrne, *et al.*, 2000; Ori *et al.*, 2000; Semiarti *et al.*, 2001; Iwakawa., *et al.*, 2002), but reactivated during *Cardamine* leaf development (Hay and Tsiantis, 2006b; Barkoulas *et al.*, 2008). In agreement, *Arabidopsis* plants ectopically expressing *BP* or *STM* display deeply lobed leaves with ectopic meristems in the sinus of the lobes, resembling compound leaves (Lincoln *et al.* 1994; Chuck *et al.*, 1996; Gallois *et al.*, 2002). It was unclear if the compound-leaf like phenotype in *bop1 bop2 as1* and *bop1 bop2 as2* triple mutants directly correlated with synergistic up-regulation of *KNOX1* genes, though it has been shown that *KNOX1* genes are up-regulated in *bop1 bop2*, *as1* and *as2* leaves individually (Byrne, *et al.*, 2000; Ori *et al.*, 2000; Semiarti *et al.*, 2001; Iwakawa., *et al.*, 2002; Lin *et al.*, 2003; Ha *et al.*, 2003; 2007; Xu *et al.*, 2003).

To test this, quantitative RT-PCR comparing *KNOX1* gene expression in 21-day-old leaves of WT, *bop1 bop2*, *as1*, *as2*, *bop1 bop2 as1*, and *bop1 bop2 as2* plants was performed.

These data showed that in mature leaves, transcripts of *BP*, *STM*, *KNAT2*, and *KNAT6* were significantly higher in *bop1 bop2 as1* or *bop1 bop2 as2* triple mutants relative to parental control plants (Student's t-test, $p<0.05$ for all). This is consistent with the synergistic leaf-petiole defects in these mutants. In contrast, *PNY* transcripts were unchanged in all of the mutants, indicating that *KNOX1* genes are the main target of BOP1/2 repression in leaves (Figure 3.3). This comparison also showed that *BP* is the main target of misexpression in all mutants.

To determine if the compound-leaf like phenotype in the triple mutants was attributable to misexpression of *BP*, we generated *bop1 bop2 as1 bp* and *bop1 bop2 as2 bp* quadruple mutants (Figure 3.2e, j). In these mutants, formation of ectopic inflorescences and flowers on leaf petioles did not occur, although leaflets still formed on the petioles. These data not only demonstrate the biological relevance of *BP* misexpression in leaves, but also indicate that other factors must be misexpressed to generate a compound-leaf like phenotype.

Considering the growth of flowers on *bop1 bop2 as1* and *bop1 bop2 as2* triple mutant petioles, we reasoned that the master floral meristem regulator LEAFY (LFY) (Schultz *et al.*, 1991; Weigel *et al.*, 1992) may be misexpressed in triple mutant leaves. We also hypothesized that *WUSCHEL* (*WUS*) may be involved, because ectopic expression of *WUS* results in ectopic floral development on stems (Xu *et al.*, 2005). We examined the transcript levels of *LFY* and *WUS* in leaves of the triple mutants and their parental controls. However, we did not observe any significant change in *LFY* and *WUS* transcript levels at the 21-day-old time-point tested (data not shown).

3.3.4 BOP1/2 represses *KNAT2* and *KNAT6* expression in shoot apices

Quantitative RT-PCR has shown that BOP1/2 represses *KNOX1* transcription in leaves (Figure 3.3), but the spatial pattern of this misexpression was unknown. To study this, I compared the

expression patterns of *BP::GUS*, *KNAT2::GUS* and *KNAT6::GUS* in WT and *bop1 bop2* leaf tissues and in the shoot apices of 25-day-old seedlings grown in short days (8 h light) (Long and Barton, 2000).

In the apices of WT, *KNAT2* was expressed in the peripheral and rib zones of the meristem and stopped at the base of young leaves (Figure 3.4a). In *bop1 bop2* mutants, *KNAT2* expression was intensified and expanded, with staining in the petioles of leaves and in hypocotyls (Figure 3.4a, b), overlapping with the *BOP1/2* expression domain (Figure 3.1a, b, d). *KNAT6* expression in WT plants was focused in the meristem-leaf boundary and in stipules (Figure 3.4c). In *bop1 bop2* apices, *KNAT6* expression was up-regulated in the boundary and it expanded into the petioles of emerging leaves, hypocotyls and young leaf tips, similar to *KNAT2* (Figure 3.4c, d). Little or no misexpression of *BP* was detected in *bop1 bop2* shoot apices, similar to previous reports although it can be seen at the seedling stage (data not shown; Madiha Khan, unpublished data; Jun *et al.*, 2010). However, *BP* is misexpressed in *bop1 bop2* midvein or to the margins of petioles in mature leaves (Madiha Khan, unpublished data), suggesting that the repression of BP by BOP1/2 occurs in late stages of leaf development. A potential problem with this experiment was that the reporter genes crossed into *bop1 bop2* were not in a homozygous state, making direct comparison with the WT control lines difficult. Nevertheless, these data support the model that BOP1/2 prevent expression of *BP* and *KNAT2/6* at the base of leaves to shape simple leaves.

3.4 Discussion

The leaves of land plants differ in size and shape but are classified into two broad groups based on their degree of complexity. Simple leaves have a single flat undivided blade (no leaflet) o its petiole, while compound leaves consist of several leaflets on its petiole. The simple or compound

leaf form is species-specific, indicating that it is under strict genetic control. Simple-compound leaf deviation has been studied extensively in *Arabidopsis*, *Cardamine* (a close relative of *Arabidopsis*) and tomato. *Arabidopsis* has simple leaves, whereas tomato and Cardamine both have dissected compound leaves (reviewed in Hay and Tsaintis, 2006b; Hay and Tsiantis, 2010).

Three pathways are known to control the simple-compound leaf deviation. The first pathway is the *KNOX1* gene pathway: reactivation of *KNOX1* gene expression during leaf development causes the formation of compound leaves (Hay and Tsiantis, 2006b). The second pathway is the auxin pathway: blocking the formation of auxin maxima in the petiole or rachis of leaves facilitates the formation of smooth petioles (Barkoulas *et al.*, 2008). The third pathway is the *CUC* genes pathway: misexpression of the organ boundary genes CUC1 and CUC2 in *Arabidopsis* promote compound leaf development (Hasson *et al.*, 2011).

3.4.1 Overlapping activities of BOP1/2 and AS1-AS2 facilitate simple leaf formation

Our data demonstrate that genetic interactions between BOP1/2 and AS1, AS2 are synergistic, since *bop1 bop2 as1* and the *bop1 bop2 as2* triple mutants display much more severe growth on their petioles than do parental control plants. This outcome was also reported by Ha *et al.* (2007) but in this case, quantitative and spatial analysis of *KNOX1* genes was not performed. Moreover, Ha *et al.* (2007) did not report the ectopic growth of flowers on the adaxial side of mature triple mutant leaves. Our data indicate that meristematic activity in the triple mutants is limited to the leaf petiole. Our interpretation is that BOP1/2 and AS1-AS2 represent partially redundant mechanisms for promoting simple leaf shape in *Arabidopsis*.

3.4.2 Turning KNOX genes off is important for simple leaf development

KNOX1 genes promote and maintain meristem activity (reviewed in Barton, 2010; Hay and Tsiantis, 2010). Our qPCR data demonstrate that BOP1/2, AS1 and AS2 function redundantly to

keep *KNOX1* genes off during *Arabidopsis* simple leaf development. In *Cardamine,* which displays compound leaves, the expression domain of *STM* and *BP* are expanded to the outer cell layers of P0 to P3 leaf primordia or to the leaf petiole (Hay and Tsiantis, 2006b). Knocking out *STM* by RNAi transforms *Cardamine* compound leaves into simple leaves (Hay and Tsiantis, 2006b). Our data show that mutation of *BP* partially rescues petiole defects in *bop1 bop2 as1* and *bop1 bop2 as2* triple mutants, demonstrating the biological relevance of *BP* misexpression in these leaves. These data are consistent with the results of others showing that *KNOX1* expression domains are key determinant of leaf shape.

3.4.3 Does BOP1/2 block the formation of auxin maxima in the petiole to inhibit leaflet formation?

There is a remarkable difference between the spatial regulation of auxin maxima in simple and compound leaf formation. Auxin maxima are restricted to the lamina and do not occur in the petiole in *Arabidopsis* (which has simple leaves). In contrast, in *Cardamine* (which has compound leaves) auxin maxima form on the leaf rachis, prior to leaflet outgrowth, indicating that auxin localization facilitates lateral leaflet initiation (Barkoulas *et al.*, 2008). Polar auxin transport is mediated by a family of PIN-FORMED (PIN) auxin efflux carriers, among which PIN1 plays a key role (Hay *et al.*, 2006a). Consistent with this, *Cardamine pin1* mutants have fewer leaflets on the rachis (Barkoulas *et al.*, 2008).

Several lines of evidence suggest that leafy petioles in *bop1 bop2* might correlate with auxin overload (L. Musa, 2010). First, the petioles of *bop1 bop2* double mutants are broad and contain multiple parallel midveins, mimicking treatment of wild-type leaves with the auxin transport inhibitor Napthoxyacetic acid (NOA) (Sieburth, 1999). Second, the expression of an auxin-responsive reporter gene *(DR5::GUS)* is enhanced in *bop1 bop2* leaf petioles, similar to

the increased *DR5::VENUS* activity seen in the *Cardamine* rachis (Barkoulis *et al.,* 2008). Third, combining *bop1 bop2* and *pin1En134* mutations results in very short and broad petioles, similar to the *as1* mutant and again suggestive of auxin transport defects in the petiole. The examination of DR5::VENUS activity and PIN1::GFP activity in *bop1 bop2* petiole will help to elucidate if BOP1/2 regulate polar auxin transport or auxin accumulation in the petiole.

3.4.4 Does BOP1/2 activity prevent reactivation of *CUC* genes in the petiole to promote simple leaf formation?

Misexpression of the organ boundary gene *CUP-SHAPED COTYLEDON2* (*CUC2*) in *Arabidopsis* is a third pathway for the transformation of simple leaves into compound leaves (Hasson *et al.*, 2011). BOP1/2 are also expressed at the boundary between the leaf meristem and the SAM (Figure 3.1b) and may directly or indirectly regulate *CUC* expression. It might be interesting to test whether mutation of *CUC2* further simplifies the structure of *bop1 bop2 as1 bp* leaves and to test if *CUC* genes are ectopically expressed in *bop1 bop2* leaves.

Table 3.1 Primers used for genotyping and qPCR analysis.

Primer	Sequence 5' – 3'
Genotyping	
4H Salk RP	CGTACCCTTTGATTTTAGTATGCTG
4H Salk LP	GCACAATCTTTCGACTTCATCACC
5H Salk RP	CCCTTTTTATAATCAGCATCAAGA
5H Salk LP	TCGACGCCGAAGTAACGAGAG
bp-2dCAPs-F1	ACCCTCCTACAAGCTTACTTGGACTGCCA
bp-2dCAPs-R1	GGAGGCAGAGACAGACGGTGTTGACCGCT
qPCR	
KNAT1-F1 HAY qPCR	CCATTCAGGAAGCAATGGAGTT
KNAT1-R1 HAY qPCR	ACTCTTCCCATCAGGATTGTTGA
STM-R1 HAY qPCR	GTCAAGGCCAAGATCATGGCT
STM -F1 HAY qPCR	TGGTGCTCCAACCTTCTGAC
KNAT2-F2	TCTGAAGGACCAGCTACTACGC
KNAT2-R1	ATTTTGTCGCCTTCAGTAGGGTAAG
KNAT6- F2	CTTACTTCAAGCTTACATCGATTGC
KNAT6-R1	CGCAGTACGTTTCCATAAATTCATC
BLR-F1	TAATGTGGGTCGTGGGATTTACACC
BLR-R2	ACCTCTTGTAAACCTCGTCGAGCAT

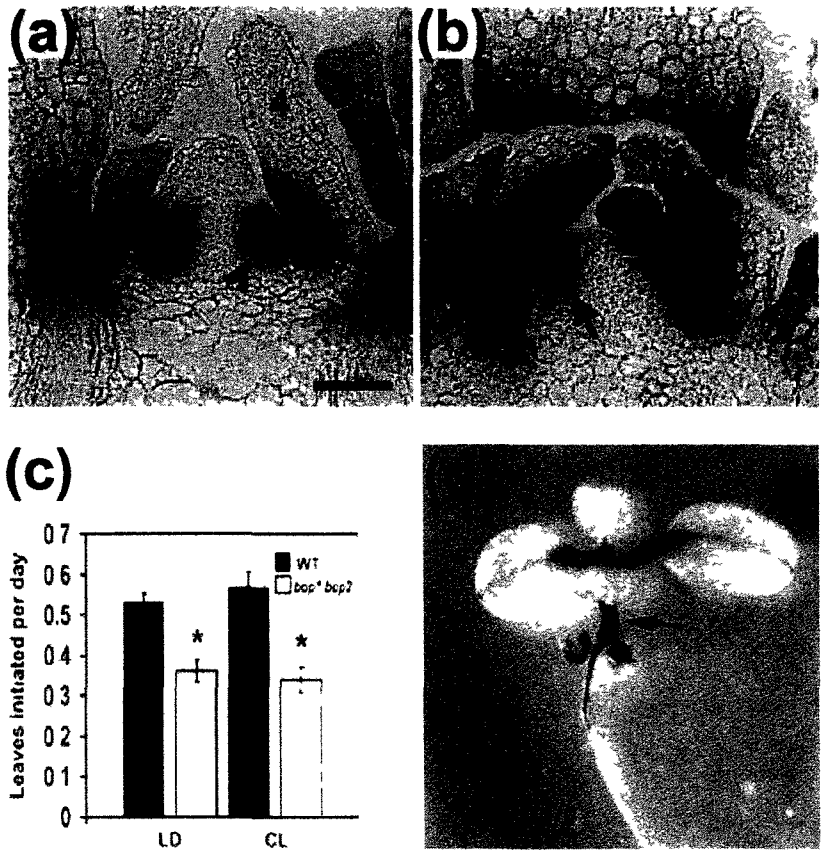


Figure 3.1 Expression pattern of *BOP2::GUS* in wild-type vegetative apices and leaf initiation rate in wild-type and *bop1 bop2* plants.

(a-b) *BOP2::GUS* expression in the apices of 25-day-old wild-type seedlings grown in short days. Expression is absent from the SAM, but detected in stage 1 leaf primordia (arrowhead). At stage 2, expression localizes to the boundary between the leaf primordia and the SAM (arrow). Numbers indicate the stage of the leaf primordia. Scale bar, 50 μm.

(c) Lower leaf initiation rate in *bop1 bop2* than in wild-type (WT) plants under both long day (LD) and continuous light (CL) conditions. Asterisks indicate significantly differences from the wild-type (Student's t-test, $p<0.05$). Error bars, s.e.m.

(d) *BOP2::GUS* expression in the WT seedlings. *BOP2* is expressed in the petiole and midvein o expanded leaves and in the seedling hypocotyl (arrowhead).

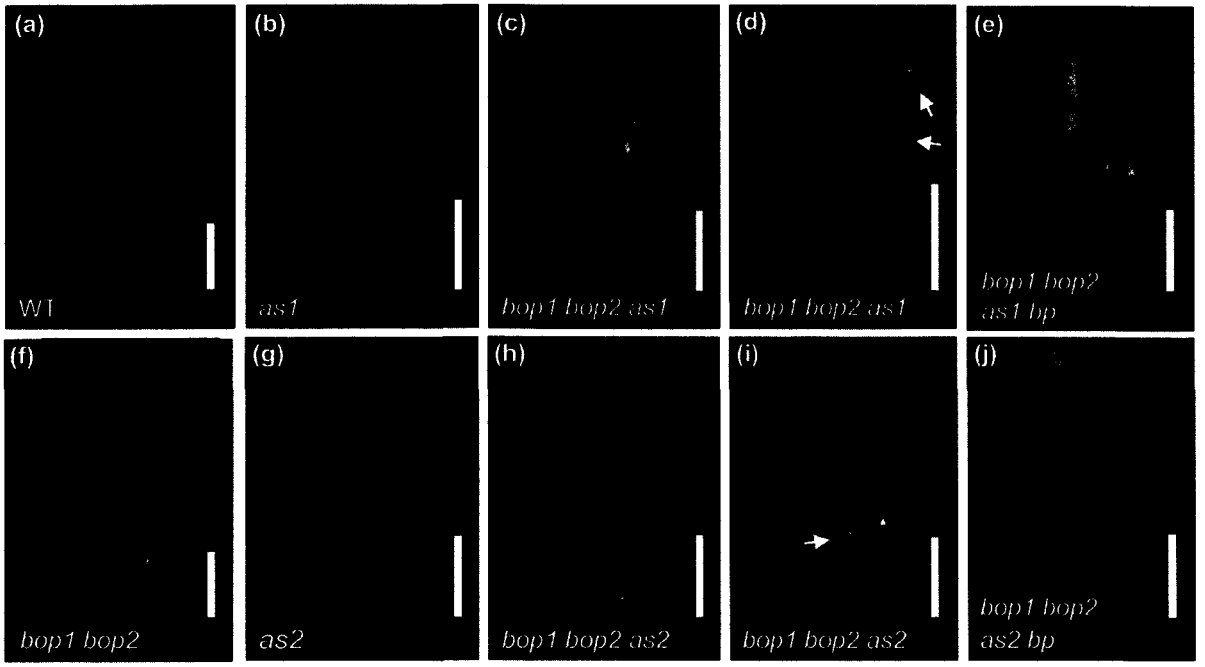


Figure 3.2 Comparison of leaf morphology in WT and mutants.

(**a**) Columbia wild-type.

(**b**) *as1* mutant.

(**c**) 3-week-old *bop1 bop2 as1* mutant; enhanced meristem activity in petiole.

(**d**) 5-week-old *bop1 bop2 as1* mutant; ectopic shoots and flowers developing from petiole (arrows).

(**e**) 7-week-old *bop1 bop2 as1 bp* mutant; only leaflets.

(**f**) *bop1 bop2* mutant.

(**g**) *as2* mutant.

(**h**) 3-week-old *bop1 bop2 as2* mutant.

(**i**) 5-week-old *bop1 bop2 as2* mutant.

(**j**) 7-week-old *bop1 bop2 as2 bp* mutant. Scale bars, 1 cm.

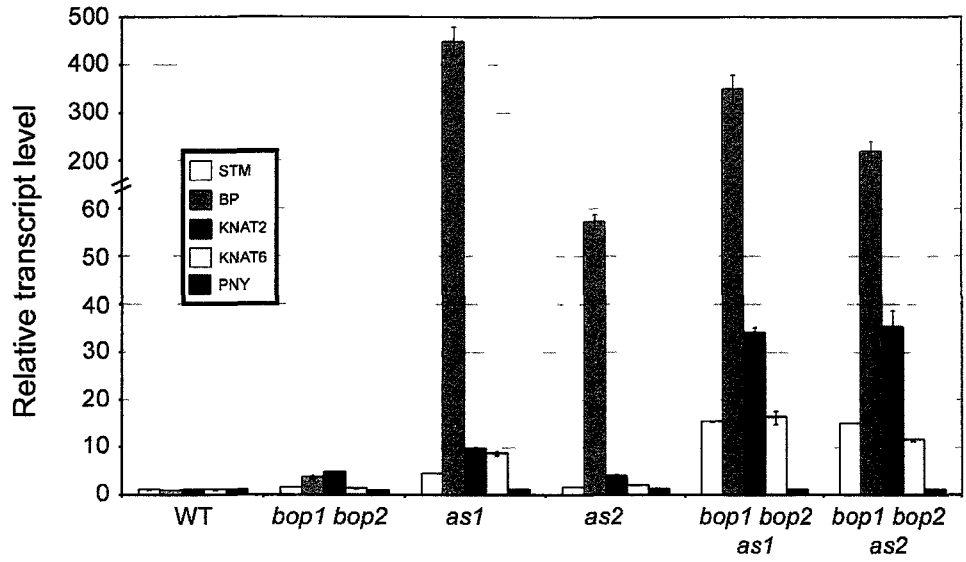


Figure 3.3 Quantitative RT-PCR analysis of class I *KNOX* genes and *PNY* in WT and mutant leaves.

Leaf tissue was collected from 21-day-old plants grown in continuous light. Relative transcript levels for *BP*, *KNAT2*, *KNAT6*, *STM*, and *PNY* are shown for the indicated genotypes. Error bars, s.e.m.

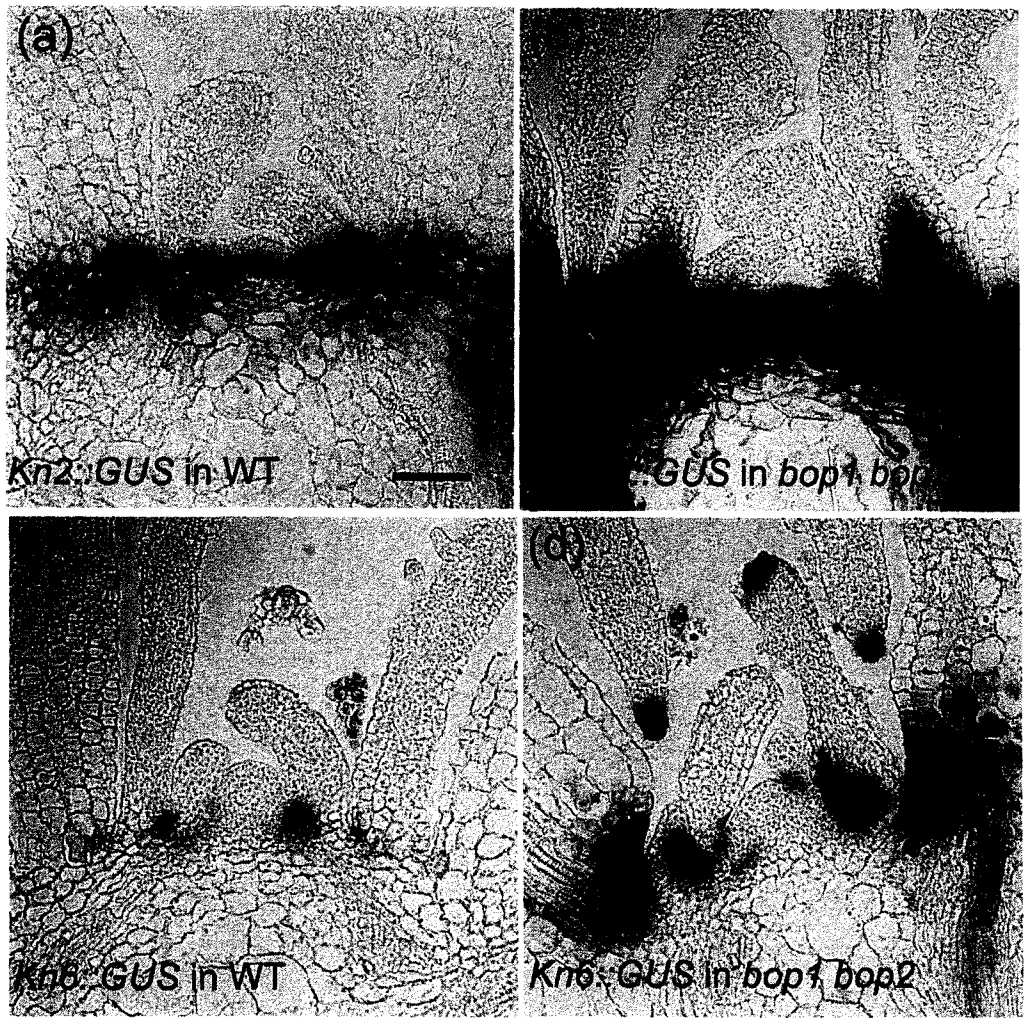


Figure 3.4 Expression patterns of *KNAT2* and *KNAT6* in WT and *bop1 bop2* apices.

(a) *KNAT2::GUS* in WT. Expression is throughout the rib and peripheral zone of the SAM and at the base of leaf primordia.

(b) *KNAT2::GUS* in *bop1 bop2*. Expression is up-regulated relative to WT control apices and expands to leaf petioles and the hypocotyls.

(c) *KNAT6::GUS* in WT. Expression localizes at the proximal and adaxial portion of leaf primordia.

(d) *KNAT6::GUS* in *bop1 bop2*. Expression is up-regulated relative to WT control apices and misexpressed at the young leaf tips and in hypocotyls. Scale bar, 50 μm.

Chapter 4

BREVIPEDICELLUS and PENNYWISE promote internode elongation in inflorescences by repressing *BLADE-ON-PETIOLE1/2* and *KNAT6*

Abstract

In *Arabidopsis* and many other plant species, the transition to flowering is tightly linked to the elongation of internodes to create the inflorescence. Internode patterning is one of the main determinants of inflorescence architecture, leading to diversity in inflorescence height and the organization of lateral structures (such as secondary shoots or flowers) on the main stem. Members of the KNOX1 (STM, BP, KNAT2 and KNAT6) and BELL (PNY, PNF, and ATH1) families of TALE homeodomain proteins share overlapping activities for the establishment and maintenance of the SAM but also control inflorescence development. Mutation of *BP* or its interacting partner *PNY* dramatically alters inflorescence architecture resulting in compact internodes, clustered or downward pointing siliques, and irregular phyllotaxy. Expression analysis showed that *KNAT2* and *KNAT6* are normally expressed in the pedicel axis close to the stem in wild-type but misexpressed in the internodes and pedicels of *bp* and *pny*. Expression analysis on *BOP1* and *BOP2* showed that they are also expressed in the pedicel axis in wild-type, and misexpressed in the pedicels and stems of *bp* and *pny*. The close expression pattern between *BOP1/2* and *KNAT2/6* suggest that these factors may have a common function. In support of this, BOP1/2 gain-of-function mutants are very short with compact internodes and clustered or downward pointing siliques, similar to *bp or pny* mutants. To investigate how *BOP1* and *BOP2* contribute to inflorescence architecture, I examined their genetic interaction with *BP* and *PNY*, in collaboration with Madiha Khan. My data show that *BOP2* and *KNAT6* expression domains are similarly enlarged in *bp* or *pny* mutants, corresponding to their mutant phenotypes. My data also provide evidence that BOP1/2 are positive regulators of *KNAT6* and that this activation may be direct. Collectively, BP-PNY negatively regulate *BOP1/2-KNAT6* in stems establishing a molecular network for the regulation of internode elongation.

4.1 Introduction

Internode elongation determines a plant's height and the organization of lateral structures on the main stem and is therefore a key determinant of inflorescence architecture. During *Arabidopsis* vegetative development, internode elongation is suppressed and compact rosettes are produced whereas during reproductive development internode elongation is activated by KNOXI and BELL homeodomain proteins (Venglat *et al.*, 2002; Byrne *et al.*, 2003; Smith and Hake, 2003; Ragni *et al.*, 2008). KNOX1 (BP, STM, KNAT2 and KNAT6) and BELL (PNY, PNF, ATH1) homeodomain proteins function redundantly during vegetative development in establishment and maintenance of the SAM (Lincoln, *et al.*, 1994; Long *et al.*, 1996; Byrne *et al.*, 2002; 2003; Belles-Boix *et al.*, 2006; Rutjens *et al.,* 2009). However, during reproductive development, antagonistic interactions between some of these KNOXI and BELL homeodomain proteins become important for internode elongation and pedicel orientation (Smith and Hake, 2003; Smith *et al.*, 2004; Ragni *et al.*, 2008; Rutjens *et al.,* 2009). Mutation of *bp* or *pny* results in reduced internodes, downward pointing or clustered siliques, altered phyllotaxy, and defects in cell differentiation, albeit in slightly different patterns for each mutant (Smith and Hake, 2003; Byrne *et al.,* 2003; Venglat *et al.,* 2002; Douglas *et al.,* 2002). The *bp pny* double mutant is extremely short (Smith and Hake, 2003) indicating overlapping activities for BP and PNY in the internodes. A second study showed that the *pny pnf* double mutant fails to bolt and produces only leaves (Smith *et al.*, 2004) indicating that the activities of PNY and PNF are redundant and required for transition of the apical meristem from SAM to IM identity and for production of internodes. Yeast-2-hybrid experiments have shown that BP forms heterodimers with PNY and PNF, suggesting a mechanism for the some of the interactions seen between BP, PNY, and PNF *in vivo* (e.g. Kanrar *et al.*, 2006). By contrast, loss-of-function in *KNAT2* and *KNAT6* or *ATH1*

rescue *bp*, *pny*, and/or *bp pny* internode elongation defects indicating that the activities of BP-PNY and KNAT2-KNAT6-ATH1 are antagonistic during reproductive development and required for development of the inflorescence (Ragni *et al.*, 2008; Rutjens *et al.*, 2009).

Several observations prompted us to test the nature of genetic interactions between BOP1/2 and BP-PNY during inflorescence development. First, strong BOP1/2 gain-of-function mutants are extremely short with downward pointing or clustered siliques (Norberg *et al.,* 2005; Ha *et al.,* 2007; Khan and Xu, data not shown), mimicking *bp pny* double mutants (Smith and Hake, 2003). Second, BOP1/2, KNAT2, and KNAT6 are co-expressed in pedicel axils where they may share a function. Third, Ragni *et al.* (2008) showed that *knat2 knat6* mutations rescue rather than enhance *bp* and *pny* mutant phenotype, revealing that the defects in these mutants are caused by misexpression of *KNAT2* and *KNAT6* in stems and pedicels.

We considered that gain-of-function BOP1/2 may interfere with internode elongation by activating *AS2*, because BOP is a direct activator for *AS2* in leaves (Jun *et al.* 2010), and AS2 is a direct repressor for *BP* (Guo *et al.*, 2008). However, mutation of *AS2* only slightly restored inflorescence height and pedicel angle in *35S::BOP1/2* plants (Ha *et al.*, 2007) and loss-of-function *as2* does not rescue *bp* inflorescence defects (Byrne *et al.*, 2002). These interactions suggested that BOP1/2 pattern internode using a mechanism that is largely independent of AS2.

I show here that BP and PNY restrict the expression domains of *BOP1/2* in pedicel axis. My data show that *BOP1/2* and *KNAT6* are co-misexpressed in *bp* and *pny* in characteristic patterns and that BOP1/2 promotes *KNAT6* expression. We will find out if *KNAT6* is a direct target of BOP1/2 activation in stems in the future.

4.2 Materials and methods

4.2.1 Plant material and growth conditions

Plant growth conditions are the same as those described in Chapter 2. Mutant alleles of *bop1-3*, *bop2-1*, *bp-1*, and *bp-2* were described in Chapter 2 and Chapter 3. The activation-tagged over-expression line *bop1-6D* was kindly provided by O. Nilsson (Norberg *et al.*, 2005). The reporter line *BOP2::GUS* is described in Chapter 2 (Xu *et al.*, 2010). All mutant combinations were constructed by crossing and confirmed by PCR genotyping where possible.

4.2.2 Primers and genotyping

Primers used for genotyping and transcript analysis are listed in Table 4.1. The strategy for genotyping *bop1-3*, *bop2-1*, *bp-1*, and *bp-2* was as described in Chapters 2 and 3. The strategy for genotyping *pny-40126*, *knat2-5*, and *knat6-1* Salk T-DNA insertion mutants was as described (www.signal.salk.edu).

4.2.3 GUS staining

Tissues were stained, embedded, and sectioned as before (Chapter 2).

4.2.4 Quantitative RT-PCR (qPCR)

RNA isolation, reverse transcription, and qPCR were performed as before (Chapters 2 and 3). qPCR was performed with triplicates and at least two biological replicates were repeated to ensure reproductivity. Primers used in the qPCR for this chapter were listed in Table 4.1.

4.2.5 *In situ* hybridization

In situ hybridizations were performed as before (Chapter 2). Primers used to create anti-sense probes for *BP*, *CUC3*, and *KNAT6* were as listed in Table 4.1.

4.3 Results

4.3.1 *BOP1/2* is misexpressed in *bp* and *pny* mutants

Ragni *et al* (2008) showed that loss-of-function *knat6* (and *knat2 knat6*) rescues *bp* and *pny* inflorescence patterning defects and that *KNAT2* and *KNAT6* GUS reporter genes are misexpressed in the stems and pedicels of *bp* and *pny* mutants. Madiha Khan in our lab found that *bop1 bop2* mutations also rescue *bp* and *pny* inflorescence patterning defects, in an identical fashion to mutation of *knat2 knat6*. To investigate if *BOP1/2* are likewise misexpressed in *bp* or *pny* mutants, I examined the expression pattern of *BOP2* in *bp* and *pny* using a GUS reporter gene driven by the *BOP2* promoter (Figure 4.1). The *BOP2::GUS* reporter line has been examined before (Xu *et al.*, 2010; Chapter 2) and the GUS activities detected by this construct are in agreement with the *BOP2* RNA presence detected by *in situ* hybridization (Hepworth *et al.*, 2005; Karim *et al.*, 2009). In wild-type (WT), *BOP2* expression was detected in the floral meristem and the adaxial side of the pedicel where it joins with stem (Figure 4.1a, b, c, d). However, in *bp-2* mutants *BOP2* expression was expanded to the abaxial side of pedicels and stems beneath the nodes (Figure 4.1f, g, h, i), consistent with the downward orientation of siliques in this mutant. Further longitudinal- and cross-sections of stem showed that *BOP2* was misexpressed in stripe cells beneath nodes in *bp-2* mutants, which become ectopically lignified later in development (Figure 4.1g, h, i, j; Douglas *et al.,* 2002; Venglat *et al.*, 2002; Mele *et al.*, 2003). In *pny* mutants, *BOP2* expression was expanded to the stems and pedicels where siliques are clustered (Figure 4.1k, l, m, n) corresponding to the *pny* mutant phenotype. Cross sections of the stem showed that *BOP2* was misexpressed in the *pny* cortex and vascular bundle cells (Figure 4.1o). Collectively, my data show that *BOP1/2* expression domains are differentially expanded in *bp* and *pny* pedicels and stems, corresponding closely to the different patterning

defects seen in these mutants. These data indicate BP and PNY restrict *BOP1/2* expression domain in the stems for proper pedicel orientation and internode elongation.

4.3.2 *KNAT6* is misexpressed in stems of *bop1-6D*, *bp* and *pny*

Overexpression of *BOP1/2* results in short plants with irregular internodes, mimicking *bp* and *pny* mutants (Norberg *et al.*, 2005; Ha *et al.*, 2007). Ragni *et al.* (2008) showed that misexpression of *KNAT6* is required for *bp* and *pny* defects prompting me to examine the pattern of *KNAT6* expression in the stems and apices of WT control plants and *bop1-6D*, *bp*, and *pny* mutants, in which BOP1 is differently misexpressed (*bop1-6D* is generated by four 35S enhancers, also see Figure 4.1). The expression pattern of *KNAT6* in *bp* and *pny* mutants has been studied before using a *KNAT6::GUS* reporter gene but this reporter is not active in the shoot apex (Ragni *et al.*, 2008) indicating that some of its control sequences are missing. To accurately determine the expression pattern of *KNAT6*, I employed *in situ* hybridization for analysis. My results showed that *KNAT6* transcripts were localized at the boundary between the FM and IM in WT (Figure 4.2a), and remained at the boundary in *bp-2* and *pny* apices (Figure 4.2e, i). However, it is expanded to FMs in *bop1-6D* apices (Figure 4.2m), similar to the *BOP1/2* expression domain in WT apices (Figure2.1; Xu *et al.*, 2010), indicating that BOP may activate KNAT6. *KNAT6* transcripts were not detected in WT stems; however, they accumulated to high levels in the stems of *bop1-6D*, *bp-2* and *pny* mutants. In *bp-2*, *KNAT6* transcript was up-regulated in the epidermal stripe cells (Figure 4.2f, g, h), similar to the pattern of *BOP1/2* misexpression in *bp-2* mutants (Figure 4.1h, i, j). In *pny* and *bop1-6D* mutants, up-regulation was strongest in the vascular bundle cells (Figure 4.2j, k, l, n, o, p). Collectively, my data demonstrate that *KNAT6* is misexpressed in *bop1-6D* floral meristems (corresponding to the *BOP1/2* expression domain, Figure 2.1) and in *bp* and *pny* stems in a pattern that corresponds to

the *BOP1/2* misexpression domain in these mutants. These data suggest that BOP1/2 is a positive regulator of *KNAT6* expression.

4.3.3 BOP1/2 promote *KNAT6* expression in the inflorescence

To further test if BOP1/2 is a positive regulator of *KNAT6* expression, I used quantitative RT-PCR to examine *KNAT2* and *KNAT6* transcript levels in the stems and pedicels of WT control plants compared to *bop1-6D*, *bp-2*, and *pny* mutants. My results showed that there was no significant difference of *KNAT2* transcript accumulation among them (data not shown); however, the transcript levels of *KNAT6* were significantly higher in *bop1-6D*, *bp*, and *pny* tissues compared to WT (Figure 4.3), consistent with the idea that BOP1/2 promotes *KNAT6* expression. When I examined *KNAT6* transcript levels in *bop1 bop2 bp* and *bop1 bop2 pny* triple mutants (they look phenotypically WT) there was a significant reduction in transcript relative *bp* and *pny* control lines (Figure 4.3). This was again consistent with the idea that BOP1/2 promote *KNAT6* expression. Moreover, inactivation *KNAT6* in *35S::BOP2* lines rescues internode elongation, confirming that up-regulation of *KNAT6* in *35S::BOP2* lines is biologically relevant (Madiha Khan, unpublished data).

I also examined the model that ectopic BOP1/2 restricts internode elongation by repressing *BP*. Ha *et al* (2007) showed by semi-quantitative RT-PCR that *BP* transcripts are reduced in the shoots and stems of *35S::BOP1/2* plants. They also showed that mutation of *as2* partially rescues pedicel orientation but not internode elongation in *35S:BOP1/2* plants (Ha *et al.*, 2007). To test if *35S:BOP2/bop1-6D* phenotypes are caused by inhibition of *BP* expression, I monitored *BP* transcript level and distribution in WT, *bop1 bop2* and *bop1-6D* apices and stems by quantitative RT-PCR and by *in situ* hybridization. Surprisingly, I did not see any significant changes in *BP* expression in response to loss or gain of BOP1/2 function (Figure 4.4a, b, c, f). To

test if boundary cell identity at the stem-pedicel junction was intact in *bop1 bop2* mutants, I used *in situ* hybridization to test for misexpression of the boundary marker *CUC3* in stems and pedicels but saw no differences relative to WT control plants (Figure 4.4d, e). Collectively, these data indicate that BOP1/2 exerts its effect through promotion of *KNAT6* expression, and that co-misexpression of *BOP1/2* and *KNAT6* is required for growth restriction in stems since misexpression of *KNAT6* alone does not restrict growth in the inflorescence (Dean *et al.,* 2004).

4.4 Discussion

Previous studies have established that BOP1/2 is a negative regulator of *KNOX1* expression in leaves (Ha *et al.*, 2003; 2007; Chapter 3). To study the genetic interaction of *BOP1/2* with *BP* and *PNY*, I made crosses of *bop1 bop2* with *pny* and *bp* mutants. The triple mutants were selected by Mahida Khan who found that *bp* and *pny* did not rescue *bop1 bop2* leaf defect; instead that *bp* and *pny* inflorescence defects are partially or fully rescued by *bop1 bop2* (data not shown). I worked together with Madiha Khanto figure out the mechanism for this rescue. In this Chapter, I examined the expression of *BOP1/2*, *KNAT6*, *BP*, and *CUC3* spatially and quantitatively in different genotypes. I found that *BOP1/2* and *KNAT6* are co-misexpressed in the stems of *bp* and *pny* in similar patterns and that ectopic BOP1/2 promotes *KNAT6* expression in stems. Experiments by Madiha Khan showed that growth restriction in *bp* and *pny* stems requires the combined misexpression of *BOP1/2* and *KNAT6*; the misexpression of just one of these factors does not block internode elongation.

4.4.1 Spatial regulation of *BOP1/2* by *BP* and *PNY* is required for internode elongation

Previous work has established that the *KNOX1* genes *STM*, *BP*, *KNAT2*, and *KNAT6* are expressed in the SAM and repressed in developing leaves by the overlapping activities of

BOP1/2, AS1, and AS2 (Byrne *et al.*, 2000; Ori *et al.*, 2000; Semiarti *et al.*, 2000; Ha *et al.*, 2003; 2007; Jun *et al.*, 2010; Chapter 3). However, in the inflorescence, BP and PNY restrict *BOP1/2* and *KNAT2/6* expression domains for appropriate internode elongation (Ragni *et al.*, 2008; this study), suggesting differences in the functions of these genes during vegetative and reproductive development. Internode elongation is attributable to the proliferation and elongation of cells in the rib zone (RZ), which lies in the L3 layer of the SAM, below the central zone (CZ) (Fletcher 2002; Chapter 1). Interestingly, *BP* is not expressed in the RZ of the SAM when internode elongation is suppressed, while it is expressed in the cortex of stems (corresponding to the RZ) when internode elongation is allowed during reproductive development (Lincoln *et al.*, 1994; this study), suggesting that expression of *BP* in the RZ promotes internode elongation. Consistent with this, loss-of-function *bp* mutants are short. Interestingly, *PNY* is not expressed in the RZ of vegetative SAM nor in the reproductive inflorescence meristem, nor is *BP* expression impaired in *pny* mutants (Byrne *et al.*, 2003; Smith and Hake, 2003; Smith *et al.*, 2004). Nonetheless, *pny* mutants have shortened internodes between some secondary inflorescences and flowers, resulting in clusters of secondary inflorescences or flowers on the main stem. This indicates that factors other than BP are involved in internode elongation. The BELL protein ATH1 may be one of these factors. During vegetative development *ATH1* is expressed throughout the SAM during which time internode elongation is suppressed. Upon floral induction, *ATH1* is down-regulated and internode elongation begins. Consistent with this, *ath1* mutants show ectopic internode elongation between successive rosette leaves while the *35S::ATH1* inflorescences are extremely short caused by reduced cell proliferation in the RZ (Gomez-Mena and Sablowski, 2008). Regarding their opposite expression patterns and functions, it is unclear if *BP* represses *ATH1* nor if *ath1* can rescue *bp*, but it is noteworthy that loss-of-function *ath1* fully rescues *pny*

internodes and partially rescues *pny pnf* flowering (Rutjins *et al.*, 2009).

4.4.2 Internode elongation and flowering is coordinated in *Arabidopsis*

During the vegetative to reproductive transition, floral inductive signals cause the vegetative meristem to undergo morphological changes that are essential for flowering (reviewed in Liu *et al.*, 2009a; Barton *et al.,* 2010). Though *pny* and *pnf* single mutants have no obvious flowering time delay or morphological change of the SAM, the *pny pnf* double mutant fails to bolt and the size of the SAM is diminished (Smith *et al.,* 2004). Expression and complementation studies showed that PNY and PNF function upstream of LFY and that FT requires the activities of PNY and PNF to promote internode elongation and flowering (Smith *et al.,* 2004; Kanrar *et al.,* 2008). Mutation in ATH1, an activator for the floral repressor FLC, can partially restore the *pny pnf* non-flowering defect, suggesting that PNY and PNF may repress ATH1 then FLC to coordinate flowering and internode elongation.

Studies also show that the size of the SAM is smaller in *pny pnf* than that of the wild-type (smith *et al.*, 2004; Ung *et al.*, 2011). *MONOPTEROS* (*MP*), an auxin response factor, is expressed in the PZ in WT inflorescence meristems and the region between MP expression domains indicates the CZ. *In situ* analysis of MP in wild type and *py pnf* showed that the size of the central zone between the *MP* expression domains is reduced in *pny pnf* double mutant compared to that of the wild type (Ung *et al.*, 2011), suggesting that the number of stem cells in *pny pnf* is reduced. Accordingly, expression of *CLAVATA3* (*CLV3*), which is expressed at the CZ and negatively modulates the meristem size, is expanded in *pny pnf* and loss-of-function in *CLV3* partially restored *pny pnf* flowering (Ung *et al.*, 2011). Floral cells and internode cells are all generated from the activity of stem cells located in the SAM, diminished stem cells in the *pny pnf* indicate loss of growth potential and inability to give rise to floral meristem and RZ cell

proliferation (which gives rise to internodes, Chapter 1). Taken together, these data indicate that PNY and PNF coordinate flowering and internode elongation by regulating the number of stem cells in the CZ. Loss-of-function *bop1 bop2* and *knat2 knat6* can rescue *pny* defects (Ragni *et al.*, 2008; this study), but it is unknown if *bop1 bop2* and *knat2 knat6* can rescue the *pny pnf* meristem size and flowering. It will be interesting to know how BOP1/2 interact with PNY/PNF to coordinate internode elongation and flowering.

4.4.3 BOP1/2 activate *KNAT6*

The qPCR analysis in stems and pedicels (Figure 4.3) showed that BOP1/2 promote *KNAT6* transcript accumulation in these tissues. Interestingly, detailed expression pattern analysis showed that *BOP1/2* and *KNAT6* are co-misexpressed in the same tissues in *bp* and *pny*, and that *KNAT6* is ectopically expressed in the floral meristems of *bop1-6D* where *BOP1* is normally expressed (Figure 4.1, 4.2). Strikingly, *BP* required for internode elongation is not significantly up- or down-regulated in *bop1 bop2* or *bop1-6D* lines suggesting that BOP1/2 exerts its effects mainly through *KNAT6*.

4.4.3 Co-misexpression of *BOP1/2* and *KNAT6* is required to block internode elongation

My data show that *BOP1/2* and *KNAT2/6* are co-misexpressed in *bp* and *pny* and that BOP1/2 promotes *KNAT6* expression but at least two lines of evidence suggest that the combined activities of BOP1/2 and KNAT6 are required to restrict internode elongation. First, *35S:KNAT6* plants are not short (Dean *et al.*, 2004). Second, *bp knat2 knat6* and *pny knat2 knat6* plants are phenotypically normal but presumably still misexpress *BOP1/2* (Ragni *et al.*, 2008). Third, inactivation of *KNAT6* restores internode elongation in *35S:BOP2* plants. Although we do not yet understand how BOP1/2-KNAT6 antagonize BP-PNY activities, it does not appear to be at the transcriptional level since *BP* and *PNY* transcripts and reporter gene expressions patterns are

normal in *bop1 bop2* and *bop1-6D* stems (this study, Madiha Khan, unpublished data). One possibility is that BOP1/2 modifies KNAT6 function in such a way that it competes for BP-PNY DNA-binding sites to antagonistically regulate the expression of target genes that control internode elongation. Presumably, if this were the case, KNAT6 would need a BELL family partner. ATH1 is a candidate for this job since ATH1 functions antagonistically with PNY-PNF in internode elongation (Gómez-Mena and Sablowski, 2008; Rutjens *et al.*, 2009). Alternatively, BOP1/2 proteins localize to both the cytoplasm and the nucleus (Hepworth *et al.* 2005; Jun *et al.* 2010) and may potentially influence PNY activity, which is essential for STM and possibly BP nuclear localization (Hackbush *et al.* 2005; Rutjens *et al.* 2009). Short internodes are typical of defects in GA biosynthesis (Achard and Genschik, 2009; Schwechheimer and Willige, 2009), however, analysis of GA 20-oxidase transcript levels in *35S:BOP2* and *bop1-6D* stems did not show a significant change (Madiha Khan, data not shown) making it uncertain if BOP1/2-KNAT6 targets GA biosynthesis. Future experiments will address the mechanism of antagonism.

Table 4.1 Primers used for genotyping, qPCR analysis, and making *in situ* hybridization probes

Primer	Sequence 5' – 3'
Genotyping	
PNY Salk_40126 RP	TTGGAATTGGAGACAAAATGTGTTA
PNY Salk_40126 LP	GGAACCAAGTTCAAACTCGAATCCA
KN2 Salk_099837 RP	CAAAAGGTGATCTCGCTGCTTTCGT
KN2 Salk_099837 LP	AATCTCTAGCGCAAAAGTTTTTGCT
KN6 Salk_054482 F2	CTTACTTCAAGCTTACATCGATTGC
KN6 Salk_054482 LP	TGCTTTCTGATCACTTCAAAAGCCT
qPCR	
KNAT1-F1 HAY qPCR	CCATTCAGGAAGCAATGGAGTT
KNAT1-R1 HAY qPCR	ACTCTTCCCATCAGGATTGTTGA
KNAT2-F2	TCTGAAGGACCAGCTACTACGC
KNAT2-R1	ATTTTGTCGCCTTCAGTAGGGTAAG
KNAT6- F2	CTTACTTCAAGCTTACATCGATTGC
KNAT6-R1	CGCAGTACGTTTCCATAAATTCATC
***In situ* probes**	
BP-LF	ATGGAAGAATACCAGCATGACAAC
BP-T7-RR	CATAATACGACTCACTATAGGCCTTCTCTGACTCAGAAGGATATG
CUC3-F1	ATGATGCTTGCGGTGGAAGATGTG
CUC3-T7-R1	CATAATACGACTCACTATAGGCTACAGCTGGAATCCTAAAGGACATGG
KNAT6-F3 (in situ)	ATGGATGGAATGTACAATTTCCATTC
KNAT6-T7-R1	CATAATACGACTCACTATAGGTCATTCCTCGGTAAAGAATGAT

Figure 4.1 Expression patterns of *BOP2* in wild-type, *bp-2* and *pny* plants.

(a) to **(d)** wild-type control; *BOP2::GUS* localized in the adaxial pedicel stem cells.

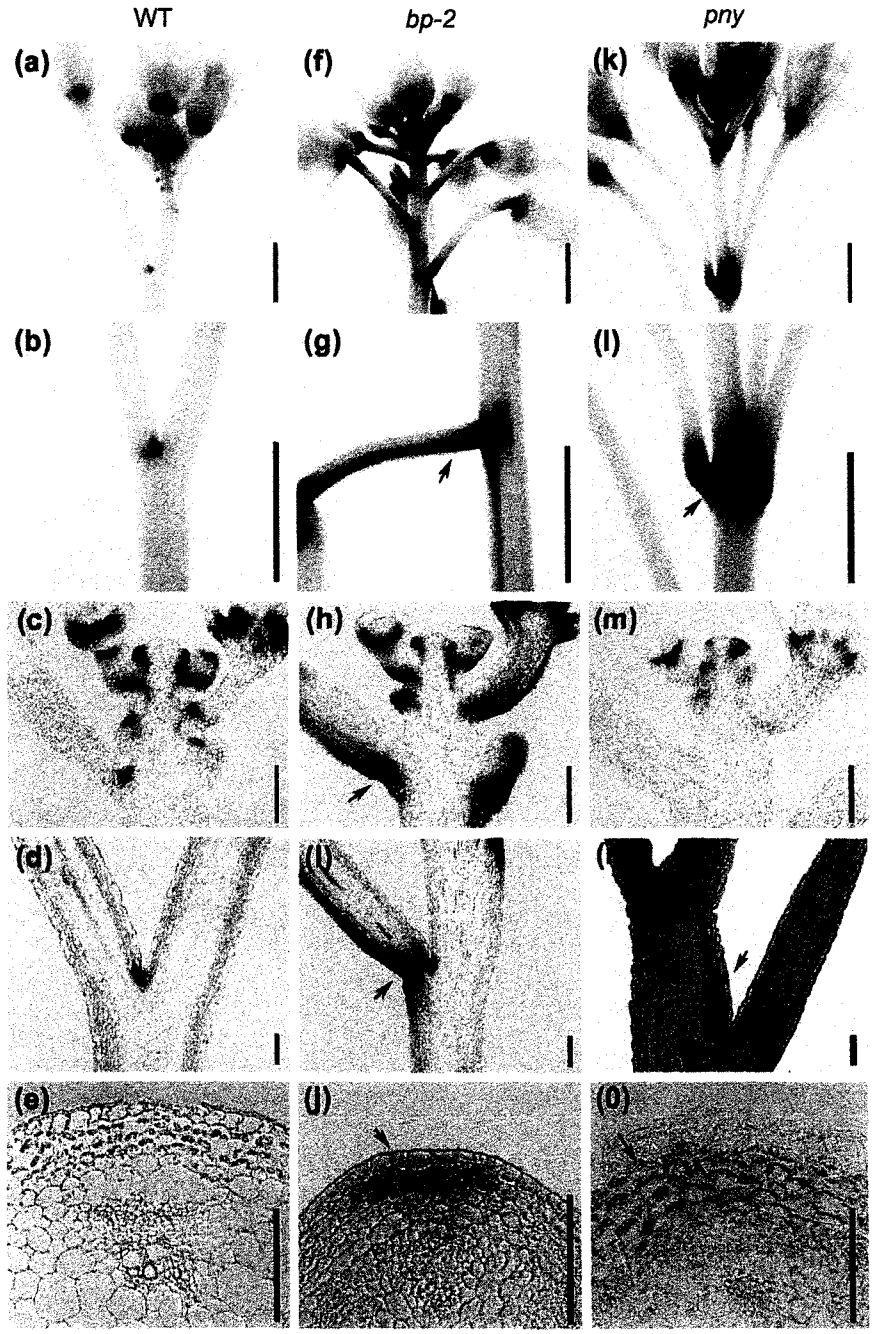
(e) Cross section of WT stem; *BOP2::GUS* expression was not detected.

(f) to **(i)** *bp-2*; ectopic expression in the abaxial pedicel cells and stem cells.

(j) Cross section of *bp-2* stem; ectopic expression in the stripe cells under the node.

(k) to **(n)** *pny*; ectopic expression in the stem and pedicel cells where internode elongation is irregular.

(o) Cross section of *pny* stem; ectopic expression in the cortex and vascular bundle cells. Scale bars, 100 μm.


WT
bp-2
pny
(a)
(f)
(k)
(b)
(g)
(l)
(c)
(h)
(m)
(d)
(i)
(e)
(j)
(o)

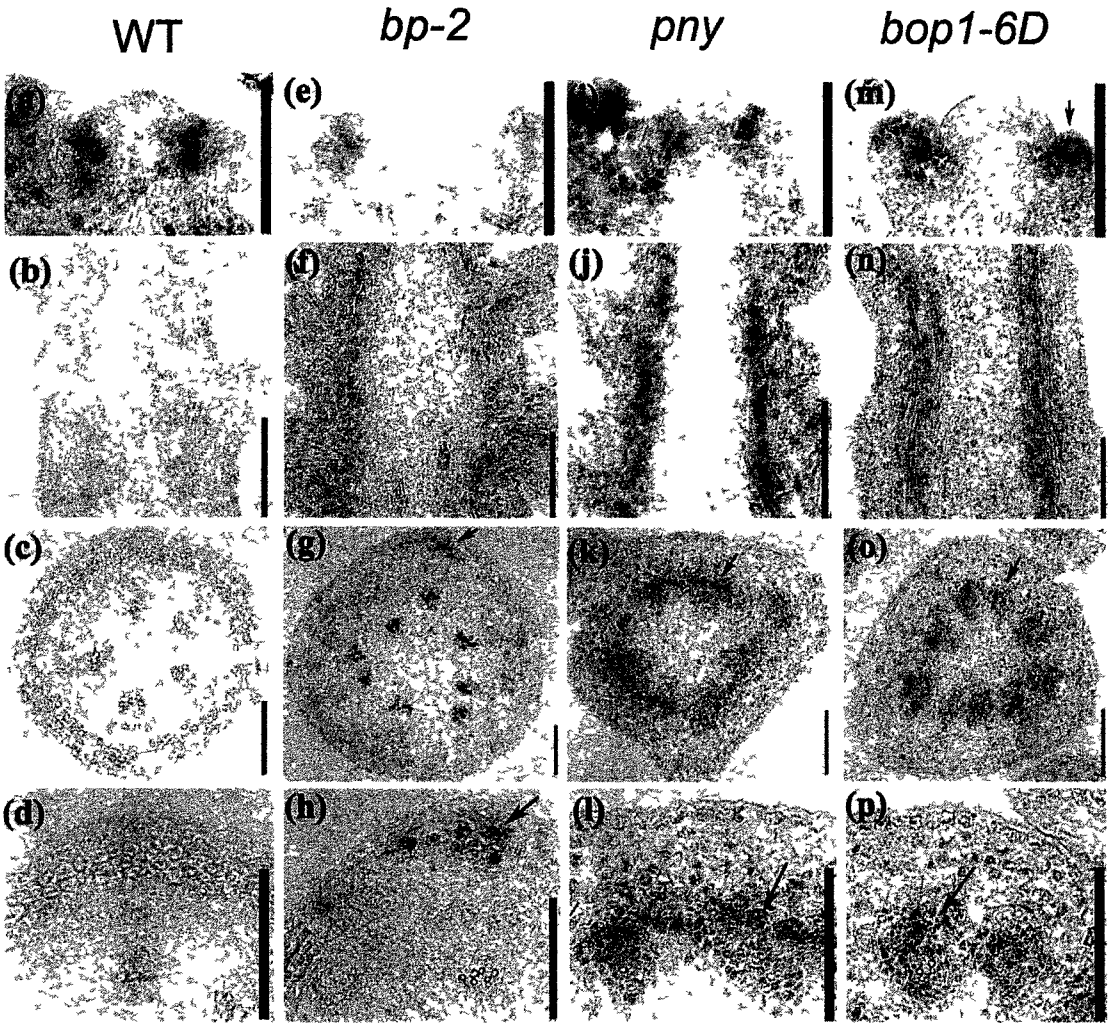


Figure 4.2 *In situ* analysis of *KNAT6* transcript accumulation in WT and mutant apices and stems.

(a-d) wild-type control; transcript localizes at the boundary between floral primordia and the inflorescence meristem.

(e-h) *bp-2*; ectopic expression in the epidermal cells and stripe cells.

(i-l) *pny*; ectopic expression in the vascular bundle cells.

(m-p) *bop1-6D*; ectopic expression in the vascular bundle cells. Scale bars, 100 μm.

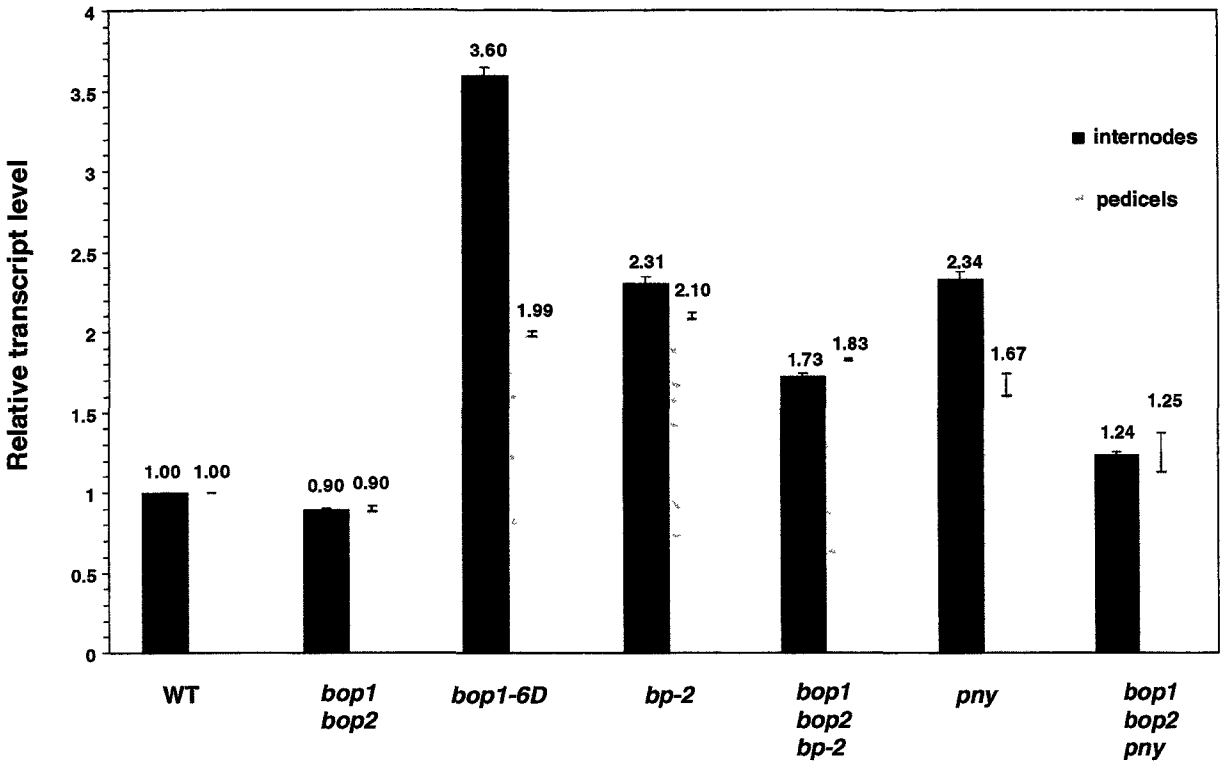


Figure 4.3 Quantitative analysis of *KNAT6* transcript accumulation in WT and mutant internodes and pedicels.

RNAs were isolated from internodes or pedicels and analyzed by qPCR for *KNAT6* expression. Genotypes are as indicated. *KNAT6* transcript level is high in *bop1-6D*, *bp-2* and *pny*, tissues compared to WT, and lower in *bop1 bop2 bp-2* and *bop1 bop2 pny* tissues compared to *bp-2* and *pny*, respectively. Values shown are mean. Error bars indicate s.e.m.

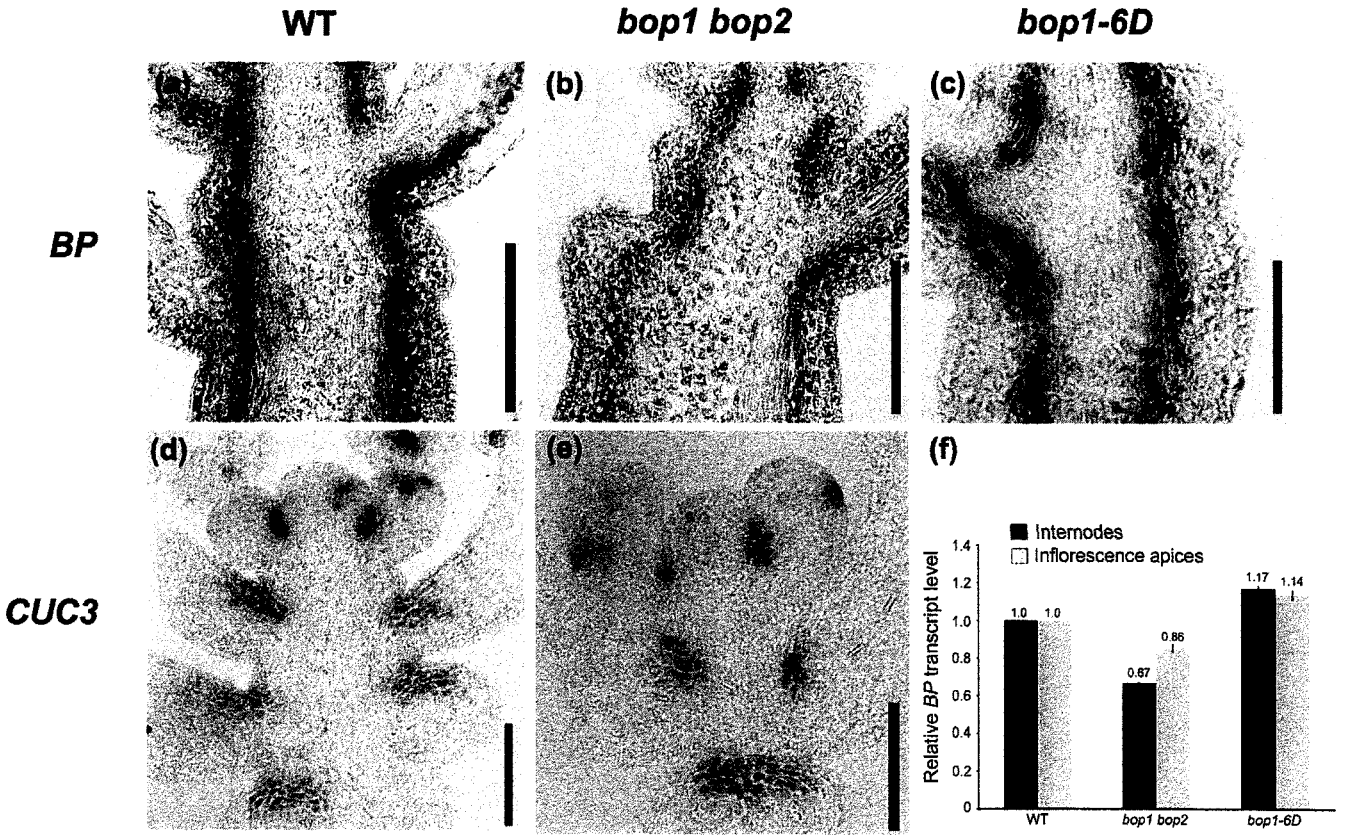


Figure 4.4 Spatial and quantitative analysis of *BP* and *CUC3* in different genotypes.

(a) to **(c)** *In situ* hybridization analysis *BP* transcript accumulation in stems of:

(a) wild-type control; high levels of *BP* transcript accumulation in stems and pedicels.

(b) *bop1 bop2*.

(c) *bop1-6D*. No significant change of *BP* transcript accumulation was detected.

(d) and **(e)** *In situ* hybridization analysis *CUC3* transcript accumulation in apices of:

(d) wild-type control and;

(e) *bop1 bop2*. *CUC3* transcripts are accumulated at the boundaries of organs. The expression pattern was the same in wild-type as in *bop1 bop2*. Scale bars, 100 μm.

(f) Quantitative RT-PCR analysis of relative *BP* transcript levels in the internodes and apices. *BP* transcript levels are similar for all genotypes. Values shown are mean. Error bars indicate s.e.m.

Chapter 5

Summary and future directions

5.1 Summary

This thesis examined the roles of the *BLADE-ON-PETIOLE* (*BOP1*) and *BOP2* genes in regulating *Arabidopsis* flower, leaf, and inflorescence architecture. I will summarize my findings and give directions for future studies in this Chapter.

My studies in the model plant species *Arabidopsis thaliana* have provided insight into the role that BOP1/2 plays in controlling floral meristem identity, leaf determinacy, and internode length.

In floral meristems, I show that BOP1 and BOP2 function redundantly with LFY, a master regulator of floral-meristem identity, to promote the expression of *AP1*, a key marker of commitment to floral fate. All three activities then converge in the floral meristem during stages 2-3 to down-regulate the expression of inflorescence identity genes including *AGL24*, *SOC1*, and *FUL* to ensure the floral determinacy. After stage 3, BOP1/2 function redundantly with LFY and AP1 to promote A-class floral patterning.

In leaves, I show that BOP1 and BOP2 in the petiole function redundantly with the leaf identity factors AS1 and AS2 to maintain the stable repression of *KNOX1* meristematic genes to facilitate the formation of a simple leaf.

In inflorescences, I show that expression domains of boundary genes *BOP1/2* and *KNAT6* are confined to pedicel axils by the overlapping activities of the KNOX1 family member BP and its interacting BELL partner PNY. My data show that *BOP2* and *KNAT6* expression domains are differentially enlarged in *bp* and *pny* mutants, corresponding to the distinctive patterns of short internodes, clustered or downward-oriented siliques, and defects in cell differentiation typical of

these mutants. My data further suggest that BOP1/2 are positive regulators of *KNAT6* expression and that this regulation may be direct.

Collectively, this work illustrates how modulation of meristematic activity and cell fate by BOP1/2 dramatically alters plant architecture. It will be interesting to determine whether BOP1/2 activity or expression patterns vary between plants species and form a platform for creating architectural diversity.

5.2 Future directions

5.2.1 What contributes to the blade growth on the petiole?

Blade growth on the petiole is a major defect in *bop1 bop2* mutants. Ha and coworkers (2007; 2010) interpret that the blade-on-petiole phenotype is caused by the combined misexpression of *KNOX1* and *YAB* abaxial identity genes and provide genetic evidence in support of this model. Our interpretation of the phenotype is that it reflects a partial conversion from simple to compound leaves. It will be very interesting to further investigate this simple to compound leaf transformation hypothesis by looking at genes that control simple-compound leaf deviation in WT and *bop1 bop2*.

5.2.1.1 Regulation of *KNOX1* genes in seedlings

I have shown in Chapter 3 that *KNAT2* and *KNAT6* are up-regulated in the SAM of *bop1 bop2* (Figure 3). However, the *KNAT2::GUS* and *KNAT6::GUS* lines that were used in this experiment were not homozygous lines. To confirm that *KNAT2* and *KNAT6* are up regulated in the SAM of *bop1 bop2* mutants, qPCR and *in situ* hybridization should be performed to re-examine *KNAT2* and *KNAT6* expression.

5.2.1.2 Examine polar auxin transport and auxin localization in the petiole

Local auxin maxima are present on the *Cardamine* rachis, and inhibition of polar auxin transport on the rachis can reduce the complexity of leaflets on *Cadarmine* petioles (Barkoulas *et al.*, 2008), indicating that local auxin maxima facilitated by polar auxin transport triggers organ (including leaflet) formation. Lama Musa from our lab has shown that the broad petiole of *bop1 bop2* resembles the phenotype of plants treated with the auxin influx inhibitor NOA. Therefore, it is very likely that BOP1/2 regulate auxin abundance and that mistransport of auxin in the petiole triggers the formation of blade-on-petiole. To investigate this, DR5::VENUS and PIN1::GFP lines could be used to study their localization in WT and *bop1 bop2* in petioles.

5.2.1.3 Examine *CUC* exp ression and genetic interaction of BOP1/2 with CUC genes

Although *BOP1/2* are expressed at the boundary between the leaf primordia and the SAM in stage 2 leaves (Figure 3.1b) their role at the boundary is not clear. Recently it was shown that misexpression of the organ boundary gene *CUP-SHAPED COTYLEDON2* (*CUC2*) in *Arabidopsis* causes transformation of simple leaves into compound leaves (Hasson *et al.*, 2011). Based on the fact that both *BOP1/2* and *CUC* are expressed at the boundary and both play roles in simple leaf formation, we hypothesized that there may be some interaction between them.

bop1 bop2 cuc1 and *bop1 bop2 cuc3* triple mutants have been constructed and these triple mutants do not show enhanced organ separation phenotypes. Since there is redundancy between *CUC* genes (*CUC1*, *CUC2*, and *CUC3*), *bop1 bop2* quadruple mutants with *cuc* genes will be constructed to confirm their mode of interaction. *CUC* and *BOP1/2* genes are both required for *KNOX1* repression in leaves (this study; Hasson *et al.*, 2011) suggesting that *BOP1/2* and *CUC* genes may function in separate or converging pathways to repress *KNOX1* genes. The phenotype of the quadruple mutants would tell if they function synergistically or not. To examine if BOP1/2

function upstream of CUC genes, the expression pattern of CUC1/2/3 could be examined in wild-type and *bop1 bop2*. If there is misexpression or down regulation of *CUC* genes in *bop1 bop2* mutant, it suggests that *BOP1/2* function upstream of *CUC* genes. Similarly, the expression of *BOP1/2* in *cuc* double mutants would tell us if *CUC* genes function upstream of *BOP1/2*.

5.2.2 Bract formation

My work in Chapter 2 has suggested that BOP1/2 are required to repress bract outgrowth in collaboration with LFY and AGL24. Liu *et al* (2009b) showed that the AGL24 in conjunction with *SOC1* and *SVP* suppress bract formation. However, the network regulating bract formation is not clear, which could be potentially interesting to study. The expression domains of *LFY*, *BOP1/2*, *AGL24*, *SOC1* and *SVP* define three distinct zones in late stage 2 floral primordia. *LFY*, *BOP1/2* and *SVP* share the floral meristem zone but partition differently. *LFY* is expressed in the adaxial part of the floral meristem with *BOP1/2* and *SVP* expression marking the boundary between the floral meristem and the cryptic bract zones (Weigel *et al.*, 1992; Hartmann *et al.*, 2000; Karim *et al.*, 2009; Xu *et al.*, 2010). *AGL24* and *SOC1* are only expressed in the cryptic bract zone (Yu *et al.*, 2004; Xu *et al.*, 2010); It should be investigated whether the activities from the floral meristem zone, cryptic bract zone and the boundary converge to facilitate growth of the flower and suppression of the bract.

The following triple or quadruple mutants *bop1 bop2 svp*, *bop1 bop2 soc1*, *bop1 bop2 agl24 svp*, *bop1 bop2 agl24 soc1*, *bop1 bop2 svp soc1*, *lfy svp agl24*, and *bop bop2 lfy agl24* could be generated see if floral development is suppressed and bract development is enhanced in these triple or quadruple mutants. RNA accumulation of *STM* in the stage 2 floral primordia of these triple or quadruple mutants would be examined to see if the meristematic cells have

changed. Also the cell division activity would be examined in the floral meristem zone and bract zone using the cell division marker histone H4 to see if the pattern of cell division has changed in the stage 2 floral primordia.

5.2.3 Direct target of BOP1/2 in the stem

My data suggested that *KNAT6* is a major transcriptional target of BOP1/2 in stems; however, we need to find out if *KNAT6* is a direct target of BOP1/2. To test this, we can make use of protein fusions to the rat glutacorticoid receptor (GR) and the yeast-one-hybrid system together with BiFC.

5.2.3.1 Identification of direct targets of BOP1 by *35S::BOP1-GR*

The introduction of a GR fusion protein into plants in combination with application of the steroid hormone dexamethasone (Dex) and cycloheximide (CHX) is an essential tool for identifying direct target genes in plants (Wagner *et al.*, 1999; Yu *et al.*, 2004; Jun *et al.* 2010). Dex is an inducer for the nuclear localization of the fusion protein and CHX is a protein synthesis inhibitor, which inhibits the synthesis of proteins allowing the identification of a direct target (Sablowski and Meyerowitz, 1998). If the transcript levels of a target gene show that is activated or repressed shortly after Dex treatment, and this activation or repression remains constant with CHX treatment, then the gene is likely a direct target of the GR fusion protein. I have constructed a *35S::BOP1-GR* fusion gene and transformed it into plants. We will treat the transgenic plants with Dex and harvest tissues 1h, 4h, and 24h after Dex treatment to see if *KNAT6* is up-regulated linearly. We will also treat the plants with Mock, Dex, CHX, and Dex+CHX regimes to see if *KNAT6* expression levels remain the same in Dex and Dex+CHX treated tissues.

5.2.3.2 Identification of the genomic region of the target that BOP-TGA complex binds

The fact that BOP1/2 proteins do not contain a DNA binding domain, they associates with TGA transcription factors in yeast (Hepworth *et al.*, 2005; Jun *et al.*, 2010; Xu *et al.*, 2010) and that BOP1 binds to its direct targets AS1 and AP1 at sites that TGA transcription factors potentially recognize and bind leads us to hypothesis that BOP1/2 may be recruited to the promoters of the genes it regulates through association with TGA factors. BOP1/2 activate *KNAT6* (Chapter4), and this activation could be direct. In addition, the *KNAT6* regulatory region contains potential TGA binding sites (http://www.athamap.de/), prompting us to examine if BOP1/2 function together with any of the TGA transcription factors to directly regulate *KNAT6* expression. The yeast-one-hybrid system could be employed to study if any of the ten TGA factors in *Arabidopsis* binds directly to the *KNAT6* promoter. If one or more TGAs bind to the *KNAT6* promoter, further deletion series on the *KNAT6* promoter could be created to test where the TGAs bind. Further, the BiFC analysis could be performed to study if the TGAs associate with BOP1/2 in plant cells. Alternatively, chromatin immunoprecipitation (ChIP) assay could be employed to examine the BOP-TGA binding sites on the regulatory region of the target gene (here refers to KNAT6).

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Appendix A. Summary of regulatory genes related to this thesis

Gene name	Protein it encodes	Expression	Biological function	Main reference
AG	MADS-box transcription factor	the centre of floral meristem from stage 3	specifies stamen and carpel identity; promotes floral determinacy	Drews *et al.*, 1991
AP1	MADS-box transcription factor	in the stage1 and 2 floral primordia; in the sepal and petal primordia	promotes FMI; specifies sepal and petal identity and represses stamen and carpel identity	Irish and Sussex, 1991; Wagner *et al.*, 1999
AP2	ERF transcription factor	in the sepal and petal primordia	specifies sepal and petal identity and represses stamen and carpel identity	Wollmann *et al.*, 2010
AP3	MADS-box transcription factor	in the petal and stamen primordia	specifies petal and stamen identity	Levin and Meyerowitz, 1995
AS1	MYB transcription factor	in the emerging leaf primordia	controls leaf morphology along the adaxial-abaxial and proximal-distal axes	Byrne *et al.*, 2000
AS2	LBD transcription factor	at the adaxial base of leaf primordia	controls leaf morphology along the adaxial-abaxial and proximal-distal axis	Iwakawa *et al.*, 2002; Xu *et al.*, 2003; Lin *et al.*, 2003
AXR1	subunit of the RUB1 ubiquitin enzyme	in the emerging leaf primordia	required in auxin signaling	Leyer *et al.*, 1993
ATH1	BELL1-like homeodomain protein	in the SAM and at the base of leaf primordia	maintains the SAM; represses internode elongation	Gomez-Mena and Sablowski, 2008; Rutjens *et al.*, 2009
BOP1/2	BTB-ankryin transcriptional co-regulators	in the stage 1 leaf primordia; localizes to the adaxial base of leaf primordia at stage 2; in stage 0, 1, 2 floral primordia and in sepal axils of later stage floral primordia	control leaf and floral architecture; bract outgrowth; and the shoot to flower transition	Hepworth *et al.*, 2005; Norberg *et al.*, 2005; Ha *et al.*, 2007
CAL	MADS-box transcription factor	in emerging floral primordia	promotes FMI	Kempin *et al.*, 1995
CLV1/2	Receptor-like proteins	in the L3 layer of SAM	negatively regulate meristem size	Clark *et al.*, 1997
CLV3	Small diffusible ligand	in the L1 and L2 layer of SAM	negatively regulates meristem size	Fletcher *et al.*, 1999

Gene name	Protein it encodes	Expression	Biological function	Main reference
CUC1/2/3	NAC transcription factors	boundaries between meristems and organs	separate organs and establish/maintain the SAM	Aida *et al.*, 1997; 1999
FD	bZIP transcription factor	SAM and floral anlagen	promotes flowering with FT	Abe *et al.*, 2005; Wigge *et al.*, 2005
FIL	YABBY transcription factor	abaxial side of leaf and floral primordia	promotes abaxial polarity	Eshed *et al.*, 2004
FLC	MADS-box transcription factor	leaves	represses flowering	Proveniers *et al.*, 2007
FT	small mobile proteins (florigen)	in leaf phloem companion cells; travels to IM	promotes flowering together with FD	Abe *et al.*, 2005; Wigge et al., 2005
FUL	MADS-box transcription factor	leaves; fruits; IM and abaxial side of floral stage 2 primordia	promotes fruit development and shoot to flower transition	Ferrandiz *et al.*, 2000
JAG	Zinc finger protein	leaf and sepal primordia	promotes cell proliferation at the distal part of sepals and petals and bract outgrowth	Dinneny *et al.*, 2004; Ohno *et al.*, 2004
KAN	GARP family transcription factor	abaxial side of leaf primordia	promotes abaxial polarity	Emery *et al.*, 2003
BP	KNOX1 homeodomain protein	in the PZ of SAM and cortex of internodes and pedicels	Establishes/ maintains the SAM; promotes internode elongation	Byrne *et al.*, 2003; Venglat *et al.*, 2002
KNAT2	KNOX1 homeodomain protein	at the base of SAM; stem-pedicel boundary	represses internode elongation	Belles-Boix *et al.*, 2006; Ragni *et al.*, 2008
KNAT6	KNOX1 homeodomain protein	at the primordia-SAM boundary; stem-pedicel boundary	establishes and maintains the SAM; represses internode elongation	Belles-Boix *et al.*, 2006; Ragni *et al.*, 2008
LFY	a unique class of plant transcription factor	Stage 0, 1, 2, floral primordia; floral organ primordia;	promotes FMI, and floral organ patterning	Weigel *et al.*, 1992
LOF	MYB transcription factor	organ boundaries	separate organs and promote axillary meristem formation	Lee *et al.*, 2009
LMI1	HD-ZIP transcription factor	leaf primordia and floral primordia	promotes FMI	Saddic *et al.*, 2006
NPR1	BTB-ankyrin transcriptional co-regulator	leaves, all tissues	mediates response to pathogen attack	Cao *et al.*, 1997
PAN	TGA transcription factor	apex of IM and floral meristems	sepal and petal patterning; FM determinancy	Das *et al.*, 2009; Maier *et al.*, 2009

Gene name	Protein it encodes	Expression	Biological function	Main reference
PHV	HD-ZIP transcription factors	adaxial side of leaf primordia	promotes adaxial polarity	Byrne, 2006
PI	MADS-box transcription factor	petal and stamen	specifies petal and stamen identity	Lamb *et al.*, 2002
PIN1	auxin efflux carrier	roots, vasculature of leaves, epidermal cells of IM, incipient primordia	facilitates polar auxin transport	Benková *et al.*, 2003; Barkoulas *et al.*, 2008
PNF	BELL-like homeodomain protein	in the SAM; in the centre of the IM and floral meristem	establishes and maintains the SAM; coordinates internode elongation and flowering	Smith *et al.*, 2004
PNY	BELL-like homeodomain protein	as a stripe between the IM and floral meristem	establishes and maintains the SAM; coordinate internode elongation and flowering	Smith and Hake 2003
SEP1/2/3/4	MADS-box transcription factors	in all floral whorls	promotes floral organ identity in all whorls	Pelaz *et al.*, 2000; Ditta *et al.*, 2004
SOC1	MADS-box transcription factor	in inflorescence meristems and the abaxial side of stage2 floral meristems	promotes flowering	Samach *et al.*, 2000
STM	KNOX1 homeodomain protein	in the SAM but absent from the incipient primordia	establishes and maintains the SAM	Long *et al.*, 1996
SPL	SQUAMOSA PROTEIN-LIKE transcription factors	in stage 1 and stage 2 floral primordia	control age-related flowering time	Wang *et al.*, 2009; Wu *et al.*, 2009
SVP	MADS-box transcription factor	in the floral meristem; and at the boundary between floral and bract meristems	represses flowering; promotes IM identity	Hartmann *et al.*, 2000
TGAs	bZIP transcription factors	in leaves, floral meristems and the inflorescence meristems	mediate plant defense response and floral development	Zhang *et al.*, 1999; Das *et al.*, 2009
UFO	F-box protein	PZ of the SAM; floral meristems and floral primordia	promotes FMI and specifies petal and stamen identity	Wilkinson and Haughn, 1995
YAB	YABBY transcription factor	abaxial side of leaf primordia	promotes abaxial polarity	Eshed *et al.*, 2004
WUS	homeodomain transcription factor	in the organization centre of SAM and floral meristems	controls stem cell activity and determinacy	Laux *et al.*, 1996

Appendix B. Genetic interactions in double mutant analysis and what they mean

Analysis of double mutant phenotypes is employed extensively as a major guide line to determine the potential mode of interaction between two genes (for example A and B). This method is especially powerful because it can give answers without knowledge of all of the genes in a pathway or the products that they encode. Interactions may be described as additive, synergistic, antagonistic, or epistatic. Criteria for these interactions are described below.

If the phenotype of the double mutant is additive (a plus b), it indicates that the two genes being analyzed function in non-interacting pathways. However, if the phenotype of the double mutant is synergistic (more than a plus b, a new phenotype may occur), it indicates that the two genes function in redundant pathways. This redundancy may be partial or complete. If A can completely compensate for B then gene function is completely redundant. In many cases however, two genes are only partially redundant since they may share a subset of developmental or cellular functions but they also each have unique functions in other processes. Closely related members of a gene family often have partial redundancy in which a shared set of functions are preserved and independent functions might also be involved (Pickett and Meeks-Wagner, 1995). When synergy occurs between closely related members of a gene family, it is often the case that the genes function together in one pathway, sharing the same task in a homologous fashion. Functional redundancy can also occur between non-homologous genes or pathways. This is often true of developmental processes, such as flowering, which are governed by multiple regulatory pathways. In this case, synergy arises when two pathways that converge at a node are disrupted (Pérez-Pérez *et al.* 2009).

When a double mutant is made, phenotypes can be enhanced (worsened) or suppressed (improved). If mutation of gene B enhances the phenotype of A, then both are functioning redundantly to control a phenotypic trait as discussed above. If on the other hand, mutation of gene B suppresses the phenotype of A, then genes A and B function antagonistically. Antagonistic interactions may be observed for example if gene B is misexpressed in mutant A, or when genes A and B might have opposite effects on the transcription of gene C, which is a master regulator of the phenotypic trait in question. The reason for the antagonistic genetic interaction must be evaluated empirically.

Double mutant analysis may also give information about the order of gene function in a linear pathway. This is most easily assayed when single mutations (a and b) produce different phenotypes from the wild-type and from each other, and the double mutant phenotype looks like one of the phenotypes produced by a single mutation (a or b). This mutation is said to be epistatic to the other. The epistatic mutation may be in either the upstream or downstream gene, depending on the nature of the two mutations and the type of regulation. In a simple linear pathway in which the regulatory interactions are all positive, the upstream mutation will be epistatic to the downstream mutation. (Avery and Wasserman, 1992).