Investigating a conserved role for BLADE-ON-PETIOLE and class I TGA bZIP transcription factors in regulation of inflorescence architecture and lignin biosynthesis in *Arabidopsis thaliana* and *Populus trichocarpa*

By

Bhaswati Devi

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Carleton University
Ottawa, Ontario, Canada

ABSTRACT

Lignin is one of the major factors in determining the woodiness of plants. Although wood and fibre formation are characteristic of trees, many herbaceous species including the model plant *Arabidopsis thaliana* (Arabidopsis) develop stems with secondarily thickened cell walls rich in lignin that provide mechanical support during flowering. BLADE-ON-PETIOLE1/2 (AtBOP1/2) are transcription factors in Arabidopsis that regulate plant architecture through modulation of growth and meristem activity. Members of this protein family have a transcriptional activation domain but lack a DNA-binding domain and require TGACG motif-binding (TGA) basic leucine zipper (bZIP) proteins as co-factors for recruitment to DNA. Arabidopsis plants that overexpress BOP1/2 are late-flowering with short internodes containing excessive lignin. Several lignin biosynthetic genes are up-regulated in the stems of BOP overexpressing lines consistent with promotion of lignin production, a characteristic of secondary growth in plants. Poplar tree has a pair of BOP-like genes (*PtrBPL1/2*) but their role in tree development is unknown. Comparison of BOP function in Arabidopsis and poplar can lead to a better understanding of developmental regulation of secondary cell wall biosynthesis in plants.

In this thesis, firstly I characterized the expression and function of *PtrBPL* genes to test for a conserved role in stem development and lignin biosynthesis. My data showed that *PtrBPL1/2* are functional homologs of *AtBOP1/2* expressed in poplar leaves, flowers, and vascular tissues of the woody stem (xylem and phloem). Arabidopsis plants overexpressing *PtrBPL1* and *PtrBPL2* had short internodes that

potentially overproduce lignin. These data support a conserved role for BOPs in tree development.

Secondly, I tested the hypothesis that Arabidopsis BOPs require class I TGA bZIP factors TGA1 and TGA4 to exert changes in inflorescence architecture and lignin biosynthesis. My data showed that *TGA1* and *TGA4* are expressed at lateral organ boundaries and in vascular tissues. I also showed that their expression in stems is regulated by the class I KNOTTED-like homeobox (KNOX) transcription factor BREVIPEDICELLUS (BP) in a manner similar to BOP1/2. However, double mutant analyses with *bp*-2 and protein interaction studies did not clearly support the hypothesis that BOP1/2 require TGA1 and TGA4 as co-factors for regulating growth patterns or lignin content in the primary inflorescence stem.

Collectively, these findings shed light on developmental functions of BOPs in Arabidopsis and poplar and their interaction with class I TGA bZIP transcription factors in regulation of inflorescence architecture and lignin biosynthesis.

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PREFACE

This thesis explores the role of BOP and class I TGA bZIP transcription factors in regulation of secondary growth and lignin biosynthesis in Arabidopsis and poplar.

I carried out the majority of work in this thesis but acknowledge that some of the data presented were obtained in collaboration with individuals listed below. I would like to thank the following for their contributions to my thesis.

Dr. Eryang Li initiated the work on poplar by cloning the poplar *BOP*-like gene that I characterized. Eryang also provided the qRT-PCR data that was used to confirm the expression profile of *BOP* genes in poplar tree and designed and initiated constructs for complementation, overexpression, and gene silencing. Shabnam Gholoobi and Madiha Khan carried forward some of this work before it was completed by me.

Michael Bush generated the TGA1:GUS and TGA4:GUS reporter genes used for analysis of expression patterns in plants. Brock Billings and Thearany Lay provided sections of GUS-stained tissues showing *TGA1* and *TGA4* reporter gene expression in the inflorescence apex and vasculature of the stem.

Natalie Woerlen generated constructs for Bimolecular Fluorescence Complementation. Denise Chabot and Keith Hubbard at Agriculture and Agri-Food Canada collected images using confocal laser scanning microscopy.

My supervisor, Dr. Shelley Hepworth, assisted in the isolation and characterization of *bp-2 tga1*, *bp-2 tga4*, and *bp-2 tga1 tga4*, *bp-2 TGA1:GUS, bp-2 TGA4:GUS* lines. I would also like to thank Dr. Hepworth for helping me to make the figures and write the thesis.

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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: shift towards wild-type phenotype

Phenotypic enhancement: worsening a mutant phenotype

Redundancy: when there is genetic compensation in the event of gene loss-of-function

Homolog: genes sharing a common ancestor in evolution

Orthologs: genes in different organisms that descend from a common ancestor (often with the same function)

Paralogs: genes that are related by a duplication event

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*

Double mutation: *bop1 bop2*

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

Protein fusion: BOP1-GR

LIST OF ABBREVIATIONS

AS1	ASYMMETRIC LEAVES1
At	*Arabidopsis thaliana*
ATH1	ARABIDOPSIS THALIANA HOMEOBOX GENE1
BELL	BEL1-like
BOP	BLADE-ON-PETIOLE
BP	BREVIPEDICELLUS
BPL	BOP-like
BiFC	Bimolecular Fluorescence Complementation
BTB/POZ	Bric-a-Brac, Tram Track, Broad Complex/POX virus and Zinc finger
bZIP	basic leucine zipper
C4H	cinnamate-4-bydroxylase
CAD	cinnamyl alcohol dehydrogenase
CCoAOMT	caffeoyl CoA-O-methyltransferase
CLV	CLAVATA
Col	Columbia (wild-type ecotype of *Arabidopsis thaliana*)
COMT	caffeic acid O-methyl transferase
CUC	CUP-SHAPED COTYLEDON
Cys	cysteine
CZ	Central zone
D35S CaMV	Double 35S Cauliflower Mosaic virus promoter
GUS	β-Glucuronidase

IM	Inflorescence meristem
KNAT	KNOTTED1-LIKE FROM ARABIDOPSIS THALIANA
KNOX	KNOTTED1-LIKE HOMEOBOX
LOB	Lateral Organ Boundary
NPR1	NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1
OC	Organizing center
PAL	phenylalanine ammonia lyase
PAN	PERIANTHIA
PCR	Polymerase chain reaction
PNF	POUND-FOOLISH
PNY	PENNYWISE
AtPRXR9GE	Arabidopsis thaliana PEROXIDASE9GE
Ptr	*Populus trichocarpa*
PZ	Peripheral zone
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RNAi	RNA interference
RZ	Rib zone
SAM	Shoot apical meristem
STM	SHOOT MERISTEMLESS
TALE	THREE-AMINO ACID-LOOP-EXTENSION
TGA	TGACG-motif binding
WT	Wild-type
WUS	WUSCHEL
YFP	Yellow fluorescent protein

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CHAPTER 1

INTRODUCTION

1.1 Thesis overview

Inflorescence architecture varies widely among flowering plants serving to organize and support the display of flowers for reproductive success. A major goal of developmental biologists is to understand the genetic basis of inflorescence patterning as this information has important applications in crop breeding. Arabidopsis (*Arabidopsis thaliana*) serves as a simple model for the systematic study of factors that regulate inflorescence architecture.

The shoot apical meristem (SAM) is a niche for stem cells located at the apex of the primary shoot. This compartment is established during embryogenesis and regulates the production and allocation of founder cells for incorporation into aerial organs and tissues (Fletcher, 2002). During vegetative development, the SAM produces leaves without internode elongation. The SAM acquires inflorescence meristem (IM) fate in response to floral inductive signals. During reproductive development, an inflorescence is produced: leaf development is repressed, internodes are elongated, and axillary meristems proliferate to form lateral branches and flowers (Bowman and Eshed, 2000; Barton, 2010). When internode elongation is ceased, secondary development begins. In Arabidopsis, this involves the differentiation of lignified interfascicular fibres which provide mechanical support. In trees, secondary growth is responsible for wood production.

My studies shed light on the role of lateral organ boundary genes in regulating stem development and lignin deposition as part of secondary growth.

My thesis is divided into two sections. In the first section, I provide evidence that Arabidopsis and poplar *BOP* genes have a conserved role in plant development. In

the second section, I investigate the potential for class I TGA bZIP transcription factors to serve as co-factors in BOP-mediated promotion of lignin biosynthesis in controlling fibre development in stems.

1.2 *Arabidopsis thaliana* as a study species

The mouse ear cress *Arabidopsis thaliana* is a member of *Brassicaceae* family related to mustard, cabbage, and canola. The species was widely adopted as a model organism in the late 1970's based on its suitability for studying problems in plant physiology, biochemistry, and development using a molecular genetics approach:

(i) Arabidopsis is small herbaceous weed (30-40 cm) with simple growth requirements, a short life cycle (6-8 weeks), and prolific seed production;

(ii) the plant is a diploid that can be self- or cross-fertilized at will;

(iii) loss and gain-of-function mutants are readily obtained using chemical mutagens, transposons, and/or *Agrobacterium tumufaciens*-mediated transformation;

(iv) the plant has a small compact genome of 125 mega base pairs organized into five chromosomes whose annotated sequence is available at www.arabidopsis.org (The Arabidopsis Information Resource); and

(v) extensive public resources including indexed libraries of T-DNA insertion mutants, seeds, and cDNA clones are available from centralized stock centers in North America, Europe, and Asia.

Although Arabidopsis has no economic value, information obtained from this plant has been successfully applied to the improvement of various crop species (Peng et al., 2000; Lavagi et al., 2012). Developmental programs in crop species turn out to be

similar to those in Arabidopsis. Thus, identification of orthologous genes is useful as a foundation for genetic engineering and for improving the speed and efficiency of marker-assisted crop breeding (Bevan and Walsh, 2005; Ferrier et al., 2011).

1.3 Modular body plan and life cycle

Plants have a modular body plan based on the production of repeating modules called phytomers by the SAM. Each phytomer has three parts: a leaf and a leaf attachment site with the potential to form an axillary meristem (collectively called a node), and an associated piece of subtending stem (internode). With each phase of the development, the meristem elaborates different structures within the module. The position of each module and its subsequent development are strongly influenced by external and genetic cues, allowing the overall architecture of a plant to unfold over developmental time and adapt to its environment (Sussex, 1989).

The life cycle of an Arabidopsis plant is broadly divided into embryonic, vegetative, and reproductive phases. Embryonic development begins at fertilization and ends with the production of a seed. The mature embryo inside a seed contains the first set of basic structures: a root, the SAM, and two embryonic leaves (cotyledons). Germination of the seed resumes growth by initiating vegetative development. The SAM produces a compact rosette of leaves without internode elongation and axillary meristems are quiescent (Fletcher, 2002; Figure 1.1). Floral inductive signals acting on the SAM cause its conversion to an IM. This switch triggers new patterns of aerial development: leaf development is repressed, internodes are elongated, and axillary meristems proliferate to form lateral branches and flowers arranged in a regular

spiral phyllotactic pattern on the primary stem (Figure 1.1; Irish, 2010; Smith et al., 2004). When primary growth ceases, the stems of most dicotyledonous plants undergo secondary growth. Meristematic activity is reinitiated between primary vascular bundles in the stem to produce the vascular cambium. The cambium produces secondary xylem, phloem, and interfascicular fibres resulting in a thicker sturdier stem reinforced with lignin.

1.4 Importance of meristems in determination of the body plan

The architectural diversity of flowering plants can be attributed to variation in the shape, size, proportion, and relative positions of organs in the aerial parts of the plant. Much of this variation can be traced to changes in the size or activity of the shoot apical meristem and its derivative meristems. These include axillary (lateral) meristems that produce side shoots, floral meristems that produce flowers, and the vascular cambium that directs secondary growth in stems.

Studies in Arabidopsis have focused on the SAM because of its central role in plant development. The SAM is located at the growing tip of the primary shoot of plants. Activity of the SAM is ultimately required to generate all aerial organs in a plant including leaves, stems, branches, and flowers. Study of the SAM and factors involved in its regulation have provided important insights into the functioning of all other meristems in the plant.

1.4.1 Organization of the SAM

The SAM is organized into three distinct zones based on differences in morphology and cell division rates: the central zone (CZ), the peripheral zone (PZ), and the rib zone (RZ) (Fig 1.2). The CZ lies at the shoot apex and contains a reservoir of enlarged,

highly vacuolated, self-renewing stem cells that divide infrequently relative to the other cells in the SAM. The progeny of these cells are displaced towards PZ of the SAM. The PZ represents a transitional zone in the SAM, where the progeny of stem cells have the potential to acquire a more specified fate. PZ cells are smaller and divide more frequently than the CZ cells, giving rise to lateral organs, which later build up the aerial parts of a plant.

The RZ extends below the CZ and is comprised of large, vacuolated cells that form the pith of the meristem. Cell division in the RZ is suppressed during vegetative development and activated during reproductive development giving rise to internodes and upward growth of plants.

Plants can maintain a pool of stem cells and continuously produce lateral organs on the flanks of the meristem by coordinating cell fate within the different zones: the progeny of CZ cells either stay in the center 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 (Bowman and Eshed, 2000; Fletcher, 2002; Barton, 2010; Bäurle and Laux, 2003).

The Arabidopsis SAM is further divided into a tunica-corpus structure consisting of two overlying layers: L1, the epidermal layer, and L2, the sub-epidermal layer (Figure 1.2). These layers are a single cell thick and remain distinct because the cells generally divide in an anticlinal orientation (perpendicular to the meristem). Cells in the underlying L3 layer, divide anticlinally and periclinally generating the stem vasculature and pith as well as the innermost cells of leaves and floral organs. Cells

from the L1, L2, and L3 layers comprise the CZ and PZ, while the RZ consists of cells from the L3 layer (Fletcher, 2002; Bäurle and Laux, 2003; Barton, 2010;).

1.4.2 Establishment of the SAM

The embryo apex is partitioned into three compartments: a central region that differentiates into the SAM, a lateral region that gives rise to the embryonic leaves, and the lateral organ boundary, a narrow band of cells that separates the embryonic leaves from the SAM (Figure 1.3). Interactions between genes expressed in these three compartments function in controlling meristem initiation, organization, and activity throughout plant development (Barton, 2010; Rast and Simon, 2008).

The SAM appears as a small mound of cells arising between the two leaf primordia at the bent-cotyledon stage of embryo development (Barton and Poethig, 1993). *WUCHEL* (*WUS*) encoding a homedodomain transcription factor is the earliest known marker of meristem fate. *WUS* transcript is first detected in inner apical cells of globular embryos at the 16-cell stage. These cells go on to form a subdomain called the Organizing Center required in maintaining the stem cell population (Barton, 2010; Takeda and Aida, 2011).

Genes expressed in the lateral organ boundary also contribute to SAM initiation. Three *CUP-SHAPED COTYLEDON* genes (*CUC1*, *CUC2*, and *CUC3*) are activated in overlapping regions of the late globular embryo. Loss-of-function studies show that all three *CUC* genes help form the SAM and cotyledon boundaries (Aida et al., 1997; Vroemen et al., 2003; Hibara et al., 2006). CUC activity promotes expression of the KNOTTED1-like homeobox (KNOX) gene *SHOOT MERISTEMLESS* (*STM*) in the SAM (Aida et al., 1999). STM confers stem cell identity and provides feedback by inhibiting

CUC expression in the meristem, restricting it to the periphery to promote establishment of lateral organ boundaries during embryogenesis (Aida et al., 1999; Barton, 2010; Takeda and Aida, 2011).

1.4.3 Maintenance of the SAM

Maintenance of the SAM during post-embryonic development is dependent on a WUSCHEL-CLAVATA (WUS-CLV) feedback loop and the overlapping activities of KNOX and BEL1-like (BELL) homeodomain transcription factors (Fletcher, 2002; Barton, 2010; Hamant and Pautot, 2010). Mutation of *CLV* genes of the feedback loop leads to accumulation of stem cells in the SAM and meristem enlargement. Mutation in *WUS* and *KNOX-BELL* genes leads to depletion of stem cells in the SAM and meristem termination (Laux et al., 1996; Mayer et al., 1998; Carlos and Fletcher, 2003; Rutjens et al., 2009; Barton, 2010). Whereas WUS acts primarily to specific meristematic cell fate in the central zone, KNOX-BELL factors maintain the meristem by inhibiting differentiation in the central and peripheral zones of the SAM (Clark et al., 1996; Endrizzi et al., 1996; Gallois et al., 2002).

1.4.3.1 WUSHEL-CLAVATA feedback loop

WUS is expressed in a small group of cells in the Organizing Center of the L3 layer directly below the CZ in the SAM (Figure 1.2). WUS transcription factor moves through small cytoplasmic channels called plasmodesmata into overlying CZ cells to promote stem cell fate and to activate a negative regulator, *CLV3*. CLV3 is a small secreted peptide that binds to CLV1 and CLV2 cell surface receptors whose products form a heterodimer. Activation of this signaling pathway restricts *WUS* transcription to the Organizing Center (Schoof et al., 2000; Carlos and Fletcher, 2003; Yadev et al.,

2011; Yadev and Reddy, 2012). The WUS-CLV feedback loop plays a central role in maintaining the stem cell population in the SAM at a constant size (Carlos and Fletcher, 2003). Mutations in *WUS* cause depletion of the stem cell population and meristem termination (Laux et al., 1996; Mayer et al., 1998). Conversely, mutations in the *CLV* genes cause enlarged shoot and floral meristems (Brand et al., 2000; Fletcher, 2002).

1.4.3.2 TALE homeodomain proteins

Three-amino acid loop extension (TALE) homeodomain proteins comprise a superfamily of transcription factors that are divided into KNOX and BELL subclasses (Hamant and Pautot, 2010). Four class I KNOX genes with overlapping functions provide meristem activity and inhibit cell differentiation in Arabidopsis: *STM*, *BREVIPEDICELLUS* (*BP*), *KNOTTED1-LIKE FROM ARABIDOPSIS THALIANA2* (*KNAT2*) and *KNAT6*. *STM* is expressed throughout the SAM and plays a central role. Strong loss-of-function *stm* mutants lack a functional SAM and terminate after the production of one or two leaves (Long et al. 1996). *BP* is expressed in the PZ and RZ (Lincoln et al., 1994) and *KNAT6* is expressed at lateral organ boundaries (Belles-Boix et al., 2006). Mutations in *bp* and *knat6* do not cause SAM defects but enhance the phenotype of weak *stm* mutants indicating their involvement (Byrne et al., 2002; Belles-Boix et al., 2006). *KNAT2* is expressed in the RZ and lateral organ boundaries but its role is unclear since mutations do not enhance the meristem defects of weak *stm* mutants (Byrne et al., 2002). Overexpression of all four class I KNOX genes promotes the ectopic development of meristems on leaves confirming a central role for these

genes in SAM formation (Lincoln et al., 1994; Chuck et al., 1996; Pautot et al., 2001; Gallois et al., 2002; Dean et al., 2004).

Three BELL homeodomain proteins encoded by *PENNYWISE* (*PNY), POUND-FOOLISH* (*PNF*), and *ARABIDOPSIS THALIANA HOMEOBOX GENE1* (*ATH1*) interact with class I KNOX factors to assist in meristem maintenance (Bellaoui et al., 2001; Byrne et al., 2003; Smith and Hake, 2003; Bhatt et al., 2004; Cole et al., 2006; Kanrar et al., 2006; Rutjens et al., 2009; Li et al., 2012). Heterodimerization of KNOX-BELL proteins is thought to be important for binding site selection and for nuclear localization of KNOX factors including STM (Cole et al., 2006). *PNY* is expressed in the CZ; *PNF* is expressed in the CZ and PZ of the SAM, whereas *ATH1* is broadly expressed in the SAM, leaf primordia, and lateral organ boundaries during the reproductive phase of development (Smith et al., 2004; Proveniers et al., 2007; Gómez-Mena and Sablowski, 2008). PNY and PNF regulate the integrity of the CZ since the expression domain of *STM* is narrower in *pny pnf* double mutants (Ung and Smith, 2011a). Triple mutants *ath1-1 pny pnf* have a phenotype similar to weak *stm* mutants (Rutjens et al., 2009). This might be due to the depletion of nuclear localized BELL-STM complexes in the SAM (Rutjens et al., 2009). These studies show that KNOX-BELL and WUS transcription factors play distinct and complementary roles in meristem maintenance.

1.4.4 Establishment and maintenance of boundaries

Lateral organ boundaries are specialized domains of restricted growth that separate meristem and organ compartments. These domains are established shortly after the emergence of lateral organs from SAM in the form of a narrow groove (Aida and

Tasaka, 2006ab; Rast and Simon, 2008). Boundaries provide meristem-organ separation and are the source of axillary meristems. Lateral organ boundaries at various junctions may be specialized for detachment or release. Abscission is the process that mediates release of spent or diseased organs from the plant body. Dehiscence is the process that mediates the release of pollen from anthers and seeds from the fruit pod (Aida and Tasaka, 2006ab, Rast and Simon, 2008). Thus, boundaries are an important determinant of plant architecture.

1.4.4.1 *CUP-SHAPED COTYLEDON* genes

Lateral organ boundary (LOB) identity is provided by the NAC-domain transcription factors CUC1, CUC2, and CUC3 (Aida and Tasaka, 2006ab). Progressive loss of CUC activity prevents establishment of the embryonic SAM and causes the fusion of cotyledons (Aida and Tasaka, 2006ab; Takeda and Aida, 2011). Inactivation of *CUC3* additionally blocks the formation of axillary meristems that give rise to lateral branches and flowers in the inflorescence (Hibara et al., 2006).

1.4.4.2 *BLADE-ON-PETIOLE* genes

BOP1 and *BOP2* encode BTB and ankyrin domain-containing transcription factors expressed at lateral organ boundaries (Ha et al., 2004; Norberg et al., 2005; Hepworth et al., 2005). Genetic analyses suggest that *BOP* genes function downstream and/or in parallel with *CUC* genes to maintain lateral organ boundaries (reviewed in Khan et al., 2014). Loss-of-function *bop1 bop2* have a mild effect on organ separation during the vegetative stage but play a central role in the overall patterning of leaves, inflorescences, flowers, and fruits at boundary junctions via regulation of meristem activity, growth, and cellular differentiation. Characteristics

of *bop1 bop2* mutants include leafy petioles, asymmetric flowers subtended by a bract, defects in axillary meristem formation, and loss of floral-organ abscission (Ha et al., 2003; Hepworth et al., 2005; Norberg et al., 2005; McKim et al., 2008; Xu et al., 2010; reviewed in Khan et al., 2014). Conversely, BOP1/2 gain-of-function mutants have short compact inflorescence due to growth restriction and stems that overproduce lignin.

How do these transcription factors regulate such a variety of processes? Recent studies have provided some answers. In leaf petioles, BOPs directly activate the expression of *ASYMMETRIC LEAVES2* (*AS2*), whose product is a LATERAL ORGAN BOUNDARY domain-containing transcription factor (Ha et al., 2007; Jun et al., 2010). AS2 functions together with the MYB transcription factor AS1 in a complex that directly represses class I KNOX genes *BP* and *KNAT2* to block re-initiation of meristem activity in leaf petioles (Guo et al., 2008; Jun et al., 2010; Lodha et al., 2013). This activity stops secondary leaflets from initiating at the base of leaves and maintains simple leaf shape (reviewed in Khan et al., 2014). Other boundary genes activated by BOP1/2 include the KNOX and BELL factors encoded by *KNAT6* and *ATH1* whose interacting products form a complex (Rutjens et al., 2009; Li et al., 2012). This module controls radial stem patterning and restricts growth to control inflorescence architecture (see sections below) as well as promotes abscission (McKim et al., 2008; Shi et al., 2011; Khan et al., 2012ab, Khan et al., 2014).

1.4.3 KNOX-BELL factors position the lateral organ boundary

An emerging theme in plant development is that Class I KNOX factors position boundary junctions by regulating the expression domain of lateral organ boundary

genes. This is first seen in embryos where STM restricts *CUC* expression to the axils of cotyledons in conjunction with auxin-based signals from the leaf (Furutani et al., 2004; Rast and Simon, 2008; Takeda and Aida, 2011). In *stm* mutants, *BOP* expression expands to the junction between the cotyledons resulting in meristem arrest (Long et al., 1996; Jun et al., 2010).

At least three BELL factors with overlapping functions (PNY, PNF, and ATH1) promote STM activity in vegetative SAM based on genetic studies. In *pny pnf* double mutants about 50% of seedlings terminate after the initiation of 5-6 leaves consistent with defects in meristem maintenance caused by reduced STM function (Rutjens et al., 2009, Ung et al., 2011b). Meristem activity is restored by inactivation of *BOP1/2*. Compatible with this finding, BOP1/2 and effectors KNAT6-ATH1 are misexpressed in *pny pnf* apices and inactivation of *KNAT6* or *ATH1* also rescues (Khan, 2013 and unpublished work). Thus, KNOX-BELL complexes maintain indeterminacy by repressing lateral organ boundary genes. As we shall see, this pattern is repeated throughout the remainder of plant development with important implications.

1.5 Reproductive development

Floral inductive signals acting on the SAM cause adoption of inflorescence meristem (IM) fate triggering flowering and internode elongation (Smith and Hake, 2003; Smith et al., 2004). Post-elongation, lignified interfascicular fibres are differentiated to provide mechanical support in the stem (Ehlting et al., 2005; Sanchez et al., 2012).

In the following sections, I will discuss how KNOX-BELL transcription factors are expressed in differentiated tissues of the stem where they promote indeterminacy

and interact with BLADE-ON-PETIOLE factors to regulate internode elongation, vascular patterning, and secondary growth.

1.5.1 Internode elongation

During the reproductive phase, STM continues to maintain the IM but expands to the stem where it is expressed in vascular tissues and contributes to internode elongation (Long et al., 1996; Kanrar et al., 2006; Sanchez et al., 2012). *BP* is down-regulated in the IM but expands to the stem and is broadly expressed in the cortex and adjacent vascular tissues where it controls internode elongation, location and number of vascular bundles, and the timing of secondary growth in conjunction with PNY (Smith et al., 2003; Venglat et al., 2002; Douglas et al., 2002; Bryne et al., 2003; Ragni et al., 2008; Khan et al., 2012ab). Expression studies suggest that BP and PNY exert their function by modulating the biosynthesis of cell wall remodeling enzymes (Mele et al., 2003; Peaucelle et al., 2011; Etchells et al., 2011; Khan et al., 2012b).

1.5.1.1 Role of KNOX-BELL factors

Loss-of-function *bp* mutants exhibit short internodes and pedicels, downward-pointing flowers, and stripes of chlorophyll-deficient epidermal tissue extending below nodes towards the base of the primary inflorescence (Venglat et al., 2002; Douglas et al., 2002). Loss-of-function *pny* mutants exhibit irregular elongation of internodes with clustered whorls of flowers on the primary stem (Bryne et al., 2003; Smith and Hake, 2003). These phenotypes are enhanced in the double mutant showing that BP-PNY have partially overlapping functions in determination of inflorescence architecture (Smith and Hake, 2003).

1.5.1.2 Interaction with *BOP* genes

Recent work shows that *bp* and *pny* defects in inflorescence architecture are caused by the localized misexpression of *BOP1/2* in the stem. This indicates a broader function for class I KNOX genes in positioning lateral organ boundaries outside of the context of the SAM. Inactivation of BOP1/2 partially rescues *bp* defects and fully rescues *pny* defects in inflorescence architecture. Moreover, transgenic plants that overexpress *BOP1* or *BOP2* cause a spectrum of defects including *bp*-like downward-pointing flowers and *pny*-like whorled clusters of flowers (Khan et al., 2012b). Overexpression phenotypes are not caused by transcriptional repression of *BP* or *PNY* (Khan et al., 2012b). The mechanism is not fully understood but requires the joint activities of boundary marker genes *KNAT6* and *ATH1*. The KNOX-BELL heterodimer formed by KNAT6-ATH1 may compete with BP-PNY binding sites to control growth patterns in the inflorescence (Khan et al., 2012ab; Belles-Boix et al., 2006; Gomez-Mena and Sablowski, 2008; reviewed in Khan et al., 2014).

1.5.2 Vascular patterning

Wild-type Arabidopsis stems have a layered structure with epidermis on the outside, followed by cortex, vascular bundles, and pith in the center. Meristematic activity resumes in the radial stem by formation of the vascular cambium, a lateral meristem that provides founder cells for the differentiation of xylem and phloem. Vascular bundles in the stem have a sandwich like structure with phloem on the top (outside), cambium in the middle, and xylem on the bottom (inside) (Figure 1.3A; Sanchez et al., 2012).

1.5.2.1 Role of KNOX-BELL factors

KNOX-BELL factors BP and PNY regulate radial patterning of the stem. Vascular bundles in *bp* mutants are often irregular in size and/or spacing. Some bundles are under developed, with xylem elements reduced or lacking in lignin (Smith and Hake, 2003). Loss-of-function *pny* mutants have additional vascular bundles resulting in reduced interfascicular space. The vascular bundles in *pny* mutants are often missing in large xylem vessels and contain only xylary fibres. Bundles seem to have more cambial cells and a larger more densely-packed cortex in comparison to wild-type (Smith and Hake, 2003). Combined mutations of *bp pny* display enhancement of the single mutant phenotypes. Vascular bundles in the double mutants are smaller and more closely packed with phloem cells forming a continuous band inside the large cortex cells and with unlignified xylem elements scattered around (Smith and Hake, 2003).

1.5.2.2 Interaction with *BOP* genes

Inactivation of *BOP1/2* genes partially rescues *bp* defects and fully rescues *pny* defects in vascular patterning. In *bop1 bop2 bp* triple mutants, vascular bundles are reestablished in a regular pattern and the stems of *bop1 bop2 pny* mutants are essentially wild-type (Khan et al., 2012b). Thus, BOP1/2 misexpression in the stem significantly alters radial patterning and vascular development in the stem.

1.5.3 Secondary development and lignin biosynthesis

Secondary (lateral) growth in the stem of woody plants occurs when primary growth in finished and is mediated by establishment of a continuous vascular cambium, a tube-like domain of meristem activity encircling the stem (Figure 1.3C; Sanchez et al.,

2012). In Arabidopsis, continuous vascular cambium forms only in the hypocotyl, at nodes, and at the base of the primary inflorescence 1-2 mm above the rosette (Sehr et al., 2010; Sanchez et al., 2012).

Secondary development in the majority of the Arabidopsis stem is limited to the secondary thickening of xylem and phloem cell walls and the differentiation of lignified interfascicular fibres in the spaces between primary vascular bundles. Interfascicular fibres complete the vascular ring and provide mechanical support. They also serve as a good model for fibre development in trees (Rogers and Campbell, 2004; Ehlting et al., 2005).

1.5.3.1 Role of KNOX-BELL factors

Class I KNOX genes play an important role in regulating secondary development based on studies in Arabidopsis, maize, tobacco, and poplar (Mele et al., 2003; Groover et al., 2006; Du et al., 2009; Townsley et al., 2012). Considering the essential role of these factors in maintaining the SAM, their activity is thought to inhibit terminal differentiation in the stem. Plants overexpressing *BP* are delayed in the differentiation of interfascicular fibres whereas in *bp* mutants, differentiation of interfascicular fibres is accelerated. Cross-sections of the *bp* stem show spatial defects in lignin deposition. Gaps are observed in the vascular ring underlying narrow stripes of epidermis and adjacent cortex in which lignin is ectopically deposited. In *pny* mutants, the vascular ring is thicker than in wild-type (Smith and Hake, 2003; Khan et al., 2012b).

1.5.3.2 Interaction with *BOP* genes

Mutation of *bop1 bop2* partially rescues *bp* defects in lignin deposition. Gaps in the vascular ring are closed and ectopic deposition of lignin in epidermal stripes, cortex, and phloem fibres is minimized. Stem patterning in *pny bop1 bop2* triple mutants is similar to wild-type (Khan et al., 2012b). In plants over-expressing BOPs, lignin is more abundant in the stem compared to wild-type, the vascular ring is thicker, and cells of the pith accumulate lignin. These data reveal a promotive role for *BOP* genes in lignin biosynthesis in opposition to BP-PNY. All of these factors are predicted to directly regulate lignin biosynthetic genes (Mele et al., 2003; Khan et al., 2012b; Khan et al., 2013; Khan et al., 2014; Etchells et al., 2010).

1.5.4 Lignin biosynthetic pathway

Lignin is a principal component of secondary cell walls in higher plants (reviewed in Rogers and Campbell, 2004). Lignin polymers are composed of p-hydroxyphenyl (H-type), guaiacyl (G-type) and syringyl (S-type) monolignols that are synthesized through the phenylpropanoid pathway starting from the amino acid phenylalanine. Monolignols are synthesized in the cytosol and then exported to the cell exterior where they are activated by cell-wall associated peroxidases and laccases for free-radical coupling into a polymer (Figure 1.4; Castellanos-Hernández et al., 2011).

1.5.4.2 BP regulation of lignin genes

Three lines of evidence suggest that BP directly regulates the expression of lignin biosynthetic genes (Mele et al., 2003). A microarray study of *bp* mutants showed the differential accumulation of transcripts encoding lignin biosynthetic enzymes including phenylalanine ammonia lyase1 [PAL1], cinnamate 4-hydroxylase1 [C4H1],

4-coumarate-CoA ligase [4CL1], cinnamyl alcohol dehydrogenase5 [CAD5] and PRXR9GE, a class III cell wall peroxidase gene. *35S:BP* plants showed lower levels of lignin biosynthetic genes *[PAL1*, *C4H*, *4CL* and *CAD1*] relative to wild-type and recombinant BP bound directly to the promoter of lignin biosynthetic genes *COMT* and *CCoAMT in vitro*. These data indicate that BP is a negative regulator of genes in the lignin biosynthetic pathway (Figure 1.4; Mele et al., 2003; Rogers and Campbell, 2004).

1.5.4.2. *BOP* regulation of lignin genes

Inactivation of *BOP1/2* in *bp-2* restores lignin gene transcripts to near wild-type levels supporting a promotive role for BOPs in regulation of lignin production. Transcript profiling identified four lignin biosynthetic genes as up-regulated in *bop1-6D* (*BOP1* overexpressing) stems [*C4H1*, *C3H1*, *CAD5*, and *PRXR9GE*] suggesting direct or indirect promotion by BOP1/2. The class III peroxidase gene *PRXR9GE* showed the greatest fold-change (15- to 20-fold) over wild type in *bp-2* and *bop1-6D* stems (Mele et al., 2003; Khan et al., 2012b). *PRXR9GE* is expressed at lateral organ boundaries and in lignified vasculature of the stem and appear to be direct target of BOP1 based on a steroid induction assay (Khan, 2013). Polymerization of monolignol subunits is unexplored as a potential key regulatory point in developmental control of secondary wall formation. Collectively, these data show reciprocal roles for BP and BOP1/2 in stem development. How these interactions apply to wood or fibre production in trees is an open question.

1.6 Link to wood and fibre development in trees

The vascular cambium is an internal ring-like lateral meristem that supports secondary growth in trees. In Arabidopsis and other dicotyledonous plants, initiation of cambial activity begins in the primary vascular bundles (fascicular cambium) and spreads out to the interfascicular regions where differentiated cells regain the ability to divide (interfascicular cambium). Ultimately, a complete tube-like domain of meristematic activity is created. Daughter cells on the outer rim produce secondary phloem (bark) whereas daughter cells on the inside make secondary xylem (wood). In forest trees, this results in the radial thickening of stems and supports the development of larger body architectures (Groover et al., 2006; Sehr et al., 2010; Sanchez et al., 2012).

Populus trichocarpa (black cottonwood) has emerged as an excellent model system for the study of wood development in trees. *Populus* is a long-lived diploid tree that is a close relative of Arabidopsis, so gene functions are often conserved. Critical genomic and molecular tools for this species are in place including DNA microarrays, transformation protocols, and a sequenced genome making the species ideal for forestry research (Jansson and Douglas, 2007).

1.6.1 Role of KNOX factors

Populus contains two class I KNOX1 genes expressed in the SAM and vascular cambium. *ARBORKNOX1* (*ARK1*) is an ortholog of Arabidopsis *STM* and *ARBORKNOX2* (*ARK2*) is an ortholog of Arabidopsis *BP* (Groover et al., 2006; Du et al., 2009). *ARK1* expression in the stem is restricted to the vascular cambium. Overexpression in poplar had the effect of inhibiting differentiation in leaves, internode elongation,

and stems were thin consistent with defects in secondary growth. Cross-sections confirmed that differentiation of secondary vascular cell types was delayed relative to wild-type plants, the boundary between the cambium and the secondary xylem was disrupted and wavy, and phloem fibres were reduced in number (Groover et al., 2006). *ARK2* was broadly expressed in the cambial zone and actively lignifying cell types before becoming progressively restricted to the cambium. *ARK2* overexpression caused shorter internodes with a thicker cambium. Extra secondary phloem was produced at the expense of phloem fibres and secondary xylem. Overall, there was a decrease in differentiation of lignified cell types caused by *ARK2* overexpression. Conversely, the stem of *amiRNA::ark2* plants (expressing an artificial miRNA construct that lowers *ARK2* transcript level) revealed the premature differentiation of secondary xylem and phloem fibres, similar to the *bp* mutant in Arabidopsis, but there was no difference in the timing of cambium formation (Du et al., 2009).

1.6.2 Potential role for *BOP* genes

The role of *BOP* genes in secondary development in trees was completely unknown. We reasoned that they may play a role in cambium functioning or lignification associated with wood or fibre development based on our studies in Arabidopsis.

BOP1 and *BOP2* belong to a small gene family in Arabidopsis whose founding member is the defense regulator *NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1)*. Transcription factors in this family 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 (Hepworth et al., 2005; reviewed in

Khan et al., 2014). The BTB/POZ domain mediates dimerization (Rochon et al., 2006) whereas the ankyrin motifs interact with TGA (TGACG-motif binding) bZIP (basic leucine zipper) transcription factors. Association with a TGA factor is essential because BTB-ankryin factors lack a DNA-binding domain (Després et al., 2003; Hepworth et al., 2005; Jun et al., 2010; Xu et al., 2010). The Arabidopsis genome encodes ten TGA factors subdivided into five clades: class I comprises TGA1 and TGA4; class II comprises TGA2, 5, and 6; class III comprises TGA3 and TGA7; class IV comprises TGA9 and TGA10; and class V comprises TGA8/PERIANTHIA (Jakoby, 2001; Gatz, 2013). In flowers, BOP1/2 form a complex with TGA8/PERIANTHIA to control sepal and petal number (Hepworth et al., 2005). Recent data show that BOP1/2 require clade I factors TGA1 and TGA4 to exert changes in flowering and plant architecture. Inactivation of *tga1 tga4* fully restores internode elongation in *35S:BOP2* transgenic plants and in *pny* mutants, and restores flowering in *pny pnf* mutants (Khan, 2013; Chisanga and Hepworth, unpublished). We hypothesized that BOPs form a complex with these factors to control lignin biosynthesis in stems.

1.7 Thesis rationale

Genome sequence data for poplar (www.phytozome.net) shows the existence of two potential *BOP* orthologs. This raises the possibility that antagonistic interactions between KNOX and *BOP* genes are preserved in trees and regulate cambium development or lignification important in wood and fibre production. This led to two hypotheses that were tested in my thesis:

1. BOP function is conserved in trees and is part of the network that regulates lignin biosynthesis in secondary growth.

2. BOPs require clade I TGA factors in regulation of lignin biosynthesis.

Figure 1.1. Architecture of the model plant *Arabidopsis thaliana*

(A) Vegetative stage: Rosette leaves are produced without internode elongation. Axillary meristems later develop in the axils of these leaves to produce paraclades. (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. Scale bar, 1.5 cm.

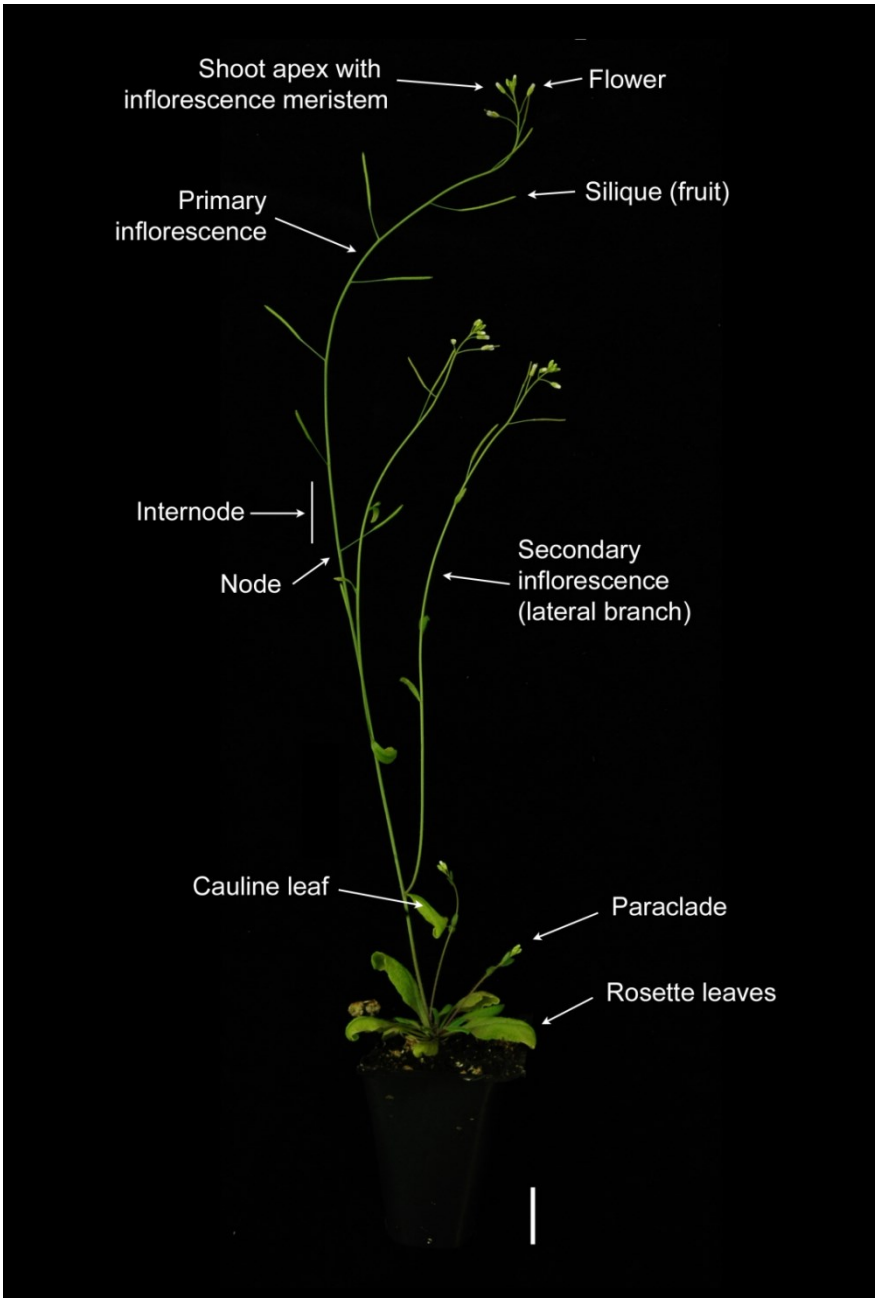

Shoot apex with inflorescence meristem
Flower
Silique (fruit)
Primary inflorescence
Internode
Node
Secondary inflorescence (lateral branch)
Cauline leaf
Paraclade
Rosette leaves

Figure 1.2 Organization of the SAM in *Arabidopsis thaliana*

Cells in the central zone of the SAM divide to replenish the population of stem cells. The central zone (red) is flanked by the peripheral zone (pale blue) where organ primordia are initiated. The rib zone (yellow) lies beneath the central zone and contains the Organizer (pink) required in maintaining stem cell identity in overlying central zone cells. The solid yellow lines show that the SAM is composed of layers. Cells in the L1 and L2 epidermal and subepidermal layers divide anticlinally (←→) contributing daughter cells to lateral organ primordia. Cells in the underlying L3 layer divide in all planes contributing mainly to the stem. As lateral organ primordia emerge on the flanks of the meristem, slowly growing cells at the junction form a narrow groove known as the lateral organ boundary (dark blue). Adapted from Wolpert et al. (2011).

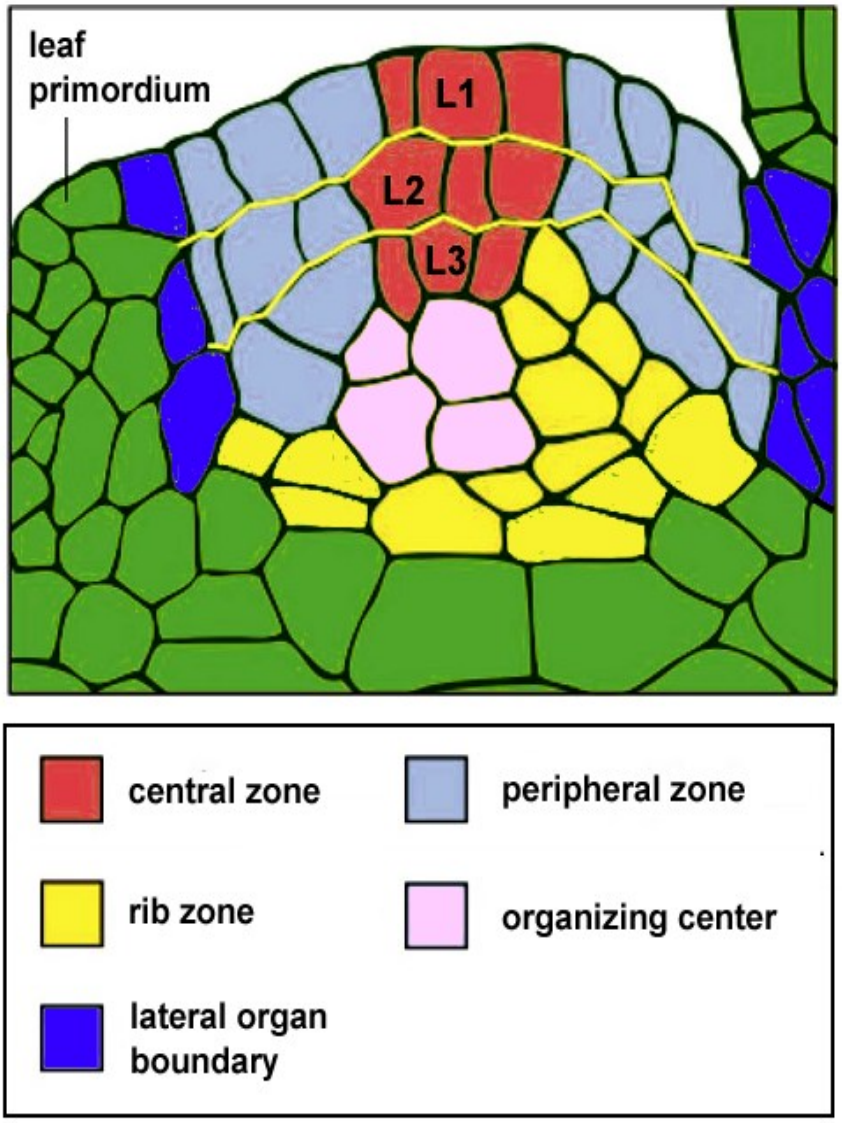

leaf
primordium
L1
L2
L3
central zone
peripheral zone
rib zone
organizing center
lateral organ
boundary

Figure 1.3 Stem vascular patterning in Arabidopsis and poplar tree

Stem cross-sections from Arabidopsis and poplar tree were stained with phloroglucinol-HCl to show lignin deposition. Right-hand panels are color-coded to show basic stem anatomy: red, cambium; yellow, phloem; blue, xylem; orange, starch sheath; green, cortex; grey, epidermis. (A) Arabidopsis, 18-cm bolting stem, young internode. Only xylem vessels in primary vascular bundles are lignified. (B) Arabidopsis, 18-cm bolting stem, old internode. Xylem and phloem in primary vascular bundles and interfascicular fibres are lignified. Right-hand panel depicts a region at the base of the stem (1-5 mm above the rosette). This is the only region to develop a continuous ring of cambium as in trees. (C) Poplar tree, mature internode. Xylem (primary and secondary) and phloem cap fibres are lignified. Vascular cambium is continuous. x, xylem; ph, phloem; if, interfascicular fibres; vc, vascular (fascicular) cambium; ic, intrafascicular cambium. Poplar stem section courtesy of http://openi.nlm.nih.gov/detailedresult.php?img=2662859_1471-2229-9-26-4&req=4. Arabidopsis stem sections provided by Eryang Li. Labelled diagrams based on Sanchez et al. (2012).

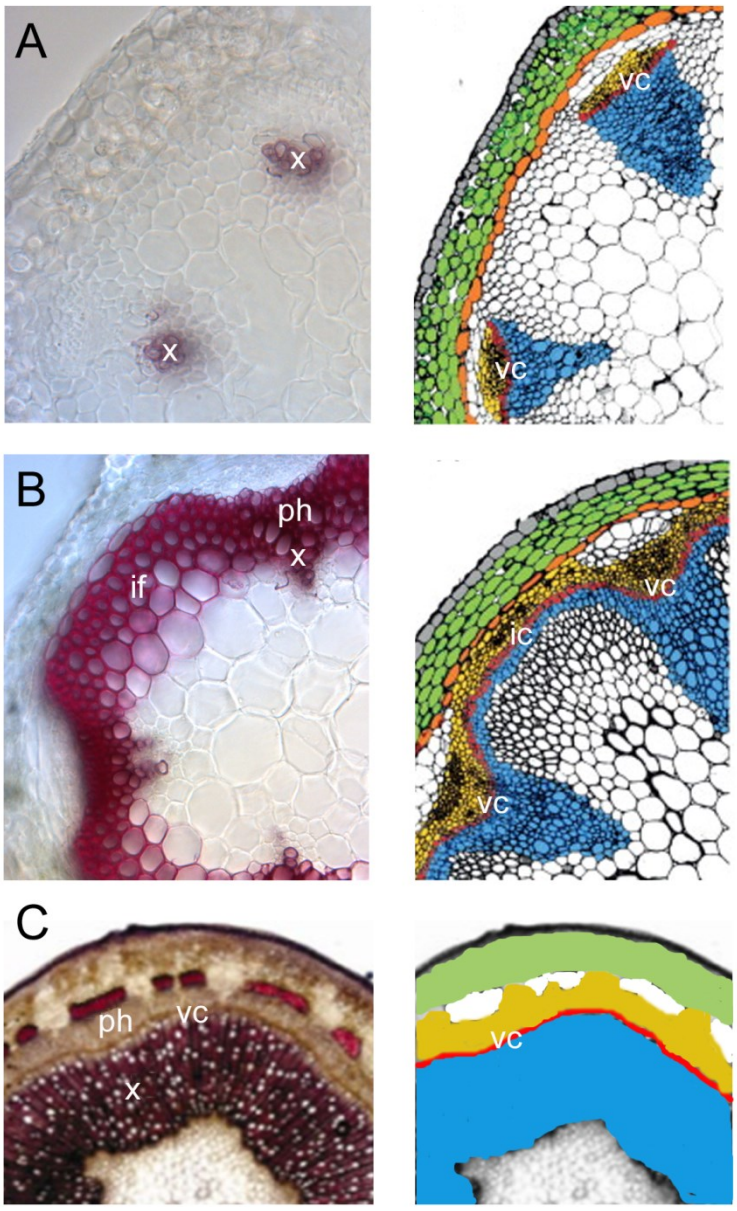
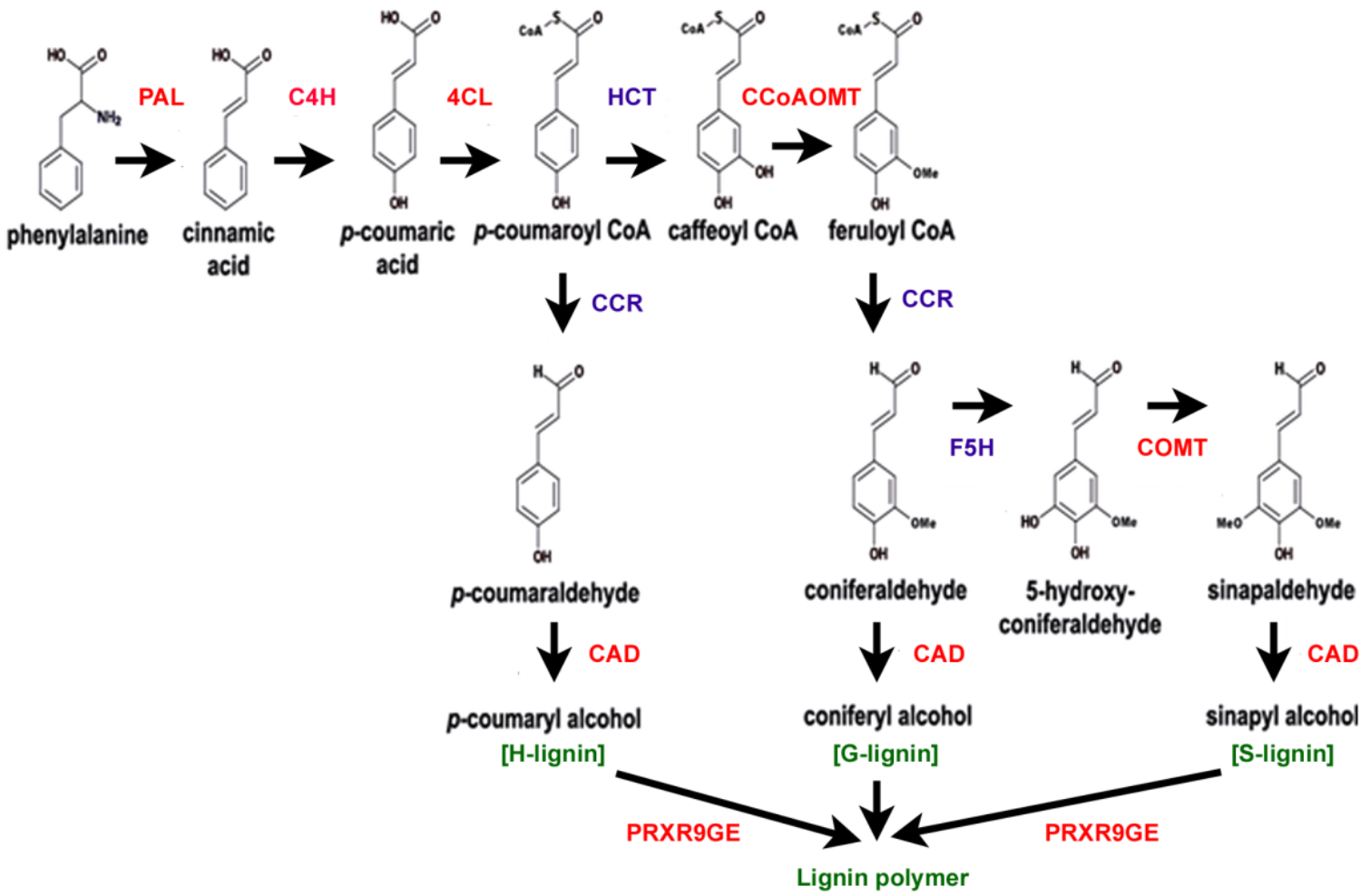

A
x
x
vc
vc
B
ph
x
if
vc
ic
vc
C
ph
vc
x
vc

Figure 1.4 Lignin biosynthetic pathway

Diagram shows enzymes and intermediates of lignin biosynthesis by the phenylpropanoid pathway. Arrows indicate a reaction in the pathway. Enzymes are shown in blue or red. Red text indicates the transcript is differentially regulated by BP (Mele et al., 2003; Khan et al., 2012b). PAL, phenylalamine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; HCT, Hydroxycinnamoyl-CoA shikimate/quinate hydroxcinnamoyl transferase; CCoAOMT, caffeoyl CoA *O*-methyltransferase; CCR, (hydroxyl)cinnamoyl CoA reductase; F5H, COMT, caffeic acid (5-hydroxy-coniferaldehyde)-*O*-methyltransferase; CAD, (hydroxyl)cinnamoyl alcohol dehydrogenase; PRXR9GE, class III cell wall peroxidase9. Monolignols are synthesized in the cytosol and exported across the plasma membrane into the apoplast where they are activated by cell-wall associated peroxidases for free-radical based coupling into lignin polymer. Figure based on Castellanos-Hernández et al. (2011).


PAL
C4H
4CL
HCT
CCoAOMT
phenylalanine
cinnamic acid
p-coumaric acid
p-coumaroyl CoA
caffeoyl CoA
feruloyl CoA
CCR
CCR
p-coumaraldehyde
coniferaldehyde
F5H
5-hydroxy-coniferaldehyde
COMT
sinapaldehyde
CAD
CAD
CAD
p-coumaryl alcohol
[H-lignin]
coniferyl alcohol
[G-lignin]
sinapyl alcohol
[S-lignin]
PRXR9GE
PRXR9GE
Lignin polymer

CHAPTER 2

MATERIALS & METHODS

2.1 Plant materials and growth conditions

Wild-type was the Columbia-0 (Col-0) ecotype of *Arabidopsis thaliana*. The double mutant *bop1-3 bop2-1,* and single mutant *bp-2*, *tga1-1,* and *tga1-4* alleles have been described previously (Hepworth et al., 2005; Venglat et al., 2002; Kesarwani et al., 2007). The strong *35S:BOP2* line and activation-tagged over-expression line *bop1-6D* were described previously (Norberg et al., 2005). The *BOP1:GUS* and *BOP2:GUS* reporter lines were also described previously (McKim et al., 2008; Xu et al., 2010). All mutant combinations were constructed by crossing and confirmed by PCR genotyping. The strategy for genotyping mutants containing T-DNA insertions from the Salk collection was as described (www.signal.salk.edu). All primers used in this study are listed in Table 2.1.

Seeds were surface-sterilized in a 50% of solution of 5% sodium hypochlorite bleach and 0.5% (w/v) sodium dodecyl sulfate prior to sowing. Seeds were sown on agar plates containing AT minimal medium (Haughn and Somerville, 1986) or on soil (Promix BX Black, Premier Horticulture, Riviere-du-Loup, QC). Soil was steam-sterilized prior to use and supplemented with 1 gram per litre of 20-20-20 plant fertilizer (Plant Pod, Product Co. Ltd, Brampton, ON). Seeds were incubated at 4°C for 2 days in the dark to break dormancy and then placed in a growth chamber to promote germination. Seedlings were transplanted at day 7 to soil in 72 well trays or in 3.5 inch square pots as appropriate. Plants in soil or *in vitro* were grown in chambers at 21°C under continuous light (24h light, intensity 100 µmol m^{-2} s^{-1}).

2.2 Construction of *BOP1:BPL1* and *BOP1:BPL2* transgenic lines

The cDNA sequences of *PtrBPL1* (*Pt6s04010*) and *PtrBPL2* (*Pt6s04190*) were amplified by polymerase chain reaction (PCR) using cDNA from mixed poplar tissues as the template, iProof as the polymerase (Biorad, Hercules, CA) and gene specific primers annealing to the 5' and 3' untranslated regions to ensure specific amplification of each ortholog (Table 2.1). The resulting products were cloned into pCR-BluntII-TOPO (Invitrogen, Carlsbad, CA). These clones were then used as template to amplify the coding sequences of *PtrBPL1* and *PtrBPL2* using gene specific primers and iProof as the polymerase. The resulting products were cloned into pCR-BluntII-TOPO to create B442 and B443 containing *PtrBPL1* and *PtrBPL2* open reading frames, respectively. Cloned inserts were sequenced to ensure fidelity. These vectors were kindly provided by Eryang Li.

To test if poplar *BOP*-like genes are functional homologs of *AtBOP1/2*, *PtrBPL1* and *PtrBPL2* were expressed under the control of the *BOP1* native promoter in Arabidopsis *bop1 bop2* mutant plants. The transgene was created in two steps. The *BOP1* promoter present in pBOP1:GUS (McKim et al., 2008) was amplified by PCR using iProof as the polymerase and primers 4H-4kb-EcoR1-F1 and 4H-4kb-Xma1-R1 that incorporated restriction sites at their 5' ends. The resulting products were digested with *Eco*R1 and *Xma*I and cloned into the corresponding sites of the binary vector pBAR (a gift from the Dangl Lab, University of North Carolina) and sequenced resulting in B149. This construct was provided by Tieqiang Hu. Next, the *PtrBPL1* and *PtrBPL2* coding sequences present in B442 and B443 were amplified by PCR using iProof as the polymerase and primers PtrBPLXbaI-F and PtrBPLSac1-R that were

suitable for both genes and incorporated restriction enzymes at their 5' ends to facilitate directional cloning. The resulting products were cloned into pCR-BluntII-TOPO and sequenced to ensure fidelity. Plasmids were digested with *Xba*I and *Sac*I to release the insert. The resulting fragments were ligated into the corresponding sites of B149 to create B487 (p*BOP1:BPL1*) and B488 (p*BOP1:BPL2*). Constructs were used to transform *bop1 bop2* mutant plants by floral dipping (Clough and Bent, 1998) using *Agrobacterium tumefaciens* (Agrobacterium) strain C58C1 pGV3101 pMP90 (Koncz and Schell, 1986). Primary transformants resistant to glufosinate-ammonium were selected on soil using the herbicide FINALE (distributed by Farnham Companies Inc, Phoenix, AZ). Complementation of leaf, flower, and abscission defects was scored in the T1 generation. Ten independent lines per construct were monitored in the T2 generation to assess transgene stability. Three independent lines per construct were made homozygous for future studies.

2.3 Construction of *D35S:BPL1* and *D35S:BPL2* transgenic lines

To create *D35S:PtrBPL1* and *D35S:PtrBPL2* constructs for analysis of overexpression phenotypes, the coding sequences present in B442 and B443 were amplified by PCR using iProof as the polymerase and primers Pt6s04010CDS-F and Pt6s04010CDS-R for *PtrBPL1* and Pt6s04190CDS-F and Pt6s04190CDS-R for *PtrBPL2*. dATP overhangs were added to products to allow transfer to the Gateway-compatible entry vector pCR8/GW/TOPO (Invitrogen, Carlsbad, CA). Clones in the correct orientation were sequenced to ensure fidelity. LR clonase© (Invitrogen, Carlsbad, CA) was used to move inserts to a pSM-3 based destination vector containing a double 35S CaMV promoter (D35S) and Nos terminator (Carl Douglas lab, unpublished). Wild-type

plants were transformed by floral dipping (see above). Hygromycin-resistant primary transformants were selected on agar plates. Phenotypes were scored in the T1 generation.

2.4 Quantitative RT-PCR (qRT-PCR)

Two µg of total poplar tissue RNA was used for reverse transcriptase synthesis using Superscript RT III as the polymerase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cDNA was a gift from the Carl Douglas lab at University of British Columbia. Reactions in triplicate containing 2 µl of 10-fold diluted cDNA, gene-specific primers (see Table 2.1) and POWER SYBR Green PCR Mastermix (Invitrogen, Carlsbad, CA) were carried out using a StepOnePlus Thermocycler (ABI Biosystems, Life Technologies, Carlsbad, CA). Poplar *C672* which encodes an elongation factor was used as the reference gene (Wang et al., 2014). Relative transcript levels were calculated according to Pfaffl (2001). Values were normalized to C672 (Wang et al., 2014) and then to young leaf. Each measurement was carried out in triplicate. Data are the average of two biological replicates for each tissue type. Error bars show standard deviation. Raw data were kindly provided by Eryang Li.

2.5 RNA hairpin constructs for *PtrBPL* gene silencing in poplar

To create a transcriptional knock-down of *PtrBPL1* and *PtrBPL2* in poplar tree, self-complementary sequences capable of forming an RNA hairpin were cloned into the intermediate vector pHannibal to prepare constructs for RNA interference (RNAi) mediated gene silencing (Wesley et al., 2001). Primers were designed based on a 300 base pair sequence in the BTB/POZ domain of *PtrBPL1* that is virtually identical in

PtrBPL2. Primers BPLRNAi-F and BPLRNAi-R incorporating *Bam*HI and *Cla*I restriction sites at their 5' ends were used to amplify a sense fragment. Primers BPLRNAiXhoI-F and BPLRNAiEcoRI-F incorporating *Xho*I and *Eco*RI restriction sites at the 5' ends were used to amplify an anti-sense fragment (Table 2.1). The fragments were digested with the appropriate restriction enzymes and ligated into the corresponding sites in pHANNIBAL. The resulting plasmid was digested with *Not*1 to release the hairpin cassette for ligation into the corresponding site of the binary vector pART27 (Gleave, 1992) ready for transformation into poplar plants.

2.6 Construction of *TGA1:GUS* and *TGA4:GUS* lines for expression analysis

To create TGA1:GUS and TGA4:GUS reporter genes, the 4-kb upstream regions of these genes including the translation start sites were amplified by PCR from genomic DNA template [BAC M1QN23 for *TGA1*; BAC T31P16 for *TGA4*] using iProof as the polymerase. Primers incorporating *Bam*HI and *Nco*I restriction sites at their 5' ends were used to facilitate directional cloning. Products were cloned into pCR-BluntII-TOPO (Invitrogen, Carlsbad, CA). Inserts were released by digestion with *Bam*HI and *Nco*I and ligated into the corresponding sites of pGCO:GUS to create translational fusions with the β-glucuronidase (GUS) gene (*uidA*) (Hepworth et al., 2002). Agrobacterium was co-transformed with pSOUP (Hellens et al., 2000). Wild-type plants were transformed by floral dipping and glufosinate-ammonium resistant primary transformants were selected on soil.

2.7 β-glucuronidase (GUS) assays

Wild-type and mutant plants expressing *TGA1:GUS* and *TGA4:GUS* reporter genes were analyzed for GUS activity as described by Sieburth and Meyerowitz (1997) with

minor changes. Stained tissues were embedded in Paraplast Plus (Sigma, St. Louis, MO) using tert-butanol instead of xylenes. Sections (10 µm) were cut from embedded tissue, affixed to glass slides, and dewaxed with tert-butanol prior to imaging.

2.8 Lignin staining

For the analysis of lignin deposition in stems, wild-type and mutant plants were grown on soil in continuous light. Hand-sections were cut from the base of five-week-old plants with a razor blade and placed in 2% phloroglucinol solution. After 5 min, five drops of concentrated hydrochloric acid were added. Two minutes were allowed for color development and images were immediately collected using an Zeiss AXIO Imager M2 light microscope equipped with an AxioCam digital camera (Zeiss).

2.9 Constructs for bimolecular fluorescence complementation (BiFC)

BiFC was used to monitor interactions between BOP and TGA factors *in planta*. Inserts corresponding to the coding regions of *BOP1*, *BOP2*, *PAN*, *TGA1*, and *TGA4* were cloned in-frame upstream of the N-terminus of YFP in pSPYNE-35S^{Gateway} and upstream of the C-terminus of YFP in pSPYCE-35S^{Gateway} (Walter et al., 2004; Arnand et al., 2007). Coding sequences of these genes were amplified by PCR from cloned cDNA templates using iProof as the polymerase and gene-specific primers (Table 2.1) that removed the stop codon to allow in-frame fusion with Yellow Fluorescent Protein (YFP). dATP overhangs were added to the resulting products to allow their transfer to the Gateway-compatible entry vector pCR8/GW/TOPO (Invitrogen, Carlsbad, CA). Inserts in the correct orientation were sequenced to ensure fidelity. LR

clonase© (Invitrogen, Carlsbad, CA) was used to move inserts to pSPYNE-35S^{Gateway} or pSPYCE-35S^{Gateway} vectors. *35S:YFP* was used as a positive controls. Agrobacterium harbouring these constructs was used to infiltrate tobacco leaves (see below). YFP fluorescence was monitored using a Zeiss LSM510 Meta confocal laser scanning microscope.

2.10 Transient expression assays in tobacco leaves

Transient expression in *Nicotiana benthamiana* (tobacco) was used to monitor BiFC interactions in leaves essentially as described in Sparkes et al. (2006). In brief, freshly streaked plates of Agrobacterium were used to inoculate overnight cultures in 5 ml of Luria Broth (LB) with selective antibiotics at 28°C. The next day, cells were pelleted by centrifugation and resuspended in 3 ml of freshly prepared infiltration buffer [10 mM $MgCl_2$, 10 mM 2-[*N*-morpholino]ethanesulfonic acid-potassium hydroxide (MES-KOH) buffer (pH 5.6), and 150 µm of acetosyringone]. To avoid co-suppression, an Agrobacterium strain expressing the viral suppressor of silencing gene p19 was included in the infiltration mixture (Voinnet et al., 2003). Cultures were mixed in a ratio of 1:1 with this strain. The mixture was incubated for 1 hour prior to infiltration of leaves. For injections, 5-6 week-old tobacco plants grown in long-days were selected with healthy green leaves. The culture was gently injected using a needleless 1 ml syringe on the flat abaxial surface of the leaf without damaging it. After 2-3 days, leaf discs (2-3 mm in diameter) were viewed under a confocal microscope to monitor for reconstitution of YFP fluorescence.

Table 2.1 List of primers

Description	Primer	Sequence 5' – 3'	Reference
Genotyping			
bop1-3	4H Salk RP	CGTACCCTTTGATTTTAGTATGCTG	Hepworth et al,. 2005
	4H Salk LP	GCACAATCTTTCGACTTCATCACC	
bop2-1	5H Salk RP	CCCTTTTTATAATCAGCATCAAGA	Hepworth et al., 2005
	5H Salk LP	TCGACGCCGAAGTAACGAGAG	
tga1-1	TGA1 Salk_028212 RP	TAGGGAATCTCCGTGTCCCCTCTGG	This study
	TGA1 Salk_028212 LP	TTCAAAACCTGGATTCATGGTTTCC	
tga4-1	TGA4 Salk_127923 RP	GAAGGTTTGAAGTTTACGAGCCTCT	This study
	TGA4 Salk_127923 LP	GCTCTGCTGAAGTTTTCCACATTCC	
T-DNA, left border	LBb1.3	ATTTTGCCGATTTCGGAAC	signal.salk.edu/cgibin/ T-DNAexpress
PtrBPL transgenics		Restriction endonuclease site is underlined	
PtrBPL1 cDNA	Pt6s04010UTR-F	ACGAGGCAACCCTAGCTAGTAAAC	Eryang Li
	Pt6s04010UTR-R	CATTTTACTTGTCTAACTCTCCTGA	
PtrBPL2 cDNA	Pt6s04190UTR-F	AGATAAATATACATATTTTTTGGAGTC	Eryang Li
	Pt6s04190UTR-R	AACTTAGGTAGGCTTGGAACTAG	
D35S:BPL1	Pt6s04010CDS F	ATGACTCTTGAAGACTCTCTAAGAACTCTG	This study
	Pt6s04010CDS R	CTAGAAGTCATGAGAGTGATCGC	
D35S:BPL2	Pt6s04190CDS F	ATGACTCTTGAAGACTCTCTAAGAACTCTG	This study
	Pt6s04190CDS R	CTAGAAGTCATGAGAGTGATGGT	
BOP1 promoter	4H-4kb-EcoRI-F1	CCGGAATTCAGGAGGGCATGTCCATATTC	Tiequang Hu
	4H-4kb-XmaI-R1	ATTCCCGGGAGCTCCTTTGTTGATTTCTTTG	
BOP1:BPL1/2	PtrBPLXba1 F	CCATCTAGAATGACTCTTGAAGACTCTCTAAGA	This study
	PtrBPLSac1 R	CCAGAGCTCCTAGAAGTCATGAGAGTGATGG	
Reporter genes			
TGA1:GUS	TGA1_4GUS_BAM_F	GAAGGATCCATATCGACCAATTAATATAGG	Mike Bush
	TGA1_4GUS_NCO_R	GGACCATGGCCATGGCCATGTTTTCCTCAACT GAAAAC	
TGA4:GUS	TGA4_4GUS_BAM_F	GAAGGATCCGTGGGCTTTCAAAACTGATGG	Mike Bush
	TGA4_4GUS_NCO_R	GGACCATGGCCATATTTCTTCAACTAGCAAC	
RNAi			
***BPL* sense**	BPLRNAiBamHI-F	CCAGGATCCATCAATGGCCAAGCTTTCTCTG	This study
	BPLRNAiCla1-R	CCAGGATCCATCAATGGCCAAGCTTTCTCTG	
***BPL* antisense**	BPLRNAiXho1-F	CCACTCGAGATCAATGGCCAAGCTTTCTCTG	This study
	BPLRNAiEcoR1-R	CCAGAATTCGTGTCAAGAGCAAGATCAACG	
qRT-PCR			
PtrBPL1	PtBPL1 RT-F	ATGGGAGAAGGCCTCAATCTCA	Eryang Li
	PtBPL1 RT-R	GTCTCTCTGGCTGCTATGGC	
PtrBPL2	PtBPL2 RT-F	AGGCAGGGCCGGCAGGGAAAACT	Eryang Li
	PtBPL2 RT-R	CCTTGATCCCATTTGACCTG	
C672	c672F	GACGGTATTTTAGCTATGGAATTG	Eryang Li
	C672R	CTGATAACACAAGTTCCCTGC	
BiFC			
pCR8/GW/TOPO-BOP1	CDSAtBOP1-F	ATGAGCAATACTTTCGAAGAATC	Natalie Woerlen
	BOP1-N-SYFP-ns-R	GAAATGGTGGTGGTGGTGATG	
pCR8/GW/TOPO-BOP2	CDSAtBOP2-F	ATGAGCAATCTTGAAGAATCTTTG	Natalie Woerlen
	BOP2-N-SYFP-ns-R	GAAGTGATGTTGATGATGGTG	
pCR8/GW/TOPO-PAN	PAN-SRDX-F	ATGCAGAGCAGCTTCAAAACCGTTC	Natalie Woerlen
	PAN-N-SYFP-ns-R	GTCTCTAGGTCTGGCTAACCA	
pCR8/GW/TOPO-TGA1	TGA1-SRDX-F	ATGAATTCGACATCGACACATTTTG	Natalie Woerlen
	TGA1-N-SYFP-ns-R	CGTTGGTTCACGATGTCGAGT	
pCR8/GW/TOPO-TGA4	TGA4-SRDX-F	ATGAATACAACCTCGACACATTTTG	Natalie Woerlen
	TGA4-N-SYFP-ns-R	CGTTGGTTCACGTTGCCTAGC	

CHAPTER 3

RESULTS

3.1 Functional characterization of *BOP*-like genes in *Populus trichocarpa*

Arabidopsis *BOP1* and *BOP2* (*AtBOP1/2*) genes are founding members of an ancient and conserved subclade of BTB-ankryin transcription factors in land plants (reviewed in Khan et al., 2014). *BOP* loss-of-function in a variety of species alters the architecture of leaves, flowers, and the inflorescence. In moss, BOPs accelerate the transition to reproductive development (reviewed in Khan et al., 2014). Overexpression of AtBOP1/2 leads to short stature and stems with an abundance of lignin, reminiscent of secondary growth in woody plants. We were therefore interested in discovering if BOP factors regulate wood or fibre production in trees.

3.1.1 The poplar genome contains two *BOP*-like genes

Populus trichocarpa (poplar) is a widely used model species for trees (Jansson and Douglas, 2007). To examine the role of BOP homologs in wood development, I searched the database Phytozome (www.phytozome.net) to identity BOP homologs in tree species including poplar. My aim was to compare tree BOP protein sequences to AtBOP1 and AtBOP2. The protein sequences were assembled into a FASTA file, aligned with a Clustal Omega and displayed using BOXSHADE. This analysis revealed that tree BOPs are highly similar to Arabidopsis BOPs (Figure 3.1). All of the proteins contained a BTB-domain at the N-terminus, a set of four ankyrin repeats at the C-terminus, and an internal domain of unknown function (DUF3420) upstream and slightly overlapping with the first ankyrin repeat. There were also two conserved polyhistidine motifs: one in the DUF3420 domain and one at the extreme C-terminus that function as putative metal-binding motifs in AtBOP1/2 (unpublished data). Two potential orthologs of *AtBOP1* (At3g57130) and *AtBOP2* (At2g41370) in poplar tree

were identified as *PtrBPL1* (POPTR-0016s04010) and *PtrBPL2* (POPTR-0006s04190). The two proteins are 93% identical to each other at the amino acid level and have 82% amino acid sequence similarity to AtBOP1 and 83% amino acid sequence similarity to AtBOP2.

3.1.2 *PtrBPL1/2* complementation of Arabidopsis *bop1 bop2* phenotype

Loss-of-function *bop1 bop2* mutations cause leafy petioles, loss of floral abscission, and flowers with a bract and extra petals on the abaxial side. To test if poplar *BPL*s are functional homologs of *AtBOP1* and *AtBOP2*, I expressed *PtrBPL1* and *PtrBPL2* under control of the *BOP1* native promoter in *bop1 bop2* double mutants and tested for complementation. In total, I obtained 203 primary transformants for *BOP1:BPL1* and 212 primary transformants for *BOP1p:BPL2* (Table 3.1) Strong or medium complementation of the leafy petiole phenotype was observed in 54% and 52% of *PtrBPL1* and *PtrBPL2* transformants, respectively. Floral organ abscission was significantly restored in 76% and 62% of *PtrBPL1* and *PtrBPL2* transformants scored, respectively. Floral patterning defects were significantly restored in 65% and 76% of *PtrBPL1* and *PtrBPL2* transformants scored, respectively (Table 3.1). Not all plants were equally complemented in all three tissues. Ten lines per construct were selected with strong complementation in all three tissues and brought into the T2 generation where plants were genotyped to confirm that they were *bop1 bop2* double mutants (Figure 3.2). Homozygous complemented lines were selected in the T3 generation and stored. These data confirm that *PtrBPL1* and *PtrBPL2* are functional homologs of Arabidopsis *BOP* genes. Both can complement *bop1 bop2* mutant phenotypes when expressed in Arabidopsis.

3.1.3 Overexpression of *PtrBPL1/2* in Arabidopsis causes short stature and evidence of altered lignin deposition in stems

Plants overexpressing AtBOP1 or AtBOP2 are late flowering with short bushy inflorescences and lignin is ectopically deposited in the stem (Figure 3.3BC; Figure 3.4ABC). To test if overexpression of PtrBPL1/2 have a similar effect, *D35S:PtrBPL1* and *D35S:PtrBPL2* constructs were used to transform Columbia wild-type Arabidopsis plants. Phenotypes were scored in the T1 generation. Of 374 primary transformants obtained for *D35S:PtrBPL1,* seven lines were recovered with a short bushy stature similar to overexpression of AtBOP1 or AtBOP2 (Table 3.2, Figure 3.3D). Of 56 primary transformants obtained for *D35S:BPL2*, one line was recovered with an overexpression phenotype similar to AtBOP1/2 (Table 3.2; Figure 3.3E). Of these, only one *D35S:BPL1* line retained a strong overexpression phenotype in the T2 generation. Stems of this plant line were analyzed for lignin deposition by staining with phloroglucinol-HCl. Sections were cut from the base of fully elongated stems with a razor blade and incubated with 2% phloroglucinol which reacts with lignin in the presence of hydrogen ions to generate a pink colour (Figure 3.4). As expected, wild-type plants have a continuous vascular ring (Figure 3.4A). In *D35S:BPL1* stems, alterations in lignin deposition were not as obvious as in *bp-2* mutants or *bop1-6D* (Figure 3.4BCD). Based on a small sample size (n=5), the vascular ring was thicker and denser than in wild-type control plants and phloem fibres were lignified (Figure 3.4AD). More intensive staining of the vascular ring in *D35S:BPL1* suggests an increase in lignin content. A more careful study in combination with analysis of cell wall chemistry is required to strengthen these findings. Overall, these data support

that poplar BOPs restrict stem growth and promote lignin biosynthesis similar to Arabidopsis BOPs.

3.1.4 Expression patterns of *PtrBPL1* and *PtrBPL2* in poplar

Arabidopsis *BOP*s are highly expressed at nodes in the inflorescence and found only at very low levels detectable by qRT-PCR in internodes of the primary stem. To determine the expression pattern of *PtrBPL1* and *PtrBPL2* in poplar tree, I first compared the expression patterns of these genes using the Botany Array Resource web-tool (www.utoronto.ca/bar) which displays publically available transcriptome data. This analysis showed that *PtrBPL1* and *PtrBPL2* are expressed in many of the same tissues as *AtBOP1* and *AtBOP2* including young leaf, roots, seedlings, and inflorescences (catkins) (Figure 3.5). Poplar *BPLs* were additionally expressed in developing secondary xylem, particularly *PtrBPL1* (Figure 3.5). These data closely agree with qRT-PCR analysis carried out on dissected poplar tissues. The qRT-PCR data also show that *PtrBPL* transcripts are most abundant in the petiole region of leaves and highly expressed in developing xylem and phloem (Figure 3.6). These data are consistent with model that *PtrBPL1* and *PtrBPL* are expressed in the woody stem where they potentially regulate secondary growth.

3.1.5 Using RNAi-mediated gene silencing to analyze *PtrBPL* loss-of-function phenotypes in poplar

To further examine the role of *PtrBPL* genes in tree development, I generated an RNA hairpin cassette for use in RNAi-mediated gene co-silencing of *PtrBPL1* and *PtrBPL2* in poplar. The construct was based on a 300 base pair sequence in *PtrBPL1* within the BTB/POZ domain that is highly conserved in *PtrBPL2*. This sequence is

predicted to achieve co-silencing of both homologs (Figure 3.1). A hairpin cassette composed of sense and antisense *BPL1* sequence under control of the 35S CaMV promoter was created in pHannibal (Wesley et al., 2001) and transferred into the binary vector pART27 (Gleave, 1992) ready for transformation into poplar tree. We are currently establishing a transformation system for poplar in tissue culture.

3.2 Testing the hypothesis that BOP1/2 require clade I TGA factors in promoting lignin biosynthesis

TGA factors are a plant-specific subclass in the bZIP superfamily of DNA-binding transcription factors (Jakoby et al., 2001). Arabidopsis TGAs are a family of ten members divided into five clades (Gatz, 2013). BTB-ankryin proteins including NPR1 and BOPs require interactions with specific TGA factors to regulate defense and flower patterning, respectively (Després et al., 2000; Després et al., 2003; Zhang et al., 2003; Hepworth et al., 2005). Clade I factors comprising TGA1 and TGA4 are identified as having dual roles in defense and development (Després et al., 2003; Ehlting et al., 2008; Song et al., 2008; Shearer et al., 2012; Alvarez et al., 2014). Unpublished data show that inactivation of *tga1 tga4* in transgenic plants overexpressing *BOP2* restores internode elongation (Khan, 2013). This suggests that BOP1/2 require clade I TGA factors to exert changes in inflorescence architecture and as potential co-factors in regulation of lignin biosynthesis.

3.2.1 Expression pattern of *TGA1* and *TGA4* in Arabidopsis plants

Reporter genes based on the bacterial enzyme β-glucuronidase (GUS) were used to monitor the expression of *TGA1* and *TGA4* in wild-type plants (Jefferson et al., 1987). Thirty to fifty transformants per construct (*TGA1:GUS* and *TGA4:GUS*) were screened

in the T1 generation to identify lines with medium to strong GUS activity. Detailed analysis of GUS staining patterns in the T2 generation showed that *TGA1* and *TGA4* expression is found at lateral organ boundaries. Both genes were highly expressed at the base of leaves, at nodes in the primary inflorescence, and at the base of floral organs before and after abscission (Figure 3.8). *TGA1* and *TGA4* expression was also abundant in leaf and stem vasculature. Cross-sections of the primary stem showed that *TGA1* is expressed in xylem whereas *TGA4* is expressed in xylem and phloem (Figure 3.8EFKL). These data show that AtBOP1/2 and clade I TGA bZIP factors have an overlapping pattern of expression at lateral organ boundaries. Given the additional expression in stem vascular tissues, we reasoned that clade I factors are good candidates as co-factors in BOP1/2 regulation of lignin biosynthesis.

3.2.2 BP regulates *TGA1* and *TGA4* expression domains in the stem

Secondary growth in trees is supported by a vascular cambium that expresses meristematic class I KNOX genes with homology to Arabidopsis *STM* and *BP* (Groover et al., 2006; Du et al., 2009). The cambium produces daughter cells for the production of secondary xylem (wood) and secondary phloem (bark). In Arabidopsis plants, mutation of *bp* causes misexpression of BOP1/2 in the stem resulting in premature lignification (Khan et al., 2012b). To test if TGA1 and TGA4 function in the same genetic pathway, we examined the expression patterns of these genes in a *bp* mutant. *TGA1:GUS* and *TGA4:GUS* transgenes were crossed into a *bp* mutant background and the resulting plants were analyzed for GUS activity. This experiment confirmed that *TGA1* and *TGA4* are misexpressed in a pattern similar to *BOP1/2* in *bp* inflorescences. Expression in the *bp* mutant expanded to the underside of nodes

continuing towards the base of the plant in thin stripes of abnormally patterned epidermal tissue (Figure 3.9; Khan et al., 2012b). These data identify BP as a negative regulator of *TGA1* and *TGA4* expression in the primary inflorescence stem.

3.2.3 Partial rescue of *bp* inflorescence defects by *tga1 tga4*

Inflorescence patterning defects in *bp* mutants include short internodes, downward-pointing flowers, and abnormal distribution of vasculature bundles in the stem. Vascular bundles in the *bp* mutant vary in size and spacing compared to wild-type. The xylem elements in some bundles are missing or reduced (Smith and Hake, 2003; Venglat et al., 2002; Douglas et al., 2002). Inactivation of *BOP1/2* partially rescues *bp* phenotypes including pedicel orientation (Khan et al., 2012b; Figure 3.10A-C). I therefore tested if inactivation of *TGA1* and/or *TGA4* has a similar effect. Mutations in *tga1*, *tga4*, and *tga1 tga4* were combined with *bp-2* by crossing and the resulting plants were analyzed. No obvious change in pedicel orientation was observed in *bp-2 tga1* or *bp-2 tga4* double mutants (Figure 3.10BDE) but pedicel orientation was slightly restored in *bp-2 tga1 tga4* triple mutants (Figure 3.10BF). Measurement of pedicel orientation is required to confirm these data.

3.2.4 Partial rescue of *bp* lignin defects by *tga1 tga4*

Previous studies have shown that BP is a negative regulator of lignin biosynthesis in the plant stem. A number of complex patterning changes occur in *bp* mutants: interfascicular and phloem fibres in the stem are prematurely lignified. In addition, the vascular ring is discontinuous with lignin ectopically deposited in the cortex below and epidermal tissues above gaps in the vascular ring (Smith and Hake, 2003; Mele et al., 2003; Khan et al., 2012b). Inactivation of *BOP1/2* closes the gaps in the

vascular ring and partially rescues ectopic deposition of lignin (Khan et al., 2012b). Stems of *bp-2 tga1*, *bp-2 tga4*, and *bp-2 tga1 tga4* double and triple mutants were sectioned and stained for lignin to test if inactivation of clade I TGA bZIP factors has a similar effect. No obvious change in lignin pattern was observed in *bp-2 tga1* double mutants. Based on small sample size (n<10), the number of gaps in *bp-2 tga4* double and *bp-2 tga1 tga4* triple mutants was reduced in comparison to *bp-2* mutants (Figure 3.11). Lignification of the epidermal stripes was not assessed by this experiment because internodes were not fully mature at the time of sectioning.

3.2.5 BiFC fails to show a direct interaction of BOPs with clade I TGA factors

The expression patterns of BOP1/2 and clade I TGA factors overlap at lateral organ boundaries raising the possibility that BOPs interact with TGA1/4 as co-factors in regulating lignin biosynthesis. BiFC was used to test if these proteins form a complex. BOP or TGA proteins were fused at the C-terminus in-frame to N-terminal or C-terminal halves of YFP (n-YFP or c-YFP). Agrobacterium cultures containing these constructs were mixed in pairs and used to infiltrate the underside of tobacco leaves for transient expression in epidermal cells. Interacting proteins that bring the two halves of YFP back together restore florescence that can be monitored by confocal microscopy. No fluorescence was detected when n-YFP or c-YFP fusions were expressed singly in plants (Figure 3.12A-H). *35S:YFP* served as a positive control with yellow fluorescence detected in the cytoplasm and nucleus (Figure 3.12M). Yellow fluorescence in the nucleus was detected for BOP2-BOP2 and BOP2-PAN interactions consistent with previously shown data (Figure 3.12IN; Hepworth et al., 2005; Jun et al., 2010; Xu et al., 2010). Yellow fluorescence in the nucleus was also detected for

PAN-PAN and TGA1-TGA1 interactions but not for TGA4-TGA4 interactions (Figure 3.12JKL). No yellow fluorescence was detected for BOP2 interactions with TGA1 or TGA4 (Figure 3.12OP). Similar results were obtained for interactions with BOP1 (data not shown). Thus, it remains unclear if there is a direct physical interaction between BOP1/2 and class I TGA factors despite an overlapping pattern of expression at lateral organ boundaries and clear genetic evidence that BOP1/2 require these factors to exert changes in inflorescence architecture.

Stock	Construct	Leaf (n=203 transformants)				Abscission (n=160 scored)				Flower (n=71 scored)		
		+++	++	+	none	+++	++	+	none	WT	partial	none
B495	***BOP1:PtrBPL1***	n=42	68	5	88	92	31	20	17	46	6	19
		Leaf (n=212 transformants)				**Abscission (n=182 scored)**				**Flower (n=62 scored)**		
		+++	++	+	none	+++	++	+	none	WT	partial	none
B496	***BOP1:PtrBPL2***	n=46	64	27	75	87	26	39	30	47	6	9

Table 3.1 Summary of Arabidopsis *bop1 bop2* complementation by *BOP1:PtrBPL1* and *BOP1:PtrBPL2*

Loss-of-function *bop1 bop2* mutants have leafy petioles, loss of floral-organ abscission, and flowers with a bract and extra petals on the abaxial side. Primary transformants were scored for complementation of leaf, abscission, and floral patterning defects according to the following key: WT-like (+++); moderate rescue (++); weak rescue (+); no rescue (none). Stock number, accession for Agrobacterium strains in the Hepworth lab glycerol collection.

Stock No.	Construct	Number of transformants	*bop1-6D* like phenotype	*pny*-like phenotype
B472A	*D35S:BPL1*	338	7	85
B472B	*D35S:BPL1*	36	0	15
B474A	*D35S:BPL2*	35	0	11
B474B	*D35S:BPL2*	21	1	6

Table 3.2. Summary of overexpression phenotypes in Arabidopsis plants transformed with *D35S:PtrBPL1* and *D35S:PtrBPL2*

Wild-type Arabidopsis plants were transformed with *D35S:PtrBPL1* and *D35S:BPL2* constructs. Overexpression phenotypes were scored in the T1 generation. Two major phenotypes were observed consistent with the known spectrum of BOP gain-of-function phenotypes: a *pny*-like phenotype: short bushy plants (<12 cm high) with clusters of fruits caused by moderate *BOP* misexpression in the stem; a *bop1-6D-like* phenotype: extremely short bushy plants (<7 cm high) that are late-flowering with a wide leaf and ectopic lignin production in the stem caused by strong constitutive *BOP1* expression. Stock number, accession for Agrobacterium strains in the Hepworth lab glycerol collection.

Figure 3.1 Alignment of Arabidopsis and tree BOP proteins for comparison of conserved motifs

Predicted protein sequences were aligned with Clustal Omega using BOXSHADE output (www.ch.embnet.org/software/BOX_form.html). Conserved BTB/POZ, DUF3420, and ankyrin domains are as indicated. Polyhistidine tracts functioning as putative metal-binding domains are highlighted in red.

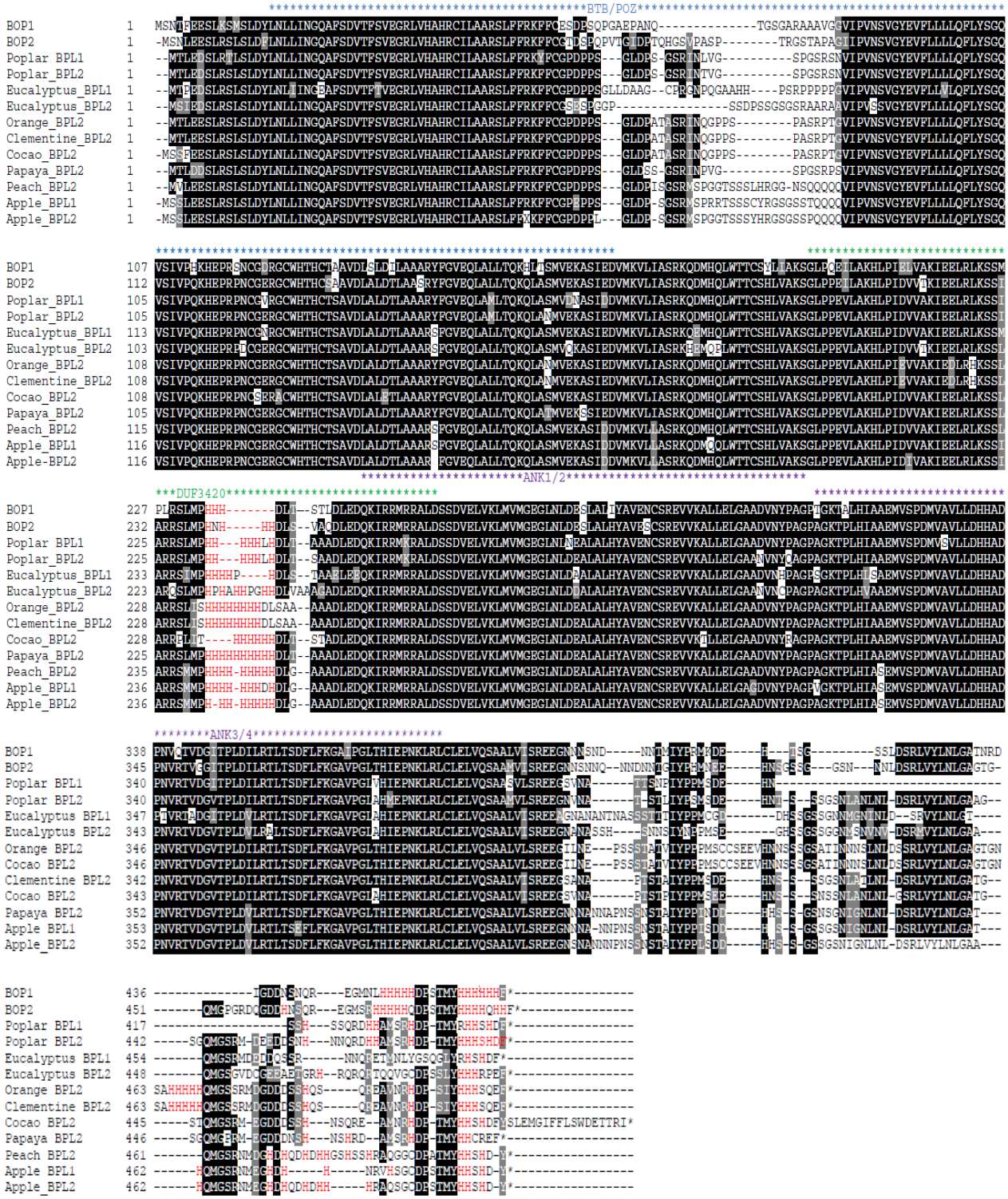

BTB/POZ
ANK1/2
DUF3420
ANK3/4
BOP1
BOP2
Poplar_BPL1
Poplar_BPL2
Eucalyptus_BPL1
Eucalyptus_BPL2
Orange_BPL2
Clementine_BPL2
Cocao_BPL2
Papaya_BPL2
Peach_BPL2
Apple_BPL1
Apple-BPL2

Figure 3.2 Complementation of Arabidopsis *bop1 bop2* leaf and floral phenotypes with *PtrBPL1* and *PtrBPL2*

(A) Schematic of *BOP1:PtrBPL1/2* constructs used for complementation testing. The plasmid backbone was the binary vector pBAR1 conferring kanamycin resistance (Kan R) in bacteria and glufosinate-ammonina resistance (Bar R) in plants. *PtrBPL1* and *PtrBPL2* coding regions were expressed under the control of a 4-kb *AtBOP1* promoter (Xu et al., 2010). (B-D) Complementation of *bop1 bop2* phenotypes with *BOP1:BPL1* and *BOP1:BPL2* transgenes. Representative complemented plants are shown in the T2 generation. (B) Wild-type control plants. (C) *bop1 bop2* mutant. (D) *BOP1:BPL1* in *bop1 bop2*; smooth petiole and normal floral-organ abscission. (E) *BOP1:BPL2* in *bop1 bop2*; smooth petiole and normal floral-organ abscission. Scale bars, 1.5 cm.

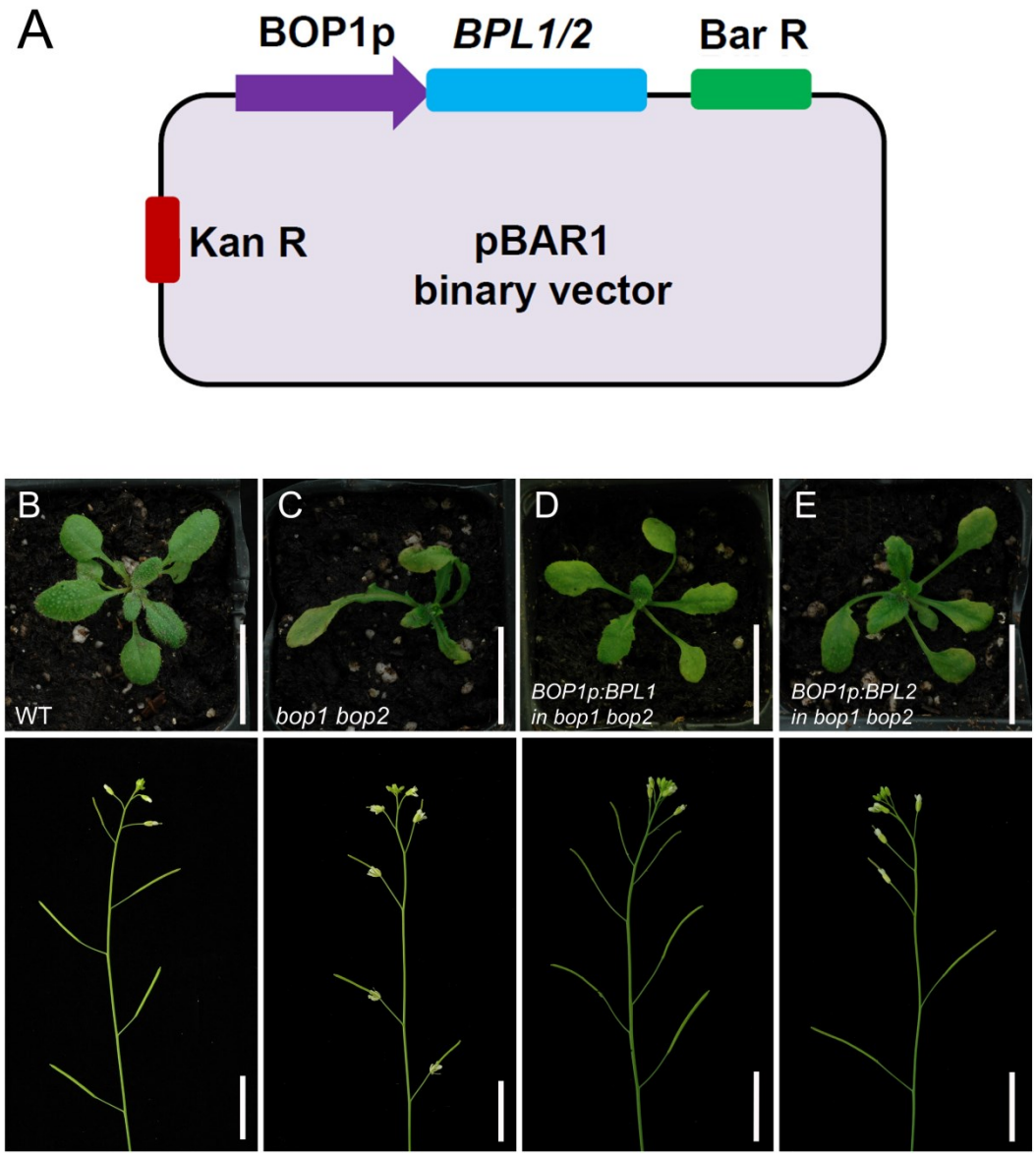
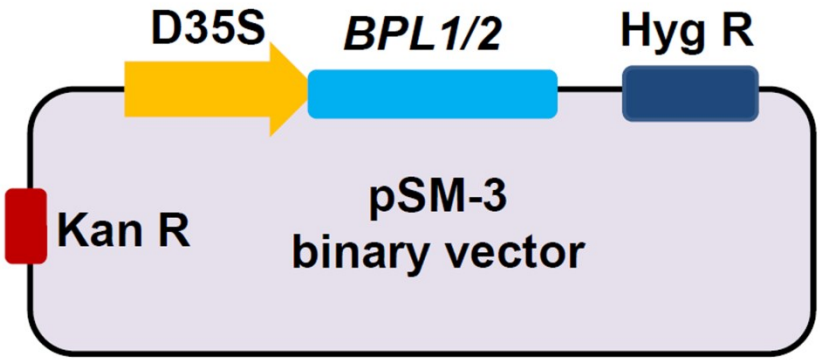

A
BOP1p
BPL1/2
Bar R
Kan R
pBAR1
binary vector
B
C
D
E
WT
bop1 bop2
BOP1p:BPL1
in bop1 bop2
BOP1p:BPL2
in bop1 bop2

Figure 3.3 Overexpression of *PtrBPL1* and *PtrBPL2* in Arabidopsis gives phenotypes similar to overexpression of *AtBOP1/2*

(A) Schematic of *D35S:BPL1/2* constructs used for overexpression. The plasmid backbone was the binary vector pSM-3 conferring kanamycin resistance (Kan R) in bacteria and hygromycin resistance (Hyg R) in plants. *PtrBPL1* and *PtrBPL2* coding regions were expressed under the control of a double 35S (D35S) CaMV promoter. (B-E) Gain-of-function phenotypes generated by *D35S:BPL1* and *D35S:BPL2* in wild-type plants. Representative plants are shown in the T1 generation. (B) Wild-type control plants. (C) *D35S:BOP2* control plants. (D) *D35S:BPL1*; short and bushy with clusters of fruits (arrows). (E) *D35S:BPL2*; bushy plants with mild clustering (arrow). Genotypes are as indicated. Scale bars, 1.5 cm.

A


D35S
BPL1/2
Hyg R
Kan R
pSM-3
binary vector


B
C
D
E
WT
D35S:BOP2
D35S:PtrBPL1
D35S:PtrBPL2

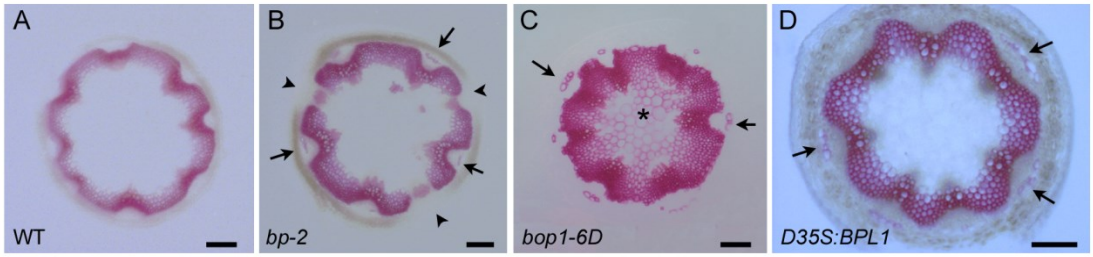


Figure 3.4 Comparison of lignin deposition patterns in wild-type plants, *bp-2* mutants, and transgenic plants overexpressing *AtBOP1* or *PtrBPL1*

Cross-sections from the base of fully elongated stems were stained with phloroglucinol-HCl to reveal lignin. Representative sections are shown for: (A) Wild-type. (B) *bp-2*; note gaps in the vascular ring (arrowheads) and premature lignification of phloem fibre cells associated with the primary vascular bundles (arrows). (C) *bop1-6D*; a strong *AtBOP1* overexpression line. Note the dense vascular ring compared to wild-type. Arrow, premature lignification of phloem fibre cells similar to *bp-2*. Asterisk, ectopic lignification of pith. Image kindly provided by Madiha Khan. (D) *35S:BPL1*; a dense vascular ring and evidence of lignified phloem fibres (arrows) similar to *bop1-6D*. Scale bars, 100 µm.

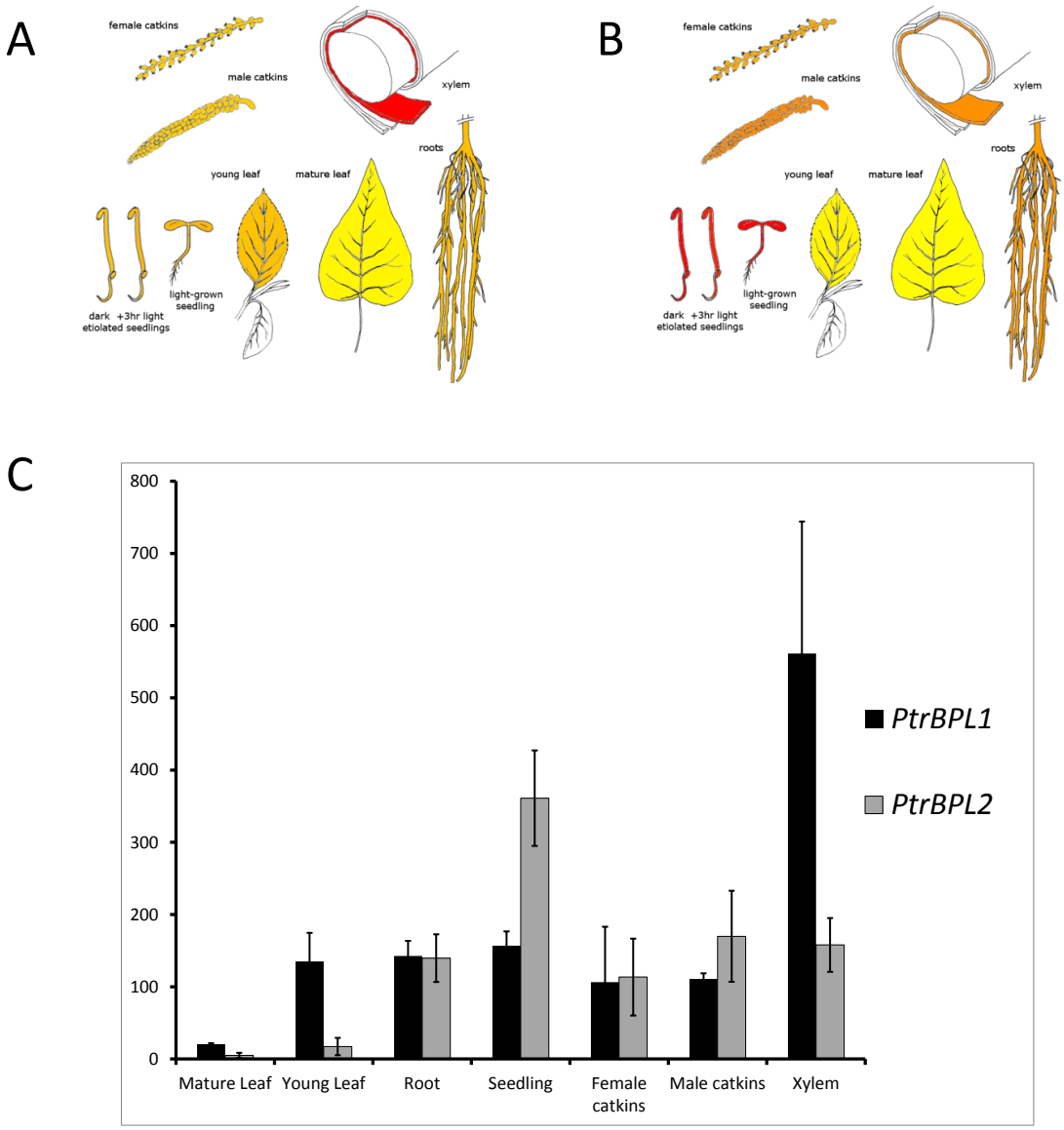


Figure 3.5 *In silico* analysis of *PtrBPL1* and *PtrBPL2* expression in dissected poplar tissues

Transcriptome data obtained from The Botany Array Resource (www.bar.utoronto.ca). Red, high expression; orange, medium expression; yellow, lower expression. (A) eFP browser output for *PtrBPL1*. (B) eFP browser output for *PtrBPL2*. (C) Relative transcript levels for *PtrBPL1* and *PtrBPL2* in different poplar tissues.

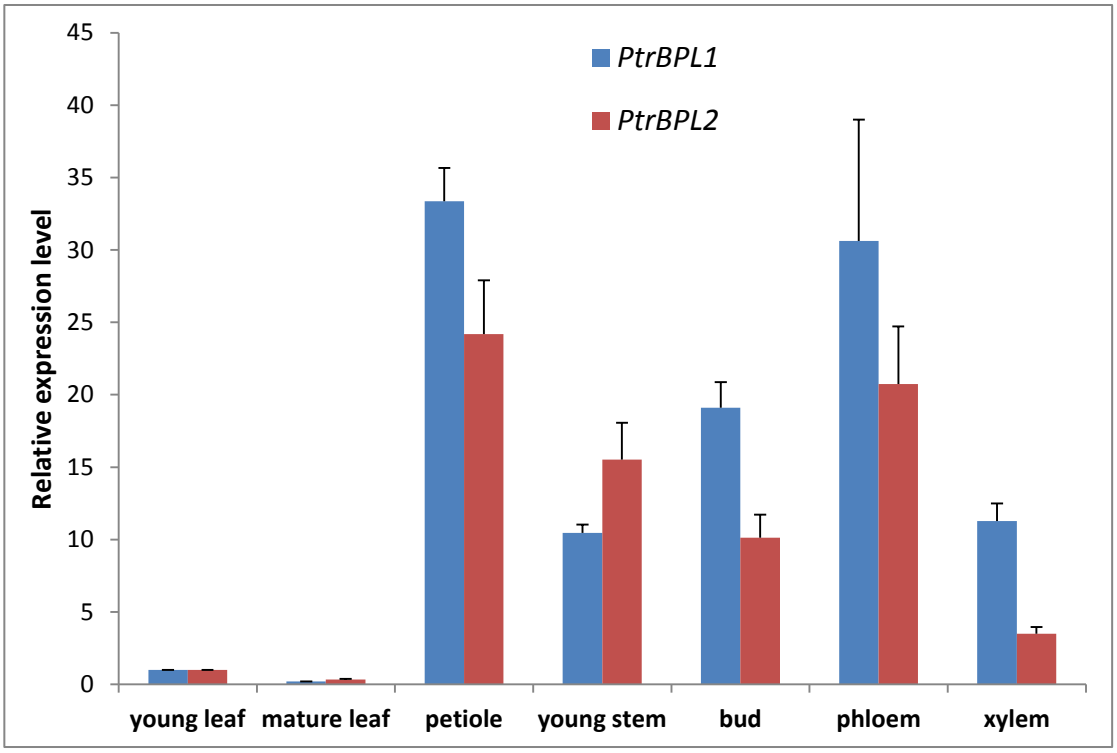


Figure 3.6 qRT-PCR analysis of *PtrBPL1* and *PtrBPL2* relative transcript levels in dissected poplar tissues

Relative abundance of *PtrBPL1* and *PtrBPL2* transcript was measured using qRT-PCR in dissected poplar tissues. Data represent the average of two biological replicates with each measurement performed in triplicate. Values were normalized to young leaf. Error bars, SD. Raw data kindly provided by Eryang Li.

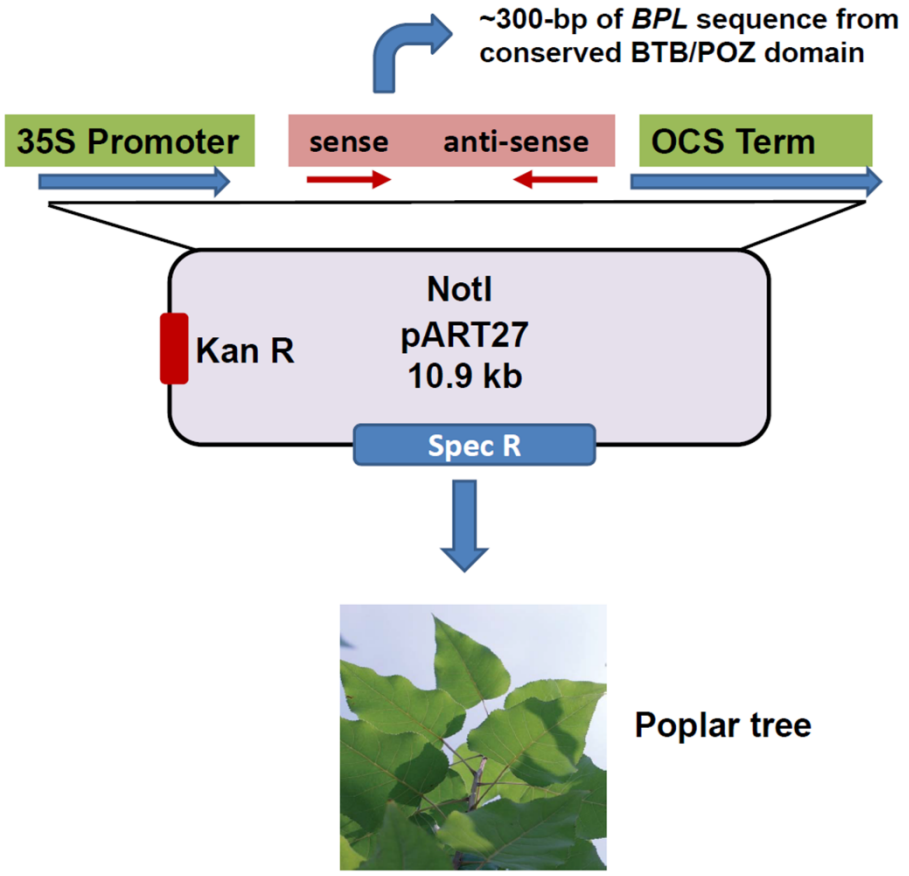
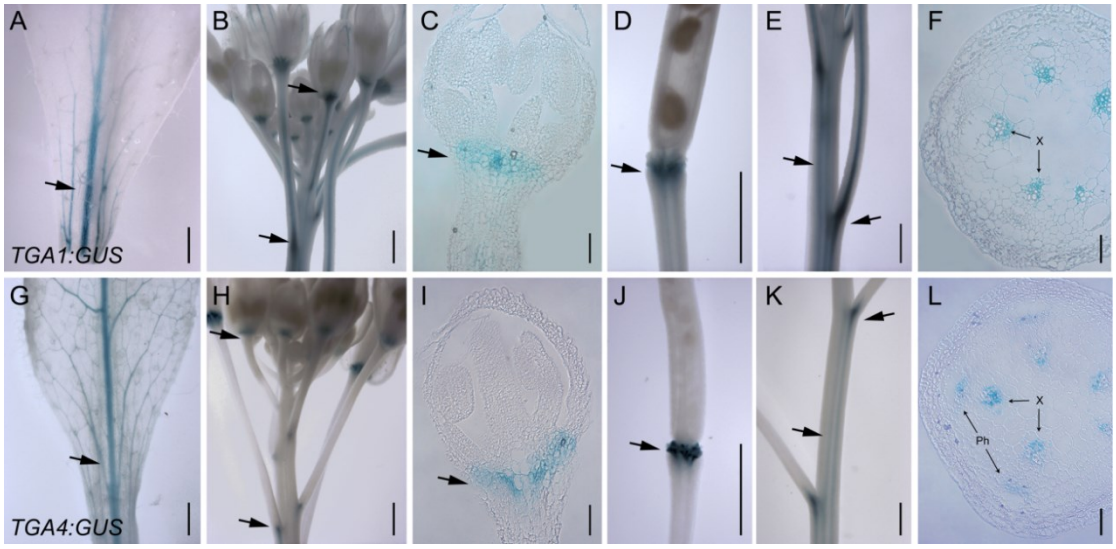


Figure 3.7 RNA hairpin construct for RNAi-mediated silencing of *PtrBPL1* and *PtrBPL2* in poplar

A highly conserved 300-base pair region in the BTB/POZ domain of PtrBPL1 was selected for co-silencing of *PtrBPL1/2* in transgenic poplar. Fragments were cloned in sense and anti-sense orientations in the polylinker of pHannibal to create an RNA hairpin controlled by the 35S CaMV promoter. The transgene cassette was released by digestion with NotI and ligated into the corresponding site of binary vector pART27 for transformation into poplar. pART27 confers resistance to spectinomycin in bacteria (Spec R) and resistance to Kanamycin (Kan R) in plants.

Figure 3.8 *TGA1:GUS* and *TGA4:GUS* expression patterns in wild-type plants

A-F, *TGA1:GUS*. (A) Leaf; expression at the base of the leaf in the vasculature of the petiole (arrow). (B) Inflorescence; expression at lateral organ boundaries including the base of floral organs and axils of pedicels (arrows). (C) Base of floral organs; expression appears at the base of young flowers after the appearance of floral organs (arrow). (D) Abscission zone; strong expression at the scar following detachment of floral organs (arrow). (E) Expression in vasculature of the primary stem (arrow). (F) Cross-section of stem; arrows denote expression in xylem (X). Scale bars, 1 mm. G-L, *TGA4:GUS*. (G) Leaf; expression at the base of the leaf in the vasculature of the petiole (arrow). (H) Inflorescence; expression at lateral organ boundaries including the base of floral organs and axils of pedicels (arrows). (I) Base of floral organs; expression appears at the base of young flowers soon after the floral organs begin to form (arrow). (J) Abscission zone; strong expression at the scar following detachment of floral organs (arrow). (K) Expression in vasculature of the primary stem (arrow). (L) Cross-section of stem; arrows denote expression in xylem (X) and phloem (Ph). Scale bars, 1 mm. Images (C) (F) (I) and (L) kindly provided by Thearany Lay.


A
B
C
D
E
F
TGA1:GUS
X
G
H
I
J
K
L
TGA4:GUS
X
Ph

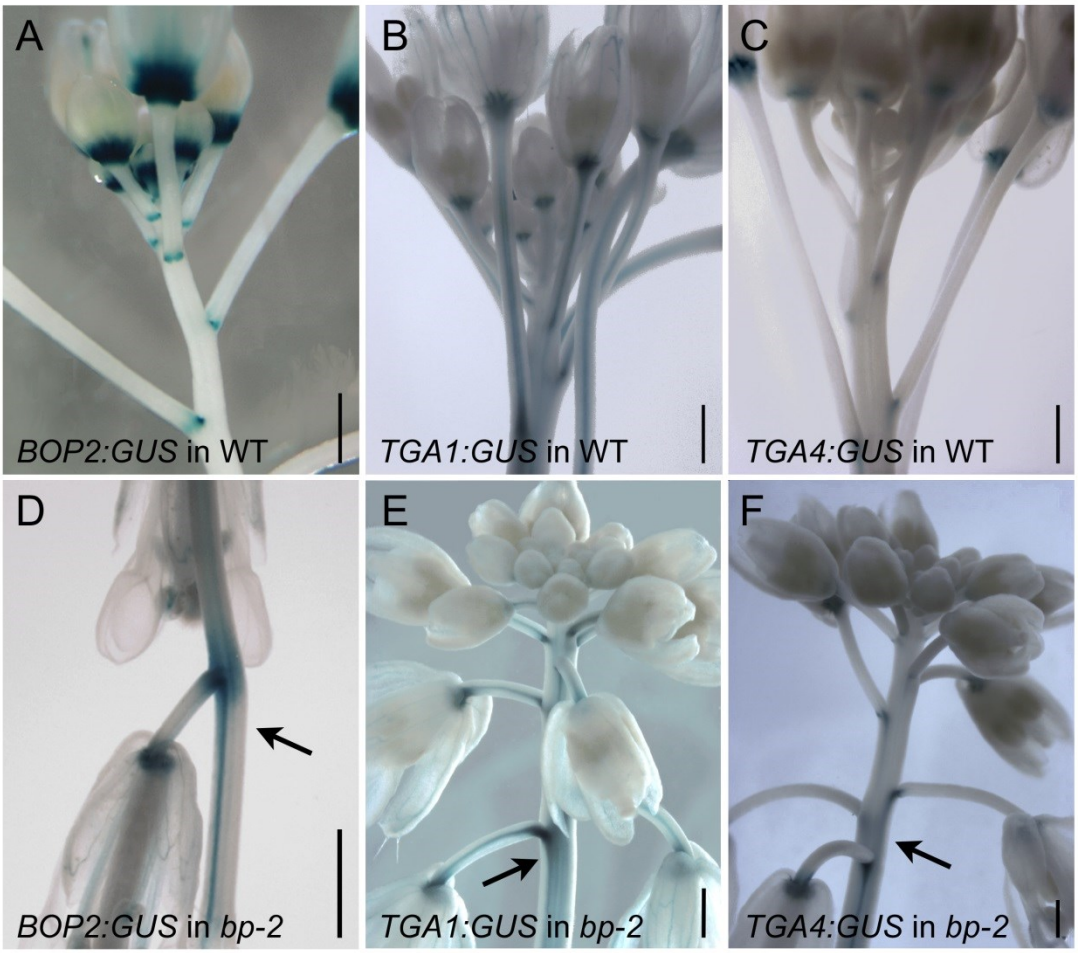


Figure 3.9 *BOP2:GUS*, *TGA1:GUS*, and *TGA4:GUS* expression patterns in wild-type and *bp-2* inflorescences

(A-C) Wild-type plants: (A) *BOP2:GUS*. (B) *TGA1:GUS*. (C) *TGA4:GUS*. All three reporters are strongly expressed at the base of the floral organs and in the axils of the floral pedicels. *TGA1:GUS* and *TGA4:GUS* are additionally expressed in the vasculature of the stem. (D-E) *bp-2* mutants: (D) *BOP2:GUS*. (E) *TGA1:GUS*. (F) *TGA4:GUS*. The expression domain of all three reporters is expanded. In *bp-2* mutants, misexpression is observed at the underside of nodes where growth is restricted and in stripes of epidermal tissue lacking chlorophyll that originate below the node and extend toward the base of the stem (arrows). Scale bars, 1 mm.

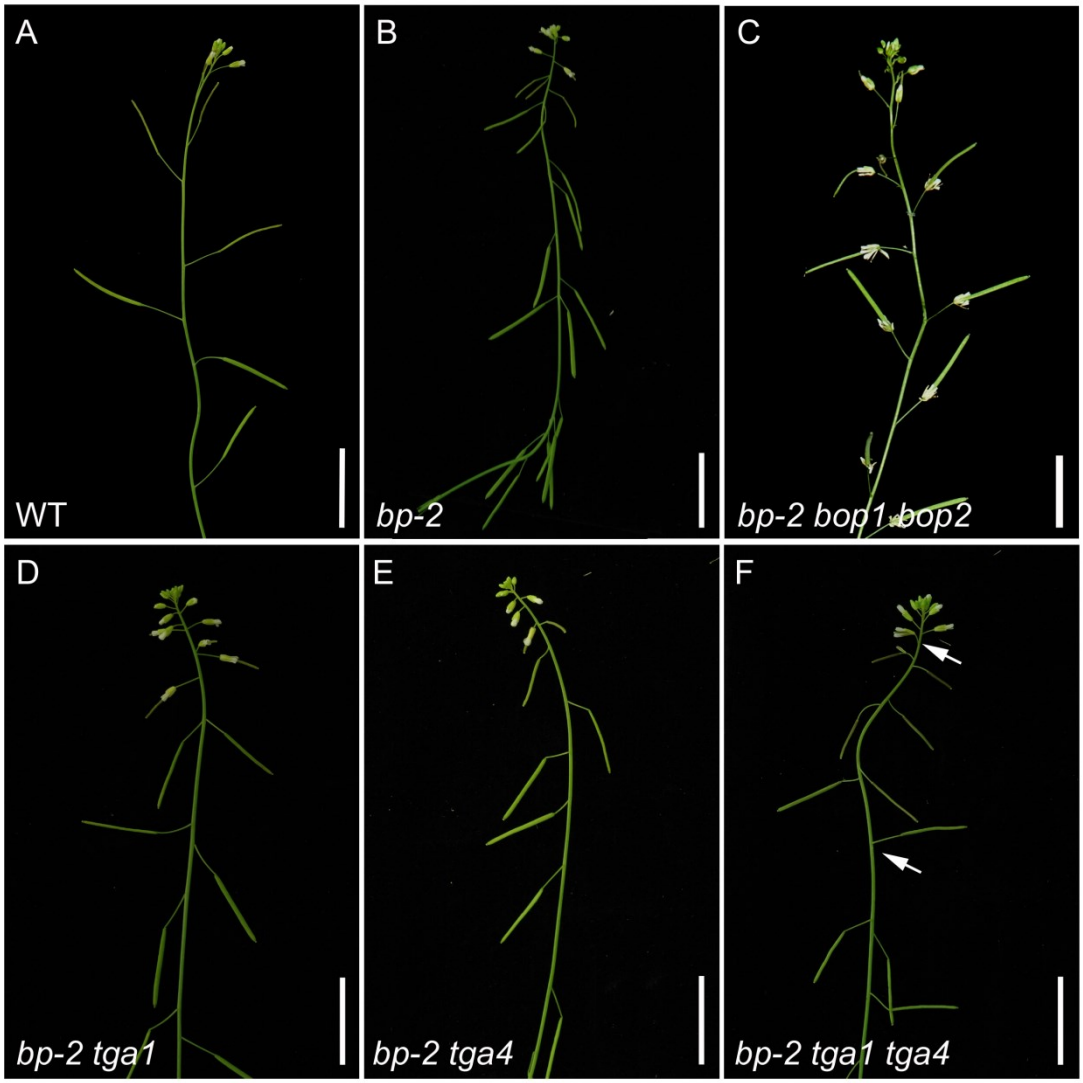


Figure 3.10 Phenotypic suppression of *bp-2* pedicel orientation by *tga1 tga4*

(A) Wild-type control. (B*) bp-2* mutant. (C) *bop1 bop2 bp-2* mutant; partial rescue of pedicel orientation. (D) *bp-2 tga1*. (E) *bp-2 tga4*. (F) *bp-2 tga1 tga4*; slight potential rescue of pedicel orientation compared to *bp-2* (arrow).

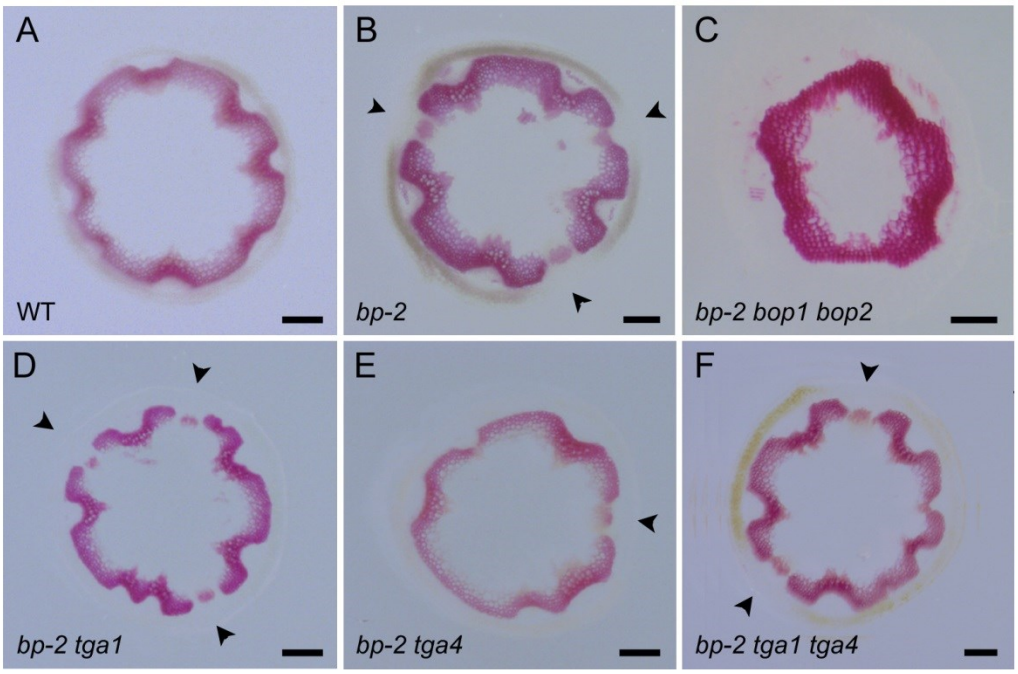
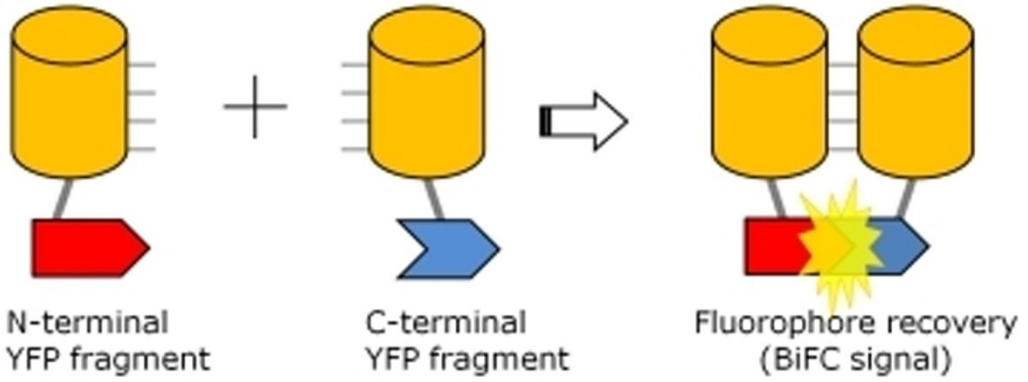


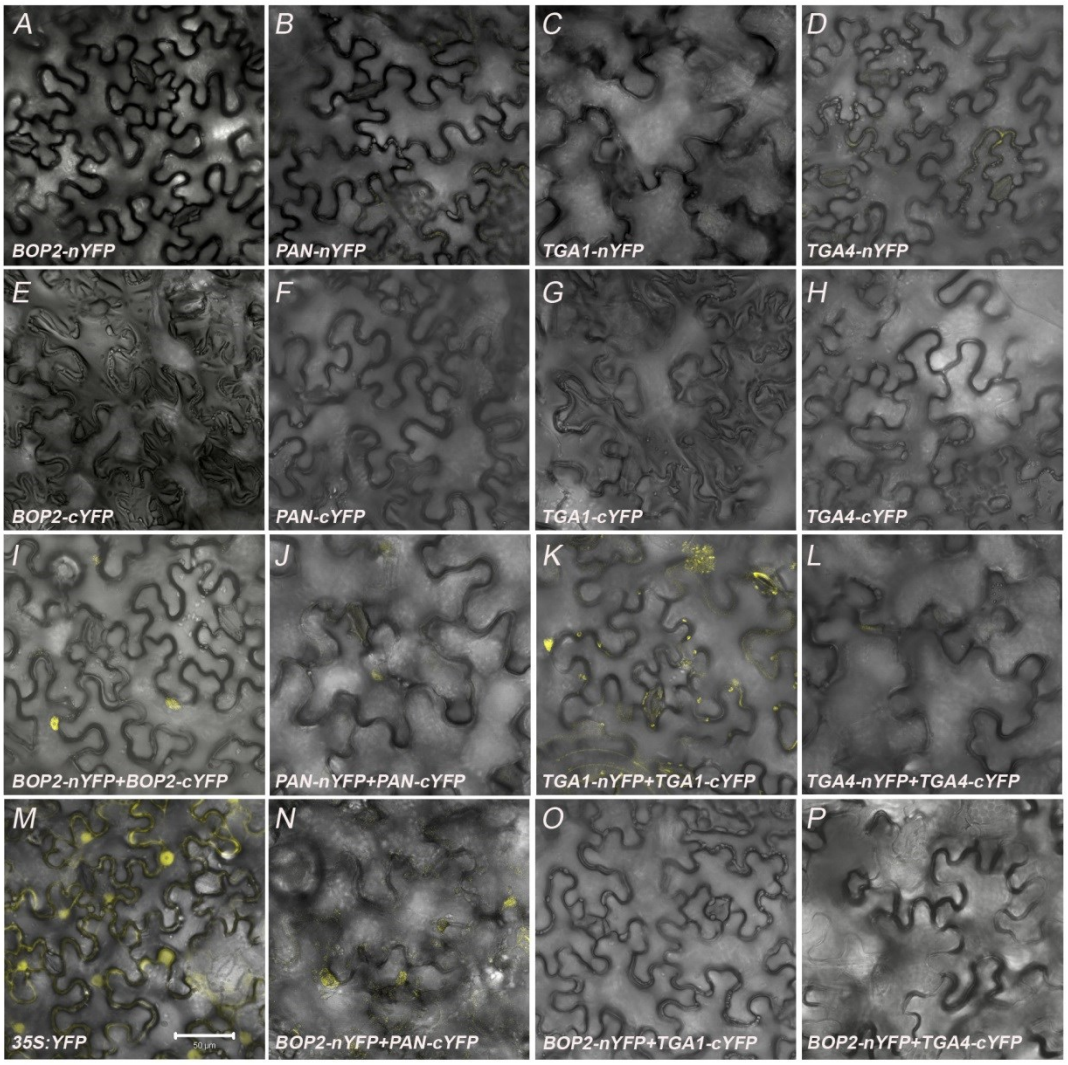
Figure 3.11 Lignification pattern in wild-type and mutant stems

Cross-sections at 1 cm above the base of 4-5 week old plants were stained with phloroglucinol-HCl to reveal lignin. (A) Wild-type control. (B) *bp-2* mutant. Note gaps in the vascular ring (arrowheads) and premature lignification of phloem fibres atop primary vascular bundles. Lignin deposits also within pith. (C) *bp-2 bop1 bop2* mutant; gaps in the vascular ring are closed. Image kindly provided by Madiha Khan. (D) *bp-1 tga1*; similar to *bp-2.* (E) *bp-2 tga4*; potentially fewer gaps in the vascular ring. (F) *bp-2 tga1 tga4*; potentially fewer gaps in the vascular ring. Scale bars, 100 µm.

Figure 3.12 Bimolecular fluorescence complementation fails to detect direct interaction between BOP and clade I TGA factors

(TOP) The BiFC technique was used to investigate BOP1 and BOP2 interactions with clade I TGA factors *in planta*. The N-terminus of YFP (nYFP) or C-terminus of YFP (c-YFP) cloned in-frame downstream of BOP2 or TGA factors (PAN, TGA1, or TGA4) individually. Constructs were expressed transiently in tobacco leaves singly or in pairs. Reconstitution of YFP fluorescence was examined 2-3 days after infiltration by confocal laser scanning microscopy. Adaxial surface of leaves was imaged. Genotypes are indicated in the lower right of panels. (A-P) Yellow fluorescence in the nucleus was detected for interactions of BOP2 with BOP2 and with PAN. Yellow fluorescence in the nucleus was also detected for PAN-PAN and TGA1-TGA1 interactions but not for TGA4-TGA4 interactions nor for BOP2 interactions with TGA1 or TGA4. As negative controls, no fluorescence was detected when n-YFP or c-YFP fusions were expressed singly in plants. Yellow fluorescence in the cytoplasm and nucleus was detected for *35S:YFP* as the positive control. Scale bar, 100 µm. Images kindly provided by Keith Hubbard/Denise Chabot.


N-terminal
YFP fragment
C-terminal
YFP fragment
Fluorophore recovery
(BiFC signal)


A
BOP2-nYFP
B
PAN-nYFP
C
TGA1-nYFP
D
TGA4-nYFP
E
BOP2-cYFP
F
PAN-cYFP
G
TGA1-cYFP
H
TGA4-cYFP
I
BOP2-nYFP+BOP2-cYFP
J
PAN-nYFP+PAN-cYFP
K
TGA1-nYFP+TGA1-cYFP
L
TGA4-nYFP+TGA4-cYFP
M
35S:YFP
50 µm
N
BOP2-nYFP+PAN-cYFP
O
BOP2-nYFP+TGA1-cYFP
P
BOP2-nYFP+TGA4-cYFP

CHAPTER 4

DISCUSSION

BTB-ankryin proteins are transcriptional co-activators present in all land plants. The Arabidopsis genome contains six family members subdivided into two subclades. NPR1 and its three closest homologs are essential in plant defense whereas BOP1 and BOP2 are regulators of plant development (reviewed in Khan et al., 2014). Both subclades are present basal land plants lacking a vascular system suggesting that BOPs play a fundamental role in plant development preceding the evolution of vascular tissues.

In *Arabidopsis thaliana*, a herbaceous weed, *BOP* genes primarily regulate development that occurs at junctions in the plant, also known as lateral organ boundaries. Boundaries are a domain of restricted growth that separate differentiating lateral organs from the meristem and modulate the production of lateral meristems that give rise to nectaries, stipules, flowers, and lateral branches. Lateral organ boundaries at some locations in the plant are specialized for separation events like abscission or dehiscence, processes which involve thickening of cell walls and deposition of lignin to resist dehydration and pathogen attack (reviewed in Aida and Tasaka, 2006ab; Khan et al., 2014).

In wild-type Arabidopsis plants, *BOP1/2* are expressed in vasculature of the leaf and root but not in the stem. Gain-of-function studies show that overexpression of BOPs in the stem restricts internode elongation and accelerates the development of lignified phloem and interfascicular fibres and ectopic deposition of lignin in the pith. These phenotypes suggest a potential role for BOPs in controlling wood and fibre production in forest trees, a hypothesis that is explored in this thesis using poplar as a model system.

4.1 *PtrBPL1* and *PtrBL2* are orthologs of the Arabidopsis *BOP* genes

Land plant genomes encode at least one *BOP* gene with the majority of species containing two or three. In the moss *Physcomitrella patens*, a pair of *BOP* genes are expressed at branch points in vegetative filaments where they promote reproductive bud meristem formation (Saleh et al., 2010). BOP activity in higher plants has been mainly studied in Arabidopsis, but to some extent in tobacco (abscission zone formation), pea and *Medicago truncatula* (leaf and floral patterning, nodulation) and in barley (tillering, spike density, and floral patterning) revealing highly conserved functions in plants (Wu et al., 2012; Gourlay et al., 2000; Yaxley et al., 2001; Couzigou et al., 2012; Khan et al., 2014; McKim, personal communication). This thesis examines the function of *BOP* genes in trees. The BOXSHADE alignment of BOP-like proteins from tree species including poplar showed approximately 80% amino acid sequence similarity to Arabidopsis BOPs suggesting a high degree of functional conservation. *PtrBPL1/2* expressed from the Arabidopsis *BOP1* promoter complemented leaf, flower, and abscission phenotypes when transformed into *bop1 bop2* double mutants (Table 3.1; Figure 3.2). The overexpression of *PtrBPL1/2* in Arabidopsis produced dwarf bushy plants with clustered flowers similar to overexpression of *AtBOP1* (*bop1-6D*) or *AtBOP2* (*35S:BOP2*) (Table 3.2; Figure 3.2; Khan et al., 2012b). *35S:PtrBPL1* plants tentatively had a thicker denser vascular ring and lignified phloem fibres suggesting increased lignin content (Figure 3.4). These data indicate that *PtrBPL1/2* functions are overlapping and homologous to *AtBOP1/2*. The next step is to test how loss and gain-of-function in poplar affects leaf and floral patterning and/or cambium function.

4.2 *PtrBPL1/2* are expressed in xylem and phloem consistent with a role in secondary growth

In silico and qRT-PCR analysis of dissected poplar tissues revealed a wide and overlapping distribution of expression for *PtrBPL1* and *PtrBPL2* (Figures 3.5 and 3.6). The resolution of this experiment was insufficient to conclude that these genes are expressed at lateral organ boundaries but enrichment of *PtrBPL* transcript in the petiole of leaves is consistent with this hypothesis. *PtrBPL1/2* expression was also enriched in seedlings, young stem, and developing secondary xylem and phloem consistent with a role in vascular patterning and/or secondary growth. Whole mount *in situ* hybridization will be used in future to examine gene expression in shoot apices and the developing stem (vascular cambium, primary vascular bundles, and secondary xylem/phloem).

4.3 Clade I bZIP TGA factors are potential BOP co-factors

BTB-ankyrin proteins contain a transcriptional activation domain at the C-terminus but lack a DNA-binding domain. Biochemical assays and genetic screens have identified TGA bZIP transcription factors as co-factors in recruitment of BTB-ankryin proteins to DNA (e.g. Després et al., 2000; Zhang et al., 2003; Hepworth et al., 2005). TGA factors are a plant-specific subclade of bZIP transcription factors. The Arabidopsis genome contains ten TGAs with overlapping roles in development, adaptation to stress, and defense against pathogens. Phylogenetic analysis divides Arabidopsis TGA factors into five clades (Jakoby et al., 2001; Gatz, 2013). Clade I comprises TGA1 and TGA4 identified as having roles in development and defense (Després et al., 2003; Kesarwani et al., 2007; Shearer et al., 2012; Alvarez et al.,

2014). In plant defense, biochemical studies showed that redox-sensitive Cys residues in TGA1 and TGA4 are reduced in response to pathogen attack and salicylic acid accumulation enabling interaction with NPR1 and activation of the defense gene *PR1* (Després et al., 2003). The significance of this finding is debated given that loss-of-function *tga1 tga4* mutants exhibit NPR1-independent defects in basal defense, which is based on chemical and structural deterrents to pathogen including secondary thickening of cell walls (Kesarwani et al., 2007; Shearer et al., 2012). TGA4 was also identified as binding to the adaptor protein CONSTANS, a key positive regulator of flowering (Samach et al., 2000; Song et al., 2008). TGA4 and CO appear to form a complex that directly interact at the promoter of *FT* in leaves to induce flowering. Consistent with these data, *TGA4* is expressed in the vasculature of leaves according to a diurnal rhythm that peaks during the night (Song et al., 2008). Double mutants *tga1 tga4* are also reported as having a curved petiole (Shearer et al., 2012) and altered root transcriptional response to nitrates exposure (Alvarez et al., 2014).

My data show that *TGA1* and *TGA4* genes are expressed in vasculature and enriched at lateral organ boundaries, including the petiole of leaves, axil of pedicels, and base of floral organs where abscission occurs (Figure 3.8). Within the inflorescence apex, there was a delay in activation of these genes at the boundary in comparison to boundary identity markers like *CUC3*, *BOP1/2* or *KNAT6* (Ha et al., 2004; Belles-Boix et al., 2006; Hibara et al., 2006). *TGA1/4* expression was not evident at the base of flowers until after the emergence of the floral organs, coinciding with specification of the abscission zone (McKim et al., 2008). *TGA1* and *TGA4* reporters were also expressed in vasculature of leaves as seen previously in Song et al. (2008) and in

vascular bundles of the stem. *TGA1* expression was restricted to xylem elements in the primary vascular bundles whereas *TGA4* was expressed in xylem and phloem. Neither were expressed in the cambium. I did not get a chance to analyze GUS expression patterns in seedlings to see if they were expressed in the root vasculature. I also did not analyze at the base of the mature stem to see if expression expanded to intrafascicular regions during secondary growth.

Although *TGA1* and *TGA4* expression domains were clearly expanded in *bp* mutants (Figure 3.9) inactivation of these genes did not significantly alter pedicel orientation or the pattern of lignin deposition (Figures 3.10 and 3.11). This suggests that misexpression of clade I TGA factors plays a minor role in conditioning the *bp* phenotype. TGA factors in other clades may play a greater role in mediating BOP function in this developmental context. In studies of the *pny* mutant, loss-of-function *tga1* partially rescues internode clustering defects. Complete rescue occurs in the *tga1 tga4* double mutant, similar to *pny bop1 bop2* triple mutants (Khan et al., 2012b; Chisanga and Hepworth, unpublished). In studies of the *pny pnf* mutant, loss-of-function *tga4* rescues flowering and internode initation, with complete rescue of clustering defects in quadruple mutant with *tga1 tga4*, identical to *pny pnf bop1 bop2* quadruple mutants. Loss-of-function *tga1 tga4* restores internode elongation in dwarf *35S:BOP2* lines further supporting that BOPs require TGA1 and TGA4 to exert changes in inflorescence architecture. These data also show that TGA1 and TGA4 do not have identical functions in development. They function closely in the same genetic pathway as BOP1/2 in regulating flowering and internode elongation,

but have a lesser role in the pathway that directs lignin biosynthesis and vascular patterning in *bp* mutants.

4.4 Visualizing BOP-TGA interactions *in vivo*

Given that BOPs and clade I TGA factors function in some of the same genetic pathways, I looked for evidence of complex formation *in planta*. Previous experiments in yeast failed to reveal an interaction (Hepworth et al., 2005) so I used BiFC in tobacco leaves, also known as split YFP. I observed a fluorescent signal in the nucleus for BOP2-BOP2 interactions and very weakly for BOP2-PAN interactions but no signal was detected for BOP2-TGA1 or BOP2-TGA4 interactions. Biochemical studies show that NPR1 interaction with clade I TGA factors in plant defense requires the reduction of Cys260 and Cys266 residues in the TGA1 C-terminus. *In vivo*, this is carried out by an enzyme activity induced by salicylic acid in response to pathogen attack. A reduced state for TGA1 and TGA4 is mimicked by conversion of Cys260 and Cys266 to alanine or serine allowing a constitutive interaction in yeast (Després et al., 2003). S-nitrosylation or S-glutathionylation of these same residues has a similar effect (Lindemayr et al., 2010). Similar modifications may promote BOP interaction with TGA1 and TGA4 *in vivo*.

4.5 Hormonal control of secondary development

Converging lines evidence indicate that BOPs modulate the abundance of jasmonic acid to regulate development and defense. Jasmonic acid is a lipid-based signaling molecule that retards growth and promotes defense against abiotic stresses (e.g. mechanical or wounding) and defense against pathogens (e.g. chewing insects, a variety of hemibiotrophic and necrotrophic pathogens) (Wasternak and Hause,

2013). Jasmonates also promote abscission in various plant species (Kim et al. 2013 and references therein) and secondary growth in Arabidopsis and tobacco stems (Sehr et al. 2010; Henrich et al. 2013). A recent studied showed that *bop1 bop2* mutants primed with methyl jasmonate are more sensitive to *Pseudomonas syringae* DC3000 pathogen than are wild-type plants receiving the same treatment. Conversely, plants overexpressing BOP1/2 and primed with methyl jasmonate show increased resistance to pathogen compared to wild-type (Canet et al. 2012). In our lab, a microarray experiment comparing internodes from wild-tpe versus BOP-o/e plants provides additional insight, showing the upregulation of numerous genes involved in cell wall modification and defense along with genes involved in jasmonate biosynthesis and signaling. In contrast, genes involved in gibberellin biosynthesis and signaling important for flowering and internode elongation were down-regulated. Studies in tobacco show that high levels of jasmonic acid applied to stems restricts internode elongation and promotes secondary thickening in part by inhibiting giberrellin biosynthesis (Heinrich et al. 2013). Thus, BOPs may promote secondary growth by modulating jasmonic acid hormone pathways.

4.6 Concluding remarks

Populus trichocarpa is a good model system for studying tree biology including wood development that cannot be truly evaluated in annual model plants like Arabidopsis. A greater understanding of the factors that influence wood and fibre development including the interaction of BLADE-ON-PETIOLE genes with class I KNOX genes will ultimately help to guide genetic improvement strategies in forest trees and energy crops.

CHAPTER 5

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

5.1 General conclusions

This thesis investigated the role of BLADE-ON-PETIOLE genes as conserved regulators of lignin biosynthesis in the context of secondary growth in the stem of flowering plants. I also tested the hypothesis that clade I TGA bZIP factors function in the same genetic pathway as AtBOP1/2 genes and serve as interacting co-factors in the regulation of lignin biosynthesis during secondary development in the stem.

My data showed that poplar *PtrBPL1* and *PtrBPL2* complement the developmental phenotypes of Arabidopsis *bop1 bop2* mutants identifying these as functional homologs of AtBOP1/2. Similar to their Arabidopsis counterparts, *PtrBPL1* and *PtrBPL2* are expressed in poplar leaves and flowers, but additionally in vascular tissues of the woody stem (secondary xylem and phloem). Arabidopsis plants overexpressing *PtrBPL1* and *PtrBPL2* had short internodes with preliminary evidence of lignin overproduction. These data support a conserved role for BOPs in tree development and in promotion of secondary growth, although the mechanism is yet unclear.

Secondly, I tested the hypothesis that Arabidopsis BOPs require clade I TGA bZIP factors TGA1 and TGA4 to exert changes in inflorescence architecture and lignin biosynthesis. My data showed that *TGA1* and *TGA4* are expressed at lateral organ boundaries and in vascular tissues. I also showed that their expression in stems is regulated by the class I KNOX transcription factor BP in a manner similar to BOP1/2. However, double mutant analyses with *bp*-2 did not clearly support the hypothesis that BOP1/2 require TGA1 and TGA4 as co-factor for regulating growth patterns or

lignin content of the primary inflorescence stem nor was a direct interaction between BOP1/2 protein and TGA factors detectable using BiFC in tobacco leaves.

5.2 Future Directions

5.2.1 Follow up analysis of secondary growth in *Arabidopsis* plants expressing the *35S:PtrBPL1* transgene

Preliminary evidence suggests that secondary growth is altered in *35S:PtrBPL1* Arabidopsis plants but this remains to be verified in part due to a small number of plant lines (n=1) with a stable overexpression phenotype transmitting to the T2 generation. Additional lines will be screened to isolate a stable homozygous line for *35S:PtrBPL1* with a strong overexpression phenotype. Stems of these plants will be sectioned (base and middle) and stained with toluidine blue and phloroglucinol to look for changes in lignin deposition and/or the lateral thickness of the vascular cambium relative to wild-type and *AtBOP1* overexpressing plants (see Sehr et al. 2010). Cell wall thickness will be quantified by measuring images obtained using transmission electron microscopy (see Wang et al. 2014). Dried stems will be sent for chemical analysis to test for changes in cell wall chemistry (total lignin and relative abundance of lignin monomers) (see Wang et al. 2014).

5.2.2 Experiments in poplar

An important next step will be to examine the developmental role of *PtrBPL1* and *PtrBPL2* in poplar by making transgenic loss-of-function and gain-of-function mutants (Ma et al. 2004). The RNAi construct that I made can be used to transform poplar in tissue culture to generate a loss-of-function phenotype. Gain-of-function

plants will be generated by introducing *D35S:BPL1* and *D35S:BPL2* constructs into poplar. Analysis of these plants will give important insights into the development role of BOP genes in trees. Similar experiments were used to identify roles for poplar class I KNOX genes *ARBORKNOX1* and *ARBORKNOX2* in SAM maintenance and cambium function (Groover et al. 2006; Du et al. 2009).

5.2.3 Class I bZIP factors as BOP-interacting cofactors in regulation of gene expression

BOP overexpression in *bp* mutants leads to the upregulation of lignin biosynthetic genes and the ectopic production of lignin. Recent work has identified boundary-related KNOX (KNAT2, KNAT6) and BELL (ATH1) transcription factors as important mediators of BOP1/2 function. Double mutant analyses suggest that ATH1-KNAT2 and ATH1-KNAT6 activities in this pathway mediate separate components of BOP function. Rescue of *bp-2* lignin defects by *bop1 bop2* is comparable to rescue by *ath1 knat6* and *knat2 knat6* (Khan et al. 2012a) Loss-of-function *ath1* or *knat2* have little or no impact and must be combined with *knat6* to reveal their activity in lignin biosynthesis (Li et al. 2011; Khan et al. 2012a). To uncover a role for TGA1/4 in lignin biosynthesis, we can combine the *tga1 tga4* double mutant with *knat6* to test if these genes function in conjunction with ATH1 in regulation of stem development.

ATH1 was recently identified as a direct transcriptional target of BOP1/2 (Khan, 2013). BOP1/2 may be recruited by TGA1 and TGA4 to bZIP sites in the *ATH1* promoter based on chromatin immunoprecipitation experiments. Future work will focus on optimizing the BiFC technique to test this hypothesis. In my previous attempt, the geometry of the interaction may fail to bring the two halves of YFP

close enough together to restore fluorescence. Fusing YFP to N-terminus of BOP and/or TGAs factors may work better. Alternatively, the interaction may be stabilized by post-translational modifications affecting the conformation of BOP1/2 or TGA1/4. For example, the defense hormone salicylic acid binds directly to the C-terminus of NPR1 to trigger a conformational change that exposes its transcriptional activation domain (Wu et al. 2012b). TGA1 and TGA4 only bind to NPR1 in their reduced form which is triggered by treatment with oxidizing agents include hydrogen peroxide or the addition of salicylic acid (Despres et al. 2003). Similar treatments or spray treatment with methyl jasmonate may allow us to detect an interaction.

Collectively, these studies will pave the way to a greater understanding of the developmental mechanisms that govern the growth and development of forest trees.

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