1 Introduction

1.1 Secondary growth in plant stems and the activity of vascular cambium

Secondary growth is additional growth that thickens the stem and root after primary growth, usually elongation growth, is completed (Evert, 2006). In trees, secondary growth is represented by the stem or root diameter. Secondary growth is a result of cell division and differentiation in the vascular cambium. The vascular cambium consists of a mantle of cells between the phloem and xylem to which it gives rise. In contrast to the shoot apical meristem (SAM) that occupies the shoot tip, the cambium is displaced towards the outer side of the plant axis and is therefore considered as a lateral meristem. Vascular cambial cells are derived mostly from procambial cells which develop during vascularization in the primary stem (Raven et al. 1999). The cambial cells divide periclinally to produce xylem and phloem. Daughter cells of the cambium differentiate to the outer side into phloem and to the inner side into xylem to produce radial files of cells that meet at the cambial zone (Figure 1A). The phloem ensures the transport of photoassimilates from source leaves to sink tissues such as the shoot apical meristem and the stem; whereas the xylem transports mainly water and mineral solutes from the root to the shoot. Xylem of trees, commonly referred to as wood, is an important source of fixed carbon used for woody materials and industrial purposes such as timber, pulp, furniture, fibers, and also as energy source or for other products (films, adhesives, etc).

During secondary growth, cambial daughter cells develop and specialize to xylem cells (Figure 1 B). Xylem cells undergo progressive stages of differentiation; (1) elongation/ enlargement, (2) secondary cell wall deposition, and (3) programmed cell death before being mature xylem (Turner et al. 2008). The hallmark of mature xylem is secondary cell wall deposition. Secondary cell wall formation contributes to a large extent to the biomass of wooden tissues. The major compounds of secondary cell walls are cellulose, hemicelluloses and lignin. The wood of economically important poplar trees typically consists of 45 % of cellulose, 25 % hemicelluloses and 20 % of lignin (Timell et al. 1969; McDougall et al. 1993).

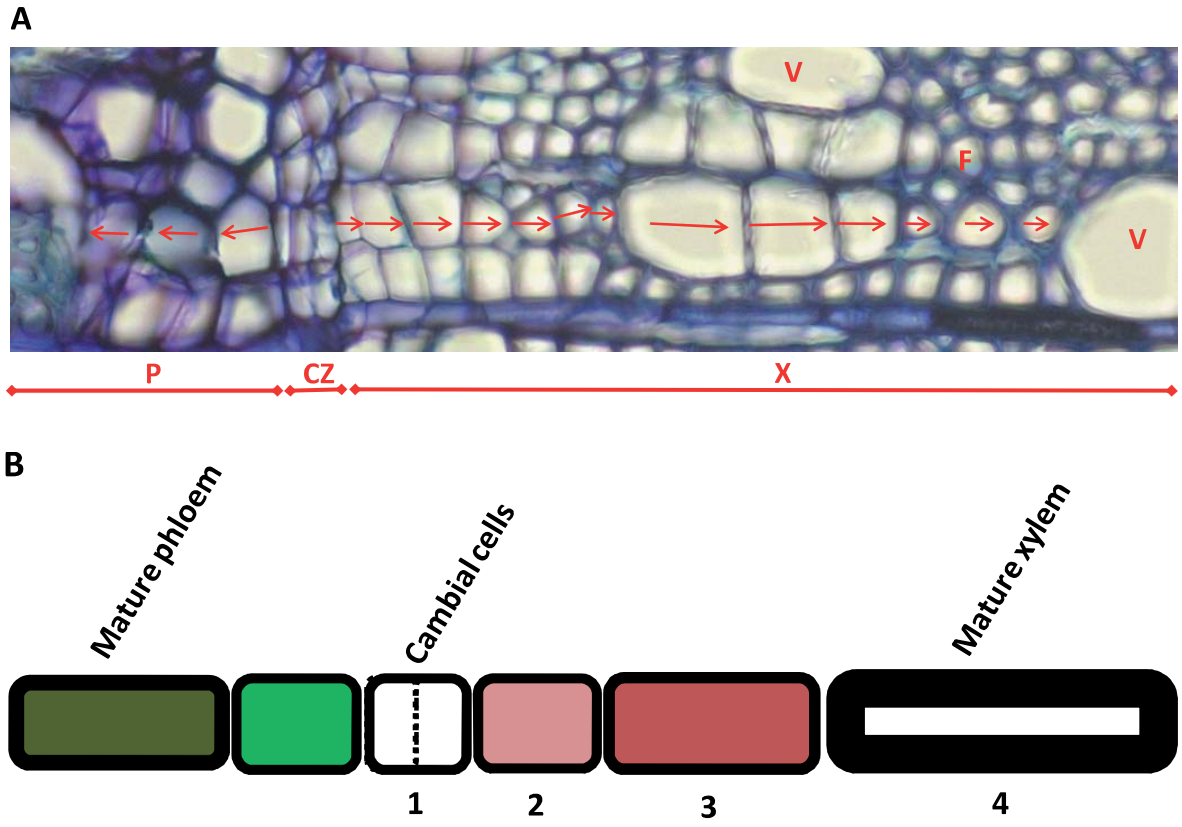

Figure 1. (A) Formation of xylem and phloem from cambial cell divisions in poplar (*Populus x canescens*). (CZ) Cambial zone, (X) xylem, (P) phloem, (V) vessel elements, (F) fibers and (red arrows) radial files of cambial derivates. (B) Differentiation and maturation of cambial daughter cells, schematically. (1) Cell division, (2) cell enlargement, (3) secondary cell wall deposition, (4) programmed cell death.

1.2 *KNOX* (*Knotted-1 Like homeobox*) gene function in plant development

KNOTTED1-like homeobox (*KNOX*) genes are families of homeobox genes identified in all monocot and dicot species and subsets of these genes regulate meristem function in all higher plant (Scofield and Murray, 2006). Homeobox genes encode proteins containing a conserved DNA-binding homeodomain motif that is found in transcription factors from all eukaryotes. Most homeobox genes encode transcription factors, which function in developmental processes. The first homeobox gene identified by mutation is *ANTENNAPEDIA* in *Drosophila melanogaster*. Mutations in *ANTENNAPEDIA* cause a homeotic conversion of organs, with antennae replaced by legs (Gehring, 1987). After that, many homeobox genes were found to play an important role in eukaryote development.

In plants, the first homeobox gene was identified in maize and called *ZmKN1* (*Zea mays KNOTTED1*) (Vollbrecht et al. 1991). Dominant mutations in *ZmKN1* inhibit leaf differentiation and cause the formation of knot-like meristematic structures in the vicinity of leaf veins suggesting *ZmKN1* to play an important role in regulation of meristematic fate (Smith et al. 1992; Sinha et al. 1993). Homologues homeobox genes were therefore termed as *KNOTTED1-like* or *KNOTTED1-like* homeobox (*KNOX*) genes (Lincoln et al. 1994; Long et al. 1996). Thereafter different classes of homeobox genes have been identified in plants like the *WOX (WUSCHEL* related homeobox*)* gene family members involved in early embryonic patterning in *Arabidopsis* (Haecker et al. 2004), the *BELL* family genes (Reiser et al. 1995) and the *HD-ZIP* (homeodomain protein containing a leucine zipper) (Sessa et al.1993).

Based on phylogenetic analyses of amino acid and nucleotide sequences, there are eight members of *KNOX* genes divided into two sub families in *Arabidopsis* (Scofield and Murray, 2006). The subfamily *KNOX* I comprises *STM, KNAT1, KNAT2* and *KNAT6* and the subfamily *KNOX* II comprises *KNAT3, KNAT4, KNAT5* and *KNA7* (Figure 2).

A well-characterized member of the class I *KNOX* genes is *SHOOT MERISTEMLESS* (*STM*), which is expressed in the centre of the shoot apical meristem (SAM) but not in the newly formed leaf primordia and in the incipient leaf (Long et al. 1996). Loss-of-function mutations in *STM* lead to premature differentiation of meristematic cells and eventually to cessation of the SAM (Long et al. 1996); but its simultaneous over-expression together with the homeodomain transcription factor *WUSCHEL* induces meristem formation at ectopic places (Lenhard et al. 2002). These findings indicate that *STM* is a critical regulator of differentiation, whose expression is required to keep cells in an undifferentiated state. The other characterized members of the class I *KNOX* genes fulfill partly redundant functions to *STM* and are generally suggested to be involved in preventing differentiation of the tissue where they are expressed (Scofield and Murray, 2006). In contrast to class I *KNOX* genes, the members of class II *KNOX* genes are only scarcely described and functional data is mostly lacking.

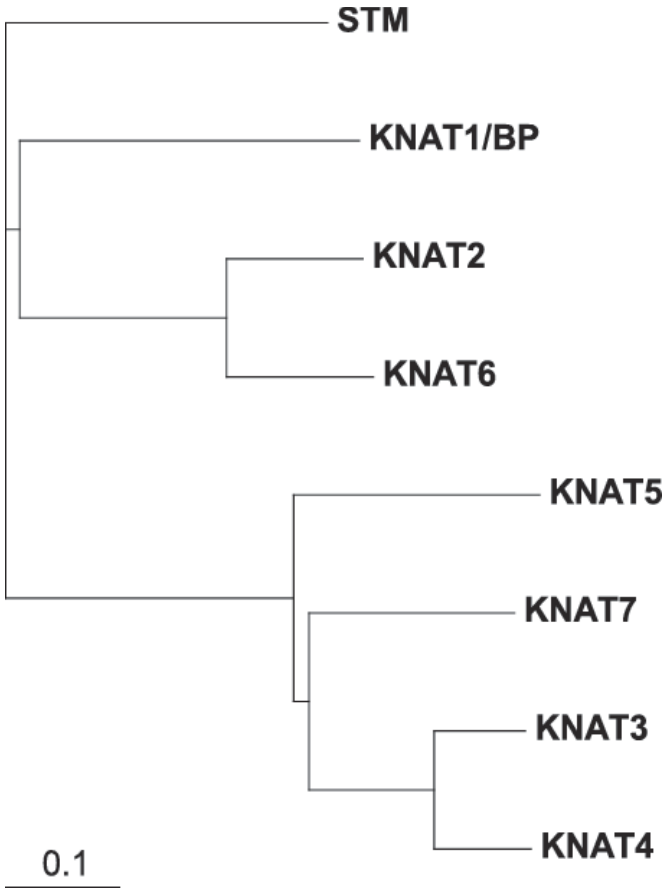

Figure 2. Phylogenetic relationship of the eight *Arabidopsis* KNOX proteins. Tree is consistant with published data (Scofield and Murray, 2006). Tree was drawn by using Treview (Sunaryo and Fischer, 2009)

1.3 Molecular and genetic control of secondary growth, xylem differentiation and secondary cell wall deposition

Although abundant data has been collected to address the genetic control of cambial activity and differentiation, the mechanism behind is still little known. In the model tree poplar however, evidence for an involvement of *KNOX* genes in controlling differentiation of cambial daughter cells has been recently found. High resolution transcript analyses of the poplar cambium had been exploited and showed several *KNOX* genes with strong cambial expression (Hertzberg et al. 2001; Schrader et al. 2004). Furthermore, the current understanding of the regulation of differentiation in vascular development was greatly enhanced by the study of the poplar *KNOX* gene *ARBORKNOX1* (*ARK1*) and *ARBORKNOX2* (*ARK2*), which are close homologues of the *Arabidopsis STM* and *BREVIPEDICELLUS(BP/KNAT1)*, respectively. *ARK1* was shown to be expressed in the cambium and over-expression of *ARK1* leads to inhibition of differentiation of vascular

cells (Groover et al. 2006). This is in line with the proposed role for *KNOX* genes in keeping cells undifferentiated. The *KNAT1* homolog, *ARK2*, was also shown to be expressed in the cambial zone and to be involved in cambial daughter cell differentiation, since downregulation of the endogenous gene by artificial miRNA-suppression led to additional secondary growth and premature secondary xylem formation (Du et al. 2009). Despite this progress, the functional analysis of gene families in poplar is strongly restricted by the long regeneration time of transgenic poplar, by the difficulty to construct loss-of-function alleles and by a steep developmental apical-basal gradient in the young stem.

The *Arabidopsis* hypocotyl has previously been shown to be a suitable model for secondary growth and xylem differentiation similar as it occurs in angiosperm trees and has therefore been suggested as a model for wood formation (Chaffey et al. 2002; Nieminen et al. 2004). Importantly, the hypocotyl does not have an apical-basal developmental gradient, as it occurs in stems (Sibout et al. 2008). However functional studies of *KNOX* gene function in regard to secondary growth of the vascular cambium in the hypocotyl have not yet been performed. In contrast to the hypocotyl, *KNOX* gene function has been addressed in the *Arabidopsis* inflorescence. Ko and Han (2004) reported that *STM* is expressed in the inflorescence stem harvested from three different stages of development: immature, intermediate, and mature. *KNAT7* was also observed to be expressed in the same tissues. Mele et al (2003) investigated the regulation of differentiation in vascular development in inflorescence stems by studying mutants of *KNAT1*/*BP* (*BREVIPEDICELLUS*). *knat1* mutants show an increase in lignification among various developmental defects of the cambial daughter cells; whereas over-expression of *KNAT1* leads to a decrease in lignin deposition (Mele et al. 2003). Other homeodomain transcription factors from class III *HD-ZIP* and *KANADI* gene family members such as *NAC, AP2, MADS*, and *MYB* have been reported to regulate cambial cell differentiation and activity in *Arabidopsis* (Zhao et al. 2005). Moreover, a leucine zipper (HD-ZIP) gene family, comprising amongst others, *ATHB-8*, *ATHB-9*, and *ATHB-14*, has been reported to play an important role in vascular development (Roberts and McCann, 2000).

Xylem cells comprise xylem vessels, fibers, parenchyma cells, and radial ray cells (Evert, 2006). One of the most studied type of xylem cells concerning cell differentiation in plants are the tracheary elements. Using the *Zinnia* model system the specification of xylem vessels has being studied and environmental factors and hormones such as light,

auxin, cytokinin, ethylene, brassinosteroids and phytosulfokine (Robert and McCann, 2000) have been shown to influence xylem vessel fate.

The formation of secondary cell wall in the xylem involves various biochemical processes including cellulose biosynthesis and lignin formation. The *Arabidopsis IRREGULAR XYLEM1 (IRX1*) and *IRX3* were reported to be secondary cell wall–specific cellulose synthase genes (Brown et al. 2005). In recessive mutants of those genes, collapsed vessel elements can be found in the inflorescence, which are likely due to reduced cellulose biosynthesis in the secondary cell wall. Other genes including *IRX5, 6, 7, 8, 9, 10, 11, and 12* also showed a distinct *irx*-like phenotype (Brown et al. 2005). Additionally, *MYB58* and *MYB63* have been reported to play an important role in lignin formation (Zhou et al. 2009). Previously, Zhong et al (2008) also reported that some MYB and NAC transcription factors, as *SECONDARY WALL ASSOCIATED NAC DOMAIN PROTEIN1* (*SND1*), as well as *KNAT7* are involved in secondary cell wall formation in the inflorescence, especially required for fiber differentiation. Furthermore, *NAC SECONDARY WALL THICKENING PROMOTING FACTOR1 (NST1)* and *SND1* were reported to play a redundant role in fiber differentiation (reviewed by Zhong and Ye, 2007).

1.4 Working hypothesis

In the vascular cambium similar decisions as in the SAM have to be taken; an equally tight balance between meristematic cells and cells, which undergo differentiation, is required. Some daughter cells of the cambial meristem differentiate into xylem or phloem, whereas others stay undifferentiated and maintain the pool of meristematic cells. *KNOX* genes might be also key players of cambial cell division and differentiation in *Arabidopsis* hypocotyls.

1.5 Objectives

The objectives of this work are:

1. To address the involvement of *KNOX* genes in secondary growth of *Arabidopsis* hypocotyls.

2. To figure out the action of *KNOX* genes on cambial cell divisions and differentiation during secondary growth of the *Arabidopsis* hypocotyls.

3. To address the function of *KNOX* genes in secondary wall formation, e.g. cellulose biosynthesis and lignin deposition, in the *Arabidopsis* hypocotyls.

4. To identify downstream targets of *KNOX* function.