Genetic Analysis of Co-ordination of Flowering and Regulation of Inflorescence Architecture in *Arabidopsis thaliana*

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ABSTRACT

In the model plant *Arabidopsis thaliana,* meristem acquisition of inflorescence fate promotes both the production of flowers at the expense of leaves and the elongation of internodes to generate the inflorescence. Competence to respond to floral-inductive signals requires the activities of two BELL homeodomain proteins: PENNYWISE (PNY) and POUND-FOOLISH (PNF). Inactivation of these genes blocks meristem conversion to an inflorescence meristem fate, but their mode of action remains unclear. My data provide evidence that the ectopic activity of four lateral organ boundaries genes acting in the same pathway blocks the conversion to IM fate in *pny pnf* double mutants. This pathway is defined by *BLADE-ON-PETIOLE1/2 (BOP1/2), ARABIDOPSIS THALIANA HOMEOBOX GENE1 (ATH1),* and *KNOTTED1-LIKE FROM ARABIDOPSIS THALIANA6 (KNAT6).* My data are consistent with the model that ectopic BOP1/2 activity in meristems promotes the misexpression of *ATH1* and *KNAT6* whose products form a transcription factor complex that antagonizes competence to flower.

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GLOSSARY OF GENETIC TERMS

Loss-of-function - loss or reduction of activity

Gain-of-function - ectopic or increased activity

Phenotypic suppression - towards or to the wild-type phenotype

Phenotypic enhancement – worsening a mutant phenotype

Redundancy –when there is genetic compensation in the event of a loss

GENETIC NOMENCLATURE IN *ARABIDOPSIS THALIANA*

Wild-type gene - *BOP1*

Wild-type protein - BOP1

Loss-of-function mutant (homozygous) - *bop1*

Loss-of-function mutant (heterozygous) - *bop1/+*

Gain-of-function (dominant) mutant - *bop1-6D*

Promoter fusion to a gene coding region - *BOP1::BOP1*

Protein fusion - BOP1:GR

LIST OF ABBREVIATIONS

AGL24 - AGAMOUS-LIKE24

AP1 – APETALA1

ATH1 – ARABIDOPSIS THALIANA HOMEOBOX GENE1

BELL - BEL1-like

BOP – BLADE-ON-PETIOLE

BP – BREVIPEDICELLUS

bZIP – basic leucine zipper

CLV - CLAVATA

CO - CONSTANS

CUC - CUP-SHAPED COTYLEDON

CZ - central zone

EMF1 - EMBRYONIC FLOWER1

FLC - FLOWERING LOCUS C

FRI - FRIGIDA

FT - FLOWERING LOCUS T

FUL - FRUITFULL

GA - gibberellin

GUS - β-Glucoronidase

IM - inflorescence meristem

KNAT – KNOTTED1-LIKE FROM ARABIDOPSIS THALIANA

KNOX - KNOTTED1-LIKE homeobox

LFY - LEAFY

MAF - MADS AFFECTING FLOWERING

NPR1 - NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1

PAN - PERIANTHIA

PNF – POUND-FOOLISH

PNY - PENNYWISE

PZ - peripheral zone

RZ - rib zone

SAM – shoot apical meristem

SOC1 - SUPPRESSOR OF OVER-EXPRESSION OF CONSTANS1

SPL - SQUAMOSA PROMOTER BINDING PROTEIN-LIKE

STM - SHOOT MERISTEMLESS

SVP - SHORT VEGETATIVE PHASE

TALE – three-amino-acid-loop-extension

TFL - TERMINAL FLOWER1

WUS - WUSCHEL

CHAPTER 1 INTRODUCTION

1.1 Overview

In plants, aerial organs and tissues are derived from the shoot apical meristem (SAM) found at the tip of the primary shoot. During vegetative development in *Arabidopsis thaliana,* the meristem produces leaves without internode elongation, generating a compact rosette. In response to endogenous and environmental floral-inductive signals, the SAM acquires an inflorescence meristem (IM) fate. Whereas the SAM directs the production of leaves, the IM directs the production of internodes, lateral branches, and flowers that comprise the inflorescence (reviewed in Fletcher, 2002).

In *Arabidopsis thaliana,* flower production and internode elongation are concomitant processes and their formation is likely to be co-ordinately regulated to optimize the timing and display of flowers for reproductive success. Three-amino-acid-loop-extension (TALE) homeodomain proteins are an atypical superclass of homeodomain proteins that are conserved among eukaryotes (plants, animals, and fungi) and characterized by a three-amino acid extension to the loop connecting the first and second helices of the homeodomain (Bürglin, 1997). The *Arabidopsis thaliana* TALE proteins PENNYWISE (PNY) and POUND-FOOLISH (PNF) were previously shown to be required for meristem competence to flower (Smith *et al.,* 2004), and thus co-ordinate the formation of both flower

formation and internode elongation but their molecular mode of action remains unclear. My work sheds light on this important question in plant developmental biology.

1.2 *Arabidopsis thaliana* as a Model Species

Flowering plants rapidly diversified from a common ancestor approximately 150 million years ago, making *Arabidopsis thaliana* closely related to several hundred thousand different plant species. *Arabidopsis thaliana* (mouse ear cress) was originally adopted as model system in the 1970's based on its usefulness as a genetic system: the plant is a self-fertilizing diploid with a small genome, simple growth requirements, high seed set, and is easily transformable using *Agrobacterium.* It was widely adopted by the plant community as a model organism in the 1980's. The *Arabidopsis* genome was the first plant genome to be fully sequenced and the information, along with numerous research tools including seeds and clones, are publically available at little or no cost (www.arabidopsis.org) (reviewed in Meyerowitz, 1989; Somerville & Koornneef, 2002). Developmental programmes similar to those in *Arabidopsis* along with many orthologous genes found in other plant species has allowed *Arabidopsis* studies to serve as a foundation for applied work in commercially important plant species (Bevan & Walsh, 2005).

1.2.1 Life Cycle of *Arabidopsis*

Unlike animals, where the body plan is completed during embryogenesis, plant organs and tissues are continually produced over the life cycle of plant. Thus, plant development is broadly divided into three phases during which different structures are produced: embryonic development, vegetative development, and reproductive development. Transition between these phases is tightly controlled. Embryonic development begins at fertilization and is completed during seed development. Germination triggers the onset of vegetative development, in which *Arabidopsis* produces a compact rosette of leaves (Figure 1.1). At the onset of reproductive development, lateral branches and flowers are formed at the expense of leaves, and internodes are elongated to produce an inflorescence (Figure 1.1). Thus, although all aerial structures of the plant are generated by the SAM—different structures are elaborated according to the phase of development (Steeves & Sussex, 1989; reviewed in Fletcher, 2002).

1.3 SAM Organization and Maintenance

1.3.1 Organization of the SAM

The SAM is located at the growing tip of the primary shoot of plants. The central zone (CZ) of the SAM contains a pool of self-renewable stem cells whose progeny are displaced towards the peripheral zone (PZ) of the SAM where they

are incorporated into developing organs. The rib zone (RZ) is located below the CZ (Figure 1.2) and contains cells that form the pith of the meristem and give rise to cells that form the bulk of the stem (reviewed in Fletcher, 2002). Shortly after primordia form in the PZ, they are physically separated from the meristem by a small band of cells with boundary identity. This creates three compartments within the shoot apex: the meristem, the primordia, and the boundary separating the two. Interactions between genes expressed in each of these compartments plays an important role in establishing, maintaining, and regulating the SAM organization and activity (reviewed in Rast & Simon, 2009).

1.3.2 Establishment and Maintenance of the SAM

Aerial tissues are derived post-embryonically from the SAM. Maintenance of the SAM requires a fine balance between division of pluripotent stem cells of the CZ and loss of cells to the PZ of the SAM and subsequent integration into lateral organs. Establishment of the embryonic meristem requires the activities of *CUP-SHAPED COTYLEDON* (*CUC*) genes associated with the meristem-lateral organ boundary. After establishment of the embryonic SAM, maintenance is achieved by the activities of a WUSCHEL-CLAVATA (WUS-CLV) signaling loop and the overlapping activities of KNOX and BEL1-like (BELL) homeodomain transcription factors (reviewed in Fletcher, 2002; Barton, 2010; Hamant & Pautot, 2010).

1.3.2.1 Establishment of the SAM

Three NAC-domain containing transcription factors: CUC1, CUC2, and CUC3, are expressed in lateral organ boundaries and function redundantly to establish the embryonic SAM (Aida *et al.*, 1997; Vroemen *et al.*, 2003). Lateral organ boundaries are sites of restricted growth between the meristem and emerging lateral organs that permit meristem-organ and organ-organ separation. Genetic studies also show that lateral organ boundaries genes contribute to post-embryonic SAM maintenance (reviewed in Aida & Tasaka, 2006). Gain-of lateral organ boundary gene function typically causes ectopic growth restriction. Conversely, loss-of lateral organ boundary gene function typically causes ectopic growth between organs and defects in lateral organ differentiation (reviewed in Aida & Tasaka, 2006). Loss of CUC activity prevents the establishment of the embryonic SAM and results in fused cotyledons as growth restriction is abolished in meristem-cotyledon boundaries (Aida *et al.*, 1997). CUC activity in globular stage embryos promotes expression of the KNOTTED1-like homeobox (KNOX) gene *SHOOT MERISTEMLESS* (*STM*) in the SAM (Aida *et al.*, 1999). *STM* expression in the SAM confers stem-cell identity (Barton & Poethig, 1993) and provides feedback by inhibiting *CUC2* expression, restricting its expression to the periphery to promote establishment of the lateral organ boundaries during embryogenesis (Aida *et al.*, 1999).

1.3.2.2 WUS-CLV Feedback Loop

Following its establishment during embryogenesis, the SAM is maintained during vegetative development by activity of the CLV1/2/3 and WUS feedback loop. The WUS transcription factor is expressed in a small group of cells in the RZ of the meristem, directly beneath the CZ, known as the organizing center. WUS protein is mobile and functions non-cell autonomously to confer stem cell identity to cells of the CZ where it also stimulates production of the signalling ligand CLV3 (Yadav *et al.*, 2011). CLV3 binds a dimer of receptors encoded by *CLV1* and *CLV2* expressed in cells surrounding the CZ, leading to repression of *WUS* and controlling the size of its expression domain, thus preventing over-proliferation of the stem cell population (reviewed in Carles & Fletcher, 2003).

1.3.2.3 KNOX and BELL Homeodomain Proteins

The TALE superfamily of homeodomain transcription factors is divided into BELL and KNOX subgroups, whose products form heterodimers with high affinity for DNA and regulate several processes in development, including meristem maintenance (Smith *et al.*, 2002; reviewed in Hamant & Pautot, 2010). The class I KNOX homedomain protein STM plays a central role in maintaining the meristem. Strong *stm* loss-of-function mutants are unable to replenish their stem cell population and initiate only a few leaves (Long *et al.*, 1996). Double

mutant analyses shows that two other class I KNOX genes expressed in the meristem and lateral organ boundaries respectively, *BREVIPEDICELLUS* (*BP*) and *KNOTTED1-LIKE FROM ARABIDOPSIS THALIANA* (*KNAT6*), function redundantly with *STM* in meristem maintenance since their inactivation enhances the meristem defects of weak *stm* mutants (Belles-Boix *et al.*, 2006; Byrne *et al.*, 2002). *KNAT2* is also expressed in meristem RZ, but its role in meristem maintenance is unclear since inactivation does not enhance the meristem defects of weak *stm* mutants (Byrne *et al.*, 2002). However, over-expression of class I KNOX genes causes the formation of ectopic meristems in leaf tissue, confirming the central role of KNOX genes in SAM formation (Lincoln *et al.*, 1994; Chuck *et al.*, 1996; Pautot *et al.*, 2001; Gallois *et al.*, 2002; Dean *et al.*, 2004).

The BELL homoedomain proteins PNY, PNF, and ARABIDOPSIS THALIANA HOMEOBOX GENE1 (ATH1) also play a key role in maintaining the meristem as *ath1 pny pnf* loss-of-function results in an *stm* loss-of-function phenotype (Rutjens *et al.*, 2009). PNY, PNF, and ATH1 interact with STM and are proposed to facilitate STM nuclear localization to promote SAM maintenance (Rutjens *et al.*, 2009). Furthermore, PNY and PNF may also promote boundary maintenance and regulate the expression domains of *WUS* and *CLV3* to promote SAM maintenance (Ung *et al.*, 2011). Interestingly, PNY-PNF and ATH1 activities

begin to diverge at the transition to flowering. ATH1 represses flowering (Proveniers *et al.*, 2007) whereas PNY-PNF promote the floral transition (Smith *et al.*, 2004).

1.4 Transition from Vegetative to Reproductive Development

1.4.1 The Decision to Flower

The transition from vegetative to reproductive development in plants is a tightly controlled developmental decision that is timed to ensure optimal reproductive success. This transition to flowering is controlled by input from several pathways that relay endogenous and environmental signals. The major pathways are the autonomous pathway that mediates internal and age-related flowering signals, the vernalization pathway that promotes flowering after exposure to prolonged cold, and the long day pathway that promotes flowering in response to long day photoperiods (reviewed in Turck *et al.*, 2008). Signals from these flowering time pathways converge to regulate the expression of a small number of floral integrator genes including *LEAFY* (*LFY*), *FLOWERING LOCUS T* (*FT*), and *SUPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) (reviewed in Parcy, 2005). Floral integrator expression in the shoot apical meristem confers inflorescence meristem identity and promotes the transition to flowering (reviewed in Parcy, 2005; Liu *et al.*, 2009). At this transition, the

meristem switches from production of leaves to production of flowers that are separated on the primary stem by elongated internodes. How floral integrators promote flower development is well studied but genetic studies have not uncovered a role for these genes in initiating internode elongation. Floral integrators promote the expression of master regulators of floral development, encoded by *LFY* and *APETALA1* (*AP1*) in lateral organ primordia (reviewed in Henderson & Dean, 2004; Putterill *et al.*, 2004).

Long day photoperiods are a powerful stimulus for flowering in *Arabidopsis*. Long day photoperiods stimulate up-regulation of the zinc finger transcription factor encoded by *CONSTANS* (*CO*), a central member of the pathway that promotes flowering in response to photoperiod (Putterill *et al.*, 1995). Loss-of-function *co* mutants exhibit a significant delay in flowering time in long day photoperiods (Koornneef *et al.*, 1998). Furthermore, constitutive expression of *CO* promotes early flowering in both long day and short day photoperiods (Onouchi *et al.*, 2000). Following accumulation of CO in long day photoperiods, CO activates the expression of the floral integrators *SOC1* in the shoot apex and *FT* in the leaves (Samach *et al.*, 2000). FT protein is translocated via the phloem from the leaves to the SAM where it exerts is function (Abe *et al.*, 2005; Wigge *et al.*, 2005; Corbesier *et al.*, 2007). In the SAM, SOC1 directly activates the expression of *LFY*, a floral integrator and master floral meristem identity gene in

lateral organ primordia (Liu *et al.*, 2008). LFY directly activates expression of the floral meristem identity gene *AP1* to further promote floral fate of lateral organ primordia (Wagner *et al.*, 1999). A complex of FT protein with the bZIP protein FD is a second direct activator of *AP1* expression. These interactions describe how floral integrators SOC1 and FT-FD promote flowering in response to floral inductive conditions by activating the expression of key floral meristem identity genes.

1.4.2 Formation of Flowers

Following establishment of floral meristem identity, LFY and AP1 in the floral meristem promote the expression of floral organ identity genes that encode transcription factors that serve to promote differentiation of the various floral organs (Savidge *et al.*, 1995; Wagner *et al.*, 2004; reviewed in Liu *et al.*, 2009). In *Arabidopsis,* floral meristems are determinant structures, producing a finite number of floral organs. Genes conferring IM identity are directly repressed by AP1 in floral meristems to promote determinacy (Yu *et al.*, 2004; Liu *et al.*, 2007; reviewed in Liu et al. 2009). In *ap1* mutants, failure to down-regulate *SHORT VEGETATIVE PHASE* (*SVP*), *AGAMOUS-LIKE24* (*AGL24*), and *SOC1* in the floral meristem causes the partial conversion of flowers to inflorescences (Liu *et al.*, 2007). LFY and AP1 in floral meristems also down-regulate *TERMINAL FLOWER1* (*TFL1*) expression to confer determinacy. In the IM, TFL1 conversely

inhibits *LFY* and *AP1* expression so that the IM remains indeterminate and capable of producing numerous floral meristems. In *tfl1* mutants, the IM is replaced by a single floral meristem (Liljegren *et al.*, 1999; Ratcliffe *et al.*, 1999). Thus, LFY and AP1 are key regulators of floral meristem identity.

1.4.3 Elongation of Internodes

The switch to flowering in *Arabidopsis* is tightly linked to the onset of internode elongation to allow formation of the inflorescence. Internode elongation requires the polarized growth and proliferation of cells in the meristem RZ (Vaughn, 1955; reviewed in Fletcher, 2002). Following their elongation, differentiation of lignified interfascicular fibres with secondary thickened cell walls provides mechanical support to the stem (Nieminen *et al.*, 2004; Ehlting *et al.*, 2005). Both internode elongation and secondary differentiation of fortified tissue are dependent on the activities of BP and PNY in the stem following the transition to flowering (Lincoln *et al.*, 1994; Douglas *et al.*, 2002; Venglat *et al.*, 2002; Byrne *et al.*, 2003; Mele *et al.*, 2003; Smith & Hake, 2003). Internode elongation is also promoted by the plant hormone gibberellic acid (GA) that is synthesized in leaves and promotes cell elongation (reviewed in Ross *et al.*, 1997; Ross *et al.*, 2002; reviewed in Achard & Genschik, 2009; Dayan *et al.*, 2012).

How internode elongation and flower formation are mechanistically linked is an area of active study. Loss-of-function mutations in genes conferring floral meristem identity impair formation of flowers but elongate internodes normally indicating that acquisition of floral meristem identity and internode elongation are regulated separately. In contrast, acquisition of IM identity is more tightly linked to internode elongation. For example, both flowering and internode elongation are blocked in plants over-expressing the floral repressor FLOWERING LOCUS C (FLC) (Michaels & Amasino, 1999; Sheldon *et al.*, 1999). Similarly, mutations in genes that delay flowering time also delay internode elongation (Lee *et al.*, 2000).

1.5 Meristem Competence to Flower

1.5.1 FLC

Vegetative SAM acquisition of reproductive IM fate requires meristem competence to respond to floral inductive signals. Early in vegetative development, flowering is repressed as the meristem is not competent to initiate flowering. This is evidenced by mutants such as *embryonic flower1* (*emf1*) that initiate flowering immediately upon germination, resulting in a terminal flower phenotype (Bai & Sung, 1995). The MADS-box transcription factor FLC is a key repressor of flowering and regulator of competence to flower (Michaels &

Amasino, 1999; Sheldon *et al.*, 1999). Constitutive expression of *FLC* can delay or completely block flowering in a dosage-dependent manner (Michaels & Amasino, 1999; Sheldon *et al.*, 1999; Werner *et al.*, 2005). FLC forms a complex with the closely related MADS-box factor SVP (Hartmann *et al.*, 2000; Helliwell *et al.*, 2006; Li *et al.*, 2008) and directly represses the expression of the key floral integrator genes *FT, SOC1,* and *FD* (Hepworth *et al.*, 2002; Helliwell *et al.*, 2006; Searle *et al.*, 2006). Thus, expression of several floral integrator genes are antagonistically regulated by FLC and signals that promote flowering to regulate flowering time (Samach *et al.*, 2000; Hepworth *et al.*, 2002; reviewed in Boss *et al.*, 2004; Helliwell *et al.*, 2006; Figure 1.3). Thus, down-regulation of *FLC* allows the induction of floral integrator gene expression and flowering.

1.5.2 Regulators of *FLC* Expression

1.5.2.1 Vernalization Pathway

Various alleles of *FLC* and *FRIGIDA* (*FRI*) - a scaffold protein that functions in activation of *FLC* expression by recruiting general transcription factors and chromatin remodelling factors (Choi *et al.*, 2011) - dictate the winter or summer annual habits of different *Arabidopsis* ecotypes. In winter-annual ecotypes of *Arabidopsis* with functional alleles of *FLC* and *FRI* plants must be exposed to prolonged cold (vernalization) to promote and maintain silencing of *FLC* in order

to flower. Conversely, in summer-annual ecotypes of *Arabidopsis* with loss-of-function or weak alleles of either *FRI* or *FLC* (*e.g.* Col-0 or L*er*) flowering occurs without a requirement for vernalization (Johanson *et al.*, 2000; Shindo *et al.*, 2005; Werner *et al.*, 2005).

1.5.2.2 Autonomous Pathway

In summer-annual accessions lacking a vernalization requirement, *FLC* expression is down-regulated by members of the autonomous pathway. Mutant analyses revealed that the members of the autonomous pathway promote the floral transition independently of photoperiod cues. Mutations in genes of the autonomous pathway cause delayed flowering regardless of photoperiod as *FLC* expression is not down-regulated (reviewed in Simpson, 2004). Thus, the autonomous pathway permits flowering by reducing levels of *FLC* (reviewed in He & Amasino, 2005).

Following silencing of *FLC* by vernalization or the activity of the autonomous pathway, the meristem is competent to respond to floral inductive signals generated during long day photoperiods and other environmental stimuli including temperature and light quality (reviewed in Henderson & Dean, 2004; Putterill *et al.*, 2004).

1.5.3 Meristematic Regulators

1.5.3.1 PNY-PNF

The BEL1-like TALE homeodomain transcription factors PNY and PNF promote competence to flower by a mechanism that is only partly understood (Smith *et al.*, 2004). Three BEL1-like factors, PNY, PNF and ATH1 are co-expressed in the vegetative SAM where they are essential for maintenance of the meristem (Byrne *et al.*, 2003; Gómez-Mena & Sablowski, 2008; Rutjens *et al.*, 2009). At the floral transition, *PNY* and *PNF* are up-regulated in the meristem whereas *ATH1* expression diminishes (Smith *et al.*, 2004; Kanrar *et al.*, 2006; Gómez-Mena & Sablowski, 2008). Loss-of-function *pny pnf* double mutants fail to complete floral evocation and are non-flowering. While the transcripts of some floral integrators such as *AGL24* accumulate normally, transcripts of other floral integrator genes such as *SOC1* and *FT* fail to accumulate in *pny pnf* shoot apices. Furthermore, master regulators of floral-meristem identity, *LFY* and *AP1*, are not expressed (Smith *et al.*, 2004; Kanrar *et al.*, 2008). Thus, although the *pny pnf* SAM responds in part to long day floral inductive conditions as evidenced by changes in the morphology of the meristem and up-regulation of some floral integrator gene expression (Smith *et al.*, 2004), the transition to reproductive development is incomplete indicating that the SAM is not competent to flower. Interestingly, inactivation of ATH1 restores flowering and partially restores internode elongation in *pny pnf* mutants, suggesting that down-regulation of ATH1 in the

vegetative SAM is a prerequisite for meristem competence to flower (Rutjens *et al.*, 2009). Constitutive expression of *LFY* restores flower formation in *pny pnf* mutants, but fails to restore internode elongation (Kanrar *et al.*, 2008). Similarly, constitutive expression of *FT* in *pny pnf* mutants rescues flowering and minimally rescues internode elongation (Kanrar *et al.*, 2008). Previously, ATH1 was identified as a positive regulator of *FLC*, a strong floral repressor (Proveniers *et al.*, 2007). These data suggest that prolonged expression of *ATH1* in *pny pnf* meristems antagonizes competence to flower by affecting levels of *FLC*. Thus, it appears that ATH1 functions in opposition to PNY-PNF in regulating of competence to flower. How PNY-PNF regulate competence to flower remains an important question.

1.5.3.1.2 SPL and miRNA156

Age-related endogenous signals that regulate vegetative to reproductive phase change in plants are also important regulators of flowering. A subset of SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors regulates a recently discovered endogenous flowering time pathway in *Arabidopsis* (Wang *et al.*, 2009). SPL transcription factors function both downstream and with FT-FD in the shoot apex to promote the expression of floral meristem identity genes and the floral transition (Schmid *et al.*, 2003; Wang *et al.*, 2009). SPL3 promotes flowering by directly activating the expression of

floral meristem identity genes *LFY, AP1,* and *FRUITFULL* (*FUL*) (Wang *et al.,* 2009; Yamaguchi *et al.,* 2009). SPL9 promotes flowering by direct activation of the floral integrator *SOC1* (Wang *et al.,* 2009). The proposed redundancy of SPL transcription factor family indicates that other members of this family serve similar functions (Wu & Poethig, 2006; Wang *et al.,* 2008). *SPL* expression is post-transcriptionally regulated by miRNA156. As plants age, miRNA156 levels gradually decline, yielding gradually increasing levels of *SPL* expression in both short day and long day photoperiods that promote flowering (Schmid *et al.,* 2003; Cardon *et al.,* 1997; 1999; Rhoades *et al.,* 2002 Wu & Poethig, 2006; Wang *et al.,* 2009). Lal *et al.* (2011) recently proposed that PNY and PNF control the abundance of miRNA156 and SPL in the meristem to promote competence to flower. However, the mechanism of this regulation remains unknown (Lal *et al.,* 2011).

1.6 PNY-BP Interaction and Function

As described above, PNY plays an important role in regulating meristem maintenance and competence to flower (Smith *et al.,* 2004; Rutjens *et al.,* 2009). In addition, PNY and its interacting partner, the KNOX homeodomain protein BP, regulate inflorescence architecture by promoting the elongation of internodes. BP and PNY further promote correct vascular patterning and the differentiation of lignified interfascicular fibres in expanded internodes (Mele *et al.,* 2003; Smith

& Hake, 2003). At the floral transition, *BP* and *PNY* expression diminishes in the SAM and becomes concentrated at the base of the IM in the rib zone and in the cortex of stems (Lincoln *et al.*, 1994) corresponding to their roles in internode differentiation. Mutations in *bp* and *pny* both disrupt internode patterning resulting in distinct alterations in inflorescence architecture (Douglas *et al.*, 2002; Venglat *et al.*, 2002; Byrne *et al.*, 2003; Smith & Hake, 2003). Mutations in *bp* cause short plants with compact internodes and downward-oriented pedicels (Douglas *et al.*, 2002; Venglat *et al.*, 2002) whereas loss-of-function mutations in *PNY* cause short plants with irregular internode elongation resulting in clusters of flowers on the primary stem (Byrne *et al.*, 2003; Smith & Hake, 2003). BP and PNY form heterodimers *in vitro* (Smith *et al.*, 2002; Cole *et al.*, 2006) and are predicted to regulate a set of common genes that control of internode elongation and patterning (Smith & Hake, 2003). Nevertheless, internode defects are enhanced in *bp pny* double mutants, resulting in severely dwarfed plants with random phyllotaxy and pedicel orientation (Smith & Hake, 2003). These data reveal that BP and PNY perform some of the same functions, but have separate functions as well. Consistent with this, BP controls the developmental timing of lignin deposition in stems and is proposed to regulate several lignin biosynthetic genes (Mele *et al.*, 2003; Khan *et al.*, 2012). Conversely, PNY has been shown to regulate the expression of *PME5*, encoding a pectin methylesterase, whose activity is required to loosen the cell wall structure to promote cell elongation required for internode elongation. Loss-of-function *pme5* mutants have clusters of fruits

along the inflorescence similar to *pny* mutants (Peaucelle *et al.*, 2011). Future work is needed to confirm direct targets of BP and PNY regulation.

1.6.1 BP and PNY Repress Boundary Gene Expression in the Stem

Two recent studies have shed light on how BP and PNY regulate inflorescence architecture. Ragni *et al.* (2008) showed that BP and PNY are negative regulators of *KNAT2* and *KNAT6* in stems and pedicels. In wild-type inflorescences, expression of these genes is limited to the axil of pedicels, a type of lateral organ boundary (Belles-Boix *et al.*, 2006; Ragni *et al.*, 2008). Inactivation of *KNAT2* and *KNAT6* in *bp* and *pny* mutants confirmed that ectopic expression of primarily *KNAT6* in the stems is the cause of decreased apical dominance and short internodes (Belles-Boix *et al.*, 2006; Ragni *et al.*, 2008). Khan *et al.* (2012) showed that BP-PNY also prevent *BLADE-ON-PETIOLE1* (*BOP1*) and *BOP2* misexpression in stems and pedicels (Figure 1.4). Khan *et al.* (2012) showed that BOP1/2 function downstream of BP-PNY in an antagonistic manner. BOP1/2 both promote the expression of *KNAT6* and require KNAT6 function to exert changes in inflorescence architecture suggesting that BOP1/2 induce the expression of one or more boundary genes that oppose the functions of BP and PNY in stems (Khan *et al.*, 2012). Given that no decrease in *BP* or *PNY* transcript was apparent in BOP1/2 gain-of-function mutants, it was suggested that BP-PNY and BOP1/2 antagonistically regulate a common set of target genes involved in internode elongation and fortification. In support of this model,

Khan *et al.* (2012) showed that BOP1/2 and BP antagonistically regulate a set of lignin biosynthetic genes that contribute to differentiation of interfascicular fibres during the internode maturation process.

1.7 BLADE-ON-PETIOLE1/2

BOP1/2 encode redundant NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1-like (NPR1) BTB-ankyrin transcriptional co-activators that are expressed in lateral organ boundaries (Ha *et al.*, 2004; Hepworth *et al.*, 2005; Jun *et al.*, 2010). Similar to NPR1, BOP1/2 are recruited to DNA via interactions with transcription factors, likely TGACG motif-binding (TGA) transcription factors, a subclass of bZIP transcription factors (Hepworth *et al.*,2005; Jakoby *et al.*, 2002). Loss-of-function studies show that BOP1/2 activity promotes the formation of simple leaves, floral organ abscission, and floral meristem fate (Hepworth *et al.*, 2005; McKim *et al.*, 2008; Xu *et al.*, 2010). In contrast, BOP gain-of-function in leads to variations in inflorescence architecture. In severe cases, plants are late flowering with short compact inflorescences (Khan *et al.*, 2012; this study). Recently, Saleh *et al.* (2011) showed that *BOP*-like genes in *Physcomitrella patens* promote the juvenile-to-adult transition.

1.71 Relationship of BOP1/2, PNY-PNF, and KNAT6

The BEL1-like transcription factors, PNY and PNF are co-expressed in the vegetative SAM where they contribute to both boundary and meristem maintenance (Byrne *et al.*, 2003; Smith *et al.*, 2004; Kanrar *et al.*, 2006). *PNY* and *PNF* are up-regulated in the meristem during the floral transition (Smith *et al.*, 2004; Kanrar *et al.*, 2006). Unlike *PNF, PNY* is expressed in stems and negatively regulates the expression of the lateral organ boundaries genes *BOP1/2* and the *KNOX* gene *KNAT6.* Thus, PNY restricts *BOP1/2* and *KNAT6* expression to pedicel axils to promote internode elongation and inflorescence patterning (Byrne *et al.*, 2003; Smith *et al.*, 2004; Ragni *et al.*, 2008; Khan *et al.*, 2012).

Thesis Rationale

Given that *pny* defects in inflorescence architecture are caused by localized misexpression of *BOP1/2* in subdomains of the stem (Khan *et al.*, 2012), we reasoned that *pny pnf* defects in competence to flower might be caused by localized misexpression of *BOP1/2* in the apical meristem. If this were true, we further hypothesized that BOP1/2 might exert its function through ATH1 and KNAT6. Several lines of evidence support this hypothesis. First, both *ATH1* and *BOP1/2* are co-expressed in lateral organ boundaries (Ha *et al.*, 2004; Gómez-Mena & Sablowski, 2008; Khan *et al.*, 2012). Second, both *bop1 bop2* and *ath1* loss-of-function mutants are lacking in floral-organ abscission showing that they have a common function (Hepworth *et al.*, 2005; Gómez-Mena & Sablowski, 2008; McKim *et al.*, 2008). Third, both *BOP1/2* and *ATH1* gain-of-function plants have short internodes and are late flowering (Proveniers *et al.*, 2007; Gómez-Mena & Sablowski, 2008; this study). My thesis was therefore designed to test the model that *pny pny* defects in meristem competence to flower are the result *BOP1/2* gain-of-function in the meristem leading to the formation of an ATH1-KNAT6 boundary complex whose function is antagonistic to PNY-PNF.

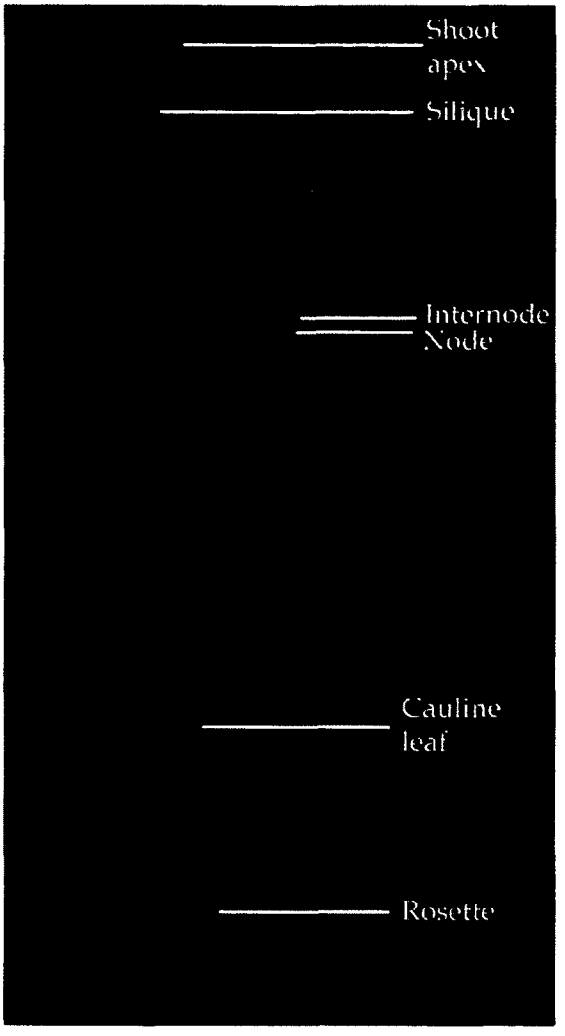

Figure 1.1. *Arabidopsis* developmental stages. Aerial tissues are derived from the SAM that produces repeating modules consisting of a leaf, a potential axillary meristem, and an internode. Internode elongation is repressed during vegetative development to generate a rosette of clustered leaves. Internodes of the stem expand following the transition to flowering. Axillary meristems originate from the axils of leaves and specify either lateral branches or flowers. Inflorescence architecture is altered by differential elaboration of these modules.

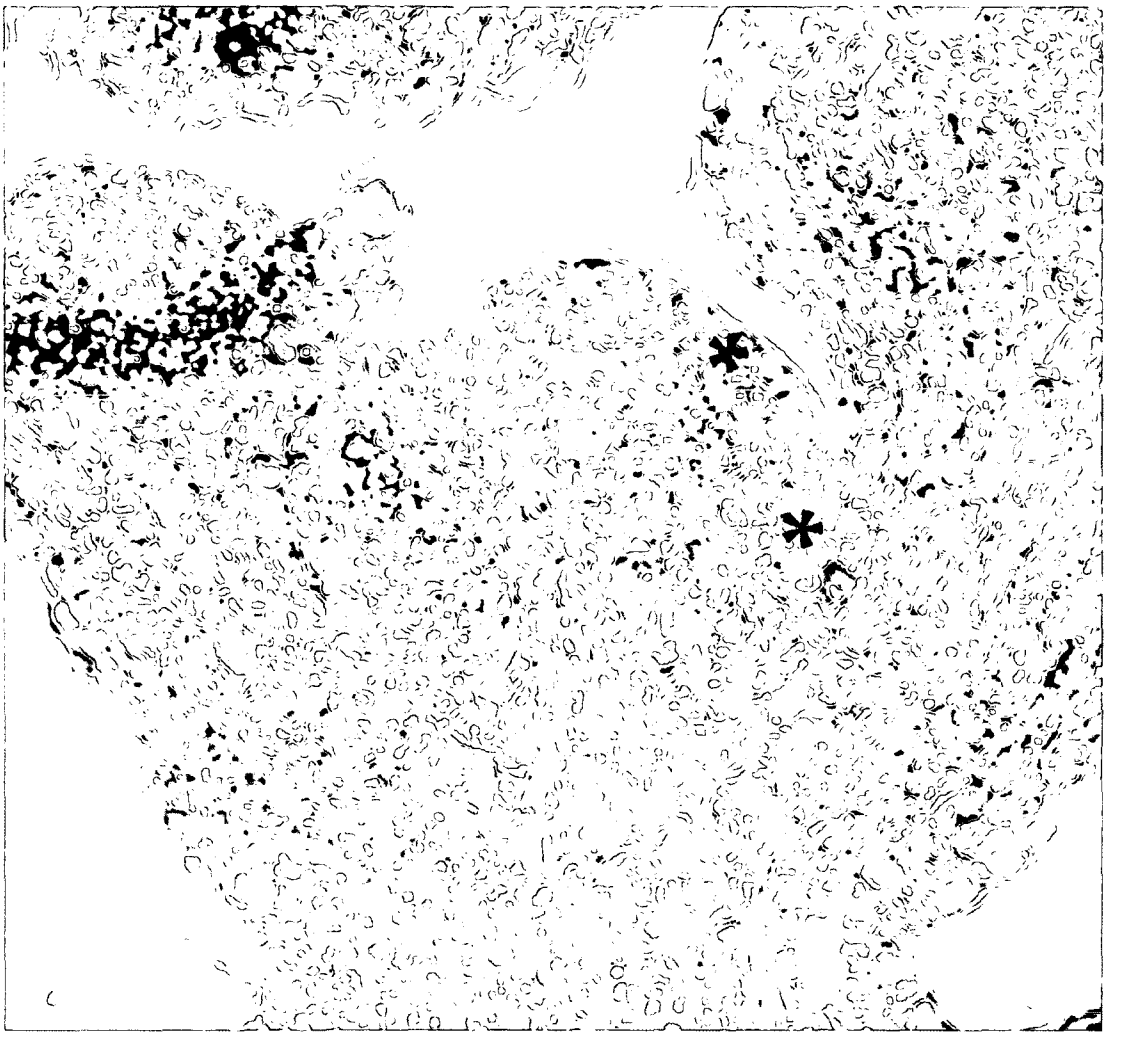

Figure 1.2. Organization of the reproductive SAM. A self-maintaining population of stem cells is located in the central zone (CZ). Lateral organs are derived from cells of the peripheral zone (PZ). The (RZ) provides cells that form the pith of the meristem and the interior of internodes. Asterisks: (upper) meristem-organ boundary and (lower) stem-pedicel boundary. PE: pedicel. FM: floral meristem.

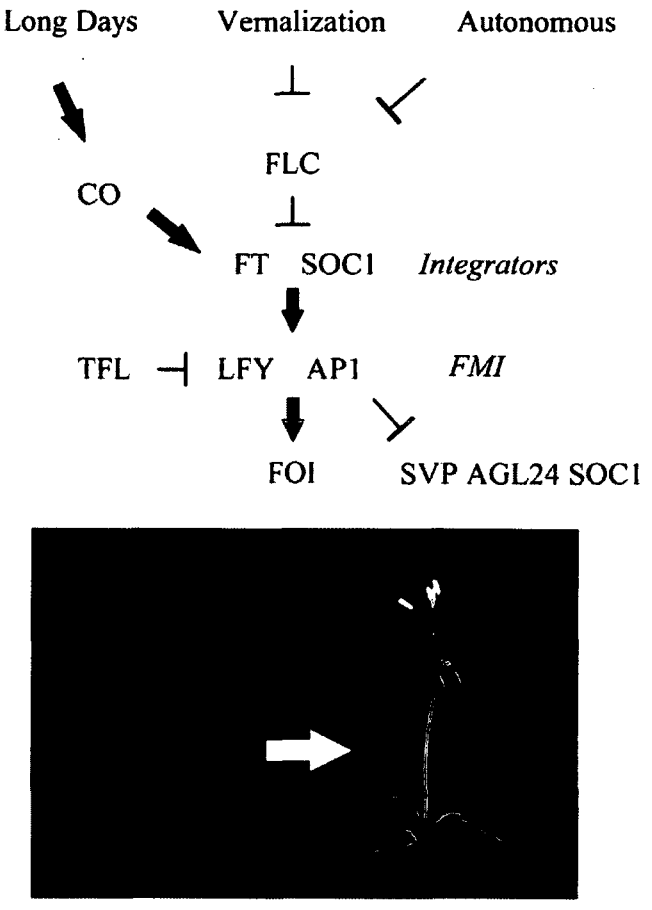

Figure 1.3. Key regulators of the floral transition. Three main signaling pathways determine flowering-time: the long day pathway (photoperiod), the vernalization pathway (cold), and the autonomous pathway (internal and age-related signals). Competence to flower requires silencing of *FLC* expression by the autonomous or vernalization pathways. This permits up-regulation of *FT* and *SOC1* genes with floral integrator activity. *FT* and *SOC1* are antagonistically regulated by FLC (autonomous and vernalization pathways) and CO (long day pathway). The floral integrators promote the up-regulation of *LFY* and *AP1* that confer floral-meristem identity (FMI) and floral organ identity (FOI). Inflorescence identity genes *SVP, AGL24,* and *SOC1* are down-regulated by AP1 in floral meristems to promote determinacy. TFL represses *LFY* and *AP1* in IMs to maintain indeterminacy.

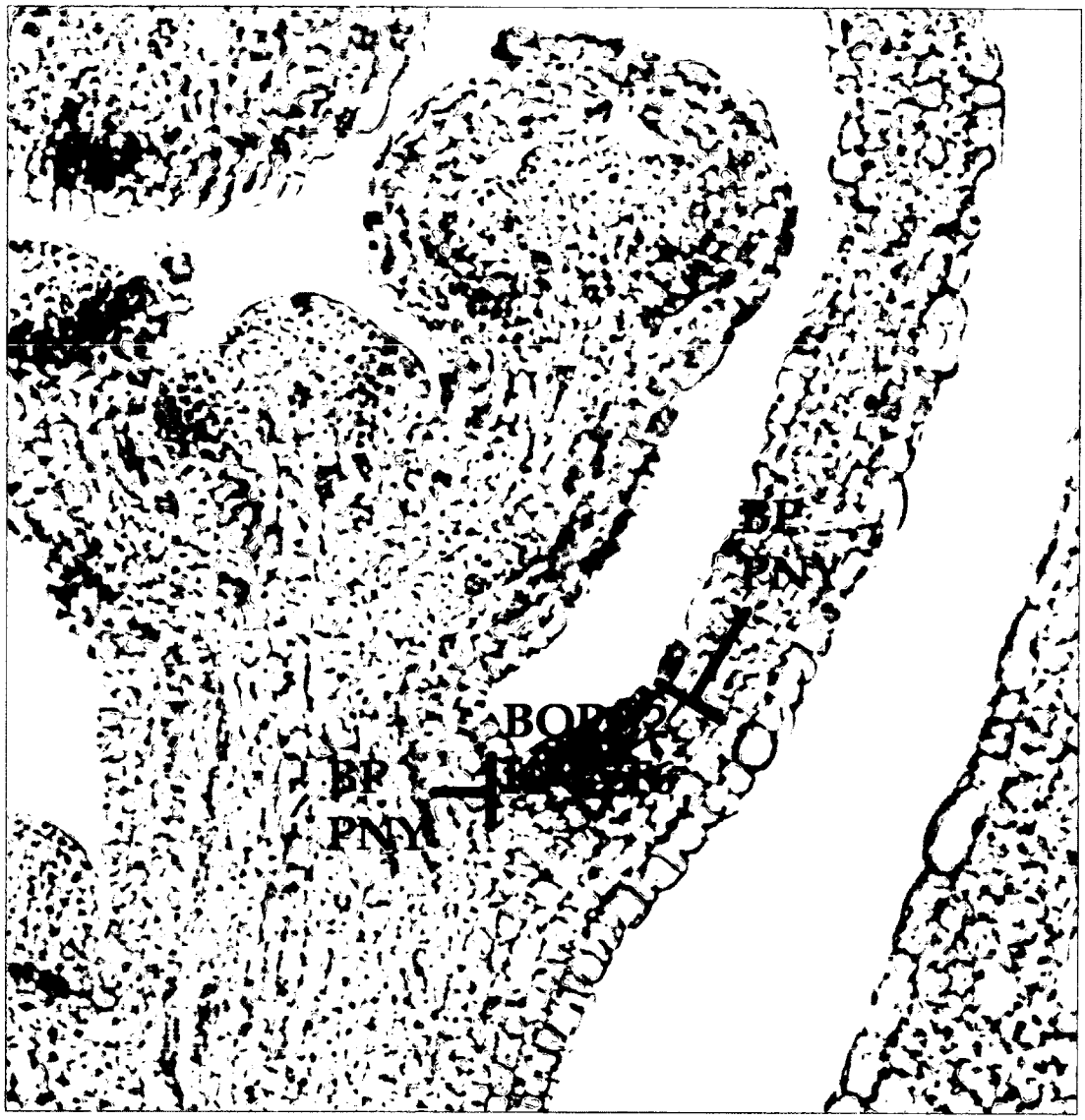

Figure 1.4. Proposed role of PNY-BP in regulation of inflorescence architecture. PNY-BP negatively regulate *BOP1/2* in stems and pedicels. BOP1/2 promote the expression of *KNAT6* in stem-pedicel boundaries to restrict growth to alter pedicel orientation and length of internodes.

CHAPTER 2 MATERIALS & METHODS

2.1 Plant Material and Growth Conditions

Arabidopsis thaliana mutants and transgenic lines used in this study were in the Columbia-0 (Col-0) background. Mutant alleles were obtained from the Arabidopsis Biological Resource Center. Loss-of-function *ath1-3*, *bop1-3*, *bop2-1*, *knat2-5*, *knat6-2*, *pny*, and *pnf* T-DNA insertion lines were previously described (Smith & Hake, 2003; Smith *et al.*, 2004; Hepworth *et al.*, 2005; Belles-Boix *et al.*, 2006; Gómez-Mena & Sablowski, 2008). Triple and quadruple mutants were generated by crossing and confirmed by PCR-based genotyping. The *BOP2::GUS* transgene (Xu *et al.*, 2010) was introgressed into mutant backgrounds by crossing. *35S::BOP2* and *bop1-6D* transgenic plants were obtained from O. Nilsson (Norberg *et al.*, 2005). The *bop1-3* knock-down mutant contains a T-DNA insertion in the promoter and a low amount of functional BOP1 protein is likely synthesized.

All seeds were surface-sterilized in a solution of 5% sodium hypochlorite and 0.5% (w/v) sodium dodecyl sulphate (SDS) prior to sowing. Seeds were sown on agar plates containing AT minimal medium (Haughn & Somerville, 1986) or on soil (Promix MPV, Premier Horticulture, Rivière-du-Loup, QC). Soil was steam-sterilized prior to use and supplemented with 1g/L of 20-20-20 plant

fertilizer (Plant Pod, Plant Product Co. Ltd, Brampton, ON). Seeds were incubated at 4°C for 2 days to break dormancy and then placed in a growth chamber to promote germination. Seedlings were transplanted to soil in 72 well trays or in 3.5 inch square pots as appropriate. Plants were grown in a controlled environment chamber in continuous light at 22°C (100μmol m^{-2} s^{-1}) except where noted below.

2.2 Genotyping

Genomic DNA re-suspended in 100 μl of molecular biology grade purified water (Fisher) was isolated from leaf tissue for PCR genotyping according to the method of Edwards *et al.* (1991). Two μl of genomic DNA was used as template in a standard 20 μl PCR reaction. Alternatively, leaf tissue was pressed onto filter paper (Whatman N^0 3) and a 2 mm filter core was used as template for PCR. The strategy for genotyping mutants containing T-DNA insertions from Salk collection (Table A1.1) was as described (www.signal.salk.edu) except that primers PNF-LP and LBb1.3 were used to detect the *pnf* T-DNA insertion. The genotyping test for *ath1-3* was as previously described (Gómez-Mena & Sablowski, 2008). Primers used for genotyping are listed in Table 2.1.

2.3 Phenotypic Analyses

Seven-week-old plants grown in continuous light were used for quantitative phenotypic analyses of inflorescence architecture. Internode lengths were measured for the first 8 pedicels counting acropetally. Paraclades were counted as the number of stems originating from the rosette. Thirty-six plants per genotype grown in continuous light were used for measurement of flowering time. Flowering time was determined by recording the date of the first apex.

2.4 β-Glucoronidase (GUS) Activity Assays

Wild-type and mutant plants expressing the *BOP2::GUS* reporter gene (Xu *et al.*, 2010) were grown in short days (8 hours light) for 3 weeks and transferred to continuous light to synchronize flowering according to the protocol of Smith *et al.* (2004). Shoot apices were harvested for analysis of GUS activity on day 15 following transfer to continuous light. Tissue was harvested into cold 90% acetone, washed in staining buffer (50mM Na_2HPO_4 / NaH_2PO_4 buffer, pH 7.0, 0.2 % Triton X-100, 4mM K_4 [$Fe(CN)_6$], 4mM K_3 [$Fe(CN)_6$]), and vacuum infiltrated with staining buffer containing 0.96 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) for 15 minutes. Samples were incubated in staining buffer at 37°C for 6 hours to allow for production of blue precipitate. Following colour development, samples were cleared to remove chlorophyll in 20%, 35%, and 50%

ethanol for 30 minutes each and fixed in formaldehyde-acetic acid-alcohol (FAA) (50% ethanol, 3.7% formaldehyde, 5% acetic acid) for 3 hours at room temperature. Tissue was then embedded in paraffin wax for sectioning. For this, tissues were infiltrated with 70%, 80%, and 90% ethanol solutions for 30 minutes each followed by overnight incubation in 95% ethanol with 0.5% Eosin-Y. Finally, samples were incubated in 100% ethanol 3 times for 30 minutes each. Next, apices were incubated in 25% *tert*-butanol (in ethanol), followed by 50% *tert*-butanol for 30 minutes each at room temperature. Samples were incubated in 100% *tert*-butanol at 60°C overnight. Apices were incubated in 50% Paraplast embedding media (Sigma) in *tert*-butanol at 60°C overnight followed by incubation in 100% paraplast at 60°C for two days with two wax changes per day. Finally, apices and wax were poured into heated petri dishes, cooled, and the wax permitted to solidify. Apices were sectioned longitudinally with a microtome to a thickness of 12 µm and heated overnight at 42°C to allow adhesion to Superfrost glass slides (Fisher). Sections were incubated twice in *tert*-butanol for 10 minutes each at 60°C, 3 minutes in 100% ethanol at room temperature, and dewaxed in xylene for 5 minutes at room temperature. Next, sections were incubated in 95%, 85%, 70%, 50%, and 30% ethanol for 3 minutes each, followed by incubation in water until mounting in 50% glycerol. Micrographs were acquired immediately using an AXIO Imager M2 microscope (Zeiss) equipped with an AxioCam digital camera (Zeiss).

2.5 *In situ* Hybridization

In situ hybridization was carried out essentially as described in Hooker *et al.* (2002). To generate DNA templates for the production of *BOP2* sense and anti-sense RNA probes by *in vitro* transcription, primers incorporating a binding site for T7 polymerase were used. The *BOP2* anti-sense probe to detect transcript was generated by amplification of the full-length *BOP2* coding sequence by PCR using primers designated *BOP2 in situ F* and *BOP2 in situ R* (the latter incorporates the T7 polymerase binding site CATAATACGACTCACTATAGG at the 5′ end of the *BOP2* coding sequence). The *BOP2* sense control probe was generated by PCR amplification of the full-length *BOP2* coding sequence with primers designated; *BOP2 sense F* and *BOP2 sense R*. The forward primer adds the T7 polymerase binding site upstream (5′) of the ATG start codon of *BOP2*. *CER6* control anti-sense and sense probes were similarly generated by PCR amplification of *CER6* cDNA with primers designated *CER6 T7R1* and *CER6 F1* for the anti-sense probe and *CER6 T7F1* and *CER6 R1* primers for the sense control probe. *CER6* was used as a positive control for *in situ* hybridization experiments because hybrization gives a strong epidermal-specific signal (Hooker *et al.*, 2002; Hepworth *et al.*, 2005). Probe templates were amplified by PCR using cloned cDNAs as template (Hooker *et al.*, 2002; Hepworth *et al.*, 2005) and gel-purified prior to use.

In vitro transcription to create probes containing ribonucleotides labelled with digoxygenin (DIG) was performed according to manufacturer's instructions (Roche) using 1 µg of template for transcription. Template DNA was removed following transcription by digestion with 20 units of DNase I (RNase free, Roche) for 15 minutes at 37°C. The digestion was terminated by the addition of ethylenediaminetetraacetic acid (EDTA) (pH 8.0) to a final concentration of 0.0167 M. Synthesized RNA was precipitated in the presence of 0.01 M LiCl and 75% ethanol overnight at -20°C and harvested by centrifugation at 14,000 RPM at 4°C for 20 minutes. The harvested RNA was re-suspended in 100 µl of sterile molecular biology grade water (Fisher). Probes were then subjected to carbonate hydrolysis to generate fragments of approximately 180-bp. Probe yield was then quantified using the DIG Nucleic Acid Detection Kit according to the manufacturer's instructions (Roche).

Wild-type and mutant plants for analysis were grown in short days (8 hours light) for 3 weeks followed by 15 days in continuous light (Smith *et al.*, 2004) prior to harvesting tissue. Genotyping was performed to identity *pny pnf* double mutants in the population. Shoot apices were harvested and fixed in FAA (50% ethanol, 3.7% formaldehyde, 5% glacial acetic acid) for 3 hours following application of vacuum to samples in fixative for 15 minutes. Samples were embedded in paraffin wax as described (Xu *et al.*, 2010) and a microtome was

used to prepare longitudinal sections of 12 μm thickness through the apical meristem as described previously (Xu *et al.*, 2010). 20 ng of RNA probe was used per slide in the hybridization reaction.

2.6 Yeast Two-Hybrid Constructs and Assays

Yeast two-hybrid assays were used test for protein-protein interactions using a previously described system (Kohalmi *et al.*, 1998). Bait and/or prey plasmids containing the full-length coding sequences of *BOP1*, *BOP2*, and *KNAT2* were as previously described (Bellaoui *et al.*, 2001; Hepworth *et al.*, 2005). A full-length cDNA for *KNAT6S* was a gift from Yasunori Machida. A full-length cDNA corresponding to *ATH1* was amplified by RT-PCR using iProof as the polymerase (BioRad) and Col-0 wild-type cDNA derived from apices as the template. Gene-specific primers used to amplify *ATH1* incorporated Sal*I* and Not*I* restriction enzyme recognition sites at their 5′ ends to facilitate directional cloning into the prey plasmid pBI-881. The initial PCR product was gel-purified and subcloned into pCR2.1, using a TOPO blunt-end cloning system (Invitrogen) to create the intermediate plasmid pTOPO-ATH1. The *ATH1* coding sequence in this vector was sequenced to verify the absence of mutations. A fragment corresponding to *ATH1* was then excised by digestion with Sal*I* and Not*I* and cloned into the corresponding sites of pBI-881 (Kohalmi *et al.*, 1998) to create pBI-881/ATH1 (AD:ATH1). The junction between GAL4AD and ATH1 in this vector was sequenced to verify integrity of the reading frame. A similar strategy was

used to create pBI-881/KNAT6S (AD:KNAT6) described in Khan *et al.,* 2012. Primers for cloning are listed in Table 2.1.

The yeast strain YPB2 (Kohalmi *et al.,* 1998) was co-transformed with bait and prey plasmid using the method of Gietz & Woods (2002). Interactions were quantified using ortho-nitrophenol-β-galactosidase (ONPG) liquid culture assays as described (Clontech Yeast Protocols). DB:ATH1 and AD:BOP1 were not analyzed for interaction.

2.7 Quantitative Reverse Transcriptase PCR (qRT-PCR)

Internodes between the first and eleventh siliques of 5-week-old plants were harvested for the isolation of total RNA. One μg of RNA was used as template for the synthesis of total cDNA using Superscript III reverse transcriptase (Invitrogen) as the polymerase. Quantitative qRT-PCR was performed as previously described (Khan *et al.,* 2012). Synthesized cDNA was diluted 10-fold prior to use as template. ATH1-qPCR-F1 and ATH1-R2 primer pairs were used for detection of *ATH1* transcript. The ATH1-R2 primer spans an intron to prohibit amplification of genomic DNA. Values were normalized to *GAPC* and wild-type control as described in Khan *et al.* (2012). Three technical replicates were performed per biological replicate. Madiha Khan repeated the experiment with two sets of independently isolated RNA with similar results obtained.

Table 2.1. Sequences of oligonucleotides used in this study.

Name	Sequence (5′ – 3′)	Reference
Creation of Y2H constructs		
ATH1-SalI -F	AAGGGGTCGACGATGGACAACAACAAC	This study
ATH1-NotI-R	GCTGCGGCCGCTGTTATTTATGCATTGC	This study
SalI-KNAT6-F1	CTTGTCGACGATGGATGGAATGTACAATTT	This study
KNAT6-NotI-R1	TAAGCGGCCGCTCATTCCTCGGTAAAGA	This study
Genotyping		
AG12-LP	GTATTTGGAAATTGCGTGTCG	This study
AG12-RP	TTTACGAGGGTTTTCGATCTTC	This study
ATH1-LP	CCAATACCGGTTTTTCAGACATGA	Gómez-Mena & Sablowski (2008)
ATH1-RP	GGCGGGTTTCGGATCTACATT	Gómez-Mena & Sablowski (2008)
BLR-Salk-RP	TTGGAATTGGAGACAAAATGTGTTA	Smith & Hake (2003)
BLR-Salk-LP	GGAACCAAGTTCAAACTCGAATCCA	Smith & Hake (2003)
PNF-LP	TGCATGAGTTCCATATATATAGCAA	Smith *et al.* (2004)
PNF-RP	TCCGATCGGTATGTGTTGTGTTCCC	Smith *et al.* (2004)
qRT-PCR		
ATH1-qPCR-F1	ATACTCGCTCGATTATTCATCTCGA	This study
ATH1-R2	ATCGATCATCCAACCATTTGAAGAAG	This study
GAPC-P1	TCAGACTCGAGAAAGCTGCTAC	Khan *et al.* (2012)
GAPC-P2	GATCAAGTCGACCACACGG	Khan *et al.* (2012)
***in situ* probe synthesis**		
BOP2-insitu-F	ATGAGCAATCTTGAAGAATCTTTGAGA	This study
BOP2-insitu-R	CATAATACGACTCACTATAGGCTAGAAGTGATGTTG ATGATGG	This study
BOP2-sense-F	CATAATACGACTCACTATAGGATGAGCAATCTTGAA GAATCTT	This study
BOP2-sense-R	CTAGAAGTGATGTTGATGATGGTGATG	This study
CER6-T7-R1	GATAATACGACTCACTATAGGGTTATTTGAGTACACC	Hooker *et al.* (2002)
CER6-F1	ATGCCTCAGGCACCG	Hooker *et al.* (2002)
CER6-T7-F1	GATAATACGACTCACTATAGGATGCCTCAGGCACCG	Hooker *et al.* (2002)
CER6-R1	TTATTTGAGTACACC	Hooker *et al.* (2002)

T7 binding and restriction endonuclease sites are underlined.

CHAPTER 3 RESULTS

3.1 Inactivation of *BOP1/2* Rescues *pny pnf* Flowering and Internode Elongation Defects

PNY (and BP) restrict *BOP1/2* expression to pedicel axils in the primary inflorescence stem where growth is restricted (Khan *et al.*, 2012). In *pny* mutants, *BOP1/2* are misexpressed in the stem cortex resulting in compressed internodes, altered phyllotaxy, and loss of apical dominance (Khan *et al.*, 2012). Given that *bop1 bop2* loss-of-function rescues *pny* inflorescence defects (Khan *et al.*, 2012), we reasoned that *bop1 bop2* loss-of-function mutations might similarly rescue flowering and internode elongation in *pny pnf* double mutants. We therefore constructed *pny pnf bop1 bop2* quadruple mutants and analyzed the resulting phenotypes in comparison to wild-type and mutant control plants. Rescue of flowering and internode elongation were observed in *bop1 bop2 pny pnf* quadruple mutants (Figure 3.1.1). To monitor this rescue in more detail, plant height, internode length, and number of paraclades (a measure of apical dominance) were scored for 20 plants per genotype. Average plant height and paraclade number in *bop1 bop2 pny pnf* quadruple mutants were similar to wild-type control plants (Students t-test, p=0.64528; p=0.84934 respectively). Furthermore, the distribution of internodes lengths in *bop1 bop2 pny pnf* was similar to the distribution in wild-type, *bop1 bop2*, and *pnf* plants indicating extensive rescue of *pny pnf* flowering, internode elongation, and apical

dominance defects by *bop1 bop2* loss-of-function (Figure 3.1.2). *bop1 bop2 pnf* triple mutants showed no defects other than those associated with *bop1 bop2* loss-of-function (data not shown). These data reveal that BOP1/2 block flowering and inhibit internode elongation in the *pny pnf* mutant background.

3.2 *BOP2* Expression is Expanded in *pny pnf* Meristems

Given that *BOP1/2* are misexpressed in *pny* mutant stems causing irregular clusters of pedicels (Khan *et al.*, 2012), I first examined the expression pattern of *BOP2* in *pny pnf* mutant apices and control plants using a *GUS* reporter gene under control of the *BOP2* promoter (Figure 3.2.1). Plants were examined for GUS activity 15 days after their transfer from short day to continuous light photoperiods to ensure synchronous induction of flowering . As expected, staining for GUS activity in wild-type, *pny*, and *pnf* control apices showed that *BOP2::GUS* expression was excluded from the IM and found only at the boundary separating the IM and floral meristems (Figure 3.2.1 left panels; Xu *et al.*, 2010). *BOP2* promoter activity was expanded into the CZ and RZ of *pny pnf* apical meristems. These expression patterns were confirmed by *in situ* hybridization to *BOP2* transcript (Figure 3.2.1 right panels). *BOP2* sense RNA probe used as a control showed no hybridization in wild-type control apices and hybridization to *CER6* control probe gave a strong epidermal signal (data not shown). These data indicate that PNY and PNF function redundantly to exclude

BOP1/2 expression from the CZ and RZ of the shoot apical meristem following exposure to floral inductive photoperiods.

3.3 Inactivation of *ATH1* and *KNAT6* also Rescues *pny pnf* Flowering and Internode Elongation Defects

In Khan *et al.* (2012), we showed that *BOP1/2* gain-of-function in stems promotes the expression of *KNAT6* and that BOP1/2 requires KNAT6 activity in part to exert changes in inflorescence architecture. Compatible with this, *knat6* mutations alone or in combination with *knat2* rescue *pny* inflorescence defects (Ragni *et al.*, 2008). Given that class I KNOX proteins like KNAT6 heterodimerize with BELL homeodomain proteins to permit high affinity DNA binding (Smith *et al.*, 2002), I speculated that KNAT6 may require ATH1 as a co-factor to block flowering in *pny pnf* mutants. Several lines of evidence support this model. First, *ath1-1* loss-of-function partially rescues *pny* inflorescence defects and flowering of *pny pnf* mutants (Rutjens *et al.*, 2009). Second, ATH1 and KNAT6 form heterodimers in yeast and *in planta* (Rutjens *et al.*, 2009; Li *et al.*, 2012). Third, *ATH1* and *KNAT6* are co-expressed in lateral organ boundaries at the base of shoots (Gómez-Mena & Sablowski, 2008; Ragni *et al.*, 2008). To further test if ATH1 and KNAT6 are components of a linear pathway controlled by BOP1/2 that block flowering in *pny pnf* mutants, I constructed *pny pnf knat2, pny pnf knat6,* and *pny pnf knat2 knat6* mutants and analyzed their resulting inflorescence and

flowering phenotypes (Figure 3.3.1). This analysis showed that inactivation of *KNAT6* (but not *KNAT2*) rescued *pny pnf* flowering and internode elongation defects (Figure 3.3.1). Interestingly, flowering and internode elongation were similar to wild-type in *ath1-3 pny pnf* triple mutants (Figure 3.3.1). To further examine this rescue, quantitative analysis was performed on 20 plants per genotype. This analysis revealed that rescue of *pny pnf* mutant flowering and internode elongation defects by *ath1-3* loss-of-function was complete. The average primary stem length of *ath1-3 pny pnf* mutants was significantly greater than that of wild-type control plants (Figure 3.3.2; Students t-test, p = 0.0474088). Loss of *KNAT6* rescued the *pny pnf* flowering defects and partially suppressed the *pny* internode elongation defects of *pny pnf* double mutants (Figure 3.3.1; Figure 3.3.3; Students t-test, p = 0.0080180). Inactivation of *KNAT2* in the *pny pnf knat6* background fully rescued the internode elongation defects of the triple mutant (Figure3.3.1; Figure 3.3.3; Students t-test, p = 0.9733270). However, inactivation of *KNAT2* alone failed to rescue flowering in *pny pnf* (Figure 3.3.1) or indeed *pny* inflorescence defects (Ragni *et al.*, 2008). Collectively, my results indicate that BOP1/2, ATH1, and KNAT6 function in the same pathway to antagonize PNY PNF function in meristem transition to IM fate.

3.4. BOP1/2 Function Upstream of ATH1 and KNAT6

3.4.1 ATH1 and KNAT6 Form a Complex that is Unlikely to Include BOP1/2

BOP1/2 encode transcriptional co-regulators that contain BTB and ankryin motifs involved in protein-protein interaction but lack a DNA binding motif (Ha *et al.*, 2004; Hepworth *et al.*, 2005). Rather, BTB-ankryin proteins interact with TGAGC motif-binding (TGA) bZIP transcription factors for recruitment to DNA (Zhang *et al.*, 1999; Després *et al.*, 2000; Hepworth *et al.*, 2005) and possibly other classes of DNA-binding proteins. I therefore performed yeast two-hybrid assays to examine if BOP1/2 might interact directly with ATH1 or KNAT6 to form a transcriptional complex. As expected, when BOP1 or BOP2 as bait (DB:BOP1 and DB:BOP2) were co-expressed with the TGA factor PERIANTHIA as prey (TA:PAN) an interaction was detected (Figure 3.4.1; BOP2 data not shown; Hepworth *et al.*, 2005). However, when BOP1 or BOP2 baits were co-expressed with ATH1 as prey (AD:ATH1) or KNAT6 as prey (in Khan *et al.*, 2012) no significant increase in β-galactosidase activity was observed (Figure 3.4.1; BOP2 data not shown; Khan *et al.*, 2012). These data suggest there is no direct interaction between BOP1/2 and KNAT6 or ATH1 (Figure 3.4.1; Khan *et al.*, 2012). In contrast, an interaction was readily detected between ATH1 and KNAT6, consistent with published results (Figure 4.3.1; Rutjens *et al.*, 2009; Li *et al.*, 2012). We concluded that BOP1/2 is unlikely to form a transcriptional complex with ATH1 and KNAT6.

3.4.2 BOP1/2 Gain-of-Function Promotes *ATH1* Expression and Delays Flowering

Given that BOP1/2 promotes *KNAT6* expression in stems (Khan *et al.*, 2012), I examined if BOP1/2 also promotes *ATH1* expression in stems. To test this, I used qRT-PCR to compare the relative *ATH1* transcript levels in internodes of wild-type control plants and transgenic plants expressing high constitutive levels of *BOP1* (*bop1-6D*) or *BOP2* (*35S::BOP2*) transcript (Figure 3.4.2). The activation-tagged *bop1-6D* line contains four *35S* enhancers upstream of *BOP1* whereas the *35S::BOP2* line was generated by transformation of plants with a *35S::BOP2* construct (Norberg *et al.*, 2005). *ATH1* transcript levels were dramatically up-regulated in both *bop1-6D* and *35S::BOP2* internodes (25 & 20-fold respectively) indicating that BOP1/2 promote the expression of *ATH1* in stems. To test if *BOP1* over-expression also delays flowering similar to *ATH1* gain-of-function (Proveniers *et al.*, 2007), 36 plants each of Columbia wild-type and *bop1-6D* were scored for flowering time (Figure 3.4.2). This analysis showed that *bop1-6D* gain-of-function mutants flowered significantly later than wild-type controls (Students t-test, $P = 4.4371 \times 10^{-20}$). Given that both *BOP1* and *ATH1* gain-of-function inhibits internode elongation and delays flowering (Figure 3.4.2; Norberg *et al.*, 2005; Proveniers *et al.*, 2007; Gómez-Mena & Sablowski, 2008), ATH1 likely functions downstream of BOP1/2 to perturb these processes.

Consistent with this view, *ath1*-3 loss of function restores internode elongation in *35S::BOP2* transgenic plants (Khan & Hepworth, unpublished results). These data support the model that BOP1 and BOP2 exert their function through ATH1-KNAT6.

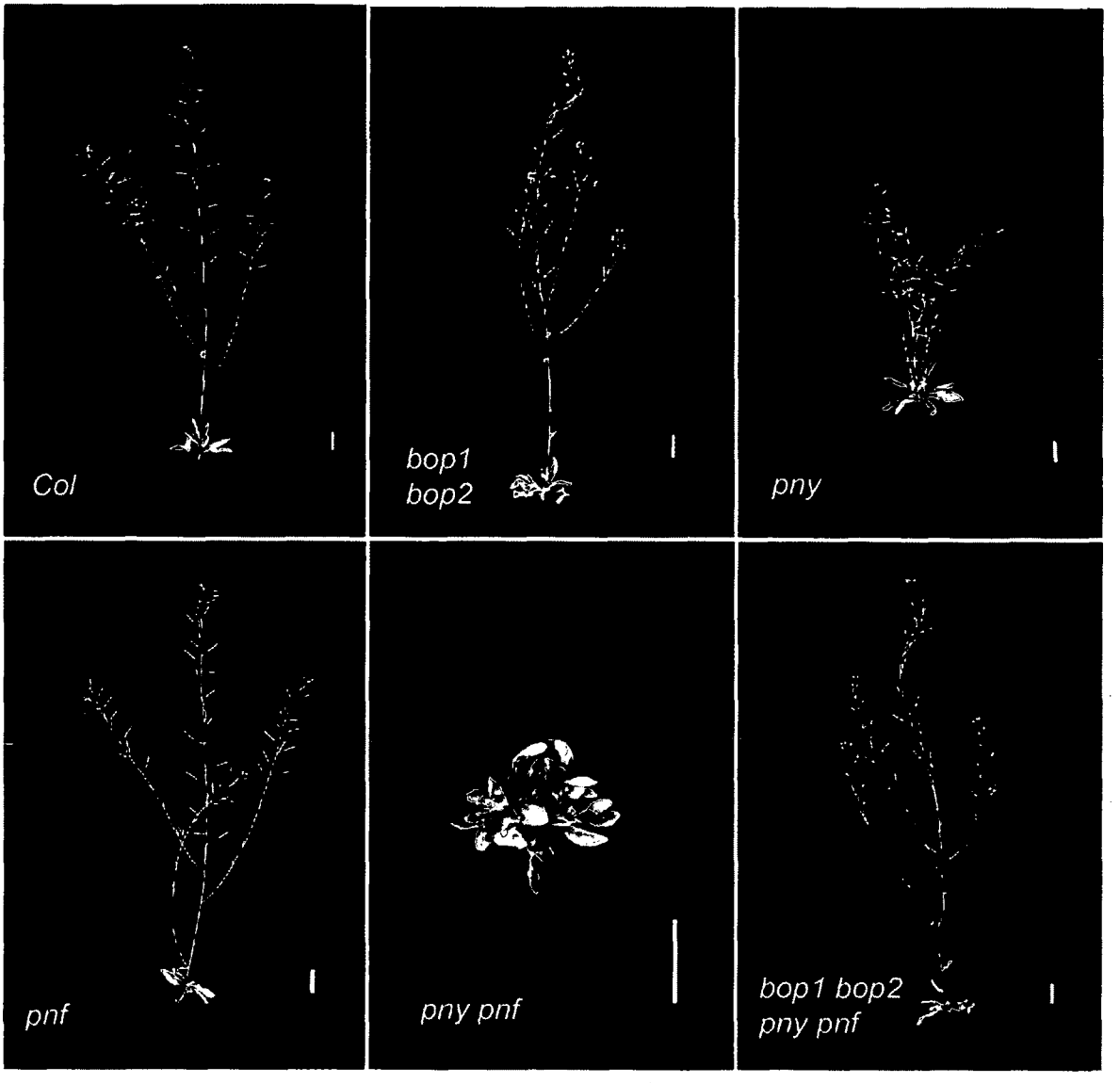

Figure 3.1.1. Inflorescence initiation, apical dominance, and plant height are restored in *bop1 bop2 pny pnf* quadruple mutants. Representative seven-week-old plants are shown. Genotypes are indicated in panels. Scale bars, 2cm.

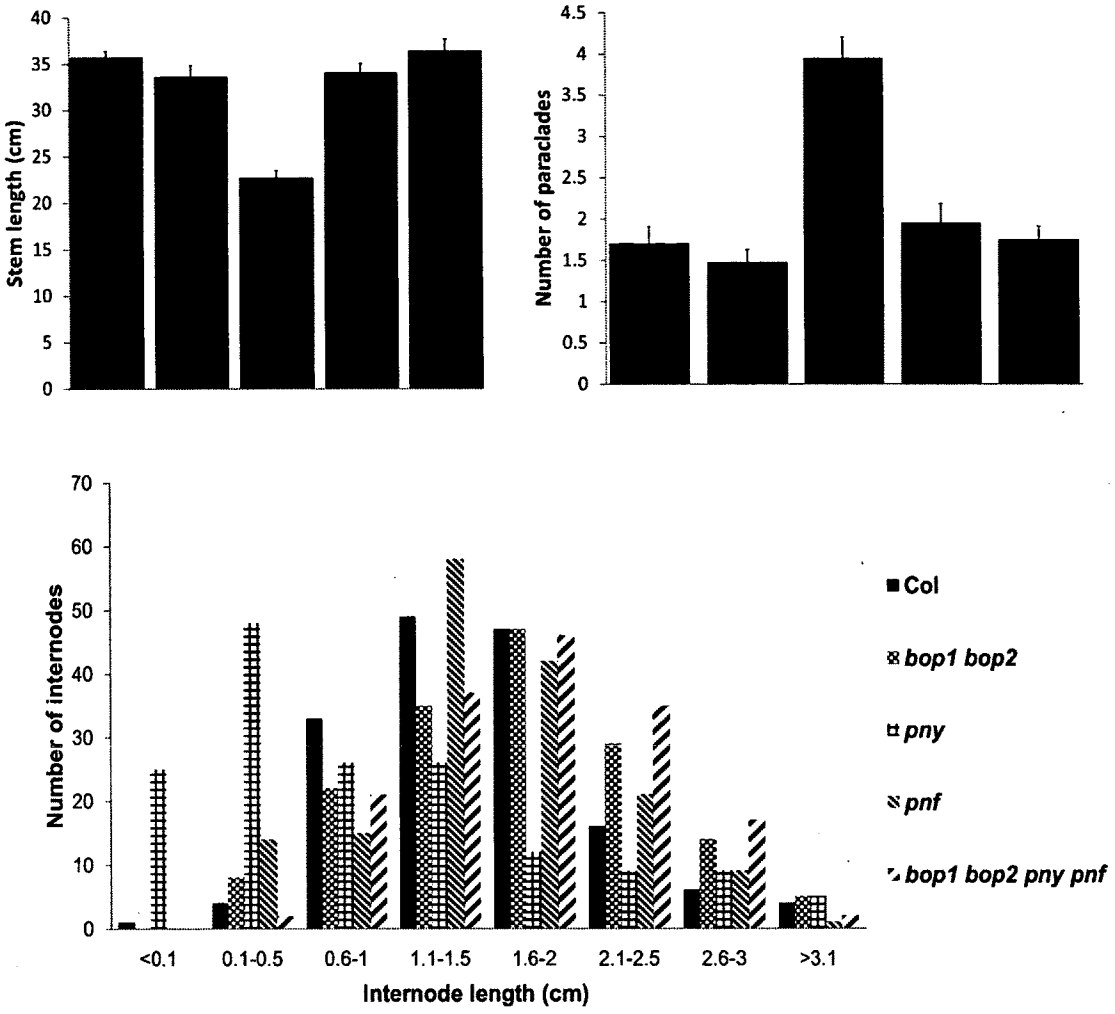

Figure 3.1.2. Loss-of-function *bop1 bop2* fully rescues plant height, apical dominance (paraclades) and internode elongation defects in *pny pnf* mutants. Twenty plants per genotype were grown in continuous light conditions for 7 weeks prior to phenotypic analysis. Error bars represent S.E.M. (n=20).

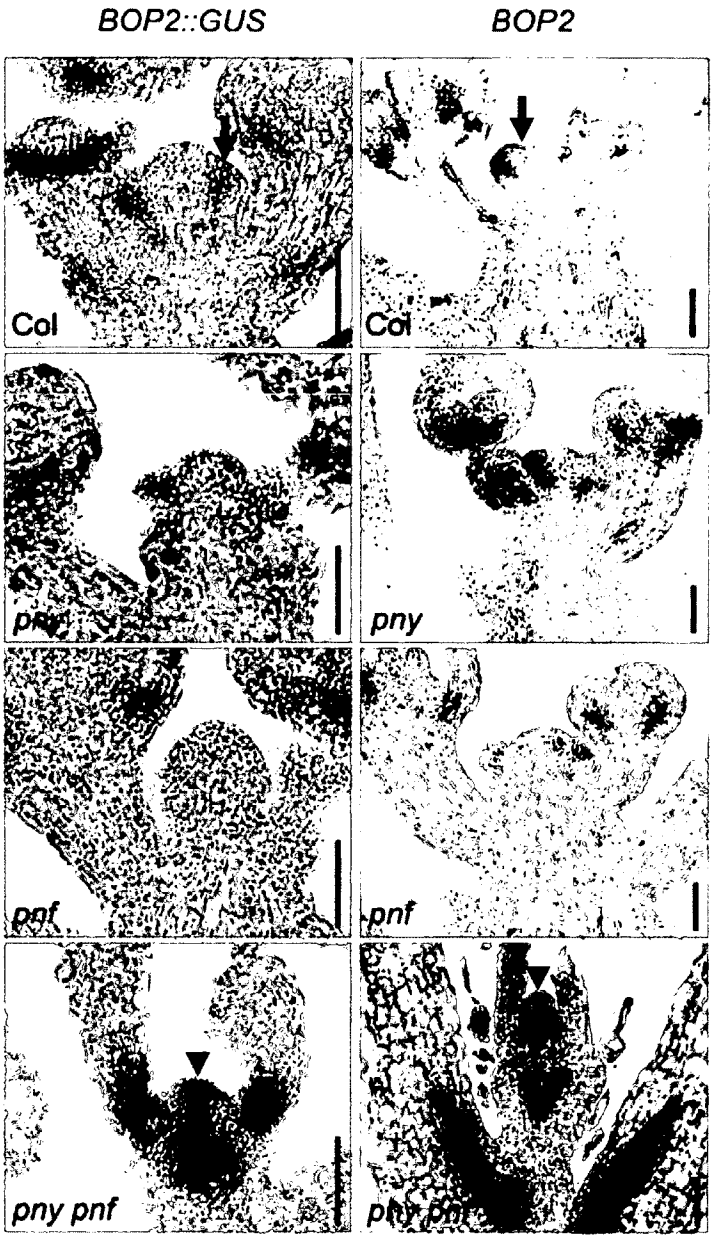

Figure 3.2.1. *BOP2* promoter activity and *BOP2* transcript expression in *pny pnf* plants exposed to floral inductive conditions. (Left) *BOP2:GUS* expression. Blue colour indicates GUS activity. (Right) *BOP2 in situ* hybridization. Purple colour indicates sites of probe hybridization to *BOP2* mRNA. Arrows, *BOP2* expression only at the IM-floral meristem boundary in wild-type plants. Arrowheads, *BOP2* misexpression in the CZ and RZ in *pny pnf* meristems. Scale bars, 50 μm.

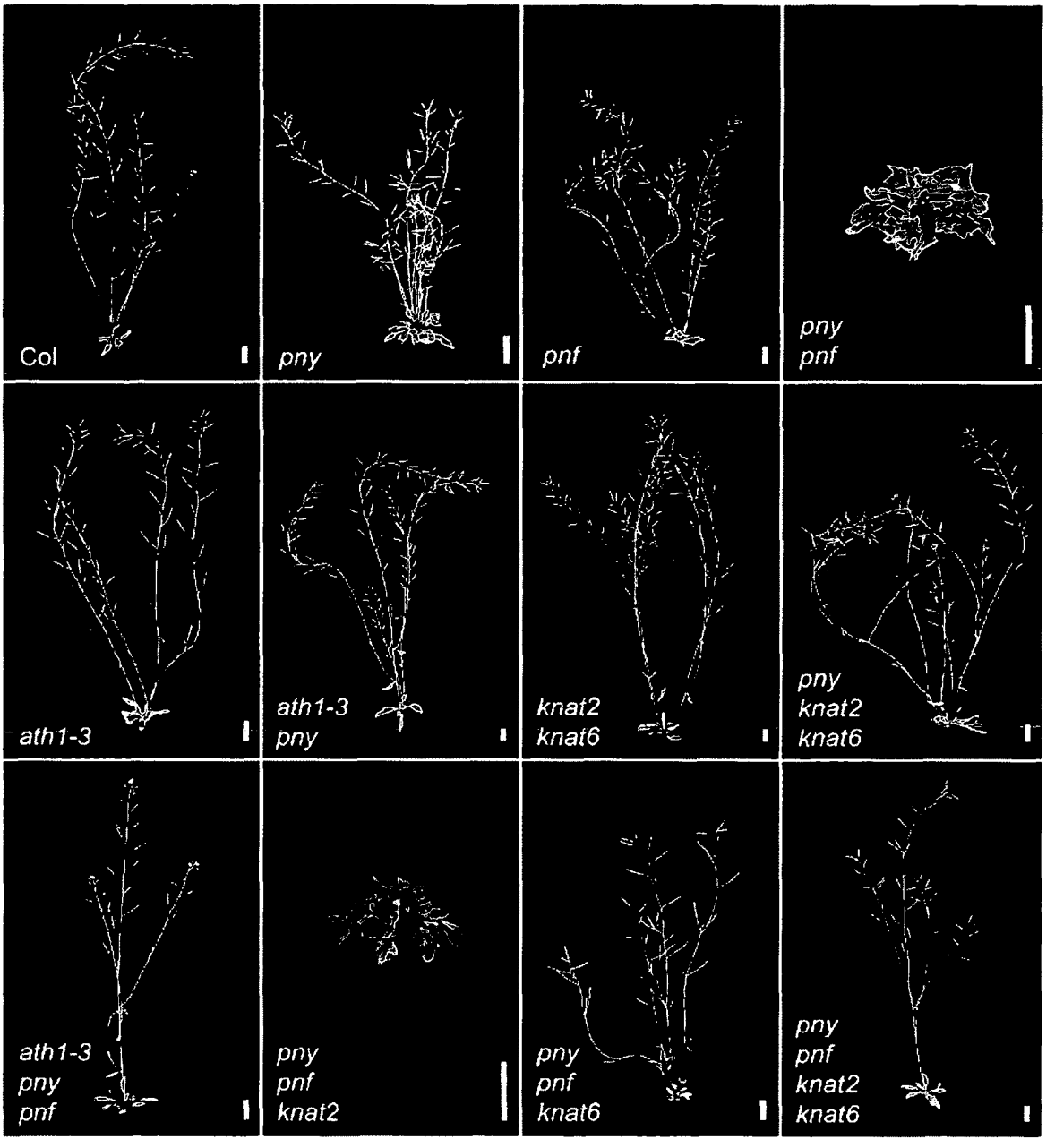

Figure 3.3.1. Loss-of-function *ath1-3* and *knat6* mutations restore flowering and internode elongation in the *pny pnf* double mutant background. Loss-of-function *knat2* mutation fails to restore flowering and internode elongation in the *pny pnf* background. Representative 7-week-old plants are shown. Genotypes are as indicated in panels. Scale bars, 2cm.

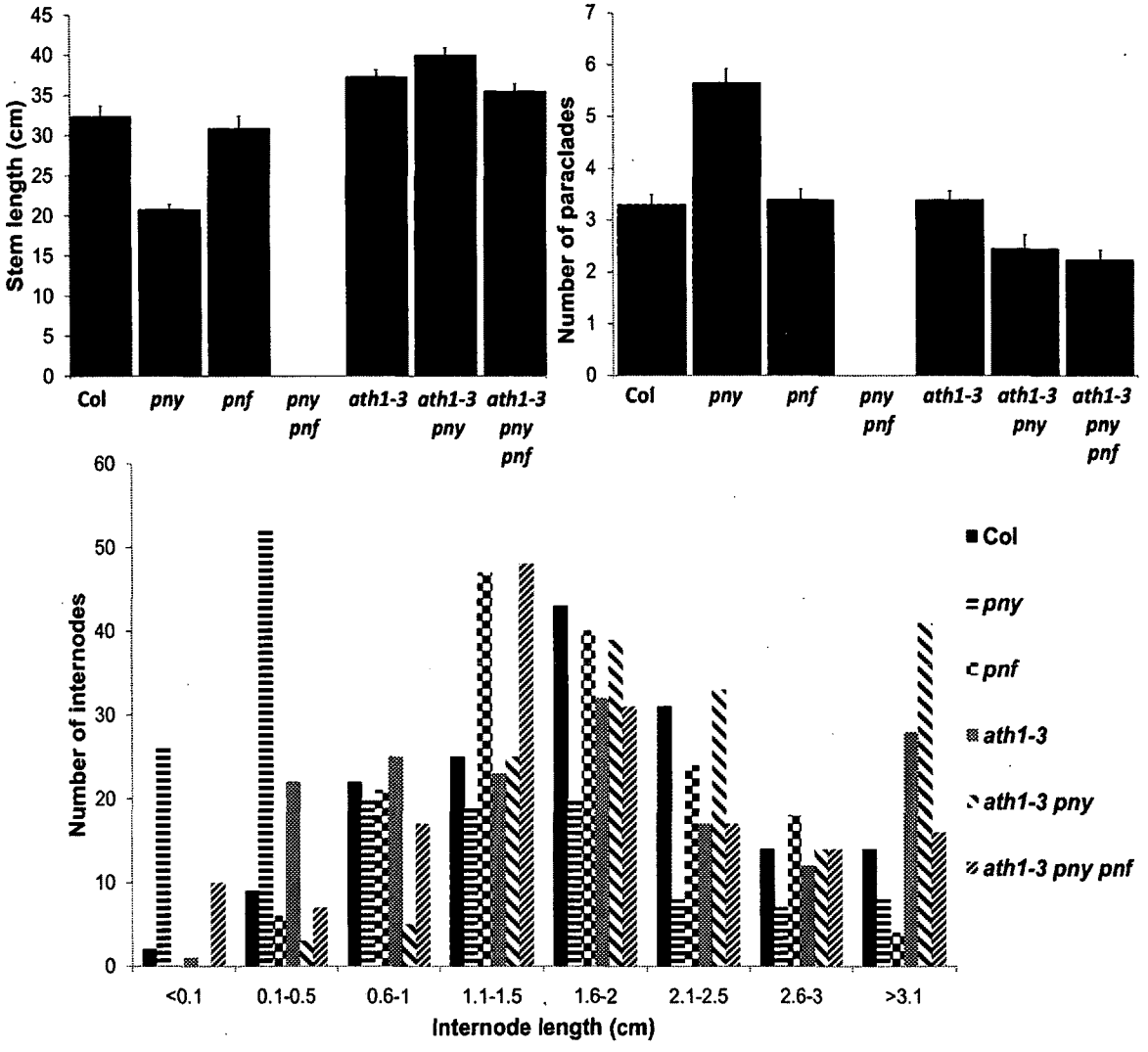

Figure 3.3.2. Loss-of-function *ath1-3* mutation fully rescues stem length, apical dominance, and internode elongation defects of *pny pnf* double mutants. Error bars, S.E.M (n=20).

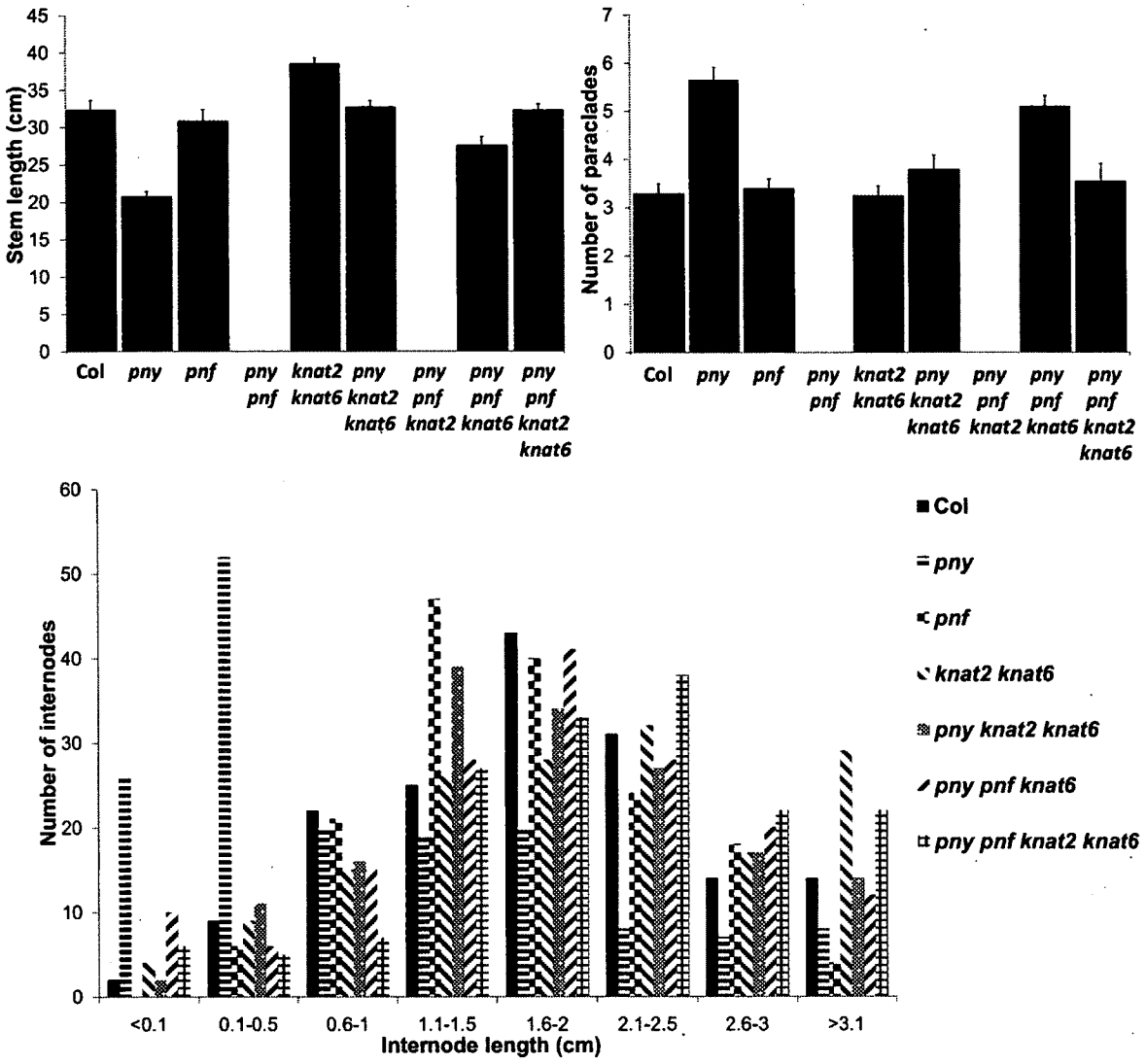

Figure 3.3.3. Inactivation of KNAT6 rescues plant height, apical dominance, and internode elongation defects in *pny pnf* double mutants. Inactivation of both KNAT2 and KNAT6 fully suppresses *pny pnf* defects. Inactivation of *KNAT2* alone does not rescue *pny* mutant defects. Error bars, S.E.M (n=20).

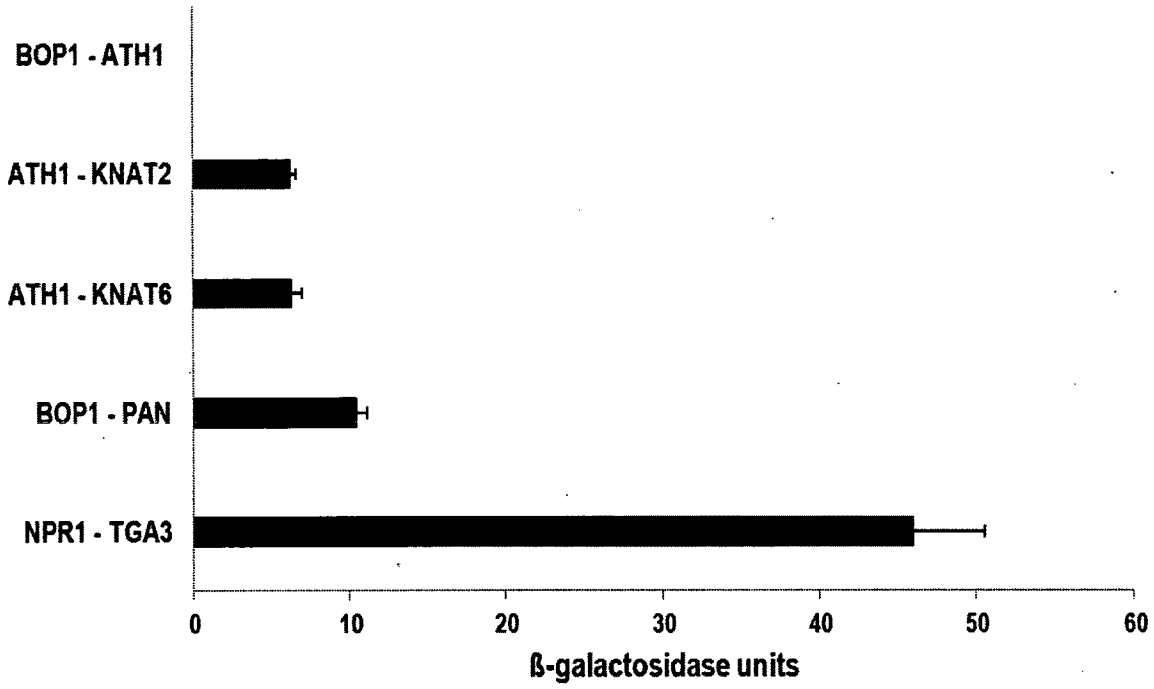

Figure 3.4.1. Quantitative analyses protein-protein interactions in yeast as monitored using a two-hybrid assay. β-galactosidase units represent LacZ reporter gene expression upon bait and prey interaction. Interactions pairs are listed on the Y-axis (Bait - Prey). Error bars represent standard deviation of three technical replicates. Interactions between BOP1-PAN and NPR1-TGA3 were used as positive interaction controls (Hepworth *et al.*, 2005).

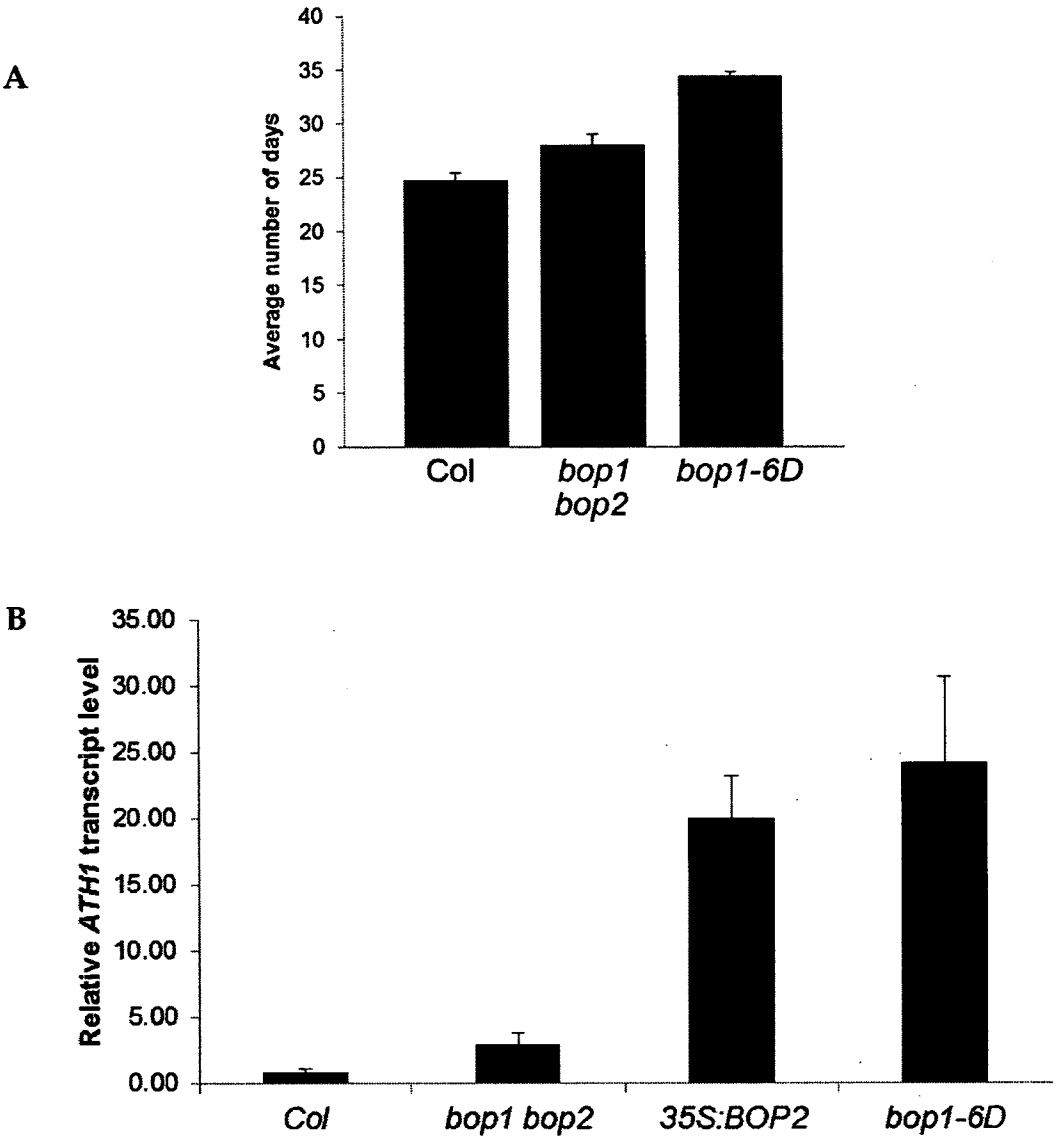

Figure 3.4.2. Constitutive expression of *BOP1* delays flowering and promotes *ATH1* expression in internodes. (A) *BOP1* gain-of-function delays flowering (n=36 plants). Error bars, S.E.M. (B) qRT-PCR analysis of relative *ATH1* transcript levels in internodes of 5-week-old plants for the genotypes indicated. Error bars represent S.E.M. of three technical replicates for one biological replicate.

CHAPTER 4 DISCUSSION

The transition to reproductive development in plants is tightly controlled to optimize pollination and seed set. In *Arabidopsis thaliana,* this transition is seen as the switch from making leaves to the start of internode elongation and production of flowers to form the inflorescence. For this transition to occur, the vegetative SAM must acquire IM fate (Figure 1.3). Competence to flower requires the activities of two BELL homeodomain proteins: PNY and PNF expressed in the meristem. Loss-of-function *pny pnf* mutants are non-flowering and produce only leaves (Smith *et al.,* 2004). Here, I use a genetics approach to show that PNY and PNF are negative regulators of the lateral organ boundaries genes *BOP1* and *BOP2*. Moreover, I show that inactivation of *bop1 bop2* as well as *knat2 knat6* and *ath1-3* rescues the non-flowering phenotype of *pny pnf* double mutants. I provide evidence that *BOP1/2* misexpression in the meristem blocks flowering by promoting the expression of *ATH1* and *KNAT6* whose products form a BELL-KNOX heterodimer (Rutjens *et al.,* 2009) that antagonizes the floral transition.

PNY and PNF in the Meristem Repress *BOP1/2* to Promote Flowering

My data show that *BOP1/2* misexpression blocks flowering and internode elongation in *pny pnf* double mutants. First, analysis of *BOP2::GUS* expression and *BOP2 in situ* hybridization shows that in *pny pnf* plants induced to flower,

BOP2 is misexpressed in the CZ and RZ of the meristem (Figure 3.1.2). No such misexpression is seen in *pny* or *pnf* single mutants, showing that their regulation of *BOP2* expression is redundant. While *BOP1* expression was not tested, these genes have an identical pattern of expression (Hepworth *et al.*, 2005; Xu *et al.*, 2010; Khan *et al.*, 2012). Compatible with this finding, inactivation of *bop1 bop2* rescues the non-flowering phenotype of *pny pnf* mutants (Figure 3.1.1). This result is analogous to the role of PNY in stems, where its activity limits *BOP1/2* expression to stem-pedicel boundary (Khan *et al.*, 2012). These data reveal that ectopic BOP2 activity in the SAM perturbs competence to flower (Figure 3.1.1; Figure 3.2.1). It would be interesting to determine if *BOP1/2* are direct targets of PNY-PNF repression and whether this repression requires STM.

Compatible with our finding that BOP gain-of-function is the cause of *pny pnf* non-flowering defects, flowering is significantly delayed in *bop1-6D* gain-of-function lines (Figure 3.4.2) indicating that BOP1/2 likely antagonize PNY-PNF activities in regulating competence to flower (Smith *et al.*, 2004). In *bp* mutants, BOP1/2 is proposed to antagonize BP activity through reciprocal regulation of target genes, supported by the identification of several lignin biosynthetic genes that are repressed by BP and promoted by BOP1/2 in stems (Khan *et al.*, 2012). A similar mechanism may apply to BOP1/2 antagonism of PNY and PNF in the SAM. *PNY* and *PNF* are up-regulated in the IM relative to the vegetative SAM

and absent in differentiating lateral organ primordia (Byrne *et al.*, 2003; Smith & Hake, 2003; Smith *et al.*, 2004; Kanrar *et al.*, 2006). In addition, inflorescence development is sensitive to PNY and PNF dosage (Kanrar *et al.*, 2006). However, it is not known if up-regulation of *PNY* and *PNF* expression during the transition to flowering is important for co-ordinating internode elongation and the production of flowers.

BOP1/2, ATH1, and KNAT6 Function in One Pathway

Studies with BOP1/2 gain-of-function plants show that BOP1/2 promote *KNAT6* expression and require KNAT6 activity to exert changes in inflorescence architecture. However, over-expression of *KNAT6* alone does not perturb inflorescence architecture suggesting that KNAT6 requires a co-factor provided by BOP1/2 to function (Khan *et al.*, 2012). My data provide evidence that BOP1/2, ATH1, and KNAT6 function in the same genetic pathway to antagonize competence to flower: mutations in all three genes restores flowering in *pny pnf* mutants (Figure 3.1.1; Figure 3.3.1). An important next step will be to examine the expression patterns of *ATH1* and *KNAT6* in *pny pnf* apices.

Four lines of evidence support that BOP1/2 exerts its function by promoting the expression of *ATH1* and *KNAT6* whose products form a complex (Figure 3.4.1).

First, *BOP1/2*, *ATH1*, and *KNAT6* are all expressed in shoot lateral organ boundaries (Gómez-Mena & Sablowski, 2008; Ragni *et al.*, 2008; Khan *et al.*, 2012). Second, *ATH1* and *KNAT6* transcripts are dramatically up-regulated in internodes of plants constitutively expressing *BOP1/2* (20-25-fold and 3-5-fold, respectively; Khan *et al.*, 2012; this study). Third, mutations in *ath1-3* and *knat6* partially restore internode elongation in *35S::BOP2* plants (Khan *et al.*, 2012; Khan & Hepworth, unpublished data). Finally, ATH1 and KNAT6 form a BELL-KNOX heterodimer in yeast and *in vivo* (Rutjens et al. 2009; this study; Li *et al.*, 2012).

A linear pathway comprising BOP1/2 and ATH1-KNAT6 (Figure 4.1.1) may also promote floral organ abscission, which occurs at the basal boundary of floral organs following fertilization of flowers. Whereas *bop1 bop2* mutations completely block floral organ abscission (Hepworth *et al.*, 2005; McKim *et al.*, 2008), mutations in *ath1* cause a partial block and mutations in *knat2 knat6* cause a delay in floral organ abscission (Gómez-Mena & Sablowski, 2008; Shi *et al.*, 2011). In *bop1 bop2* mutants, cells specialized for detachment of floral organs are improperly specified (McKim *et al.*, 2008). These similarities suggest that BOP1/2 activation of *ATH1* and *KNAT6* may represent a conserved module that operates in boundaries throughout the plant at different stages of development. All three genes are similarly expressed in the valve margin of fruits (also a

boundary) where their activity opposes that of BP-PNY in the adjacent replum (Gómez-Mena & Sablowski, 2008; Ragni *et al.*, 2008; Khan *et al.*, 2012).

Does ATH1-KNAT6 Promote *FLC* Expression to Block Flowering?

Previously, it was shown that *35S::ATH1* plants are late flowering through promotion of *FLC* expression (Proveniers *et al.*, 2007). In addition, loss-of-function *ath1-1* mutants flower earlier that wild-type in short day photoperiods (Proveniers *et al.*, 2007). In wild-type plants, *ATH1* expression in the meristem is down-regulated at the floral transition, presumably as part of a mechanism that promotes flowering (Gómez-Mena & Sablowski, 2008). Thus, ATH1-KNAT6 and PNY-PNF may antagonistically regulate *FLC* expression, either directly or indirectly, to modulate competence to flower.

BOP1 gain-of-function causes a significant delay in flowering-time relative to wild-type plants (Figure 3.4.2). Given that constitutive expression of *ATH1* promotes *FLC* expression to delay flowering (Proveniers *et al.*, 2007) it follows that BOP1/2 acting through ATH1-KNAT6 may antagonize competence to flower by maintaining high levels of *FLC* expression in floral-inductive conditions. FLC gain-of-function causes a range of delays in flowering time; likely a result of various levels of FLC activity in individual transgenic lines

(Michaels & Amasino, 1999; Sheldon *et al.*, 1999; Werner *et al.*, 2005). Furthermore, several FLC gain-of-function mutants (Sheldon *et al.*, 1999) appear to phenocopy the complete block in flowering of *pny pnf* loss-of-function mutants (Smith *et al.*, 2004). Interestingly, constitutive expression of *ATH1* in *Lolium perenne* caused delayed or complete inhibition of flowering (van der Valk *et al.*, 2004), indicating that sufficient dosage of ATH1 activity can completely inhibit flowering. Therefore, in *pny pnf* mutants, sufficient ATH1 activity may completely inhibit flowering through promoting *FLC* expression. Attenuated transcript levels of direct targets of FLC repression: *SOC1* and *FT* (Hepworth *et al.*, 2002; Helliwell *et al.*, 2006; Searle *et al.*, 2006) in *pny pnf* double mutants (Smith *et al.*, 2004; Kanrar *et al.*, 2008) supports the notion that FLC gain-of-function blocks flowering in *pny pnf* loss-of-function mutants. Furthermore, constitutive expression of *FT* restored flower production in the *pny pnf* mutant background (Kanrar *et al.*, 2008). Together, these studies support that FLC gain-of-function as a result of ectopic *BOP1/2* expression blocks flowering in *pny pnf* mutants. It would be interesting to determine the mechanism of BOP1/2-induced ATH1-KNAT6 regulation of *FLC* expression.

Alternatively, ATH1-KNAT6 may regulate the expression of miRNA156 or members of the *SPL* gene family to block flowering in *pny pnf* mutants. A recent study by Lal *et al.* (2012) indicates that PNY-PNF may promote flowering

through repression of miR156, a key negative regulator of SPL mRNA accumulation. The SPL module is part of an autonomous flowering-time pathway that inhibits flowering during juvenile plant development (Wu & Poethig, 2006; Wang *et al.*, 2009). Constitutive expression of *SPL4* rescues flowering in *pny pnf* mutants, but internode elongation remained repressed, similar to constitutive expression of *LFY* or *FT* (Kanrar *et al.*, 2008; Lal *et al.*, 2011). Furthermore, *SPL4* and *SPL5* transcripts fail to accumulate in *pny pnf* mutants in floral inductive photoperiods (Lal *et al.*, 2011). Given that *SPL* genes are regulated downstream of FT and thus FLC (Schmid *et al.*, 2003; Helliwell *et al.*, 2006), ATH1-KNAT6 likely indirectly regulate *SPL* genes via regulation of *FLC* expression.

Control of Internode Elongation by ATH1

Down-regulation of *ATH1* expression in long day photoperiods permits elongation of internodes following the floral transition and reduces activation of *FLC* expression to yield a meristem with competence to flower (Proveniers *et al.*, 2007; Gómez-Mena & Sablowski, 2008). However, the mechanism of ATH1 regulation of inflorescence architecture has yet to be elucidated. In wild-type plants, it is unlikely that BOP1/2 and KNAT6 function with ATH1 to co-ordinate flowering and internode elongation at the floral transition as *BOP1/2* and *KNAT6* expression is excluded from the SAM (Ha *et al.*, 2004; Belles-Boix *et al.*, 2006).

Given that *KNAT2* and *ATH1* are co-expressed in the RZ (Pautot *et al.*, 2001; Gómez-Mena & Sablowski, 2008) an ATH1-KNAT2 heterodimer may inhibit internode elongation prior to the floral transition. Recently, reduced expression of the pectin methylesterase encoded by *PME5* was shown to correlate with reduced elongation of stem epidermal and pith cells resulting in short internodes in *blr-6* (*pny*) mutants (Peaucelle *et al.*, 2011). Constitutive expression of *ATH1* similarly inhibits elongation of pith cells (Gómez-Mena & Sablowski, 2008). These studies indicate that ATH1-KNAT6 via BOP1/2 gain-of-function in *pny* stems may negatively regulate the production of pectin methylesterases in internodes to restrict growth.

Conclusion

These data support the model that misexpression of a boundary module comprised of BOP1/2 and its transcriptional targets *ATH1* and *KNAT6* in *pny pnf* meristems blocks competence to flower. Direct targets of the ATH1-KNAT6 heterodimer have yet to be identified, but floral repressors such as FLC or regulators of *FLC* are a possibility. A similar module is likely to operate in *bp* and *pny* mutant stems to inhibit internode elongation and promote differentiation of interfascicular fibres, in the valve margin of fruits to promote dehiscence, and at the base floral organs to promote abscission. Future studies will determine if *ATH1* and *KNAT6* are direct transcriptional targets of BOP1/2 in lateral organ boundaries.

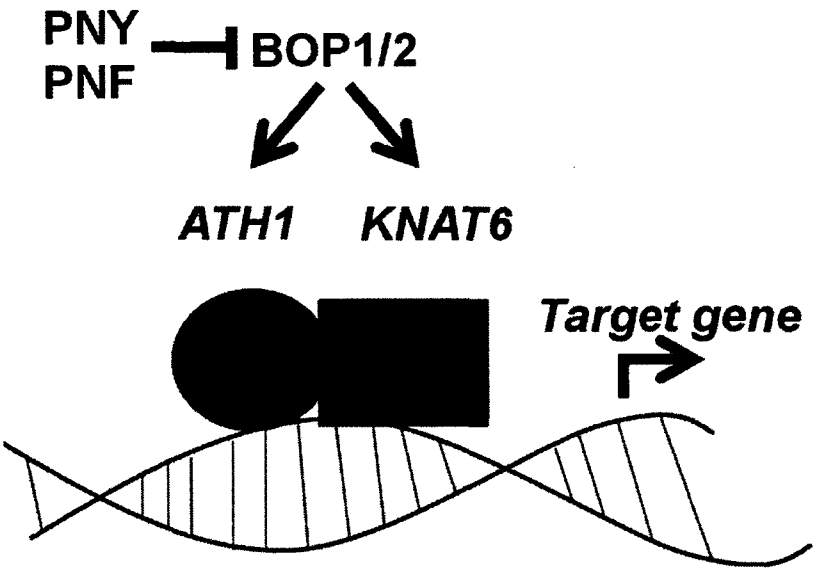

Figure 4.1.1. Model of regulation of inflorescence architecture and competence to flower by PNY-PNF. PNY-PNF are negative regulators of *BOP1/2* expression in the SAM of plants exposed to floral inductive conditions. In lateral organ boundaries, BOP1/2 are proposed to promote the expression of *ATH1* and *KNAT6* that encode members of a BELL-KNOX heterodimer. This transcription factor complex is predicted promote expression of target genes that restrict growth restriction and regulate competence to flower.

CHAPTER 5 FUTURE DIRECTIONS

Is *ATH1* Misexpressed in *pny pnf* and *35S::BOP2* Shoot Apical Meristems?

In wild-type apices, *ATH1* is down-regulated at the transition to flowering (Gómez-Mena & Sablowski, 2008). Given that *ATH1* transcript is up-regulated 20-25-fold in *bop1-6D/35S::BOP2* internodes (Figure 3.4.2), we predict that *ATH1* may be similarly up-regulated in *pny pnf* and *bop1-6D/35S::BOP2* apical meristems. To test if *pny pnf* and plants constitutively expressing *BOP1/2* maintain *ATH1* expression in the meristem, I performed qRT-PCR to quantify *ATH1* cDNA levels from mRNA isolated from *pny pnf* mutants and controls grown in short days or short days plus 3 long days as previously described (Gómez-Mena & Sablowski, 2008). However, this method lacked sensitivity to determine if *ATH1* expression was indeed maintained in *pny pnf* meristems. Micro-dissection of *pny pnf* apices may eliminate background RNA from whole rosettes. Alternatively, *in situ* hybridization can be used to examine the spatial distribution of *ATH1* transcript in *pny pnf* and *bop1-6D/35S::BOP2* apices relative to wild-type control plants before and after exposure to floral inductive conditions.

Is *ATH1* Directly Regulated by BOP1/2?

Steroid-inducible forms of BOP1 (BOP1:GR) have been generated to determine if BOP1/2 activation of *ATH1* is direct or indirect. I have already made a

BOP1::BOP1:GR construct that codes for a translational fusion of BOP1 protein to the *Rattus norvegicus* glucocorticoid receptor hormone-binding domain (Lloyd *et al.*, 1995). *BOP1::BOP1:GR bop1 bop2* transformants have been generated and addition of the steroid hormone dexamethasone shown to complement *bop1 bop2* leaf and floral-organ abscission defects (Hepworth *et al.*, 2005; data not shown), confirming that the fusion protein is active and that its activity is inducible. In the future, *35S::BOP1:GR* transgenic plants should be used to examine if BOP1/2 induces *ATH1* expression. Transformation of an *ATH1::GUS* reporter (Proveniers *et al.*, 2007) into *35S::BOP1:GR* plants will reveal if the *ATH1* promoter contains regulatory elements that respond to hormone-induced BOP1:GR activity. If *ATH1::GUS* responds to BOP1:GR induction, chromatin immunoprecipitation should be used to confirm direct association of tagged BOP1 to the *ATH1* promoter.

What are the downstream targets of BOP1/2 induced ATH1-KNAT6?

Interestingly, constitutive *BOP1/2* expression delays flowering (this study). This suggests that ectopic ATH1-KNAT6 activity prevents accumulation of a flowering activator or promotes expression of a floral repressor. Over-expression of *ATH1* causes extreme late flowering in a C24 genetic background by activating *FLC* expression (Proveniers *et al.*, 2007). Therefore, it is most plausible that BOP1/2 gain-of-function promotes *FLC* expression or expression of a related floral repressor (*e.g.* SVP or one of five MADS AFFECTING FLOWERING (MAF)

genes) (Li *et al.*, 2008; Ratcliffe *et al.*, 2001; Ratcliffe *et al.*, 2003). Over-expression of floral repressors in the meristem of *pny pnf* and *bop1-6D/35S::BOP2* apices can be monitored using qRT-PCR and by *in situ* hybridization. It would be interesting to determine if *flc* loss-of-function rescues flowering and internode elongation in *pny pnf* mutants.

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APPENDIX I MUTANTS USED IN THIS STUDY

Table A1.1. Mutants used in this study.

Allele	Mutagen	Description	Reference
ath1-3	T-DNA	Fourth exon; null	Proveniers *et al.*, 2007
bop1-3	T-DNA	Promoter; knock-down	Hepworth *et al.*, 2005
bop1-6D	activation tag T-DNA	over-expression	Norberg *et al.*, 2005
bop2-1	T-DNA	5'UTR; null	Hepworth *et al.*, 2005
knat2-5	T-DNA	Third exon; null	Belles-Boix *et al.*, 2006
knat6-2	T-DNA	Second intron; null	Belles-Boix *et al.*, 2006
pnf-96116	T-DNA	First intron; null	Smith *et al.*, 2004
pny-40126	T-DNA	First intron; null	Smith & Hake, 2003

APPENDIX II SEGREGATION ANALYSES

Table A2.1. Segregation analysis of F2 populations generated for isolation of mutants for this study.

Genotype	Phenotype	Observed	Expected	N	X^2
BOP2::GUS/+ pny/+ pnf/+	*pny*	5	9	48	
	pny pnf	4	3		
					2.1111
ath1/+ pny/+	*ath1*	10	17.75	71	
	pny	4	13.31		
					9.8959*
ath1/+ pny/+ pnf/+	*ath1*	17	17.5	70	
	pny	1	8.75		
	pny pnf	0	3.28		
					10.1585*
pny pnf/+ knat2/+ knat6/+	*pny*	56	119.25	212	
	pny pnf	0	9.9		
					43.4479*
pny/+ pnf/+ knat2/+	*pny*	42	32.6	174	
	pny pnf	13	10.9		
					3.1150
pny/+ pnf/+ knat6/+	*pny*	45	28.1	200	
	pny pnf	0	9.3		
					19.4640*

*Unexpected segregation ratios may be caused by rescue of *pny* and/or *pny pnf* phenotypes by elimination of one copy of *KNAT6* or *ATH1* (heterozygous).

APPENDIX III GENETIC INTERACTIONS - DOUBLE MUTANT ANALYSIS

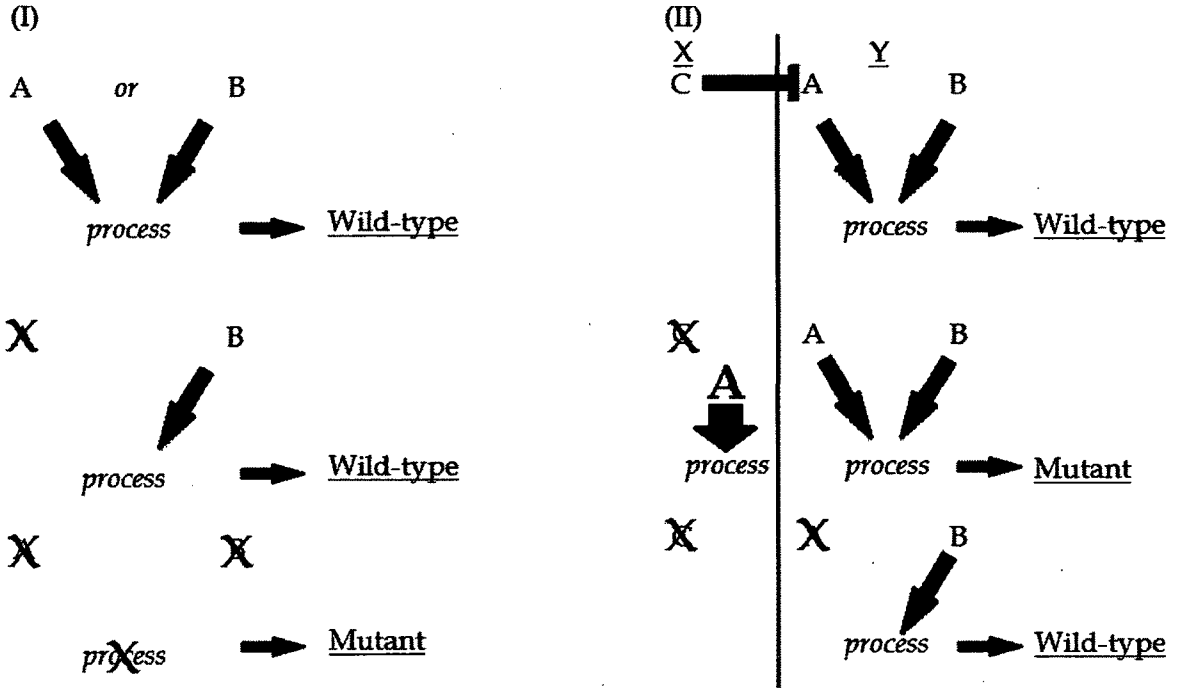

Figure A.3. Double mutant analysis.
(I) A and B function redundantly. Redundant transcription factors are able to compensate in the event of a loss of the other. In this case, both A and B must be eliminated to disrupt the downstream process and generate a mutant phenotype. Elimination of only one transcription factor fails to disrupt the process.
(II) C is a repressor of A but not B. In this case, C excludes A activity and thus the downstream process from domain X. Loss of C allows A gain-of-function and thus the process to occur in domain X causing a mutant phenotype. Elimination of both A and C (double mutant) prevents A gain-of-function and allows normal (wild-type) development as B normally regulates the process in domain Y. Arrows represent promotion and blunt bars indicate repression.