Investigating the role of boundary genes in plant vascular cambiums

By

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ABSTRACT

Meristems play an essential role in plant growth and development. Shoot and root apical meristems are responsible for primary elongation growth in shoots and roots, respectively. Secondary or radial growth that follows is dependent on the vascular cambium, a circular meristem that produces secondary xylem (wood) and secondary phloem (inner bark). Little is known about the vascular cambium, despite its importance to wood formation in trees. Class I KNOX homeodomain transcription factors are important regulators of meristem maintenance in plants. Members of this class, including BREVIPEDICELLUS (BP) maintain the shoot apical meristem in part by preserving boundaries that keep stem cells separate from differentiating organs. The role of boundary genes in the vascular cambium is mostly unknown. Here, I provide evidence that spatial regulation of boundary genes by Class I KNOX genes is important for different reasons in the vascular cambiums of stem and root-hypocotyl in *Arabidopsis thaliana*, a model plant species. In particular, BP repression of boundary genes including *BLADE-ON-PETIOLE1* and *2* is important for the differentiation of reproductive-phase secondary xylem in Arabidopsis root-hypocotyl but not stem. *Populus trichocarpa* (Poplar) is a model tree that contains two *BOP*-like genes, designed as *PtrBPL1* and *PtrBPL2*. Transgenics were created to examine the expression and function of these genes in poplar. My data are consistent with a role for boundary genes in vascular cambiums and shed light on mechanisms controlling secondary growth in trees.

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PREFACE

This thesis explores mechanisms governing regulation of secondary growth in Arabidopsis and poplar.

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Natalie Woerlen, Eryang Li, Veronique Pautot, and Shelley Hepworth designed the research. Natalie Woerlen initiated the work on Arabidopsis root-hypocotyl secondary growth and wrote the first draft of the published manuscript. Adina Popescu and I contributed equally to addressing reviewer comments. Laura Corrigan assisted me in quantitative analysis of root secondary growth. Dr. Eryang Li and Bhaswati Devi initiated the work on poplar and partially made constructs for functional analysis of *PtrBPL1* and *PtrBPL2* in Arabidopsis and poplar. Jhadeswar Murmu finished construction of the RNAi construct for knockdown of *PtrBPL1/2* expression in poplar. I would like to thank my supervisor Dr. Shelley Hepworth for making figures and editing this 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 toward wild type phenotype

Phenotypic enhancement: worsening a mutant phenotype

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

Homolog: genes sharing a common ancestor in evolution

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

Paralog: genes that are related by a duplication event within the genome of an organism

GENETIC NOMENCLATURE IN *ARABIDOPSIS THALIANA*

Wild type gene: *BOP1*

Wild type protein: BOP1

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

Loss-of-function mutant (hemizygous): *bop1/+*

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

Double mutant: *bop1 bop2*

Promoter fusion to a gene coding region: *35S:BOP1*

Protein fusion: *BOP1-GR*

LIST OF ABBREVIATIONS

AS1	ASYMMETRIC LEAVES1
At	*Arabidopsis thaliana*
ATH1	ARABIDOPSIS THALIANA HOMEOBOX GENE1
ARK	ARBORKNOX
BAP	6-benzylaminopurine
BELL	BEL1-like
BOP	BLADE-ON-PETIOLE
BP	BREVIPEDICELLUS
BPL	BOP-like
BTB/POZ	Bric-a-Brac, Tram Track, Broad Complex/POX virus and Zinc finger
CIM	Callus Induction Medium
CLE	CLAVATA/ESR related
CLV	CLAVATA
Col	Columbia (wild-type ecotype of *Arabidopsis thaliana*)
CUC	CUP-SHAPED COTYLEDON
CTAB	cetyl trimethylammonium bromide
CZ	central zone
ddH_2O	distilled deionized water
D35S CaMV	double 35S Cauliflower Mosaic Virus promoter
DNA	deoxyribose nucleic acid
EDTA	ethylenediaminetetraacetic acid
FAA	formaldehyde-acetic-acid-alcohol

GA	gibberellin
GUS	β-Glucuronidase
h	hour
HCl	hydrochloric acid
IBA	indole-3-butyric acid
KNAT	KNOTTED1-LIKE FROM ARABIDOPSIS THALIANA
KNOX	KNOTTED1-LIKE HOMEOBOX
LB	Lysogeny Broth
LOB	Lateral Organ Boundary
MES	2-(*N*-morpholino) ethanesulfonic acid
miR	microRNA
MS	Murashige and Skoog
NAA	1-naphthaleneacetic acid
NAC	NAM, ATAF, and CUC
NPTII	neomycin phosphotransferase II
OC	Organizing center
PCR	Polymerase chain reaction
PNF	POUND-FOOLISH
PNY	PENNYWISE
Ptr	*Populus trichocarpa*
PZ	peripheral zone
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RAM	Root apical meristem

RIM	Root induction medium
RNAi	RNA interference
RZ	rib zone
SAM	Shoot apical meristem
SEM	Shoot elongation medium
SDS	sodium dodecyl sulphate
STM	SHOOT MERISTEMLESS
TALE	THREE-AMINO ACID-LOOP-EXTENSION
T-DNA	transfer DNA
TDZ	thiadiazuron
UBQ	UBIQUITIN
VC	vascular cambium
WT	wild-type
w/v	weight / volume
WOX	WUSCHEL-RELATED HOMEOBOX
WUS	WUSCHEL
X-gluc	5-bromo-4-chloro-3-indoxyl-β-D-glucuronide
2ip	6-(γ, γ-dimethylallylamino)purine

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CHAPTER 1: INTRODUCTION

1.1 Thesis overview

Plant growth and development relies on meristems. Primary meristems at the shoot and root tips are responsible for vertical growth of shoots and roots, respectively. Vascular meristems called procambia derived from these meristems provide primary tissues of the vascular system: xylem and phloem. Secondary growth that follows relies on the vascular cambium, a ring-like meristem derived from procambium. The vascular cambium allows plant organs to thicken in circumference through the production of secondary xylem (wood) and secondary phloem (bark). Knowledge of the vascular cambium is incomplete despite its importance to wood formation in trees (Nieminen et al., 2015).

Class I KNOX homeodomain transcription factors are important regulators of meristem activity in land plants (Tsuda and Hake, 2016). Members of this class maintain the SAM in part by establishing low-growth regions called boundaries that separate forming organs from the meristem. Disruption of boundaries terminates the stem cell population and impairs organ formation and patterning (Hepworth and Pautot, 2015). The vascular cambium is a source of stem cells much like the apical meristem of shoots. How do mechanisms responsible for maintenance of the SAM apply to the vascular cambium? My thesis investigates the role of boundary genes in plant secondary growth using *Arabidopsis thaliana* (thale cress) and *Populus trichocarpa* (poplar tree) as study species.

1.2 *Arabidopsis thaliana* as a model plant species

Arabidopsis thaliana (Arabidopsis) was introduced as genetic model for plant biology studies in the late 1970's (Somerville and Koorneef, 2002). This plant is a member of the

Brassicaceae family, which includes economically important species like broccoli, cabbage, horseradish, mustard, and oilseed rape (Somerville and Koorneef, 2002; Koornneef and Meinke, 2010). Arabidopsis was chosen as a model species based on characteristics that make it easy to work with in a lab. Adult plants are relatively small (30-40 cm in height) with simple growth requirements and a short life cycle (6-8 weeks) (Meyerowitz, 1989; Somerville and Koorneef, 2002). The plant is a self-fertilizing diploid with prolific seed production. Loss and gain-of-function mutants are easy to make using chemical mutagens, transposons, or Agrobacterium-mediated transformation, which can also be used to create transgenic plants. The Arabidopsis genome is simple and compact, containing five chromosomes whose fully-annotated sequences can be accessed through The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). Stock centers in North America, Europe, and Asia are set up to distribute seeds, cDNA clones, indexed libraries of T-DNA insertion mutants and other resources for a small cost (Koornneef and Meinke, 2010). Arabidopsis has proved to be one of the most important discovery tools for plant biology in the last twenty years. Although lacking in economic value, genome composition and developmental programs are highly conserved with crop species. Information gained from Arabidopsis has been successfully applied to the improvement of agronomic traits in numerous crop species (Ferrier et al., 2011; Lavagi et al., 2012).

1.3 *Arabidopsis thaliana* life cycle

The life cycle of a flowering plant has embryonic, vegetative, and reproductive phases (Figure 1.1). Embryonic development begins at fertilization. Division of the fertilized egg produces an embryo within a seed (Wolpert et al., 2015). The mature embryo contains the first basic set of plant structures: one or two leaves (cotyledons), a stem (hypocotyl), and a primary root

(radicle). Post-embryonic development begins at germination and is divided into two phases: vegetative and reproductive. In Arabidopsis, the vegetative phase is dominated by the production of leaves. By contrast, the reproductive phase is dominated by the production of lateral branches and flowers. Stem elongation during the reproductive phase distributes these structures to produce an inflorescence (Figure 1.1). Each flower is composed of four whorls of floral organs: sepals, petals, stamens, and carpels. Two fused carpels at the center of the flower form the gynoecium (female reproductive organ). The gynoecium contains three tissues from top to bottom: the stigma, style, and ovary which gives rise to ovules internally. Pollen landing on the stigma fertilize the ovules, which mature into seeds, thus completing the life cycle (Wolpert et al., 2015).

1.4 Meristems

Post-embryonic growth of a plant relies on meristems. Meristems are specialized niches for stem cells. Different types of meristems can be found in different parts of the plant. Within the meristem, continuous division of stem cells replenishes the meristem and provides cells for organ production. Daughter cells exiting the meristem differentiate into all of the specialized cell types that make up the plant body (Aichinger et al., 2012).

1.4.1 Primary growth

The vertical growth of plants depends on two meristems that originate in the embryo: the shoot apical meristem (SAM) and the root apical meristem (RAM) (Figure 1.2). The SAM produces all of the above-ground structures in a plant. By contrast, the RAM produces the root system (Barton, 2010; Wolpert et al., 2015). Vascular procambial meristems provide the primary vascular tissue of roots and shoots: xylem and phloem.

The vascular anatomy of shoots and roots differs (Figure 1.2). In the stem, individual vascular bundles are arranged in ring. Each bundle has a sandwich-like structure. Procambial cells at the center of each "sandwich" divide to make daughter cells that differentiate as xylem on the inside and phloem on the outside (Sanchez et al., 2012). Vascular tissues in the root and hypocotyl instead form a central bundle with procambium interspersed between xylem and phloem (Miyashima et al., 2013; Nieminen et al., 2015). Regardless of origin, xylem provides mechanical support and transports water and solutes from the roots to the leaves. Phloem transports sugars from photosynthesis, signaling molecules, and metabolites from leaves to the rest of the plant (Miyashima et al., 2013; Nieminen et al., 2015).

1.4.2 Secondary growth

Primary growth is followed by secondary growth in which roots and shoots grow in circumference. This thickening relies on the vascular cambium, a ring-like lateral meristem derived from procambium (Nieminen et al., 2015). Vascular cambium cells divide to produce secondary xylem (wood) on the inside and secondary phloem (inner bark) on the outside. Secondary xylem consists of non-lignified xylem parenchyma interspersed with lignified xylem vessels and fibres and secondary phloem consists of sieve-elements and their companion cells, phloem fibres, and phloem parenchyma (Miyashima et al., 2013; Nieminen et al., 2015). Arabidopsis is a popular model for studying wood development based on extensive secondary growth in the inflorescence stem, hypocotyl, and roots (Esau, 1965; Chaffey et al., 2002; Beck, 2010; Nieminen et al., 2015). All three tissues establish prominent vasculature similar to angiosperm trees (Ragni and Hardtke, 2014; Nieminen et al., 2015; Barra-Jiménez and Ragni, 2017).

1.4.2.1 Inflorescence stem

Primary vascular in the Arabidopsis inflorescence stem is organized as individual bundles (Figure 1.2, A-C). During secondary growth, meristematic attributes of the fascicular cambium (originating within primary vascular bundles) extends laterally into the interfascicular regions (spaces between bundles) to close the ring (Sanchez et al., 2012). A complete ring of cambium is formed only at the base of the inflorescence stem (oldest part). In middle sections of the stem, differentiation of lignified interfascicular fibres completes the vascular ring. In top sections of the stem, secondary growth is limited to the vascular bundles. Therefore, secondary growth in the inflorescence stem is described by a gradient (Altamura et al., 2001; Sehr et al., 2010; Sanchez et al., 2012; Nieminen et al., 2015; Barra-Jiménez and Ragni, 2017).

1.4.2.2 Root-hypocotyl

Primary vascular in the root and hypocotyl is organized centrally (Figure 1.2, C-F). During secondary growth, procambium cells proliferate and rearrange to form a continuous ring of vascular cambium that produces secondary xylem on the inside and secondary phloem on the outside (Ragni and Hardtke, 2014; Nieminen et al., 2015). The vascular cambium in hypocotyl is completed a few days after germination and remains active throughout development (Chaffey et al., 2002; Ragni et al., 2011). The vascular cambium of roots forms the same way (Dolan et al., 1993; Busse and Evet, 1999; Chaffey et al., 2002). Two phases of secondary growth are distinguished in root and hypocotyl (Figure 1.3). During phase I, there is equal production of xylem and phloem. The xylem that is formed during this proportional phase of growth (xylem I) is composed of non-lignified parenchyma and lignified vessel cells. The transition to flowering triggers a switch to phase II in which xylem production is expanded relative to phloem (Chaffey et al., 2002; Sibout et al., 2008). Xylem fibres differentiated from parenchyma are formed

exclusively during this expansion phase of growth (xylem II), which is seen as a dense ring of lignified fibers interspersed with vessels (Chaffey et al., 2002; Sibout et al., 2008; Ragni and Hardtke, 2014; Nieminen et al., 2015).

1.5 Conserved patterns in plant development

The SAM and the vascular cambium fulfill the same basic function of providing stem cell activity. Recent studies indicate that the maintenance and activity of these two meristems are controlled by similar transcription factors and hormones (Schrader et al., 2004; Miyashima et al., 2013; Nieminen et al., 2015; Ye and Zhong, 2015). Understanding the role of factors that maintain the SAM and their functions in the vascular cambium is an area of active study.

1.6 Organization of the SAM

The Arabidopsis SAM is a rounded dome-like structure (Figure 1.4). It has three histologically distinct cell layers: L1, L2, and L3 (Fletcher, 2002; Barton, 2010). The epidermal (L1) and sub-epidermal (L2) layers constitute the tunica (covering) and the inner (L3) layers constitute the corpus (body). Fate mapping show that each of these cell layers gives rise to different tissues in the plant body (Fletcher, 2002; Stahl and Simon, 2005; Barton, 2010). Cells in the L1 and L2 layers divide in a plane perpendicular the surface. The L1 layer forms the plant epidermis and the L2 layer forms the plant mesoderm and germline. Cells in the L3 layer divide in all planes, forming the inner tissue of leaves, stems, branches, and flowers that includes the vascular system (Fletcher, 2002; Stahl and Simon, 2005; Barton, 2010; Wolpert et al., 2015).

The SAM is further organized into functional domains: the central zone (CZ), peripheral zone (PZ) and rib zone (RZ) (Figure 1.4). The CZ contains a group of pluripotent stem cells.

Daughter cells from division of CZ cells are displaced laterally into the PZ where organogenesis takes place. The RZ provides cells for upward growth of the stem (Fletcher, 2002; Barton, 2010; Wolpert et al., 2015). A subdomain of the RZ called the organizing center (OC) controls the size of the CZ (Barton, 2010; Aichinger et al., 2012; Wolpert et al., 2015). Differential regulation of activities in the CZ, PZ and RZ are responsible for changing production of leaves, stems, branches, and flowers throughout the life cycle (Fletcher, 2002; Sanchez et al., 2012; Bencivenga et al., 2016).

1.7 Meristem-organ boundaries

Continuous activity of the SAM requires separation of the stem cell population from emerging lateral organs (Hepworth and Pautot, 2015). Low-growth regions called boundaries are rapidly established at meristem-organ interfaces for this function (Figures 1.4). Impairment of boundaries causes termination of the SAM and/or fusion of organs (Aida and Tasaka, 2006a, 2006b). As differentiation proceeds, the boundary extends to encircle the base of the organ where it takes on a patterning role (Figure 1.5A). Boundaries are the source of axillary meristems that make lateral branches and flowers. Boundaries are also sites for the regulated detachment of organs from the plant body (Hepworth and Pautot, 2015).

1.8 SAM maintenance

Maintenance of the SAM relies on two interconnecting mechanisms: a WUSCHEL-CLAVATA (WUS-CLV) feedback loop and the overlapping activities of KNOX and BELL three amino-acid-loop-extension (TALE) homeodomain transcription factors (Hamant and Pautot, 2010; Aichinger et al., 2012). These two modules play distinct and complementary roles (Gallois et al., 2002; Lenhard et al., 2002; Aichinger et al., 2012).

1.8.1 WUSCHEL-CLAVATA feedback loop

Stem cell maintenance in the SAM depends in part on a negative-feedback loop between the homeodomain transcription factor WUSCHEL (WUS) and the secreted peptide ligand CLAVATA3 (CLV3) (Aichinger et al., 2012). WUS protein is produced in the Organizing Center of the SAM and moves into overlying outer layers of the CZ where it promotes stem cell fate and activates *CLV3* expression (Yadav et al., 2011). CLV3 signaling represses *WUS* expression thereby creating a negative feedback loop that controls the size of the stem cell population (Schoof et al., 2000; Yadav et al., 2011; Aichinger et al., 2012). Paralogs of WUS and CLV3 control stem cell behaviour in the RAM (WOX5 and CLE40) and in cambia meristems (WOX4 and CLE41/44) suggesting that different classes of meristems are preserved by similar mechanisms (Stahl et al., 2009; Hirakawa et al., 2010; Aichinger et al., 2012).

1.8.2 KNOX-BELL homeodomain proteins

Three-amino-acid-loop-extension (TALE) proteins are a superfamily of homeodomain transcription factors found in eukaryotes (Bertolino et al., 1995; Bürglin, 1997). Plant TALEs are divided into KNOTTED1-like (KNOX) and BELL-like (BELL) subclasses, whose dimerization is important for function (Hamant and Pautot, 2010). KNOX-BELL heterodimer composition governs site-specific DNA binding (Smith et al., 2002) and regulates nuclear localization ((Bhatt et al., 2004; Cole et al., 2006; Lee et al., 2008).

In Arabidopsis, four Class I KNOX genes contribute to SAM initiation and maintenance: *SHOOT MERISTEMLESS (STM)*, *BREVIPEDICELLUS*, also called *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA1* (*BP*/*KNAT1*), *KNAT2,* and *KNAT6* (Scofield and Murray, 2006; Hamant and Pautot, 2010).

STM is expressed in all subdomains of the SAM except at sites of organ initiation consistent with essential roles in meristem initiation, meristem maintenance, and the initiation of boundaries (Endrizzi et al., 1996; Long et al., 1996; Landrein et al., 2015; Balkunde et al., 2017). Strong *stm* mutants lack a SAM or terminate growth after forming a few leaves (Endrizzi et al., 1996; Long et al., 1996). STM directly and indirectly contributes to the expression of other Class I KNOX genes in subdomains of the SAM (Belles-Boix et al., 2006; Scofield et al., 2013; Scofield et al., 2014). *BP* is expressed in the PZ and RZ (Lincoln et al., 1994), *KNAT2* is expressed in the RZ and organ boundaries, and *KNAT6* is expressed in organ boundaries (Belles-Boix et al., 2006). Mutations in *BP* enhance the phenotype of weak *stm* mutants and genetic experiments show that BP can compensate for missing STM activity in the CZ (Byrne et al., 2002; Scofield et al., 2013). Mutations in *KNAT6* also enhance the phenotype of weak *stm* mutants in organ separation and SAM maintenance indicating their involvement (Belles-Boix et al., 2006). Genetic experiments have yet to identify a role for KNAT2 in the SAM (Byrne et al., 2002).

At least three BELL members: *PENNYWISE (PNY), POUND-FOOLISH (PNF)*, and *ARABIDOPSIS THALIANA HOMEOBOX GENE1 (ATH1)* interact with STM and allow it to properly function (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). Interactions with PNY and other BELLs are required for nuclear import of STM (Cole et al., 2006). Triple mutants *ath1-1 pny pnf* have a phenotype similar to weak *stm* mutants, possibly caused by depletion of nuclear-localized STM (Rutjens et al., 2009). STM-BELL complexes maintains the SAM by repressing differentiation of the stem cell population and also by initiating and maintaining organ boundaries (Hepworth and Pautot, 2015). *ATH1*, which contributes to boundary establishment, is expressed in the SAM during vegetative development and downregulated at the transition to

flowering (Proveniers et al., 2007; Gómez-Mena and Sablowski, 2008). A specific role for ATH1 in the establishment of boundaries is shown by fused organs in the *ath1-3* mutant (Gómez-Mena and Sablowski, 2008). *PNY* and *PNF* are expressed in an overlapping domain with *STM* (Long et al., 1996; Smith et al., 2004). The SAM in *pny pnf* mutants is narrower and prematurely terminates in a small majority of seedlings (Rutjens et al., 2009; Ung and Smith, 2011). Double mutants fail to elongate a stem or produce flowers (Smith et al., 2004). These defects are caused by misexpression of boundary genes including *ATH1* and *KNAT6* in the SAM. The activity of boundary genes in the SAM prevents the organized division of cells required for stem elongation and flower initiation (Andrés et al., 2015; Khan et al., 2015; Bencivenga et al., 2016). Deletion of boundary genes restores meristem function in *pny pnf* mutants (Khan et al., 2015). STM activity in *pny pnf* mutants must be maintained by additional BELL factors in the SAM whose identities are unknown.

1.8.3 Organ boundary genes

Class I KNOX proteins STM and BP move from cell to cell (Kim et al., 2003; Kim et al., 2005). This allows low levels of protein to accumulate in boundaries, contributing to their initiation, possibly by activating genes that confer boundary identity (Landrein et al., 2015; Balkunde et al., 2017). *CUP-SHAPED COTYLEDON1/2/3* genes belonging to a group of a NAC (NAM, ATAF, and CUC) domain-containing transcription factors confer boundary identity in plants (Maugarny et al., 2016). *BLADE-ON-PETIOLE1* and *2* (*BOP1/2*) are a second group of boundary genes that function in parallel with *CUC1/2/3* to pattern developmental boundaries (Khan et al., 2014).

BOP1/2 are closely-related transcriptional coactivator proteins characterized by BTB/POZ and ankyrin domains (Hepworth et al., 2005; Jun et al., 2010; Khan et al., 2015). BOP1/2 directly and indirectly promote the expression of boundary genes including *ATH1* and *KNAT6* to exert their functions (Khan et al., 2012a; Khan et al., 2012b; Khan et al., 2015). Loss-of-function *bop1 bop2* mutations disrupt the patterning of shoot organ boundaries resulting in pleiotropic defects (Figure 1.5B). Mutant characteristics include leafy petioles, defects in the initiation and patterning of floral meristems, and loss of floral-organ abscission (Hepworth et al., 2005; Norberg et al., 2005; McKim et al., 2008; Xu et al., 2010; Khan et al., 2014). Conversely, overexpression of *BOP1/2* impairs internode elongation, disrupts vascular patterning and promotes abundant lignification in the stem (Figure 1.5C) (Norberg et al., 2005; Khan et al., 2012b; Khan et al., 2015). These gain-of-function phenotypes of BOP1/2 rely on the downstream activities of interacting homeodomain transcription factors, ATH1 and KNAT6 (Rutjens et al., 2009; Li et al., 2012; Khan et al., 2012a; Khan et al., 2012b).

1.9 Vascular cambium

1.9.1 Stem

The Arabidopsis inflorescence stem is an established model for wood development (Barra-Jiménez and Ragni, 2017). During secondary growth, fascicular cambia extend and join into a continuous ring of meristematic tissue as it happens in trees. In Arabidopsis, this process is only completed at the base of the stem (Figure 1.6) (Nieminen et al., 2015; Barra-Jiménez and Ragni, 2017).

Class I KNOX homeoproteins STM and BP are important for internode elongation and vascular patterning during the reproductive phase (Long et al., 1996; Douglas et al., 2002; Venglat

et al., 2002; Smith and Hake, 2003; Kanrar et al., 2006; Sanchez et al., 2012). Their combined activities are proposed to control the timing of stem secondary growth and maintain the vascular cambium (Mele et al., 2003; Sanchez et al., 2012).

STM is expressed in the RZ of the SAM that gives rise to the stem and in vascular bundles, including procambium, xylem, and phloem (Figure 1.7) (Long et al., 1996; Sanchez et al., 2012). *BP* is also expressed in the RZ and in select stem tissues: phloem and cortex (Figure 1.7) (Lincoln et al., 1994; Douglas et al., 2002; Venglat et al., 2002; Smith and Hake, 2003). Surprisingly, *BP* does not express in the vascular (pro)cambium. An expression pattern outside of the cambial zone suggests roles for *STM* and *BP* beyond maintenance of stem cells in the procambium.

The role of STM in stem vascular patterning is difficult to assess, because knockouts terminate the SAM (Long et al., 1996). In *bp* mutants, cortex and epidermal patterning is disturbed and the organization and spacing of vascular bundles is irregular. Some bundles are underdeveloped, with xylem elements reduced or lacking in lignin (Douglas et al., 2002; Venglat et al., 2002; Mele et al., 2003; Smith and Hake, 2003). Cross-sections of the stem show gaps in the vascular ring and spatial defects in lignin deposition. Further, lignification of interfascicular fibers and xylem is accelerated compared to wild-type (Mele et al., 2003). Interestingly, all of these *bp* defects are the result of misexpression of boundary genes in the stem. Inactivation of BOP1/2 (Khan et al., 2012b) or BOP1/2 downstream effectors: KNAT6 and ATH1 or KNAT2 in combination with KNAT6 or ATH1 (Ragni et al., 2008; Li et al., 2012; Khan et al., 2012a) greatly restores internode elongation and normal vascular patterning in *bp* mutants (Ragni et al., 2008; Khan et al., 2012a; Khan et al., 2012b). Overexpression BOP1/2 mimics *bp* defects in stem patterning showing that spatial regulation of boundary genes in the stem is important for normal vascular development. Biochemical experiments showed that BP directly represses *KNAT2* and

KNAT6 (Zhao et al., 2015). PNY, which interacts with BP and partly mediates its activity, directly represses *BOP1/2*, *ATH1*, and *KNAT6* (Andrés et al., 2015; Bencivenga et al., 2016).

1.9.2 Hypocotyl

The vascular cambium in the hypocotyl is completed a few days after germination (Sibout et al. 2008). In contrast to the stem, both *STM* and *BP* are highly expressed in the cambial zone. In addition, these genes are expressed in secondary phloem and developing and mature secondary xylem, again suggesting a role outside their traditional role in the SAM (Liebsch et al., 2014). Loss-of-function *bp* and *stm bp* mutations had little effect on the size of the cambial zone. Rather, lignification of xylem fibers and vessels was reduced, resulting in a narrower hypocotyl (Liebsch et al., 2014). These defects were partially rescued by inactivation of *BOP1/2*, which are misexpressed in the cambial zone and developing xylem of *bp* and *stm bp* hypocotyls. These data point to contrasting roles for KNOX spatial regulation of boundary genes in vascular cambiums of the stem versus the hypocotyl.

1.9.3 Poplar tree

Populus trichocarpa (western balsam poplar) is as an excellent model system for the study of wood development. This diploid tree has a fully sequenced genome and is a dicot like Arabidopsis (Jansson and Douglas, 2007). Poplar was chosen for this role in part because it grows quickly and is easier to transform and propagate *in vitro* than most other trees (Ma et al., 2004).

Secondary growth in poplar proceeds similarly to the stem of Arabidopsis plants (Figure 1.5). The vascular tissue of primary growth is derived from procambium and arranged in bundles. Formation of the vascular cambium begins by internode 3 and is complete by about the internode

6. Substantial secondary phloem and secondary xylem with well-lignified vessels and fibers are present by internode 9 (Dharmaawardhana et al., 2010). Further, the arrangement and composition secondary vasculature in poplar tree compares well to cell types and morphologies found in the Arabidopsis inflorescence stem and root-hypocotyl (Barra-Jiménez and Ragni, 2017).

1.9.3.1. Class I KNOX genes

Populus contains two Class I KNOX genes, *ARBORKNOX1* (*PtrARK1*) and *ARBORKNOX2* (*PtrARK2*), which are the functional equivalents of Arabidopsis *STM* and *BP*, respectively (Groover et al., 2006; Du et al., 2009). *PtrARK1* expression in the stem is restricted to the vascular cambium. *PtrARK1* overexpression in poplar produces defects in internode elongation and stem thickness consistent with impairment of secondary growth. Stem cross-sections show delayed differentiation of secondary vascular cell relative to wild-type plants: the boundary between the cambium and the secondary xylem is uneven and disrupted, and there are fewer phloem fibers (Groover et al., 2006). *PtrARK2* expression is less restricted to the cambium, at least initially. Expression is also observed in developing secondary xylem and phloem fibers and in actively lignifying cell types. In more mature parts of the stem, *PtrARK2* expression becomes mainly localized to the cambium (Du et al., 2009). Transgenic plants overexpressing *PtrARK2* have a thicker cambium and extra secondary phloem is produced at the expense of phloem fibres and secondary xylem. Overall, there was a decrease in lignified cell types. In plants expressing an artificial miRNA that lowered the abundance of *PtrARK2* mRNA, differentiation of secondary xylem and phloem fibers was premature similar to the Arabidopsis *bp* mutant in inflorescence stem (Du et al., 2009).

1.9.3.2 Boundary genes in trees

How boundary genes function in trees in unknown. The poplar genome contains a pair of *BOP*-like genes designated as *PtrBPL1* (POPTR-0016s04010) and *PtrBPL2* (POPTR-006s04190). These proteins are 93% identical to each other at the amino acid level and have 81.8% amino acid sequence similarity to AtBOP1 and 83% amino acid sequence similarity to AtBOP2 (Devi, 2014). Preliminary experiments found that transcripts accumulate in stem vascular tissues consistent with a vascular cambium role. In addition, *PtrBPL1* and *PtrBPL2* when expressed in *bop1 bop2* plants under the control of a *BOP1* native promoter fully correct mutant phenotypic defects, suggesting they are functional equivalents of *AtBOP1/2* (Devi, 2014). To complete this study, my role was to investigate the effect of *PtrBPL1* and *PtrBPL2* gain-of-function in Arabidopsis plants. My additional role was to generate transgenic poplar plants to investigate the role of boundary genes in tree secondary growth.

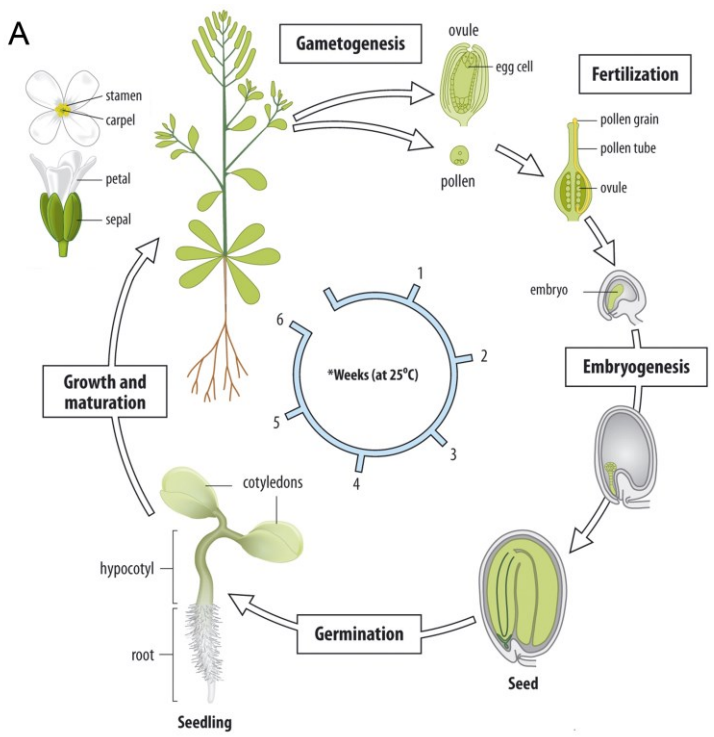
1.10 Thesis rationale and research questions

The Arabidopsis stem and root-hypocotyl are considered to be useful models for wood development in trees. These tissues are thought to have identical vascular cambia maintained by similar mechanisms as in the SAM (Figure 1.8). This assumption may be premature. Existing studies point to contrasting roles for boundary genes in the vascular cambium of Arabidopsis stem and hypocotyl. In the stem, where BOP1/2 are not normally expressed, misexpression causes gaps in the vascular ring and accelerates the formation of lignified secondary cell walls. In the hypocotyl, where BOP1/2 are expressed in secondary phloem, misexpression blocks the formation of xylem fibers. My thesis partially addresses two questions:

1. Which of these contrasting models applies to the vascular cambium of root?
2. Which model best describes the vascular cambium of trees?

Figure 1.1 *Arabidopsis thaliana* life cycle and plant architecture

(A) Plant development begins at fertilization and contains three phases: embryonic, vegetative, and reproductive. Arabidopsis embryos have a basic set of body parts: two leaves (cotyledons), a hypocotyl (stem) and a root. Post-embryonic growth is supported apical meristems formed at the shoot and root tips. (B) During vegetative growth, the plant forms a basal rosette of leaves. Stem elongation and axillary meristem outgrowth are repressed during the vegetative phase. During the reproductive phase, the plant switches to a new pattern of development. During first phase of reproductive growth, leaf development is partially repressed and branches are formed. During the second phase of reproductive growth, leaf development is fully repressed and flowers are formed. Internodes are elongated during both phases of reproductive growth so that branches and flowers are evenly dispersed along the primary axis. When vertical growth completes, the plant switches to secondary growth resulting in the radial thickening of roots and shoots. Figure adapted from Wolpert et al. (2015) and Devi (2014).


A
Gametogenesis
ovule
egg cell
pollen
Fertilization
pollen grain
pollen tube
ovule
stamen
carpel
petal
sepal
embryo
Embryogenesis
*Weeks (at 25°C)
1
2
3
4
5
6
Growth and maturation
cotyledons
hypocotyl
root
Seedling
Germination
Seed

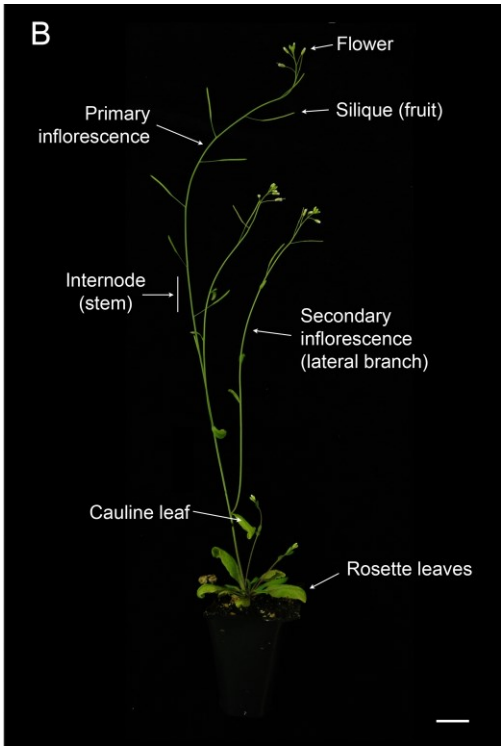
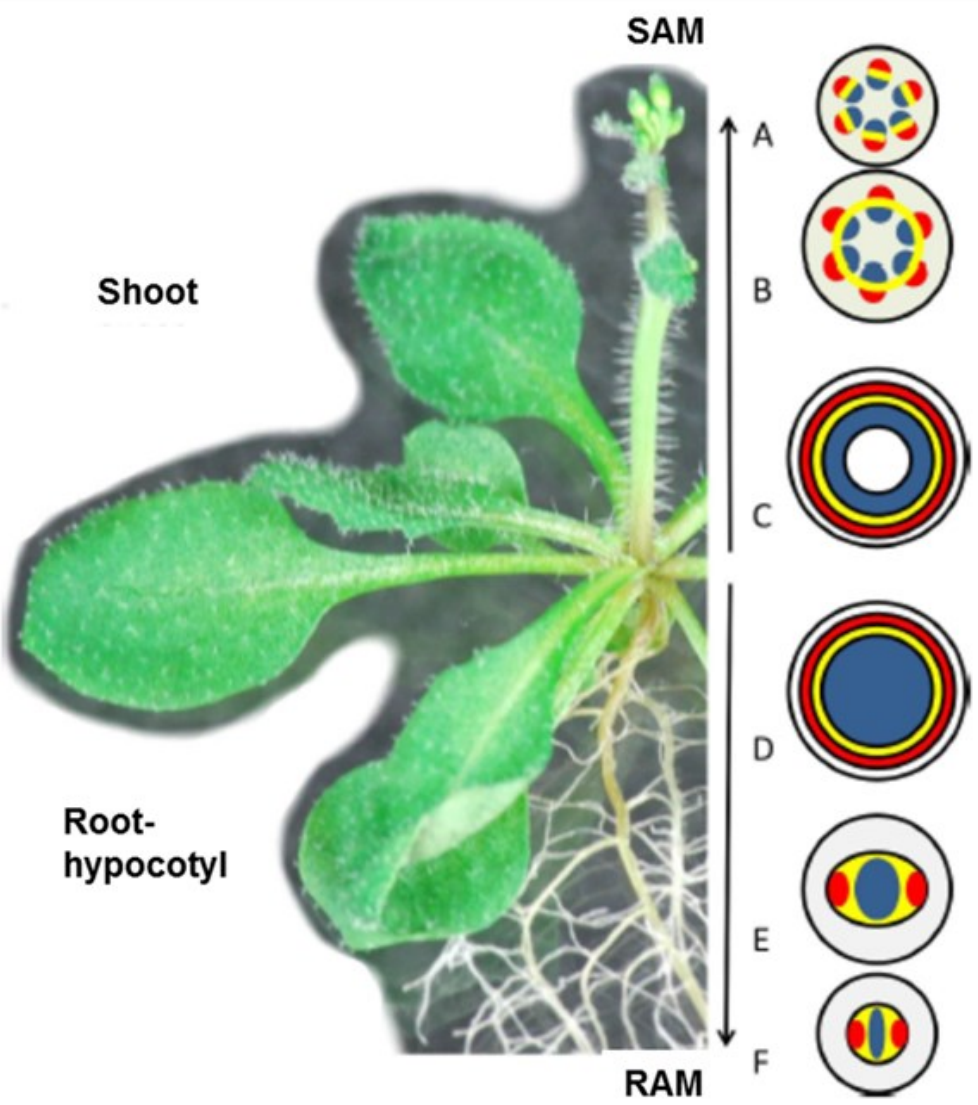

B
Flower
Silique (fruit)
Primary inflorescence
Internode (stem)
Secondary inflorescence (lateral branch)
Cauline leaf
Rosette leaves

Figure 1.2 Meristems and vascular organization in *Arabidopsis thaliana*

Primary meristems called the shoot apical meristem (SAM) and the root apical meristem (RAM) generate above and below ground parts of the plant. These meristems are the source of vascular meristems called procambium that provide the primary vascular tissues: xylem and phloem. During secondary growth, roots and shoots thicken. This requires the formation of a circular lateral meristem called the vascular cambium. (A-C) Stem. (D-F) Root-hypocotyl. (C-D) A continuous vascular cambium forms in both structures but the process is slightly different based on different organization of the primary vasculature. In stems, individual vascular bundles are arranged in a ring. In the root and hypocotyl, the vasculature is centralized. Yellow, procambium-cambium; red, phloem; blue, xylem. SAM, shoot apical meristem; RAM, root apical meristem. Image from: Nieminen et al., 2015.


SAM
Shoot
Root-
hypocotyl
RAM
A
B
C
D
E
F

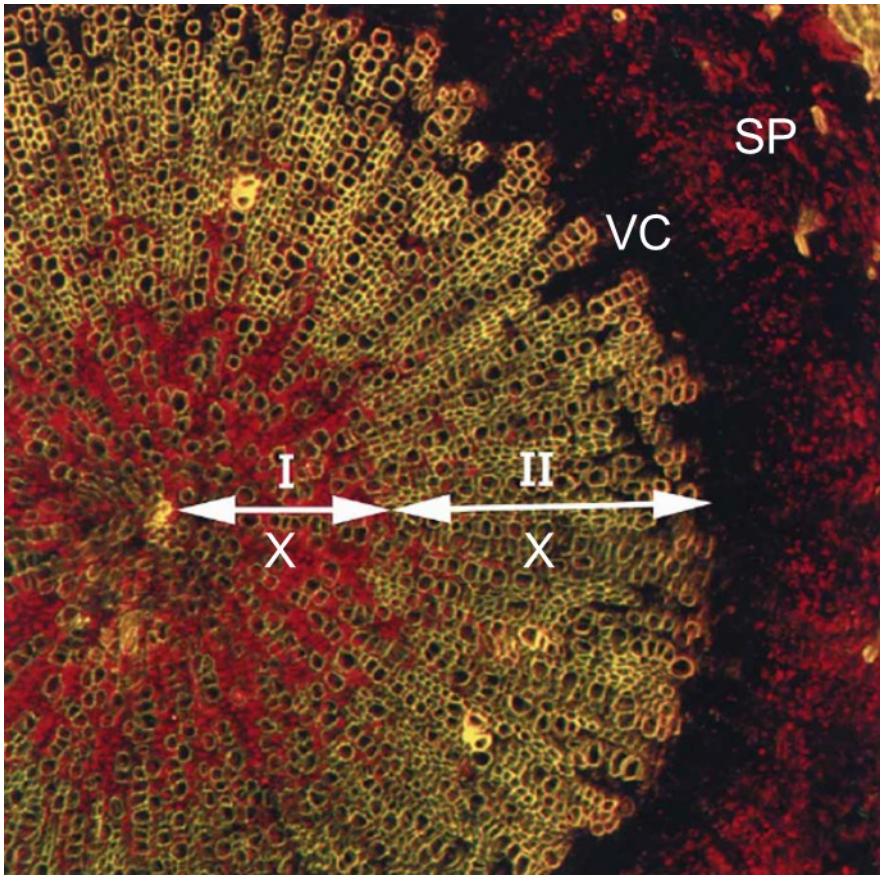


Figure 1.3 Cross-section of the Arabidopsis hypocotyl viewed by confocal microscopy

The first phase of secondary xylem development is characterized by the formation of xylem vessels (yellow, thick-wall tubes) and xylem parenchyma (red, non-lignified). Lignified xylem vessels and fibres (yellow) are formed during the second phase of xylem development. I, phase I; II, phase II; X, xylem; VC, vascular cambium, SP, secondary phloem. Image from Chaffey et al. (2002).

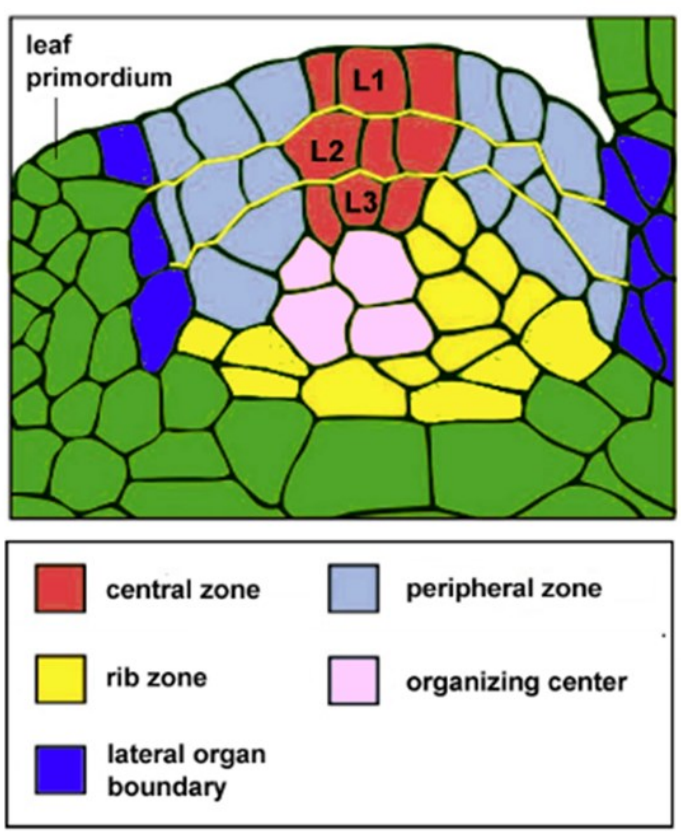


Figure 1.4 Organization of the SAM

The SAM in *Arabidopsis thaliana* contains three histologically distinct cell layers. Cells in the epidermal (L1) and subepidermal (L2) layers divide anticlinally (←→). By contrast, cells in the interior (L3) layer divide in all planes. Superimposed on these layers are three functional domains: the central zone (red), peripheral zone (light blue), and rib zone (yellow). The central zone (CZ) contains undifferentiated stem cells. Organogenesis takes place in the peripheral zone (PZ). Low-growth regions called lateral organ boundaries (dark blue) separate emerging organs from meristem. The rib zone (RZ) provides cells for upward growth of stem. The RZ also contains an organizing center (OC) that maintains the CZ. Figure modified from Wolpert et al. (2015).

Figure 1.5 *BLADE-ON-PETIOLE* loss and gain-of-function phenotypes

(A) *BOP1/2* are expressed in organ boundaries originating in the shoot apex are pleiotropic regulators of plant architecture. Boundaries are low-growth domains that separate the meristem from emerging lateral organs. Subsequently, boundaries extend to encircle the base of shoot organs serving as an attachment point to the plant body. Boundaries are the source of axillary meristems that provide lateral branches and flowers. Boundaries are also specialized zones for dehiscence (opening of anthers or seed pods) or abscission (regulated detachment of plant organs). Images from Hepworth and Pautot (2015) and Wolpert et al. (2015). (B) Loss-of-function *bop1 bop2* mutant phenotypes. From left to right: leafy petioles, defects in the initiation and patterning of flower meristems, organ fusions, and loss of floral organ abscission. Images from Hepworth et al., 2005; Khan et al., 2014.

(C) Gain-of-function *BOP1/2* phenotypes. Overexpression of *BOP1* or *BOP2* from a constitutive promoter inhibits vertical growth and accelerates stem secondary growth. Lignin (pink-stained by phloroglucinol) deposition is accelerated relative to wild-type stems with occasional gaps in the vascular ring. The vascular ring is thicker/denser and phloem fibres are lignified (arrows) compared to wild-type. Images from Khan et al., 2012b and Devi (2014).

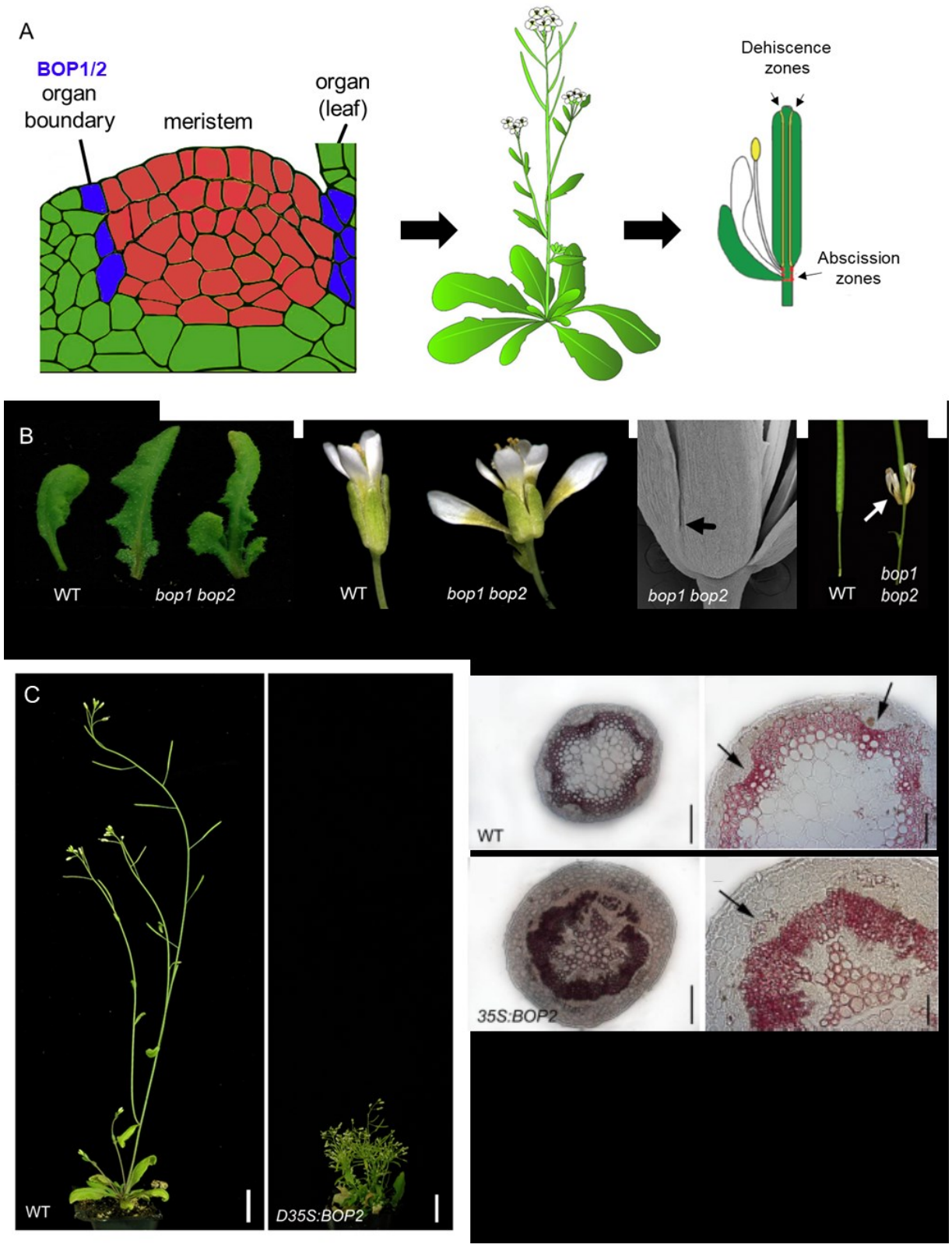
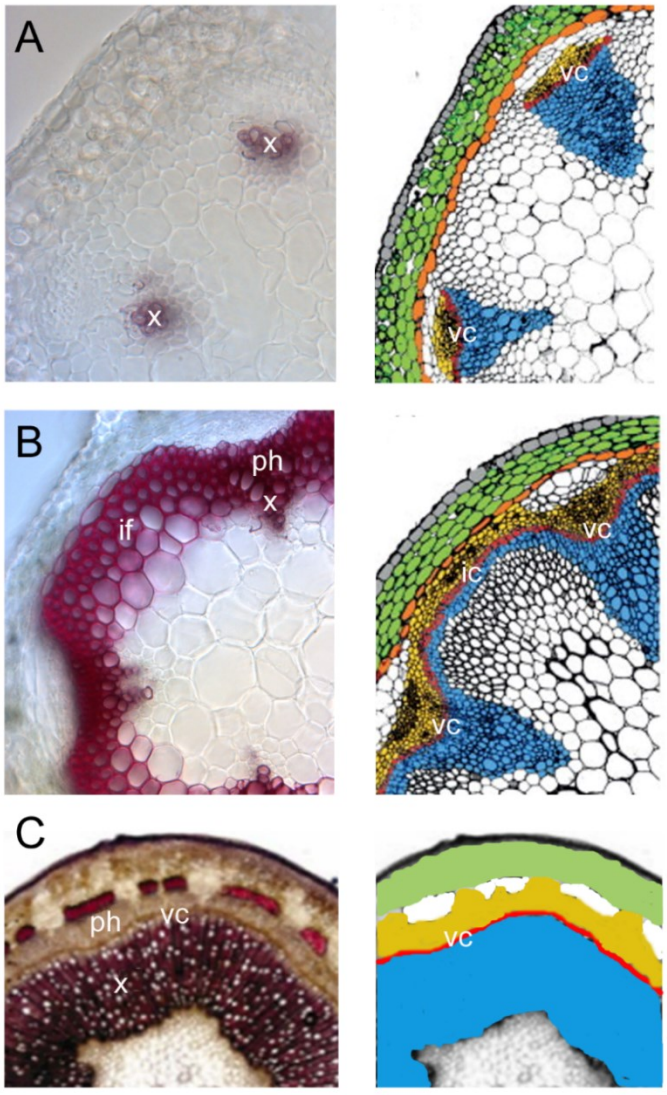

A
BOP1/2
organ
boundary
meristem
organ
(leaf)
Dehiscence
zones
Abscission
zones
B
WT
bop1 bop2
WT
bop1 bop2
bop1 bop2
WT
bop1
bop2
C
WT
D35S:BOP2
WT
35S:BOP2

Figure 1.6 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. Images based on Sanchez et al. (2012). (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 fibers 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 (Barakat et al., 2009). Xylem (primary and secondary) and phloem cap fibers are lignified. Vascular cambium is continuous. x, xylem; ph, phloem; if, interfascicular fibers; vc, vascular (fascicular) cambium; ic, intrafascicular cambium. Figure courtesy of Devi (2014).


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

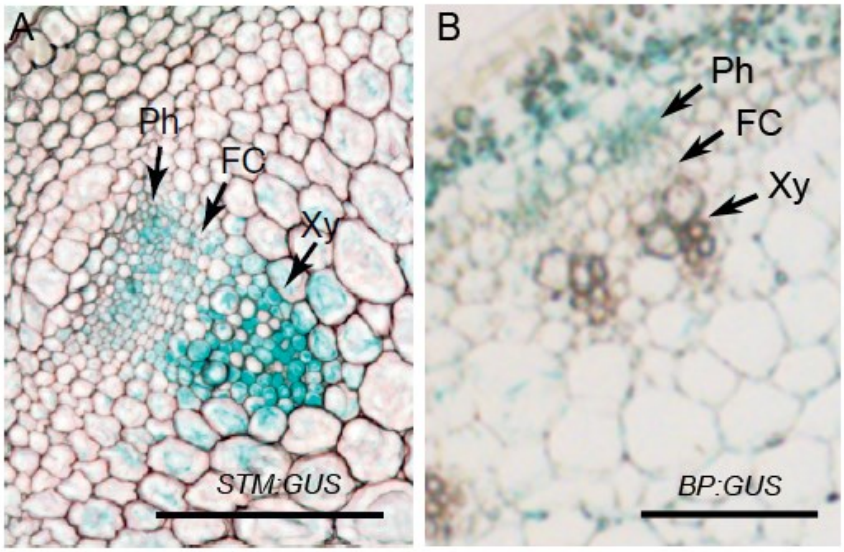


Figure 1.7 *STM* and *BP* expression pattern in stem primary vasculature

(A) *STM:GUS* reporter gene, showing expression of *STM* in the fascicular cambium, xylem, and phloem. From: Sanchez et al. (2012). (B) *BP:GUS* reporter gene, showing expression in the stem cortex and phloem. Scale bars, 100 µm.

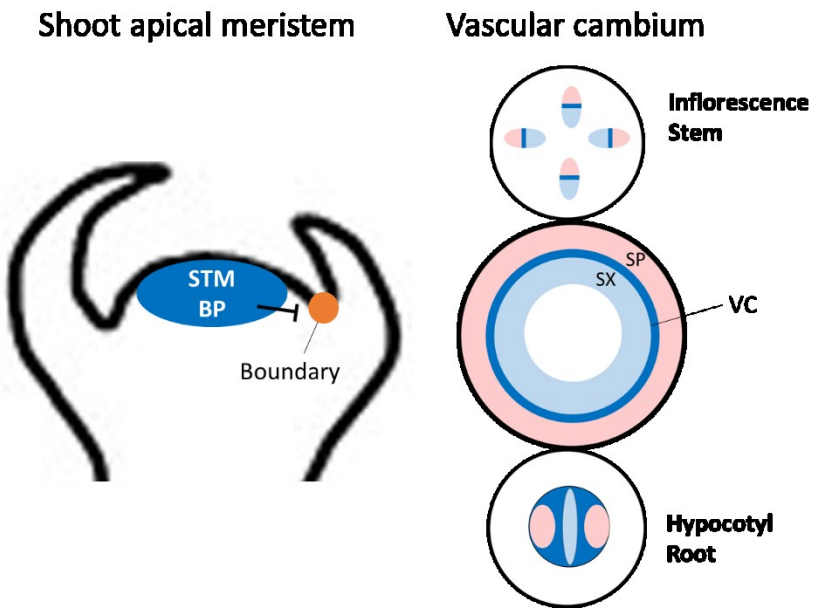


Figure 1.8 Models and research questions

The Arabidopsis stem, hypocotyl and root undergo secondary growth and are considered as good models for understanding wood development in trees. These tissues are thought to have identical vascular cambia maintained by similar mechanisms as in the SAM (Figure 1.8). This assumption may be premature. Existing studies point to contrasting roles for boundary genes in the vascular cambium of Arabidopsis stem and hypocotyl. In the stem, *KNOX* genes spatially regulate boundary genes to inhibit lignification of the vascular ring. In the hypocotyl, *KNOX* genes spatially regulate boundary genes to promote xylem fiber development. Which of these contrasting models applies to the vascular cambium of root and which model best describes the vascular cambium of trees? STM, SHOOT MERISTEMLESS; BP, BREVIPEDICELLUS; VC, vascular cambium; SX, secondary xylem; SP, secondary phloem.

Chapter 2: MATERIALS AND METHODS

2.1 Arabidopsis plant material and growth conditions

The *Arabidopsis thaliana* accession Columbia (Col-0) was used as wild-type. Plants were grown *in vitro* or on soil in growth chambers at 21°C under continuous light (24 h light, intensity 100 μmol m^{-2} s^{-1}) under long day (16h light/8h dark) photoperiods. Mutants *bop1-3 bop2-1* (Hepworth et al., 2005), *bp-2* backcrossed into a Col-0 background (Venglat et al., 2002), *knat6-2* (Belles-Boix et al., 2006) and *ath1-3* (Gómez-Mena and Sablowski, 2008) have been previously described. The strong activation-tagged overexpression line *bop1-6D* used in this study has also been described (Norberg et al., 2005). Reporter lines *BOP1:GUS* (McKim et al., 2008), *BOP2:GUS* (Xu et al., 2010), *BP:GUS* (Venglat et al., 2002), *KNAT6:GUS* (Belles-Boix et al., 2006) and *ATH1:GUS* (Proveniers et al., 2007) have also been previously described. Double or triple mutant combinations used in this study were constructed by crossing as described (Woerlen et al., 2017).

Seeds were surface-sterilized using anhydrous ethanol followed a solution of 0.5% (w/v) sodium dodecyl sulfate (SDS) and 5% sodium hypochlorite bleach. Sterilized seeds were subsequently rinsed four times with sterile distilled deionized water (ddH_2O) and sown on bacteriology plates containing *Arabidopsis thaliana* minimal medium (Haughn and Somerville, 1986). The plated seeds were then incubated for 2-3 days at 4°C in the dark to break dormancy, and then transferred to a growth chamber. When seedlings were 7-10 days old they were transplanted to steam-sterilized soil (ProMix BX, Premier Horticulture, Rivière-du-Loup, QC) supplemented with a 1 gram per liter solution of 20-20-20 plant fertilizer (Plant-Prod Inc., Brampton, ON) contained in 72-well trays or 3.5-inch square pots as appropriate. Plants were

grown to maturity in growth chambers at 21°C under continuous light (~100 μmol m^{-2} sec^{-1}) and supplemented with water and fertilizer as required.

2.2 Analysis of secondary growth

Inflorescence stem material was harvested from five-week-old plants grown on soil under long days. Sections were hand-cut from the base of the primary inflorescence stem using a razor blade (Khan et al., 2012b). Alternatively, the lower 1 cm of stem at the base of the primary inflorescence was fixed and embedded in wax for sectioning using a microtome.

Root and hypocotyl samples were harvested from seven-week-old plants grown on soil under long days. Roots were rinsed in distilled water to remove soil. Under a dissecting microscope, adventitious and lateral roots were removed using a razor blade. The apical 3 mm length piece of stem directly below the rosette leaves containing the hypocotyl (plus the first mm of secondary root) was harvested for analysis of secondary growth in the hypocotyl (Liebsch et al., 2014). An approximately one cm length piece of root directly below was harvested for analysis of secondary growth in the taproot (Woerlen et al., 2017). The tissue was fixed and embedded in wax for sectioning using a microtome.

2.3 Embedding and sectioning

Tissues were fixed by submerging in a formaldehyde-acetic-acid-alcohol (FAA: 50% pure ethanol, 5 % glacial acetic acid, 3.7% formaldehyde 41.3 % H_2O) solution for 3-4.5 hours. Samples were placed under vacuum for 15 minutes and released to remove air bubbles and to promote penetration of the fixative into the tissue. Fixation was followed by dehydration using an ethanol series. Tissue was incubated twice in 50% ethanol for 30 minutes, 60% ethanol for 30 minutes, and 70 % ethanol for 30 minutes followed by storage at 4°C until further use. To continue tissue

processing, samples were transferred to 85% ethanol for 30 minutes, followed by incubation in 95% ethanol overnight with a few drops of 1% Eosin added to stain the tissue red. The next day, the tissues were cleared by incubating in 100% ethanol for 60 minutes, followed by two changes of 100% ethanol for 30 minutes each. This was followed by a xylene series: 25% xylene:75% ethanol for 30 minutes, 50% xylene:50% ethanol for 30 minutes, 75% xylene:25% ethanol for 30 minutes, then two times 100% xylene for 30 minutes. To slowly permeate the tissue with paraffin wax (Paraplast Plus, Sigma-Aldrich, St. Louis, Missouri, USA) samples were incubated in a mixture of 50% xylene: 50% melted wax overnight at 60°C. To melt the wax, paraffin chips were placed in a large glass bottle and incubated overnight at 60°C. Six wax changes were performed over three days, spaced at least 6 hours apart. At the end, samples in liquid wax were poured into petri plates. Once solidified, the molds were stored at 4°C until sectioning.

Blocks of embedded tissue were mounted on wooden stubs. A manual rotary microtome (HM325, Microm International, Waldorff, Germany) was used cut tissue sections (25 μm thick). Sections were bound to Superfrost Plus microscope slides (Fisher Scientific, Toronto, Ontario, Canada) by incubating overnight at 42°C on a slide warmer (Model 77, Fisher Scientific, Toronto, Ontario, Canada).

2.4 Histochemical analyses

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 (in 95% ethanol) solution for 5 min. Subsequently, five drops of concentrated hydrochloric acid were added. Two minutes were allowed for color development. Samples were transferred to a glass slide and a cover slip was added. Images were

immediately collected using a stereomicroscope (SteRIO Discovery V20 equipped with an Axiocam digital camera (Carl Zeiss, North York, Ontario, Canada).

For the analysis of lignin deposition in fixed tissues, sections mounted on glass slides were dewaxed by incubating 100% xylenes for 20 minutes at room temperature. Samples were subsequently incubated in 100% ethanol and 95% ethanol for 30 minutes each. Once ready to take pictures, 95% ethanol was drained off the slides and replaced with a 2% phloroglucinol solution for ten minutes at room temperature. Drops of concentrated hydrochloric acid were added over the entire surface of the slide until pink colour development was visible. A cover slip was added and images were immediately collected using a stereomicroscope for low-resolution images (as above) or a compound microscope (Axioimager M2, Carl Zeiss, North York, Ontario, Canada) for high-resolution images.

Toluidine blue staining was carried out as previously described (O'Brien et al., 1964). Tissue sections mounted on glass slides were dewaxed as above. After that, tissue was rehydrated by dipping in 100%, 95%, 85%, 70%, 50% and 30% ethanol solutions and finally pure water for 3 min each. At the end, slides were incubated in staining solution (0.05% toluidine blue O (w/v) in benzoate buffer, pH 4.4) for approximately 45 seconds. Slides were transferred to distilled water to remove the excess stain. A cover slip was placed on the top and pictures were immediately acquired using a stereomicroscope (for low resolution images) or a compound microscope (for high resolution images).

2.5 β-Glucuronidase (GUS) staining assay

Staining for β-glucuronidase activity in Arabidopsis plants was carried out as previously described (Khan et al., 2012b) with small changes. The tissue was placed in chilled 90% acetone and kept on ice during sample collection. Samples were then placed at room temperature for 15

minutes to complete the fixation. The acetone was removed and replaced with a GUS staining solution that contained 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ and 2 mM of 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide (X-Gluc). Samples were incubated at 37°C for 3 to 24 hours until a localized blue precipitate was visible.

The staining solution was removed, and samples were processed for sectioning as described above with minor changes. Samples were dehydrated in 30% and 50% ethanol, fixed in FAA for 2-3 hours, and then transferred to 70% ethanol. *tert*-butanol was used instead of xylenes during processing to avoid dissolving the blue precipitate. For dewaxing, slides were incubated in *tert*-butanol for 45 minutes at 60°C with occasional shaking. Slides were dipped in 100% xylene for 1-3 minutes at the end to remove residual traces of wax. After that, the tissue was rehydrated by dipping slides in 100%, 95%, 85%, 70%, 50% and 30% ethanol solutions and then distilled water for 3 min each. At the end, sections were mounted in 50% glycerol and a cover slip was added. Images were collected using a compound microscope.

2.6 Poplar plant materials and growth conditions

Hybrid poplar clone INRA 717-IB4 (*Populus tremula* x *Populus alba*) was used as wild-type. This female poplar clone was kindly provided by Dr. Sharon Regan. For transformation and micro propagation, plants were grown in *vitro* and then on soil in growth chambers at 21°C under continuous light (intensity 100 μmol m^{-2} s^{-1}). After six months, poplars were transferred to six-inch pots containing soil and grown in the greenhouse under ambient temperature and natural light.

2.7 Constructs for transformation of poplar

Constructs for transformation of poplar were as previously described (Table 2.1). The reporter gene plasmid pBOP1:GUS uses kanamycin as a selectable marker in plants (McKim et al., 2008). The reporter gene plasmid pBOP2:GUS uses phosphinothricin as a selectable marker

in plants (Xu et al., 2010). Constructs for overexpression *D35S:PtrBPL1* and *D35S:PtrBPL2* use hygromycin as a selectable marker in plants (Devi, 2014).

Our construct for RNAi-mediated knock down of *PtrBPL1/2* expression in transgenic poplar has also been previously described (Devi, 2014). An RNA-hairpin cassette was designed to co-silence *PtrBPL1* and *PtrBPL2*. This construct is based on a highly conserved 359-base pair sequence in *PtrBPL1* and *PtrBPL2* that encodes part of the BTB/POZ domain. The hairpin cassette was created in pHannibal (Wesley et al., 2001) and composed of sense and antisense *BPL1* sequences under the control of a 35S promoter (Devi, 2014). The resulting cassette was verified by sequencing. Digestion of the plasmid with *Not*I was used to release a 3.6-kb fragment that was the cloned into the corresponding site of binary vector pART27 (Gleave, 1992) to create the final construct. Primers for sequencing are listed in Table 2.2.

2.8 *Agrobacterium*-mediated transformation of *Populus trichocarpa*

Transformation of poplar was carried out essentially as described (Meilan and Ma, 2006) with minor adjustments. *Agrobacterium tumefaciens* strain C58C1 GV3101 pMP90 was used for transformations (Koncz and Schell, 1986). Young leaves from growth chamber plants were cut into small pieces (≈ 0.5 -1.0 cm x 0.5 -1.0 cm) and then surface-sterilized with 70% (v/v) ethanol for 1 minute, rinsed in sterile purified water, treated with 10% (v/v) sodium hypochlorite bleach solution for 3 minutes, followed by three rinses in sterile purified water for 3 minutes each. For each construct, approximately 10 leaf discs were used per plate x 30 plates for a total of 300. Immediately after, the explants were pre cultured on Murashige and Skoog (MS) modified basal medium with Gamborg vitamins supplemented with 30 g/L (w/v) sucrose, 0.20 g/L (w/v) L-glutamine, 0.25 g/L (w/v) 2-(*N*-morpholino) ethanesulfonic acid (MES), 10 µM 1-naphthaleneacetic acid (NAA), and 5 µM 6-(γ, γ-dimethylallylamino) purine (2ip) and 3 g/L (w/v)

Gellan gum as the solidifying agent at pH 5.8. The explants were subsequently incubated at 22°C in the dark for 2 days. *Agrobacterium tumefaciens* strains harboring constructs were streaked onto Lysogeny Broth (LB) medium plates containing 15 g/L of agar as the solidifying agent and supplemented with 100 mg/L rifampicin, 40 mg/L gentamycin, and the appropriate antibiotics for each construct: 50 mg/L kanamycin for *D35S:BPL1*, *D35SS:BPL2*, and *BOP1:GUS*, 25 mg/L kanamycin and 5 mg/L tetracycline for *BOP2:GUS*, and 80 mg/L spectinomycin for *35S:RNAiPtrBPL1/2* were used. Plates were then inverted and incubated at 28°C for 2-3 days. A single isolated colony was picked and inoculated into 5mL LB liquid medium (pH 7.0) supplemented with the same appropriate antibiotics as above. The cultures were grown at 30°C with continuous shaking (250 rpm) for 2 days. The *Agrobacterium* cultures were then sub cultured by making a dilution of 1:100 into 5 mL of LB pH 5.4 supplemented with the same antibiotics as before except eliminating the rifampicin and adding 100 μM of acetosyringone. The new culture was incubated overnight at 28°C with shaking to grow the *Agrobacterium* until an ideal OD 600 of 0.7 was reached (Movahedi et al., 2014). The pre-cultured explants were submerged in the *Agrobacterium*–infective suspension and incubated for 1 hr. Excess *Agrobacteria* were immediately removed. Petri plates containing the infected leaves were sealed with surgical tape, covered in silver foil, and co-cultivated for 3 days in the dark at 22°C. After this interval, the infected leaf discs were carefully transferred to 50 ml Falcon tubes containing 40 mL sterile purified water. The explants were rinsed 5 times with sterile purified water to remove the excess *Agrobacteria.* The explants were lastly washed in 35 mL of sterile distilled water with addition of 300 mg/L of cefotaxime and 200 mg/L of timentin for 1 hr with shaking to kill any excess *Agrobacteria.* The wounded leaf discs were blotted dry on sterile filter papers to remove the surface water. The putative transformed explants were transferred to MS callus induction medium

(CIM) supplemented with 300 mg/L cefotaxime, 200 mg/L timentin, and suitable selection agent: 10 mg/L kanamycin, 5 mg/L hygromycin, or 1 mg/L phosphinothricin. To rescue leaf disks from severe *Agrobacterium* attack, they were observed every day. When bacterial re-growth contamination occurred, the leaf disks were immediately sub cultured to fresh medium. Every two weeks, the explants were sub cultured to a fresh CIM medium. Under ideal conditions, callus formed after 14-21 days. After the selected calluses were formed, they were sub cultured to MS shoot induction medium (SIM) supplemented with 30g/L (w/v) sucrose, 0.20 g/L (w/v) L-glutamine, 0.25 g/L (w/v) MES, 0.05 mg/L thiadiazuron (TDZ), 300 mg/L cefotaxime and 200 mg/L timentin at pH 5.8 and an increased concentration of the appropriate selection agent: 25 mg/L for kanamycin. However, this was not possible for experiments using phosphinothricin or hygromycin as the selecting agent since one or no transformed calli were produced, respectively. To induce shoot regeneration, the calli were subsequently incubated in growth chambers at 21°C under continuous light (intensity 100 µmol m^{-2} s^{-1}). Subculture was carried out every 2-3 weeks to a fresh medium. After the selected shoots reached approximately 1 cm with visible leaflets, they were transferred onto shoot elongation media (SEM). MS shoot elongation medium was supplemented with 30 g/L (w/v) sucrose, 0.20 g/L (w/v) L-glutamine, 0.25 g/L (w/v) MES, 0.05 mg/L 6-benzylaminopurine (BAP), 300 mg/L cefotaxime, 200 mg/L timentin, and a selecting agent (25 mg/L) kanamycin adjusted to pH 5.8. When elongated shoots were about 2 cm, they were individually separated and placed onto ½ MS root induction medium (RIM). The root induction medium was supplemented with 20 g/L (w/v) sucrose, 0.20 g/L (w/v) L-glutamine, 0.25 MES g/L (w/v), indole-3-butyric acid (IBA) 0.1 mg/L, 100 mg/L timentin and selecting agent (25mg/L kanamycin) at pH 5.8. Shoots were sub cultured every 2 weeks to a fresh medium. When the roots were well-developed, plants were transferred to soil and grown in growth chambers.

When plants were about six months old, they were moved to the greenhouse for further analysis. Transformation efficiencies were calculated using the following equation: (number of calli per total number of the explants) X 100.

2.9 Genomic DNA extraction from poplar

Genomic DNA for PCR-based genotyping was extracted from young poplar leaves using a CTAB (cetyl trimethylammonium bromide) method as previously described (Fan et al., 2015). Briefly, ~0.1 g of fresh tissue was ground in liquid nitrogen using a sterilized mortar and pestle. The resulting powder was transferred to a 1.5 ml microcentrifuge tube and 400 µl of pre-heated CTAB buffer [2% CTAB (w/v), 1.4 M NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA), and 100 mM Tris-Cl (pH 8.0)] was added to each sample. Samples were gently mixed by inversion. After incubating for 30 minutes at 65°C, 200 µl of chloroform was added. Samples were mixed by gentle inversion and incubated at room temperature for 10 minutes. After centrifugation at 16,000g for 5 minutes, the supernatant was transferred into a new tube, mixed with 300 µl of isopropanol, and incubated for 30 minutes at 4°C. Finally, the sample was centrifuged at 16,000 g for 5 minutes to collect the precipitated genomic DNA. The supernatant was removed and the pellet at the bottom of the tube was washed in 70% ethanol and dissolved in 100 µl of ddH_2O. DNA samples were stored at -20°C until further use.

2.10 Poplar genotyping

Genomic DNA from poplar was tested for integrity by PCR-amplifying a 100-bp fragment corresponding to housekeeping gene *UBIQUITIN* (Brunner et al., 2004) using primers UBQ-RT-F- 5′and UBQ-RT-R with an annealing temperature of 58ºC. The *AtBOP1:GUS* transgene was detected by PCR-amplifying a 750-bp fragment of DNA spanning the junction

between the *AtBOP1* promoter and the *GUS* reporter gene using primers P4H-F6 and GUS-1R and an annealing temperature of 56ºC. The *BOP2:GUS* transgene was detected by PCR-amplifying a 780-bp fragment of DNA spanning the junction between the *AtBOP2* promoter and the *GUS* reporter gene using primers P5H-F6 and GUS-1R and an annealing temperature of 56°C. The RNAi transgene was detected by PCR-amplifying a 923-bp fragment of DNA corresponding to part of the RNA hairpin using primers 35S-F1-Hannibal and Hannibal-Int-R and an annealing temperature of 55°C. The *BOP1:GUS* and *RNAi* constructs were also detected by PCR-amplifying a 300-bp fragment of DNA corresponding to part of the coding region of the *NPTII* gene which confers kanamycin resistance using primers NPTII-F and NPTII-R and an annealing temperature of 58ºC. See Table 2.2 for primer sequences.

2.11 Poplar GUS staining

Poplar tissue was stained for GUS according to two methods (Khan et al., 2012b; Chen et al., 2013). A third method for GUS tissue staining was also used (Jefferson et al., 1987) with some minor changes. Briefly, the samples were collected at room temperature and fixed in 0.3% formaldehyde in 10 mM MES pH 5.6 and 0.3 M mannitol for 45 minutes. The MES solution was removed and tissues were subsequently washed several times in 50 mM Na_2PO4, pH 7.0. All fixed tissues were transferred to GUS solution that contained 1 mM of X-Gluc in 50 mM Na_2PO4 (pH 7.0) and incubated for 20 minutes to several hours until observing localized blue precipitate. After staining, tissues were rinsed in 70% ethanol several times to remove chlorophyll. In each case, Arabidopsis control lines showed blue staining and poplar transgenics did not.

Table 2.1 List of constructs

Construct	Description
pBOP1:GUS	This plasmid contains ~4000 base-pairs of nucleotide sequence corresponding to the 5' promoter region of *AtBOP1* up to an including the start codon cloned in front a GUS reporter gene present in pBI101.1. The selectable marker in plants is kanamycin (McKim et al., 2008).
pBOP2:GUS	This plasmid contains 4015 base-pairs of nucleotide sequence corresponding to the 5' promoter region of *AtBOP2* up to and including the start codon plus the first 16 nucleotides of coding sequence cloned as a translational fusion with the GUS reporter gene present in pGreen 0229. The selectable marker in plants is phospinothricin (Xu et al., 2010).
pPtrBPL-RNAi	This plasmid contains an RNA-hairpin cassette based on a highly conserved 359 base-pair nucleotide sequence from the BTB/POZ domain of *PtrBPL1* and *PtrBPL2* cloned behind a universal *35S* promoter. The cassette was created in pHannibal and transferred to the binary vector pART27. The selectable marker in plants is kanamycin (Devi, 2014).
pD35S: BPL1	This plasmid contains a 1332 base-pair nucleotide sequence corresponding to the *PtrPBL1* coding sequence cloned behind a universal double 35S CaMV promoter (D35S) present a pSM-3, a pCAMBIA1390-based binary Gateway destination vector. The selectable marker in plants is hygromycin (Unda et al., 2017).
pD35S: BPL2	This plasmid contains a 1446 base-pair nucleotide sequence corresponding to the *PtrPBL2* coding sequence cloned behind a universal double 35S CaMV promoter (D35S) present a pSM-3, a pCAMBIA1390-based binary Gateway destination vector. The selectable marker in plants is hygromycin (Unda et al., 2017).

Table 2.2 List of primers

Description	**Primer**	**Sequence 5'- 3'**	**Reference**
RNAi sequencing 1st round	35S-F1-Hannibal pHannibal-Int-R	GCTCCTACAAATGCCATCATTGCGA CAAACCAGCTAGAATTACTATTATG	Bhaswati Devi
RNAi sequencing 2nd round	35S-F1-Hannibal OCS-Term-R	GCTCCTACAAATGCCATCATTGCGA GATCTGAGCTACACATGCTCAGGT	Bhaswati Devi
D35S:BPL1 PCR colony screening	Pt16s04010cdsF BPL1 CDS 360 R2	ATGACTCTTGAAGACTCTCTAAGAACTCTG CACGCCACAATTAGGCCTTGG	Bhaswati Devi
D35S:BPL2 PCR colony screening	Pt6s04190cdsF BPL2 CDS 360 R2	ATGACTCTTGAAGACTCTCTAAGATCTCTA CTCACCACAATTAGGCCTTGGC	Bhaswati Devi
Poplar genomic DNA integrity	UBQ–RT-F UBQ-RT-R	GTTGATTTTTGCTGGGAAGC GATCTTGGCCTTCACGTTGT	This study
BOP1:GUS genotyping in poplar	P4H-F6 GUS-1R	TAAGTGTTACGTGACACACG CCACCAACGCTGATCAATTCCACAG	McKim et al., 2008
BOP2: GUS genotyping in poplar	P5H-F6 GUS-1R	CATATCCCTCGTTTGTTTGG CCACCAACGCTGATCAATTCCACAG	Xu et al., 2010
RNAi genotyping in poplar	pHannibal-Int-F pHannibal-Int-R	CATAATAGTAATTCTAGCTGGTTTG CAAACCAGCTAGAATTACTATTATG	This study
NPTII genotyping in poplar	NPTII–F NPTII-R	GAGCACGTACTCGGATGGAAG GTAAAGCACGAGGAAGCGGTC	Ying Wang

Chapter 3: RESULTS

3.1 A gradient of secondary growth in Arabidopsis taproots

The role of boundary genes in root secondary growth has not been investigated. Abundant secondary growth occurs the Arabidopsis tap root, which encompasses the upper first centimeter of the primary root. Thickening of roots is presumed to follow the same principles as in hypocotyl because the primary anatomy of vasculature is similar (Dolan et al., 1993; Buss and Evet 1999; Chaffey et al., 2002). Cellular organization during secondary growth of the root is depicted in Figure 3.1A. Two phases of secondary growth are distinguished. During phase I, production of xylem and phloem is proportional (equal). The xylem produced in this phase is xylem I, which consist of xylem parenchyma interspaced with lignified vessels. The transition to flowering triggers a switch to phase II in which xylem production expands relative to phloem (Chaffey et al. 2002; Sibout et al. 2008). During phase II, xylem parenchyma cells differentiate to form lignified fibres (Chaffey et al., 2002; Sibout et al., 2008). Xylem II is seen as a dense ring of lignified fibers interspersed with vessels (Chaffey et al., 2002; Ragni and Hardtke, 2014; Nieminen et al., 2015).

To monitor the developmental gradient of secondary growth in roots, transverse sections were examined from the top, middle, and lower middle of taproots from seven-week-old plants using phloroglucinol to stain for lignin (Figure 3.1B-E). Clearly defined regions of xylem I and II were formed at all positions but root diameter was greatest, and morphology was least variable at the top. Thereafter, sections were taken from the top of the taproot (1-2 mm below the root-hypocotyl junction) to ensure consistency.

3.2 BOP1/2 loss and gain of function mutants alter xylem fibre differentiation

Functional analysis of BOP1/2 in root secondary growth was assessed by examining the phenotype of loss and gain-of-function mutants. Transverse sections of taproot from wild-type,

bop1 bop2, and *bop1-6D* (a gain-of-function line) were stained with phloroglucinol (pink) and toluidine blue (purple) to visualize lignified secondary walls. In *bop1 bop*2 double mutants, the overall organization of tissues was preserved, with a slight thickening of xylem II ring relative to wild-type (Figure 3.2ABJ). The most obvious difference was a significant enlargement of xylem II relative to wild-type control plants (Figure 3.2AB). By contrast, differentiation of xylem II including lignified fibers and vessels was missing in *bop1-6D* gain–of-function mutants (Figure 3.2C). In hemizygous *bop1-6D* plants, lignified vessels were sometimes formed (Figure 3.3). Thus, BOP1/2 repress xylem II differentiation.

3.3 BP interacts antagonistically with boundary genes to promote xylem fiber differentiation in roots

In the stem and hypocotyl, BP represses *BOP1/2* in specific subdomains to preserve normal patterns of secondary growth (Khan et al., 2012b; Liebsch et al., 2014). To test this principle in roots, secondary growth was analyzed in the taproot of *bp* and *bp bop1 bop2* mutants. Sectioning showed that xylem II differentiation in *bp-2* roots was reduced or absent (Figure 3.2DJ) similar to *bop1-6D* mutants (Figure 3.2CJ). Inactivation of *BOP1/2* significantly rescued *bp-2* defects. Taproots in *bop1 bop2 bp-2* triple mutants contained a lignified xylem II ring similar in thickness to wild-type control plants (Figure 3.2EJ). Sometimes, lignification of xylem II fibres was incomplete resulting in a ring of a lower density compared to wild type (Figure 3.3). The diameter of *bp-2* taproots was significantly narrower than wild-type (*bp-2*, 0.85 ± 0.12 mm versus wild-type, 1.55 ± 0.13 mm, *t* test $p<0.0001$), but was partially restored in *bop1 bop2 bp-2* triple mutants (Figure 3.2J). These data indicate opposing roles for BP and BOP1/2 in controlling root diameter and cell differentiation during the xylem expansion phase.

BOP1/2 regulate aerial development in part through promotion of homeobox genes *ATH1* and *KNAT6* which are co-expressed in boundaries (Belles-Boix et al., 2006; Gómez-Mena and Sablowski, 2008; Ragni et al., 2008; Khan et al., 2012b). These genes are also expressed in root vasculature (Dean et al., 2004; Belles-Boix et al., 2006; Woerlen et al., 2017). Inactivation of *ATH1* and/or *KNAT6* partially rescued *bp-2* defects in root secondary growth similar to *bop1 bop2*. Differentiation of xylem II resumed in all three mutant combination: *ath1 bp-2*, *knat6 bp-2* and *ath1 knat6 bp-2* (Figure 3.2FGHJ). Inactivation of these genes also partially corrected *bp-2* defects in root diameter (Figure 3.2I). These data suggest that BP is a general repressor of boundary genes in the root.

3.4 BP spatial regulation of boundary genes is important for differentiation of reproductive-stage xylem

Experiments carried out mainly by Natalie and Adina monitored the expression pattern of *BOP1/2*, *ATH1*, and *KNAT6* boundary genes in wild-type and *bp-2* roots during secondary growth (Figure 3.4). In wild-type plants, *BOP1:GUS* and *BOP2:GUS* reporter genes were strongly expressed in secondary phloem and absent in the vascular cambium and secondary xylem (Woerlen et al., 2017). As predicted, *BOP2* expression was strongly upregulated in the xylem parenchyma cells of *bp-2* mutant roots and in xylem II cambial derivatives. Misexpression of *BOP1* was observed in xylem vessel cells but changes in expression of this gene were mild compared to *BOP2* (Woerlen et al., 2017). *ATH1:GUS* and *KNAT6:GUS* reporters were also highly expressed in secondary phloem of wild-type plants. In *bp-2* mutants, these genes were strongly upregulated in xylem parenchyma cells and the cambial zone similar to *BOP1/2* (Woerlen et al., 2017).

Collectively, these data reveal that correct spatial regulation of boundary genes is important for secondary growth in the root-hypocotyl and stem. In roots, BP repression promotes the production of reproductive-stage xylem. However, BP repression plays a contrasting role in stem where it delays the formation of lignified interfascicular fibers that complete the vascular ring (Mele et al., 2003; Khan et al., 2012a; Khan et al., 2012b).

3.5 Analysis of *PtrBPL1* or *PtrBPL2* gain-of-function in Arabidopsis plants

Poplar tree (*Populus trichocarpa*) expresses two *BOP* orthologs in vascular tissues of the woody stem (Khan et al., 2012b; Devi, 2014). Presumably, spatial regulation of these genes by KNOX transcription factors preserves normal patterns of wood development. To partially analyze the function of these genes, I tested their gain-of-function phenotypes in Arabidopsis plants.

3.5.1 Inflorescence stem

Arabidopsis plants that overexpress *AtBOP1* or *AtBOP2* are short and bushy with abundant lignin deposition in the stem (Khan et al., 2012b). To test if *35S:PtrBPL1* and *35S:PtrBPL2* have a similar activity, overexpression lines in Arabidopsis were created (Devi, 2014). A number of transformed lines had severe defects in stem elongation in the T1 generation. Due to a small number of plant lines (n=1) with a stable overexpression phenotype transmitting to the T2 generation, I screened for additional lines (Table 3.1). The stems of 5-6 representative T1 plants were hand-sectioned and stained with phloroglucinol to look for changes in lignin deposition. Within this population, *35S:PtrBPL1* and *35S:PtrBPL2* transgenic lines often showed a denser vascular ring and lignified phloem fibers similar to lines overexpressing *AtBOP1/2* (Figure 3.5).

3.5.2 Root-hypocotyl

Arabidopsis plants that overexpress *AtBOP1* or *AtBOP2* lack xylem II features in the root (this study) and the hypocotyl (Liebsch et al., 2014). Analysis of secondary growth in the hypocotyl of *35S:PtrBPL1* and *35S:PtrBPL2* lines showed at least one *35S:PtrBPL1* transformed line (n=3) missing xylem II fibers and vessels similar to *bop1-6D* control plants (Figure 3.2). The xylem II ring in all *35S:PtrBPL2* transformed lines (n=3) was reduced in thickness compared to wild-type control plants, similar to *bp-2* mutants (Liebsch et al., 2014; Woerlen et al., 2017). These data suggest that poplar *BPL*-like genes can functionally substitute in Arabidopsis plants.

3.6 Transgenic poplars expressing *BOP1:GUS* and *BOP2:GUS* reporter genes

In poplar, *PtrBPL1* and *PtrBPL2* are expressed in young leaves, roots, seedlings, and inflorescences according to microarray transcriptome data compiled by the Botany Array Resource (www.utoronto.ca/bar). These data closely matched in-house transcript analysis by qRT-PCR carried out on dissected poplar tissues. Unlike Arabidopsis, *PtrBPL1* and *PtrBPL2* transcripts in poplar stem were detected in both xylem and phloem (Devi, 2014). Since these methods do not provide high-resolution spatial information, my role was to generate transgenic poplar expressing *AtBOP1:GUS* and *AtBOP2:GUS* reporter genes. Cis-regulatory sequences in poplar and Arabidopsis tend to be conserved (Ding et al., 2012) and so these reporters were expected to give useful spatial information about gene expression in poplar. Agrobacterium-mediated transformation of leaf disks (n=300) was used to generate 15 putative *AtBOP1:GUS* transgenic lines from callus (Table 3.2). Genotyping confirmed that 11 of 15 lines contained a transgene. These plants were transferred to soil and assayed for GUS activity using three different methods (Jefferson et al., 1987; Khan et al., 2012b; Chen et al., 2013). However, zero were positive for

GUS staining. Control lines stained positive, suggesting that the Arabidopsis reporter was not active in poplar.

Agrobacterium-mediated transformation of leaf disks was used to generate one putative *AtBOP2:GUS* transgenic line from callus growing on selection medium containing phospinothricin (Table 3.2). However, this clone tested negative for the transgene suggesting it was a false-positive. Additional transformants were not recovered despite several attempts.

3.7 Transgenic poplars expressing a construct for RNAi-mediated *PtrBPL1/2* gene silencing

RNAi-mediated gene silencing was used as an approach to down-regulate *PtrBPL1/2* expression in transgenic poplar. An RNA-hairpin cassette for co-silencing of *PtrBPL1* and *PtrBPL2* was constructed (Devi, 2014). This construct is based on a highly conserved 359-base pair sequence in *PtrBPL1* and *PtrBPL2* that encodes part of the BTB/POZ domain. A hairpin cassette composed of sense and antisense *BPL1* sequence under control of the 35S promoter was created in pHannibal (Wesley et al. 2001). This cassette was cloned into the binary vector pART27 (Gleave, 1992) and used to transform poplar tree. Thirty putative RNAi transgenic lines were transferred to soil for analysis. Twenty-six of these plants were genotyped but only two tested positive for the transgene (Table 3.3). However, the morphology of these plants and stem sections stained for lignin were similar to wild-type. No further testing was carried out.

3.8 Transgenic poplars expressing *D35S:BPL1* and *D35S:BPL2* transgenes

Agrobacterium-mediated transformation of leaf disks was used to try and generate poplar plants expressing *D35S:BPL1* and *D35S:BPL2* transgenes. Despite several attempts, no transformants were obtained for either construct, possibly due to problems with selection using

hygromycin. We cannot rule out the possibility that overexpression of *PtrBPL1* or *PtrBPL2* interferes with callus induction and shoot regeneration.

Table 3.1 Summary of overexpression phenotypes in Arabidopsis plants transformed with *D35S:PtrBPL1* and *D35S:PtrBPL2*

Stock No.	Construct	Number of transformants	*bop1-6D* like phenotype	*pny*-like phenotype
B472A	*D35S:BPL1*	443	7	93
B472B	*D35S:BPL1*	81	0	15
B474A	*D35S:BPL2*	92	0	15
B474B	*D35S:BPL2*	68	1	7

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 associated with moderate *BOP* misexpression in the stem; a *bop1-6D-like* phenotype: extremely short bushy plants (<7 cm high) associated with strong *BOP* misexpression. Stock number, accession for Agrobacterium strains in the Hepworth lab glycerol collection.

Table 3.2 Summary of poplar transformations with *AtBOP1:GUS* and *AtBOP2:GUS* reporter gene constructs

Reporter construct	Genotyping result	GUS staining result
AtBOP1:GUS		
1A	+	-
2A	+	-
3B	+	-
4A	+	-
5A	+	-
6B	+	-
7A	+	-
8A	+	-
9B	+	-
10A	+	-
11	not determined	not determined
12	not determined.	not determined
13A	+	-
14	not determined.	not determined
15	not determined	not determined
AtBOP2:GUS		
1A	-	-
1B	-	-

Leaf disks from poplar wild-type plants (n=300 per construct) were infected with *Agrobacterium* cultures from B630 (*AtBOP1:GUS*) or B639 (*AtBOP2:GUS*) in the Hepworth lab strain collection. Fifteen putative *AtBOP1:GUS* transformed calli grew on selection media containing kanamycin. Eleven plantlets regenerated from independent calli tested positive for the transgene. All lines tested negative for GUS staining. One putative *AtBOP2:GUS* transformed callus grew on selection media containing phosphinothricin. Two plantlets regenerated from this callus tested negative for the transgene.

Table 3.3 Summary of poplar plants transformed with *PtrBPL RNAi, D35S:BPL1,* and *D35S:BPL2* constructs

Construct	Genotype Result	Phenotype result
PtrBPL-RNAi		
1AB	-	
2AC	-	
3C	-	
4AB	-	
5C	-	
6ABC	-	
7BC	-	
8ABC	-	
9A	-	
10AB	-	
11C	+	**WT-like**
12ABC	-	
13B	-	
14ABC	-	
15C	-	
16	not determined	
17	not determined	
18C	-	
20B	-	
21AB	-	
22C	-	
23B	-	
24B	+	**WT-like**
25A	-	
26C	-	
28BC	-	
29AB	-	
30AB	-	
D35S:BPL1		
No transformants		
D35S:BPL2		
No transformants		

Leaf disks from poplar wild-type plants (n=300 per construct) were infected with Agrobacterium cultures from accessions B652 (*PtrBPL-RNAi*), B472/473/B667 (*D35S:BPL1*), and B474/B475/B668 (*D35S:BPL2*) in the Hepworth lab strain collection. Thirty putative *PtrRNAi* transformed calli grew on selection media containing kanamycin. Three plantlets per calli were moved to soil for further testing. Of these, 2 of 26 genotyped lines tested positive for the transgene (**bold**). Phenotypes of positive plants were scored 5-6 weeks after transfer to soil. Morphology of leaves and shoots were similar to control plants. Lignin deposition in stem sections was also similar to control plants (data not shown). No further testing was carried out. Hygromycin-resistant calli were not obtained for leaf disks infected with *Agrobacterium* containing *D35S:BPL1* or *D35S:BPL2* transgenes.

Figure 3.1 Anatomy and developmental gradient of lignin deposition in taproot of wild-type plants

(A) Organization of cell types during root secondary growth. Proliferation of procambium cells in the central vascular cylinder of the primary root gives rise to the vascular cambium (VC) (Dolan et al., 1993; Dolan and Roberts, 1995). Cambium cells divide to form secondary phloem (SP) (outwardly) and secondary xylem (XI and XII) (inwardly). x, primary xylem; XI, proportional growth phase; XII, xylem expansion phase. A separate meristem called the cork cambium (CC) derived from the pericycle layer of the central vascular cylinder in the primary root produces the outer periderm (bark) of secondary thickened roots. The original non-dividing outer layers of the root (endoderm, cortex, and epidermis) are shed (Dolan and Roberts, 1995). Modified from (Vishwanath et al., 2015). (B) Taproot of representative seven-week-old wild-type plant. Horizontal bar indicates the root-hypocotyl junction. Tissues below this junction were harvested for analysis (arrow) (C–E) Transverse sections were stained with phloroglucinol-HCl to detect lignin (pink colour). Approximate distances (mm) below the root-hypocotyl junction were as indicated. Scale bars, 2 mm (B**)**, 0.25 mm (C-E). Figure from: Woerlen et al., 2017.

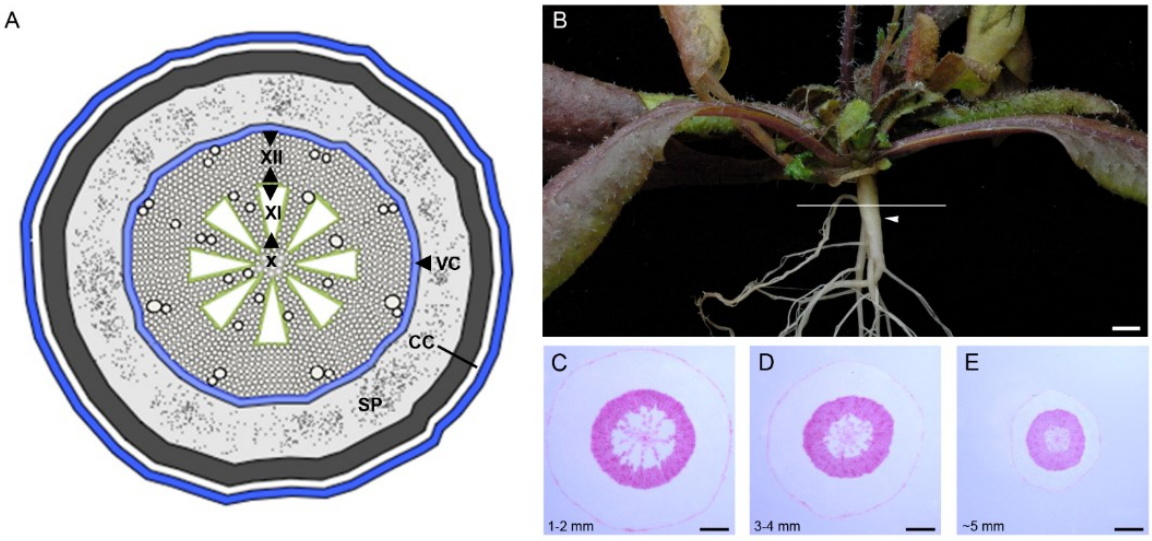
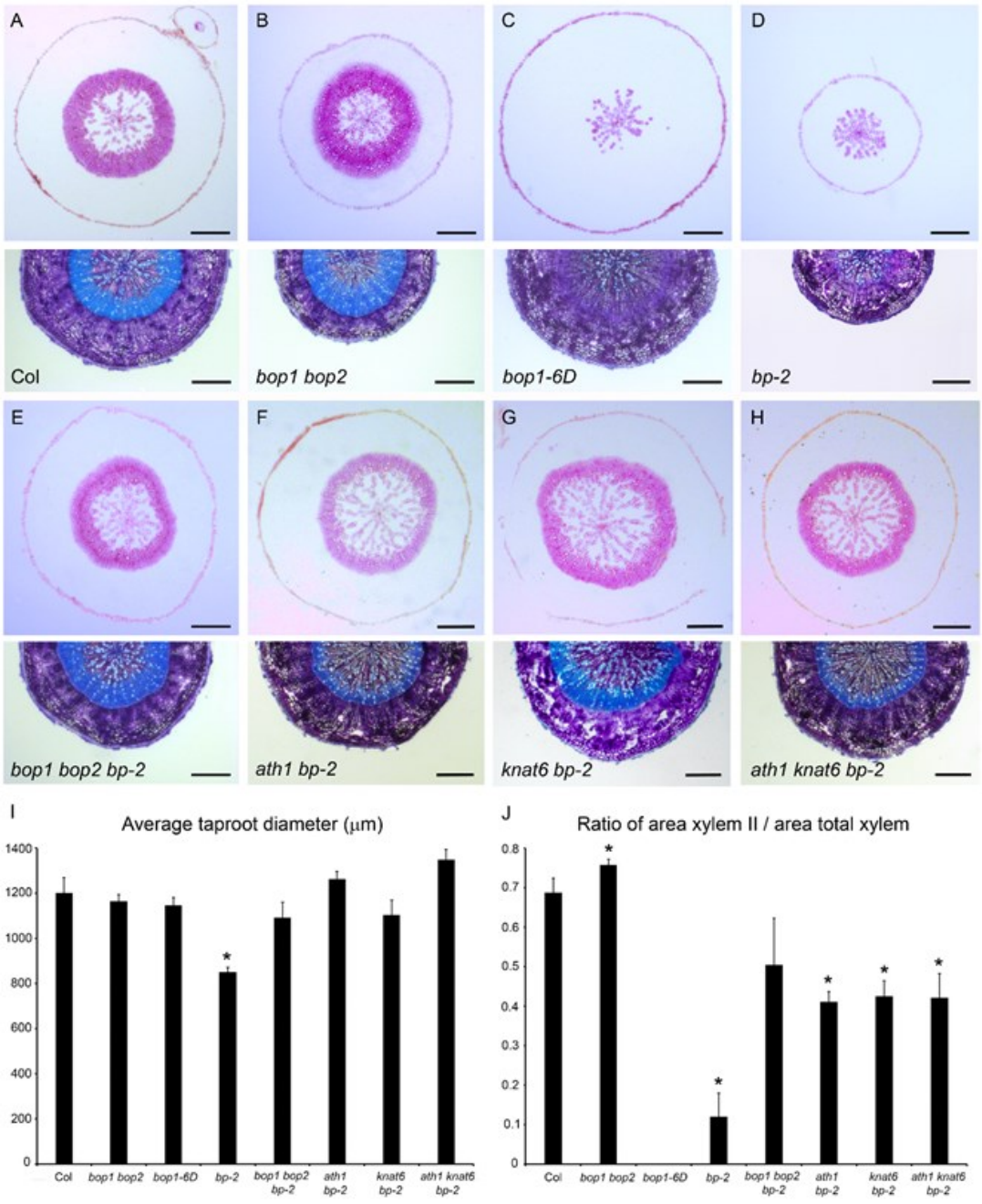

A
XII
XI
X
VC
CC
SP
B
C
1-2 mm
D
3-4 mm
E
~5 mm

Figure 3.2 Lignin deposition in wild-type and mutant secondarily thickened roots

(A–H) Transverse sections from the top of 7-week-old taproots were stained with (top) phloroglucinol-HCl to detect lignin (pink) or (bottom) toluidine blue (pH 4.0) to detect lignin (blue) and pectin (purple). Representative data are shown. (A) Col, wild-type. (B) *bop1 bop2*; lignified ring of secondary xylem II is thicker compared to wild-type. (C) *bop1-6D;* xylem II cambial derivatives are non-lignified. (D) *bp-2*; xylem II cambial derivatives are non-lignified and root diameter is reduced compared to wild-type. (E) *bop1 bop2 bp-2*, xylem II ring is restored, and root diameter is increased compared to *bp-2*. (F) *ath1 bp-2*, xylem II ring is restored, and root diameter is increased compared to *bp-2*. (G) *knat6 bp-2*, xylem II ring is restored, and root diameter is increased compared to *bp-2*. (H) *ath1 knat6 bp-2*, xylem II ring is restored, and root diameter is increased compared to *bp-2*. (I) Average taproot diameter in wild-type and mutants. (J) Average ratio between the area occupied by xylem II and total xylem area. (I-J) Data are means ± s.e.m. based on measurement of transverse sections from 7-week-old plants using 6-9 taproots per genotype. Asterisks, significantly different than wild-type, $p<0.05$, *t-test*. Scale bars, 0.25 mm. Figure from: Woerlen et al., 2017.


A
B
C
D
Col
bop1 bop2
bop1-6D
bp-2
E
F
G
H
bop1 bop2 bp-2
ath1 bp-2
knat6 bp-2
ath1 knat6 bp-2
I
Average taproot diameter (μm)
1400
1200
1000
800
600
400
200
0
Col
bop1 bop2
bop1-6D
bp-2
bop1 bop2 bp-2
ath1 bp-2
knat6 bp-2
ath1 knat6 bp-2
J
Ratio of area xylem II / area total xylem
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Col
bop1 bop2
bop1-6D
bp-2
bop1 bop2 bp-2
ath1 bp-2
knat6 bp-2
ath1 knat6 bp-2

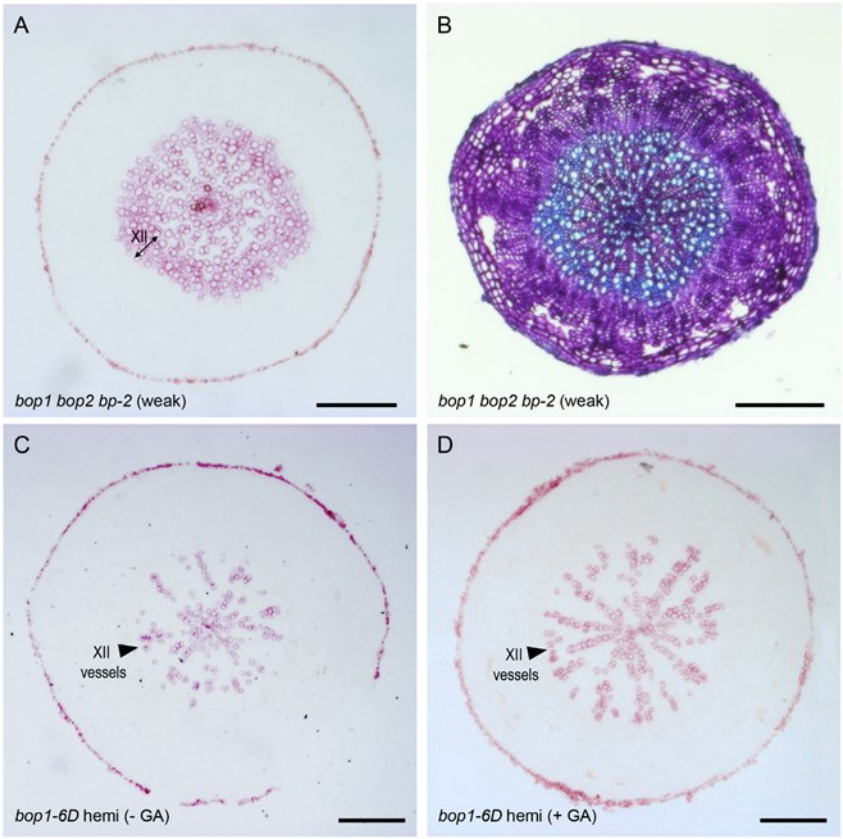
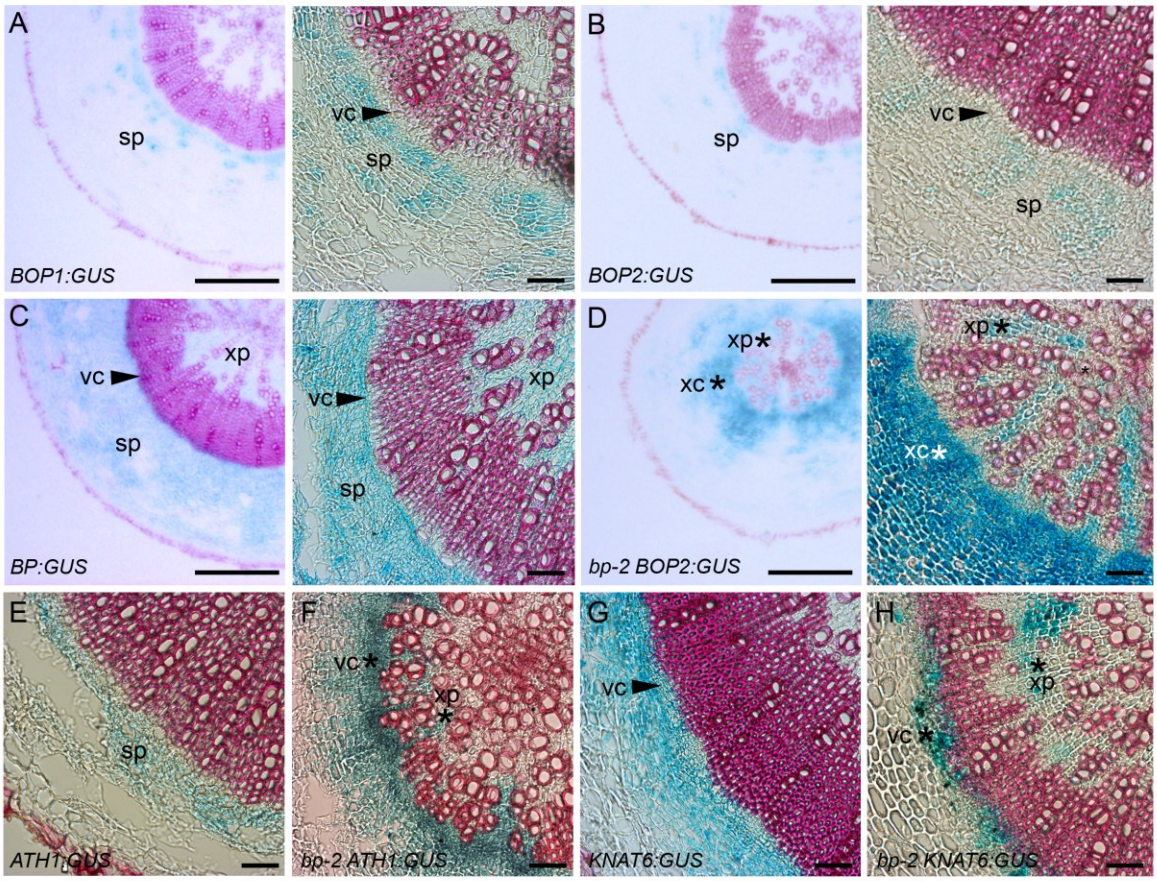


Figure 3.3 Depiction of *bop1 bop2 bp-2* mutants with a weak rescue phenotype and effect of external GA on *bop1-6D* xylem formation

(A, C-D) Transverse sections were stained phloroglucinol-HCl to detect lignin (pink) and (B) toluidine blue detect to lignin (blue) and pectin (purple). (A-B) *bop1 bop2 bp-2* mutant showing a weak rescue phenotype, xylem II vessels are lignified but fiber formation is incomplete. (C) *bop1-6D/+* hemizygous mutant untreated with GA. Arrows denote scattered formation of lignified xylem II vessels. (D) *bop1-6D/+* hemizygous mutant treated daily with a solution of 100 µm GA_3. Arrows denote scattered formation of lignified xylem II vessels similar to the mock control (C). Scale bars, 0.25 mm. Figure from: Woerlen et al., 2017.

Figure 3.4 Boundary gene expression in wild-type and *bp-2* secondarily thickened roots

GUS reporters were used to monitor *BP*, *BOP1*, *BOP2*, *ATH1*, and *KNAT6* expression (blue) in transverse sections. Phloroglucinol-HCl staining was used detect lignin (pink). (A) *BOP1:GUS*, expressed in secondary phloem (sp). Absent in the vascular cambium (vc). (B) *BOP2:GUS,* expressed in secondary phloem. Absent in the vascular cambium. (C) *BP:GUS,* arrowheads point to high levels of expression in the vascular cambium. Expressed at lower levels in xylem I parenchyma cells (xp) and secondary phloem. (D) *bp-2 BOP2:GUS,* expression was upregulated. Asterisks denote ectopic expression in xylem I parenchyma cells and xylem II cambial derivatives (xc). (E) *ATH1:GUS*, expressed in secondary phloem and at lower levels in the vascular cambium. (F) *bp-2 ATH1:GUS*, expression was upregulated. Asterisks denote increased expression in xylem I parenchyma cells and the vascular cambium. (G) *KNAT6:GUS*, expressed in secondary phloem and at lower levels in the vascular cambium. (H) *bp-2 KNAT6:GUS*, expression was upregulated. Asterisks denote increased expression in xylem parenchyma cells and the vascular cambium. Scale bars, 50 μm except 0.25 mm A-D (left panels). Figure from: Woerlen et al., 2017.


A
sp
BOP1:GUS
vc
sp
B
sp
BOP2:GUS
vc
sp
C
xp
vc
sp
BP:GUS
vc
xp
sp
D
xp*
xc*
bp-2 BOP2:GUS
xp*
xc*
E
sp
ATH1:GUS
F
vc*
xp
*
bp-2 ATH1:GUS
G
vc
KNAT6:GUS
H
*
xp
vc*
bp-2 KNAT6:GUS

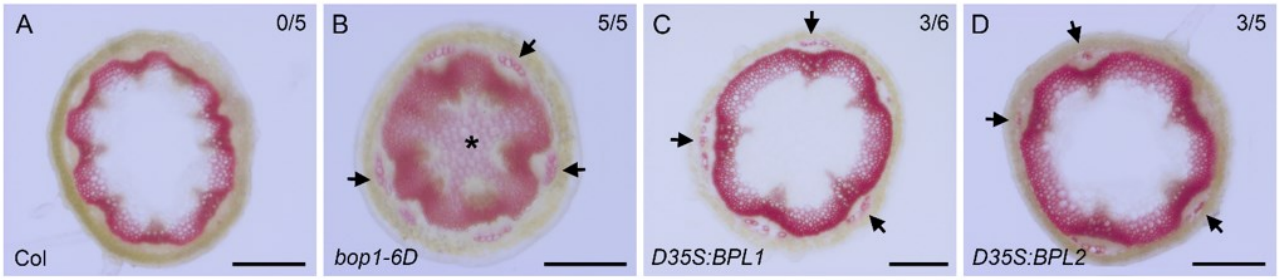


Figure 3.5 Lignin deposition in inflorescence stem of Arabidopsis plants expressing *D35S:PtrBPL1* and *D35S:PtrBPL2* transgenes

Transverse sections from the base of fully elongated stems were stained with phloroglucinol-HCl to reveal lignin (pink). Top left, number of independent transgenic lines showing *bop1-6D*-like change in lignin deposition. Representative sections are shown for: (A) Wild-type. (B) *bop1-6D*, showing a thicker denser vascular ring, lignified pith (asterisk), and lignified phloem fibers (arrows). (C) *D35S:PtrBPL1* transgenic line, showing thicker vascular ring and lignified phloem fibers (arrow). (D) *D35S:PtrBPL2* transgenic line, showing thicker vascular ring and lignified phloem fibers (arrows). Scale bars, 0.25 mm.

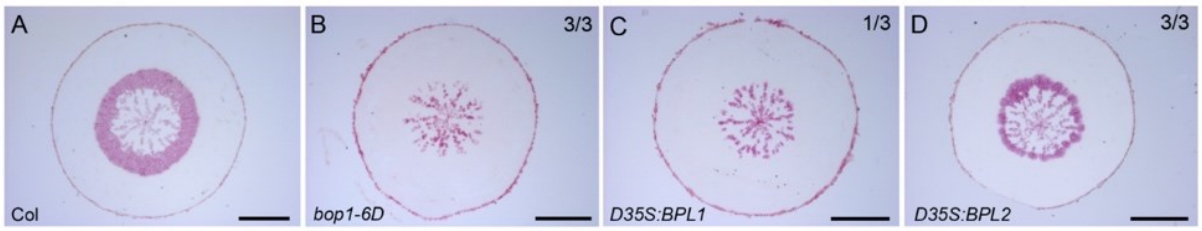


Figure 3.6 Lignin deposition in hypocotyl of Arabidopsis plants expressing *D35S:PtrBPL1* and *D35S:PtrBPL2* transgenes

Transverse sections from the middle of the hypocotyl (1.5 mm below the rosette leaves) were stained with phloroglucinol-HCl to reveal lignin (pink). Top left, number of independent transgenic lines with phenotype. Representative sections are shown for: (A) Wild-type, showing a thick ring of xylem II. (B) *bop1-6D*, xylem II fibers and vessels are missing. (C) *D35S:PtrBPL1* transgenic line, similar to *bop1-6D*. (D) *D35S:PtrBPL2* transgenic line, thickness of xylem II ring is reduced compared to wild-type. Scale bars, 0.5 mm

Chapter 4: DISCUSSION

In Arabidopsis plants, a continuous vascular cambium forms at the inflorescence stem base, hypocotyl, and root. All three tissues are considered as useful models for wood development in trees, based on secondary growth characterized by the abundant deposition of lignin (Chaffey et al., 2002; Strabala and MacMillan, 2013; Nieminen et al., 2015; Barra-Jiménez and Ragni, 2017).

Much like the SAM, the vascular cambium provides stem cell activity, but is organized differently since cambium cells are not physically separated from differentiating xylem and phloem. The SAM is maintained by Class I KNOX genes that spatially regulate boundary genes to allow continuous production of lateral organs. In previous work, we showed that Class I KNOX genes in inflorescence stem negatively regulate boundary genes and this delays the differentiation of lignified cell walls. My thesis tested the role of boundary genes in the vascular cambium of roots, since their roles may be unique in different tissues undergoing secondary growth compared to the SAM.

4.1 Spatial regulation of boundary genes is important for xylem differentiation in roots

Collectively, our work shows that correct spatial regulation of boundary genes is essential for normal root secondary growth. In wild-type roots, *BOP1/2* are expressed in secondary phloem but not in the meristematic cambial zone or xylem. Homeobox boundary genes *ATH1* and *KNAT6* follow a similar pattern. This pattern is maintained by BP, which represses boundary genes in the cambial zone and in developing xylem. Genetic interactions show that this repression is essential for the differentiation of lignified fibers and vessels during the expansion phase of secondary xylem development since inactivation of boundary genes substantially rescues *bp* patterning defects in the root. Transgenic plants that overexpress *BOP1* have nearly the same phenotype as a *bp* mutant indicating that BOP1/2 function downstream of BP in blocking xylem II differentiation.

Root development in *bp bop1 bop2* and *bp ath1 knat6* triple mutants is almost normal suggesting that other redundant factors control secondary growth. This might ensure robustness and flexibility.

4.2 Alignment of root and hypocotyl models

My work confirms that regulatory interactions governing secondary growth in the root (this study) are similar to the hypocotyl (Liebsch et al., 2014). That is, BP in the root-hypocotyl promotes the differentiation of lignified fibers via repression of boundary genes (this study; Liebsch et al., 2014). This makes sense since vascular tissues in these organs are centrally arranged and have a common origin during embryogenesis (Scheres et al., 1994; Scheres et al., 2002; De Rybel et al., 2016). Mutations that affect radial organization of the root display identical phenotypes in the hypocotyl (Scheres et al., 1995).

By contrast, vascular tissues in the inflorescence stem are derived from the SAM (De Rybel et al., 2016). The role of BP in the inflorescence stem more closely resembles its role in the SAM in that repression of boundary genes delays the differentiation of lignified fibers thereby maintaining indeterminacy until internode elongation is complete (Mele et al., 2003; Khan et al., 2012b)

4.3 Other SAM-boundary regulators

In aerial tissues, BOP1/2 are upstream regulators of KNOX-BELL boundary genes *ATH1* and *KNAT6* (Khan et al., 2012a; Khan et al., 2012b; Khan et al., 2015). KNAT6-ATH1 form a complex (Rutjens et al., 2009; Li et al., 2012) and function downstream of BOP1/2 in modulating secondary growth in the inflorescence stem (Khan et al., 2012a; Khan et al., 2012b; Khan et al., 2015). BP directly represses *KNAT6* through recruitment of the chromatin remodeling ATPase BRAHMA to sites in the promoter (Zhao et al., 2015). Regulatory interactions in the root are

probably the same.

BP exerts part of its function as a complex with PNY in the inflorescence stem (Byrne et al., 2003; Smith and Hake, 2003; Bhatt et al., 2004). Genetic studies show that these two genes have similar but distinct effects on vascular patterning in the stem (Smith and Hake, 2003). The size and spacing of vascular bundles in *bp* stems is irregular with some containing small xylem elements. These changes precede spatial and temporal defects in lignin deposition (Mele et al., 2003). Vascular bundles in the *pny* mutant are crowded with some lacking large xylem vessels (Smith and Hake, 2003). Overexpression of *BOP1/2* recapitulates both *bp* and *pny* patterning defects (Khan et al., 2012b). PNY functions as a direct repressor of *BOP1/2, ATH1,* and *KNAT6* in meristems (Andrés et al., 2015; Bencivenga et al., 2016). The significance of PNY activity in roots is unknown but may directly repress organ boundary genes in secondary xylem.

4.4 Differential impact on xylem II

Interestingly, *BOP1/2* misexpression in the root-hypocotyl have a selective impact on xylem II formation (see also Liebsch et al., 2014). The switch to xylem II happens at the floral transition in response to gibberellin (GA) influx from aerial tissues (Ragni et al., 2011). Misexpression of BOP1/2 in developing xylem may interfere with this signal. *BOP1/2* misexpression in the SAM of *pny pnf* mutants has a similar selective impact on reproductive development by blocking internode elongation and flower initiation. This deficiency correlates with reduced responsiveness to GA and changes in the *miR156*-SQUAMOSA PROMOTER BINDING PROTEIN-LIKE-*miRNA172* module and FLOWERING LOCUS T-FD flowering-time pathways (Lal et al., 2011; Andrés et al., 2015; Khan et al., 2015). External application of GA to *BOP1* overexpressing plants fails to restore xylem II formation (Figure 3.3) or stem elongation suggesting that BOP1 does not simply inhibit GA biosynthesis (Khan et al., 2015).

4.5 Lignin biosynthesis

Arabidopsis studies focusing on aerial tissues have identified BOP1/2 and BP as opposing regulators of lignin biosynthesis genes. BP binds directly to the promoter of lignin biosynthesis genes acting as a repressor (Mele et al., 2003). Also, maize and tobacco plants overexpressing *KNOTTED1* show a reduction in stem lignin content that is associated with down regulation of key genes in the lignin biosynthesis pathway (Townsley et al., 2013). BOP1/2 has an opposite effect on genes of the lignin biosynthesis pathway. At least four genes in this pathway are upregulated in *bop1-6D* stems [*cinnamyl alcohol dehydrogenase 5*, *cinnamate 4-hydroxylase 1*, *p-coumarate 3-hydroxylase 1,* and *PRXR9GE,* which encodes a class III peroxidase] (Khan et al., 2012b). Transcripts for these genes are also upregulated in *bp-2* stems and restored to normal levels by inactivation of *BOP1/2* (Khan et al., 2012b).

4.6 Comparison to trees

4.6.1 Class I KNOX genes

Phenotypic analysis of loss and gain of KNOX effects on secondary growth in poplar show closest alignment with the Arabidopsis inflorescence stem as a model. ARK1 and ARK2 are the poplar orthologs of Arabidopsis STM and BP, respectively (Groover et al., 2006; Du et al., 2009; Du and Groover, 2010). *ARK1* expression is concentrated in the cambial zone. Poplar plants overexpressing *ARK1* have short internodes and thin stems in which differentiation of lignified secondary xylem and phloem is severely inhibited (Groover et al., 2006). *ARK2* expression becomes concentrated in the cambial zone but is initially expressed in developing secondary xylem and phloem fibers similar to *BP* in the Arabidopsis hypocotyl and root (Du et al., 2009; Liebsch et al., 2014; Woerlen et al., 2017). *ARK2* overexpression causes short internodes and a thicker cambium characterized by an overall reduction in production of lignified phloem fibers and

secondary xylem. Plants expressing an artificial miRNA that lowers *ARK2* transcript abundance display premature differentiation of secondary xylem and phloem fibers, similar to the *bp* mutant in Arabidopsis inflorescence stem (Mele et al., 2003; Du et al., 2009).

4.6.2 *BLADE-ON-PETIOLE* genes

The poplar genome contains two *BOP*-like genes designed *PtrBPL1* (POPTR-0016s04010) and *PtrBPL2* (POPTR-006s04190) (Devi, 2014). Expression of these orthologs may be spatially regulated by KNOX transcription factors in trees to preserve normal patterns of wood development. Arabidopsis plants overexpressing *PtrBPL1* and *PtrBPL2* display all the same characteristic defects in stem elongation and lignin deposition associated with overexpression of *AtBOP1* or *AtBOP2* (Khan et al., 2012b). Transverse sectioning analysis of *35S:PtrBPL1/2* transgenic lines often showed a denser vascular ring and lignified phloem fibers in the stem, similar to *AtBOP1/2* lines. Secondary growth in the root-hypocotyl was especially sensitive to *PtrBPL1/2* overexpression. Formation of xylem II was reduced or blocked in virtually all transgenic lines regardless of defects in plant height. Taken together, these data show strong functional conservation between Arabidopsis and poplar BOPs.

4.6.3 Transgenic poplar

PtrBPL1 and *PtrBPL2* transcripts are found in xylem and phloem of poplar stem similar to the root-hypocotyl of Arabidopsis plants (Devi, 2014). We expect their expression to be dominant in secondary phloem but this remains to be seen. Agrobacterium-mediated transformation of leaf disks (n=300) produced eleven *AtBOP1:GUS* genotyped clones equal to a transformation efficiency of 3.7%. This aligns with 5-37% transformation efficiency reported for poplar, depending on the Agrobacterium strain and the construct (Meilan and Ma, 2006). Unfortunately, transgenic poplar expressing the *AtBOP1:GUS* reporter showed no β-glucuronidase activity. This

was a surprise, since regulatory elements between Arabidopsis and poplar are highly conserved and GUS is a common reporter gene in poplar (Ding et al., 2012; Chen et al., 2013). Further, there are numerous examples in the literature of cross-use of promoters between different plant species (Lee et al., 1995; Chen et al., 2013; Couzigou et al., 2016). No transformants were obtained for *AtBOP2: GUS* using phosphinothricin as a selection agent. This herbicide is used as a selection agent in poplar but is toxic at low concentrations (De Blocke, 1990; Confaloneiri et al., 2000; Kutsokon et al., 2013). In the meantime, *PtrBPL1* and *PtrBPL2* promoters cloned from poplar and will be fused to GUS for expression in Arabidopsis plants (Estornell et al., 2015). *In situ* hybridization is another approach that can be used to directly analyze *PtrBPL1/2* transcripts in the stem of wild-type and *ARK2* knockdown poplar plants (Groover et al., 2006; Du et al., 2009).

Attempts to create *PtrBPL1/2* loss and gain-of-function mutants in poplar were also unsuccessful. *35S:PtrBPL* RNAi lines were selected in transgenic poplar but only 2 of 26 clones contained the transgene, revealing a high number of false positives. Positive clones grown to maturity resembled wild-type and were not analyzed further. RNAi silencing in plants usually results in a range of phenotypes from weak to strong so it is important to examine a large number of transformants. Transcript levels were not tested in my lines so it is unclear if silencing was effective. Dominant negative phenotypes mimicking loss-of-function can also be generated by overexpressing transcription factors fused to an SRDX repression motif (Hiratsu et al., 2003). This approach is likely to work in poplar given that transgenic Arabidopsis plants expressing *35S:BOP1-SRDX* produce strong loss-of-function phenotypes (Kevin Xiong in the lab). No transformants were recovered for *D35S:PtrBPL1* or *D35S:PtrBPL2* in poplar using hygromycin as a selection agent. This compound is also toxic at low concentrations (Howe, 1991; Tzfira et al., 1997; Jeon et al., 2016). Remaking these constructs into an expression vector that uses kanamycin

as the selectable marker may be the best option going forward.

4.7 Final conclusion and future directions

In conclusion, the vascular cambiums formed in the Arabidopsis stem and the root-hypocotyl may not be identical. They appear to differ in patterns of expression for Class I KNOX and boundary genes. Although BP represses boundary genes in both contexts, the developmental outcome appears to differ. In the root and hypocotyl, BP excludes boundary genes from the meristematic cambial zone and developing xylem and this is essential for the differentiation of fibers and vessels during the expansion phase of secondary xylem development (Liebsch et al., 2014; Woerlen et al., 2017). In the inflorescence stem, BP repression of boundary genes in the phloem and cortex plays a contrasting role, required in delaying the formation of lignified interfascicular fibers that complete the vascular ring during secondary growth (Mele et al., 2003; Khan et al., 2012b). Testing the loss and gain-of-function of BOPs in poplar and examining their expression patterns in wild-type and *ARK1* or *ARK2* silenced lines will provide a more complete picture of how KNOX and boundary genes interact to control wood development in trees.

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