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Cytokinin signalling in the regulation of cambial development

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following four articles and one manuscript. In the text they are referred to by their Roman numerals. The published papers are reprinted with permission from the publishers.

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II) Tuskan GA, Difazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U, Putnam N, Ralph S, Rombauts S, Salamov A, Schein J, Sterck L, Aerts A, Bhalerao RR, Bhalerao RP, Blaudez D, Boerjan W, Brun A, Brunner A, Busov V, Campbell M, Carlson J, Chalot M, Chapman J, Chen GL, Cooper D, Coutinho PM, Couturier J, Covert S, Cronk Q, Cunningham R, Davis J, Degroeve S, Dejardin A, Depamphilis C, Detter J, Dirks B, Dubchak I, Duplessis S, Ehlting J, Ellis B, Gendler K, Goodstein D, Gribskov M, Grimwood J, Groover A, Gunter L, Hamberger B, Heinze B, Helariutta Y, Henrissat B, Holligan D, Holt R, Huang W, Islam-Faridi N, Jones S, Jones-Rhoades M, Jorgensen R, Joshi C, Kangasjarvi J, Karlsson J, Kelleher C, Kirkpatrick R, Kirst M, Kohler A, Kalluri U, Larimer F, Leebens-Mack J, Leple JC, Locascio P, Lou Y, Lucas S, Martin F, Montanini B, Napoli C, Nelson DR, Nelson C, **Nieminen K**, Nilsson O, Pereda V, Peter G, Philippe R, Pilate G, Poliakov A, Razumovskaya J, Richardson P, Rinaldi C, Ritland K, Rouze P, Ryaboy D, Schmutz J, Schrader J, Segerman B, Shin H, Siddiqui A, Sterky F, Terry A, Tsai CJ, Uberbacher E, Unneberg P, Vahala J, Wall K, Wessler S, Yang G, Yin T, Douglas C, Marra M, Sandberg G, Van de Peer Y, Rokhsar D. (2006) The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). Science. 313: 1596-1604.

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V) Nieminen K, Immanen J, Albert VA, Helariutta Y. High-resolution expression profiling of cytokinin signalling genes across *Populus trichocarpa* cambial zone. manuscript.

ABBREVIATIONS

AUX/IAA	*AUXIN/INDOLE-3-ACETIC ACID* genes
ABA	abscisic acid
ADP	adenosine diphosphate
AtGA20ox1	*Arabidopsis GIBBERELLIN 20-OXIDASE1*
ATP	adenosine triphosphate
AHK2	*ARABIDOPSIS HISTIDINE KINASE 2*
AHK3	*ARABIDOPSIS HISTIDINE KINASE 3*
AHK4	*ARABIDOPSIS HISTIDINE KINASE 4*
AHP6	*ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6*
CKI1	*CYTOKININ-INDEPENDENT 1*
CKX	cytokinin oxidase
CRE1	*CYTOKININ RESPONSE 1*
CYCD3	*Arabidopsis CYCLIN D3*
cZ	cis-zeatin
GA	gibberellin
GUS	β-glucuronidase
HPt	histidine containing phosphotransmitter
IAA	indole-3-acetic acid
iP	pentenyladenine
iPA	isopentenyladenosine
iPMP	isopentenyladenosine-5′-monophosphate
IPT	isopentenyl transferase
LOG	*LONELY GUY*
RR	response regulator
tZ	trans-zeatin
tZR	trans-zeatinriboside
wol	*wooden leg*
WUS	*WUSCHEL*
ZOG	zeatin-*O*-glucoside

ABSTRACT

Secondary growth of plants is of pivotal importance in terrestrial ecosystems, providing a significant carbon sink in the form of wood. As plant biomass accumulation results largely from the cambial growth, it is surprising that quite little is known about the hormonal or genetic control of this important process in any plant species.

Since their discovery as regulators of plant cell divisions, cytokinins have been assumed to participate in the control of cambial development. Evidence for this action was deduced from hormone treatment experiments, where exogenously applied cytokinin was shown to enhance cambial cell divisions in diverse plant organs and species.

The central aim of my thesis studies was to explore the function of cytokinin in the regulation of cambial development.

In my thesis work, the conservation of cytokinin signalling genes between herbaceous plants and trees was examined. Taking advantage of the sequenced *Populus trichocarpa* genome, genes involved in the signalling and homeostasis of cytokinins were identified from the genome of this hardwood tree species. The characterised gene families were then compared to their Arabidopsis counterparts. Presumably reflecting the ancient origin of cytokinin signalling system, the *Populus* genome contains orthologs for all Arabidopsis cytokinin signalling and homeostasis genes. Thus, genes belonging to five main families of isopentenyl transferases (IPTs), cytokinin oxidases (CKXs), two-component receptors, histidine containing phosphotransmitters (HPts) and response regulators (RRs) were identified from the *Populus* genome. Three subfamilies associated with cytokinin signal transduction, the CKI1-like family of two-component receptors, the AHP4-like HPts, and the ARR22-like atypical RRs, were significantly larger in *Populus* genome than in Arabidopsis. Potential contribution to the extensive secondary development of *Populus* by the members of these considerably expanded gene families will be discussed.

Representatives of all cytokinin signal transduction elements were expressed in the *Populus* cambial zone, and most of the expressed genes appeared to be slightly more abundant on the phloem side of the meristem. The abundance of cytokinin related genes in the cambium emphasizes the important role of this hormone in the regulation of the extensive secondary growth characteristic of tree species.

The function of the pseudo HPts in primary vascular development was studied in Arabidopsis root vasculature. It was demonstrated that the pseudo HPt AHP6 has a role in locally inhibiting cytokinin signalling in the protoxylem position in the Arabidopsis root, thus enabling differentiation of the protoxylem cell file. The possible role of pseudo HPts in cambial development will be discussed.

The expression peak of cytokinin signalling genes in the tree cambial zone strongly indicates that cytokinin has a role in the regulation of this meristem function. To address whether cytokinin signalling is required for cambial activity, transgenic *Populus* trees with modified cytokinin signalling were produced. These trees were expressing a cytokinin catabolic gene from

Arabidopsis, *CYTOKININ OXIDASE 2*, (*AtCKX2*) under the promoter of a *Betula CYTOKININ RECEPTOR 1* (*BpCRE1*).

The *pBpCRE1::CKX2* transgenic *Populus* trees showed a reduced concentration of a biologically active cytokinin, correlating with their impaired cytokinin response. Furthermore, the radial growth of these trees was compromised, as illustrated by a smaller stem diameter than in wild-type trees of the same height. Moreover, the level of cambial cytokinin signalling was down-regulated in these thin-stemmed trees.

The reduced signalling correlated with a decreased number of meristematic cambial cells, implicating cytokinin activity as a direct regulator of cambial cell division activity. Together, the results of my study indicate that cytokinins are major hormonal regulators required for cambial development.

1. Introduction

A substantial amount of plant biomass, in the form of wood, originates from the activity of vascular cambium. Wood is the xylem tissue of vasculature, which continuously enlarges throughout the life of dicotyledons. This developmental process, called secondary growth, is driven by the vascular cambium, a cylindrical secondary meristem. Cell divisions taking place in the stem cells of cambium produce secondary xylem (wood) and phloem (bark) and result in the radial growth of stems and roots of woody plants (Fig. 1; I).

As plant biomass accumulation results largely from secondary growth, it is surprising that so little is known about the hormonal or genetic control of this important process in any plant species. For this reason, the vascular cambium has earned the characterisation of being “the least understood plant meristem” (Groover 2005).

This situation is changing, however, due to the recent development of genomic and molecular genetic tools for the model tree genus *Populus*. These new tools, including the milestone of the *Populus trichocarpa* genome sequencing (II), will greatly facilitate research of secondary growth. Furthermore, lately Arabidopsis has been emerging as a plausible model for studies of secondary development (I). Despite being an annual herbaceous plant, Arabidopsis still displays the basic features of secondary growth present in other dicotyledons (I), although in a miniature scale as compared to tree species.

The aim of my PhD research has been to characterise the function of the plant hormone cytokinin in the secondary growth. My major research model has been *Populus* tree species, complemented by experiments carried out in Arabidopsis.

I will first describe the process of secondary development and introduce *Populus* and Arabidopsis as a model species for studies of this process. I will then sum up the current understanding about the cytokinin signal transduction pathway and its role in the regulation of plant meristem activity. In addition to cytokinin, the function of two other plant hormones, auxin and gibberellin, in the cambial meristem will also be introduced. My own results will be discussed within this context.

1.1. Plant secondary development

The growth of a plant depends on the function of its meristems, the first of which are established already during embryogenesis. The activity of the two primary meristems, in the shoot and root apex, forms primary tissues from which all plant organs develop (I, Fig. 1). Defining the location of vascular tissue development, provascular strands consisting of procambial cells form within the primary tissue. The primary vascular tissues, xylem and phloem, will later differentiate inside these strands (I, Fig. 1).

The first xylem cells to differentiate within the primary vascular bundles represent protoxylem; metaxylem cells differentiate later. The protoxylem cells differentiate in the position next to the pericyle in the root and in the innermost position of the vascular bundles in the shoot. These two primary xylem cell types can be identified based on their secondary cell wall characteristics; protoxylem cells have ring-

like (annular) or helical (spiral) cell wall thickenings, whereas the metaxylem cells have either netlike (reticulate) or porous (pitted) thickening.

After the xylem and phloem cells have differentiated inside the vascular bundles, a population of undifferentiated, pluripotent procambial cell files remains between them throughout primary development (I, Fig. 1). Later in the life of the plant, these persisting procambial cells start to divide. By giving rise to the secondary meristem, the cylindrical vascular cambium, these cell divisions mark the start of secondary development (Baucher et al. 2007).

In the shoot, in addition to the procambial cells within vascular bundles, parenchymous cells in the regions between the bundles also contribute to the formation of the complete cambium cylinder (I, Fig. 1). The part of cambium cylinder formed within the vascular bundles is called fascicular cambium and that formed in the regions between the vascular bundles is the interfascicular cambium (Esau 1965). In the root, part of the cambium is formed by the procambial cells within the vascular bundle, whereas pericycle cells give rise to the vascular cambium in the position next to the xylem poles.

In addition to the vascular cambium, plants have another secondary lateral meristem; the cork cambium, or phellogen. Pericycle cells in the root and the outermost parenchymous cells layers in the shoot give rise to this meristem, whose activity forms a protective outer layer, cork, for plant organs (Fig. 1) (Esau 1965).

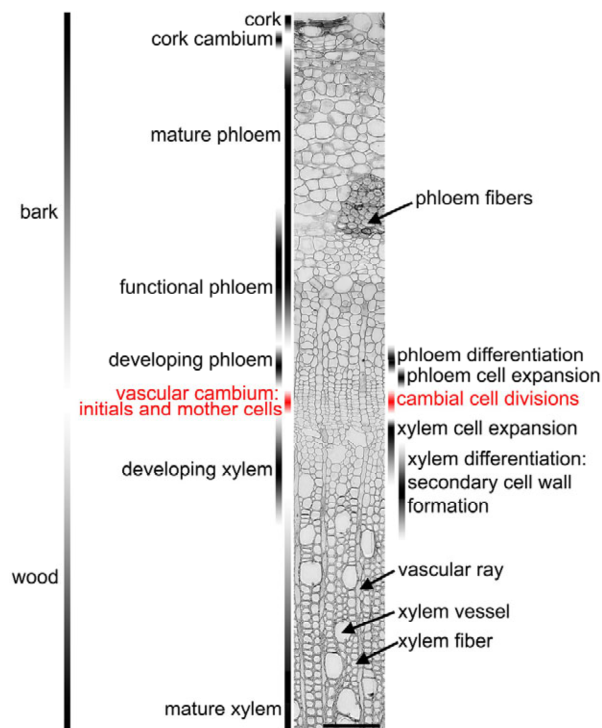

Fig. 1 Cross-section of a *Populus tremula* × *tremuloides* stem showing the organization of the cambial region. In the middle of the cambial zone reside the meristematic cells; comprised from the stem cells, and the phloem and xylem mother cells derived from them. The exact location of the cambial stem cells is not known. New phloem cells differentiate on the peripheral side of the cambium and new xylem cells on the internal side. Bark is a general name for the secondary phloem and wood for the secondary xylem tissues. The surface of the stem is covered by cork tissue, which is produced through activity of the second secondary meristem of *Populus* stem, the cork cambium. Scale bar 200 µm.

However, the majority of the secondary tissues in plants, the bulk of which consists of xylem, is formed through the activity of vascular cambium. In nearly all vascular plants, the secondary xylem (wood) consists of three general cell types: the interconnected xylem vessels, which are the actual conduits for water and solutes; the xylem fibers, which are cells with thick secondary cell walls that provide structural support; and the xylem parenchyma cells, which can later become fibers (Fig. 1). Secondary phloem (bark) consists instead of sieve elements, companion cells, fibers, and parenchyma cells. In addition to the axial vascular tissue system of xylem and phloem cells, secondary xylem has a radial system of rays arranged longitudinally relative to the long axis of the stem or root (Fig. 1). The vascular rays, consisting mostly of parenchymous cells, serve to transport substances, including photosynthesis products and water, across the stem between the secondary xylem and phloem.

Reflecting the radial organisation of the stem and root, cambium is inherently polarized, such that the daughter cells produced on one, peripheral, side will acquire phloem fates, while those produced on the other, internal, side will acquire xylem fate (Fig. 1; I, Fig. 1). A radial developmental gradient, consisting of regions of cell division, cell expansion, and cell differentiation, can be defined across the cambial zone (Fig. 1; I, Fig. 1). In the middle of the cambial zone reside the dividing cells, the cambial stem cells and the mother cells produced through their activity; on the internal side are the expanding xylem cells and the secondary cell wall forming xylem, and on the peripheral side are the expanding phloem cells which will gradually differentiate into mature phloem (Fig. 1; I, Fig. 1). In the domain of cell divisions, anticlinal divisions, which take place parallel with the radius of the organ, expand the cambial cylinder radially, whereas periclinal divisions, occurring parallel with the surface of the organ, produce new phloem or xylem cells.

1.1.1. Cambial stem cells produce new vascular tissues

At the heart of the function of vascular cambium are the meristematic initials, the stem cells. Through their division they give rise to new cells, which will ultimately differentiate into the secondary vascular tissues xylem and phloem (Fig. 1). Two types of initial cells can be identified in the cambium: fusiform initials, which give rise to the axial cell system, and ray initials, responsible for ray development (Lachaud et al. 1999) These initials differ somewhat in their morphology; the fusiform initials are highly elongated whereas ray initials are more or less isodiametric cells. (Esau 1965; Lachaud et al. 1999).

When a cambial initial divides into two, one of the new cells retains the cambial initial identity, while the other, depending on which side of the cambial initial it resides, undergoes a committed fate as a xylem or phloem mother cell. The mother cell is able to divide several times, and give rise to multiple cells that are required for secondary vascular tissues (Lachaud et al. 1999).

Since the initials and mother cells look similar (both are thin-walled, flat cells) the exact location of the cambial initials within the cambial meristem can not be identified through anatomical observation. However, a functional definition of the cambial initials

by Schrader et al. (2004) states that they are the only cells in radial files that are able to 1) produce phloem and xylem mother cells through periclinal cell divisions and 2) initiate new cell files through anticlinal divisions. In the *Populus* stem, most of the anticlinal cell divisions take place in the peripheral domain of the meristematic cambial cells; they occur close to the phloem cells (Schrader et al. 2004; Nilsson et al. 2008). This indicates that the vascular cambial stem cells are located in that domain of the cambial zone (Schrader et al. 2004). This conclusion is, however, based on the definition of the initials as the only cambial cells able to divide both periclinally and anticlinally. It may still be possible that the mother cells derived from the initials may also divide anticlinally and form new cell files. Molecular markers that would be able to differentiate between the initials and mother cells would help to confirm the exact localisation of cambial initials.

1.2. *Populus* vs. Arabidopsis as model species in secondary development studies

Woody growth resulting from extensive secondary xylem development is a defining trait of tree species. This woody growth habit, which results from the function of the cylindrical vascular cambium, is characteristic of dicotyledonous species. Monocotyledons generally do not display secondary growth and are accordingly limited in size, mostly representing herbaceous plants.

The development of cylindrical cambium is an evolutionarily ancient feature, probably already present before the divergence of gymnosperm and angiosperm plants (Rowe and Speck 2005), yet it has been gained and lost multiple times during plant evolution (Groover 2005). Reflecting this evolution history, species with various degrees of secondary growth, ranging from trees to herbs, are found within dicotyledon orders and families. This suggests that secondary growth is a quantitative trait, a measure of degree, rather than a qualitative trait that is either present or absent in certain species (Groover 2005). This is reflected by the fact that *Populus* and Arabidopsis (Chaffey et al. 2002; I), despite having strikingly different growth habits, both display secondary growth, albeit on a drastically different scale.

Due to the sheer amount of secondary growth present in trees, they seem to represent natural model organisms in which to study this process. However, working with trees presents some additional challenges, as well as some unique benefits, when compared to research using annual model species. One obvious challenge is that the traditional genetics methods are hard to implement on trees (Groover 2005). Most tree species have long generation times and can take many years or decades to become sexually mature. They also tend to grow to an inconveniently large size during this time. In addition to the long generation time, the out-crossing tendency of forest trees contributes to their highly heterozygous genomes, and trees often suffer from inbreeding depression. Due to these features, many of the traditional developmental genetic strategies used in model annuals are impractical for trees. For example, mutagenesis-based genetic screens are almost impossible to conduct with trees, as homozygous loss-of-function mutants can not be produced through inbreeding.

1.2.1. *Populus* as a model for secondary development studies

During recent years, *Populus* has emerged as the most popular model tree species. Many genomic and transgenic technologies can be directly applied to *Populus*, and they have contributed greatly to our understanding of secondary growth in the recent years.

Due to the large size and radial organisation of tree stems, significant amounts of cells from homogeneous tissue types can be harvested from the cambial zone at specific stages of development. The cambial zone can be divided into cryo-fractions representing meristematic cambial cells, developing xylem and phloem cells and mature phloem and xylem cells. This method has been successfully used in numerous studies (Schrader et al. 2004; Uggla et al. 1998; Hertzberg et al. 2001; Schrader et al. 2003; Schrader et al. 2004; Israelsson et al. 2005; IV, V).

Transformation of *Populus* is relatively straightforward, allowing the function of individual genes to be studied in detail using a transgenic approach. The primary strategy is to introduce a transgene that produces a dominant phenotype which can already be characterised in primary transformants. Although this is a powerful approach for determining gene function, the production and thorough characterisation of transgenic *Populus* trees is still somewhat laborious and time consuming, especially when compared to Arabidopsis.

1.2.2. Arabidopsis as a model for secondary development

Despite being an annual, or biannual, herbaceous species, Arabidopsis displays significant secondary growth in the inflorescence stem, hypocotyl and root (Chaffey et al. 2002; I). Therefore, the use of Arabidopsis, the most popular model plant for dicotyledons, has recently gained popularity in secondary development research (Ko et al. 2004; Pineau et al. 2005; Zhao et al. 2005; Sibout et al. 2008; reviewed in I). The process of secondary development in the Arabidopsis hypocotyl and root has been observed to occur in two phases: an early phase of proportional radial growth, in which the cambium produces both xylem and phloem at a similar rate, and a later xylem expansion phase, in which the cambium produces more xylem than phloem (Chaffey et al. 2002; Sibout et al. 2008). During the first phase, only xylem vessels and parenchyma cells differentiate in the secondary xylem, whereas during the second phase both vessels and fibers differentiate (Chaffey et al. 2002). The later phase, characterised by extensive wood formation, closely resembles the secondary growth in tree species. It is thus feasible that the mechanisms controlling secondary development in Arabidopsis have parallel mechanisms in trees.

Arabidopsis is, however, devoid of some major characteristics of tree species, one of the most obvious being the lack of perennial growth characterised by an annual cycle of cambial activity and dormancy. Furthermore, no ray cells have been observed in the Arabidopsis secondary xylem, indicating a different system from *Populus* in regulating radial transport across secondary xylem (Chaffey et al. 2002).

Thus, studies of trees and herbaceous species complement each other, and research on both is needed to fully understand the process of secondary development in plants.

1.3. Cytokinin phytohormones

Cytokinins are a class of plant hormones that are central to the regulation of cell division and differentiation in plants. They are known to control various processes in plant growth and development, including delay of senescence (Gan and Amasino 1995), control of shoot and root meristem activity (Werner et al. 2001; Werner et al. 2003, Miyawaki et al. 2004, Higuchi et al. 2004) and transmission of nutritional signals (Takei et al. 2001; Sakakibara 2006).

Naturally occurring cytokinins are N6-substituted adenine derivatives carrying either an isoprene-derived or aromatic side chain (Kakimoto 2003; Sakakibara 2006). Cytokinins can be classified into four groups: isopentenyladenine(iP)-type, trans-zeatin(tZ)-type, cis-zeatin(cZ)-type and aromatic cytokinins, depending on the identity of the side chain. Biologically active cytokinins are the free base forms (iP, tZ, and cZ), and the first steps of their biosynthesis are catalysed by the ATP/ADP isopentenyltransferases (ATP/ADP IPTs) (Miyawaki et al. 2004). A novel rice gene, *LONELY GUY,* was recently identified to function in the final step of cytokinin biosynthesis (Kurakawa et al. 2007). The LOG protein releases the bioactive free base cytokinin, iP or tZ, from the inactive cytokinin nucleotide form. The best-known cytokinin catabolic enzymes are the cytokinin oxidases/dehydrogenases (CKXs) (Werner et al. 2001; Werner et al. 2003).

1.3.1. The cytokinin signal transduction phosphorelay

The cytokinin signal transduction pathway is well known in Arabidopsis. Plants respond to cytokinins through a phosphorelay consisting of a multistep phosphate transfer between histidine and aspartate residues (Hwang and Sheen, 2001; Kakimoto 2003). The components participating in the phosphorelay are the plasma membrane located two-component histidine kinase receptors, histidine containing phosphor-transmitters (HPts), which move between the cytoplasm and nucleus, and nuclear-localised phosphoaccepting type-A and type-B response regulators (RRs) (Hwang and Sheen, 2001).

At the start of the phosphorelay, when a cytokinin molecule binds to a histidine kinase receptor, the receptor is auto-phosphorylated on a histidine residue in its transmitter domain. From the phosphorylated histidine, the phosphate is transferred to an aspartate residue in a receiver domain of the receptor. From the receiver domain of the receptor, the phosphate is then transferred to a histidine in a cytoplasm-located HPt. The phoshorylated HPt moves into the nucleus, where it transfers the phosphate to an aspartate in the receiver domain of a type-A or type-B RR (Hwang and Sheen, 2001). Phosphorylated type-B RRs act as transcription factors and induce expression of cytokinin primary response genes, including type-A RRs. In contrast to the type-B RRs, the phosphorylated type-A RRs act as inhibitors of cytokinin signalling (Lee et al. 2007 and 2008; To et al. 2007).

1.3.2. Cytokinin receptors

The Arabidopsis genome includes three cytokinin receptors CRE1/WOL/AHK4, AHK2 and AHK3, which belong to the superfamily of two-component regulators. In addition to the cytokinin receptors, this family in Arabidopsis includes five ethylene receptors, phytochromes (PHYA-E), one putative osmosensor (AtHK1), one histidine kinase (CKI2/AHK5) implicated in ethylene and ABA signalling (Iwama et al. 2007), and one histidine kinase (CKI1) of unknown, but potentially cytokinin signalling related, function (Kakimoto et al. 2003). The CKI1 and its *Populus* orthologs will be discussed in more detail in the chapter 4.3.

Compared to AHK2 and AHK3, CRE1 seem to have a unique ability among the Arabidopsis cytokinin receptors to act bidirectionally on the HPts. CRE1 acts as a kinase that phosphorylates HPts in the presence of cytokinin and as a phosphatase which dephosphorylates them in the absence of cytokinin (Mähönen et al. 2006). The *wol* mutation in the *CRE1* gene abolishes its cytokinin binding ability (Yamada et al. 2001; Mähönen et al. 2006), and thus transforms the receptor to constitutive phosphatase activity. This activity removes phosphate from HPts and therefore, in a dose-dependent manner, inhibits the phosphorelay from proceeding onwards from HPts (Mähönen et al. 2006).

1.3.3. Histidine containing phosphor-transmitters

Canonical HPts are positive components of the cytokinin signalling phosphorelay, they mediate the phosphotransfer from receptor kinases to phosphoaccepting RRs. We will take a closer look at the HPt family in chapters 4.2., 4.4. and 4.5.

1.3.4. Response regulators

The RRs can be classified into four subfamilies: 1) A-type RRs, which contain only a phospho-accepting receiver domain with the phospho-accepting aspartate residue, 2) B-type RRs, in which the receiver domain is fused to a DNA-binding domain, 3) pseudo RRs, which lack the phospho-accepting aspartate in their receiver domain, and 4) extra RRs, which, despite having an unconventional receiver domain, still contain a phospho-accepting aspartate residue (Kiba et al. 2004).

The expression of type-A RRs is induced by cytokinin and they act as repressors of cytokinin activated gene expression (D'Agostino et al. 2000; To et al. 2004). They thus represent negative feedback regulators of cytokinin signalling.

By contrast, type-B RRs are DNA-binding transcriptional activators that positively mediate cytokinin responses (Hwang and Sheen, 2001; Sakai et al. 2001). The pseudo RRs are known to participate in the regulation of light responses in Arabidopsis (Makino et al. 2000; Mizuno 2004; Murakami et al. 2004) and are not known to have any role in the cytokinin signalling.

Extra RRs represent a poorly characterized RR characterized by an atypical receiver domain amino acid sequence. They have been observed to have phosphatase activity towards HPts in an *in vitro* assay, indicating that they may interact with the cytokinin signalling phosphorelay (Kiba et al. 2004). However, as their expression is not induced by cytokinin, their

possible connection with cytokinin signalling is not known (Kiba et al. 2004).

1.4. Cytokinin signalling in the regulation of meristem activity

1.4.1. Cytokinin signalling in the function of the shoot apical meristem

Cytokinin was originally identified as an agent able to induce plant cell division (Miller et al. 1955). It was later determined that cytokinins can activate plant cell divisions through by inducting the expression of a cell cycle activator, *CYCD3* (*Arabidopsis CYCLIN D3*) (Riou-Khamlichi et al. 1999; Dewitte et al. 2007).

In accordance with their ability to stimulate plant cell divisions, cytokinins regulate the activity of plant apical meristems. Cytokinin signalling has been observed to have an opposite function in the two apical meristems: moderately cytokinin deficient plants display reduced shoot growth and accelerated root elongation (Werner et al. 2003).

Reflecting the positive role of cytokinins in the shoot apical meristem, the size of this meristem is reduced in the Arabidopsis triple cytokinin receptor mutant (Higuchi et al. 2004), as well as in mutants lacking several cytokinin biosynthetic enzymes (*atipt1;3;5;7*) (Miyawaki et al. 2006). The same phenotype is also seen in a triple mutant for three positive regulators of cytokinin signalling, the type-B response regulators (*arr1,10,12*) (Ishida et al. 2008).

Furthermore, the rice gene *LONELY GUY*, which encodes a cytokinin-activating enzyme, was recently shown to have specific expression in the shoot apical meristem (Kurakawa et al. 2007). Accordingly, the *log* mutant, in which the cytokinin signalling is reduced at the shoot apical meristem, shows a severe reduction in shoot apical meristem size (Kurakawa et al. 2007). This observation indicates that LOG function is required to maintain shoot apical meristem activity, presumably by maintaining a high concentration of bioactive cytokinins in it.

1.4.2. Cytokinin signalling in the function of the root apical meristem

Contrary to the reduced shoot growth in cytokinin deficient mutants, cytokinins have been perceived to have a negative effect on root growth. Treatment with external cytokinin inhibits root elongation (Cary et al. 1995). Supporting this observation, the elongation rate of the primary root is enhanced in mutants lacking one (*ahk3*) or two (*ahk2 ahk3*) cytokinin receptors (Riefler et al. 2006; Dello Ioio et al. 2007), or several key cytokinin biosynthetic enzymes (*atipt1;3;5;7* quadruple mutant for ATP/ADP isopentenyltransferases) (Miyawaki et al. 2006) and in transgenic plants expressing a cytokinin degrading enzyme, CKX, under the 35S promoter (Werner et al. 2003).

Dello Ioio at el. (2007) was able to show that the negative effect of cytokinins on root elongation is due to their regulatory function on root meristem length. The stem cells of the root apical meristem are located in the tip of the root. The zone where the stem cell derived vascular mother cells further divide is called the meristematic zone. Cytokinin facilitates the exit of undifferentiated meristematic cells from the meristematic zone to the elongation-differentiation zone, where they start to differentiate into various root cell types

(Dello Ioio et al. 2007). Cytokinin thus controls the length of the meristematic zone and therefore the number of dividing meristematic mother cells, whereas cytokinin treatment does not inhibit divisions of the stem cells in the root tip (Dello Ioio et al. 2007).

Some level of cytokinin signalling is still required for root meristem activity, as shown by studies of the Arabidopsis *wooden leg* (*wol*) mutant. As discussed above, this mutant represents an altered form of the CRE1 receptor with a negative activity in the cytokinin phosphorelay. In this mutant, the number of periclinal cell divisions in the root vasculature is reduced, and primary root growth is aborted (Mähönen et al. 2000; Higuchi et al. 2004). In addition, all of the cell files in the root vasculature of *wol* differentiate into protoxylem (Mähönen et al. 2000), indicating that cytokinin signalling is necessary to allow other cell types to differentiate in the vasculature (Higuchi et al. 2004).

Reflecting the negative role of the *wol* mutant form in cytokinin signalling, the *wol* root phenotype is phenocopied in mutants lacking several positive cytokinin signalling components. These include a mutant lacking all three cytokinin receptors (*cre1*, *ahk2*, *ahk3*) (Higuchi et al. 2004; Nishimura et al. 2004), a quintuple AHP mutant (*ahp1,2,3,4,5*) (Hutchison et al. 2006), and a mutant lacking three type-B Arabidopsis response regulators (*arr1,10,12*) (Argyros et al. 2008; Ishida et al. 2008). Based on these observations, some minimum level of cytokinin signalling seems to be required for the maintenance of undifferentiated stem cell activity and meristematic cell divisions in the root apical meristem.

Further supporting the role of cytokinin in the maintenance of root stem cell activity, it was recently shown that a transient antagonistic interaction between auxin and cytokinin signalling is essential for root stem cell specification during early stages of embryogenesis (Müller and Sheen 2008). In this study, auxin was shown to specify the domain of cytokinin signalling by inducing the expression of negative regulators of the phosphorelay. The introduction of an engineered dominant negative repressor of cytokinin signalling led to strong pattern deficits early in embryogenesis (Müller and Sheen 2008). In contrast to the dominant negative approach, no obvious defects in embryogenic pattern formation were seen in the triple cytokinin receptor mutant (*cre1*, *ahk2*, *ahk3*) (Higuchi et al. 2004). This indicates that cytokinin phosphorelay may be induced independently of the CRE cytokinin receptors at some stages of embryogenesis (Müller and Sheen 2008).

Based on these observations, it seems that cytokinin plays different roles in the tip of the root apical meristem and in the upper part of the root, in the position where cells exit from from the meristematic zone to the elongation-differentiation zone (Benková and Hejátko 2009). Future studies will clarify how plants balance these two somewhat contradicting roles of cytokinin in the root meristem development.

1.4.3. Cytokinin signalling in the regulation of cambial meristem activity

Since their discovery as stimulators of plant cell divisions, cytokinins have been assumed to participate in the regulation of cambial activity. Evidence for this function was

deduced from hormone treatment experiments. In these studies, when exogenous cytokinin was applied together with auxin, the treatment was shown to enhance cambial cell divisions in diverse plant organs and species (Loomis and Torrey 1964; Saks et al. 1984). However, until very recently there was no evidence whether endogenous cytokinins are required for cambial development.

A recent study published back-to-back with my *Populus* paper (IV) shows that cytokinins are required for cambial activity in Arabidopsis (Matsumoto-Kitano et al. 2008). In this work, secondary development was studied in the *atipt1;3;5;7* quadruple mutant lacking four key cytokinin biosynthetic ATP/ADP isopentenyl-transferase enzymes. Accordingly, the level of cytokinins was severely reduced in this mutant (Miyawaki et al. 2006). Both the size of the rosette and the height of the inflorescence stem were reduced, reflecting the function of cytokinins as positive regulators of shoot apical meristem activity. By contrast, root elongation was accelerated (Miyawaki et al. 2006), in accordance with the role of cytokinins in restricting the length of the meristematic zone in the root.

However, the most dramatic phenotype in the *atipt1;3;5;7* mutant was the lack of vascular cambium, and consequently radial growth, in the root (Matsumoto-Kitano et al. 2008). External application of cytokinin was able to recover the formation of the cambial cylinder and radial growth in the root in a dose-dependent manner (Matsumoto-Kitano et al. 2008). This result shows that cytokinins are central regulators of the formation and activity of the vascular cambium. In addition, the radial growth of wild-type roots was stimulated through external cytokinin application, and overexpression of *AtIPT* genes also enhanced secondary growth. These results further confirm the role of cytokinins in the regulation of cambial activity (Matsumoto-Kitano et al. 2008).

In contrast to the root, formation of fascicular and interfascicular cambium was observed in the short inflorescence stem of the *atipt1;3;5;7* mutant (Matsumoto-Kitano et al. 2008). However, the activity of both was greatly reduced, resulting in the development of a thin stem. The presence of some level of cambial activity may reflect the fact that these plants still have some functional IPTs and were not completely devoid of cytokinins (Matsumoto-Kitano et al. 2008). Supporting this hypothesis, the inflorescence stem of the quintuple mutant, which displayed some cambial activity, had slightly higher cytokinin levels than the root, which was altogether lacking cambium (Matsumoto-Kitano et al. 2008).

Another very important observation by Matsumoto-Kitano et al. (2008) was that cytokinins are transported through the plant. As the result of grafting experiments, it was shown that a cytokinin deficient root could be rescued through grafting onto a wild-type shoot, and vice versa. When an *atipt1;3;5;7* mutant shoot was grafted onto a wild-type root, the radial growth of the mutant shoot recovered to normal levels; when a wild-type shoot was grafted onto an *atipt1;3;5;7* mutant root, a cambium was formed in the mutant root and radial growth was completely recovered. This result indicates that cytokinins which were produced in an organ with functional cytokinin biosynthetic enzymes were transported into organs devoid of cytokinin biosynthesis, where they were fully functional. Furthermore, through

cytokinin concentration analyses, it was shown that tZ-type cytokinins were transported from root to shoot and iP-type from shoot to root. Interestingly, no obvious phenotypes were seen in the tZ deprived root or in the iP deprived shoots, indicating that the plant can survive solely by the shoot or root produced cytokinins (Matsumoto-Kitano et al. 2008). It remains to be seen whether the different cytokinins have specific functions in other developmental processes than secondary growth.

1.5. Other hormones in the regulation of cambial meristem function

Besides cytokinin, several other hormones, including auxin and gibberellin, have been implicated in the control of cambial activity, because exogenous application of these hormones to plant organs has a stimulatory effect on cell divisions. We will now take a look at what is known about their role in the regulation of cambial activity.

1.5.1. Auxin

Auxin is a well known hormonal regulator of secondary development. Classic hormone treatment studies have implicated auxin as a stimulator of cambial activity, since applied auxin can reactivate cambium in decapitated shoots (Snow, 1935; Digby and Wareing, 1966; Little and Bonga 1974; Little et al. 2002; Björklund et al. 2007; reviewed by Savidge 1988). The shoot apex is a major source of auxin (Sundberg and Uggla 1998), and auxin is transported from the apex downwards through the stem (Little and Savidge 1987; Björklund et al. 2007).

A radial gradient of auxin (IAA) has been detected across the cambial zone of both *Populus* and *Pinus* tree, indicating a possible role for this hormone in secondary development, s (Uggla et al. 1996, 1998; Tuominen et al. 1997). The level of IAA peaks in the dividing cambial cells, from which it decreases steeply towards differentiating phloem and more gradually towards differentiating xylem. This gradient is assumed to be formed when auxin that is transported downwards from the stem apex is differentially distributed across the cambial zone (Schrader et al. 2003). Supporting this, several genes encoding auxin transporters are expressed across the cambial zone in *Populus* (Schrader et al. 2003).

The cambial auxin gradient correlates with an expression peak of auxin signalling genes in the cambial cells (Moyle et al. 2002). Recently, however, Nilsson et al. (2008) observed that a large portion of auxin-responsive genes was expressed at a higher level in the differentiating xylem cells than in the meristematic dividing cells, where the auxin concentration is at its highest (Nilsson et al. 2008). The reason for this difference between the auxin signalling and auxin response gene expression patterns remains to be clarified.

Functional studies using transgenic *Populus* trees have further described the role of auxin in the regulation of cambial development (Nilsson et al. 2008). Nilsson et al. (2008) engineered transgenic *Populus* trees to ectopically express a stabilized form of a *Populus AUX/IAA* (*PttIAA3stabilized*), which acts as a repressor of auxin responsive gene expression, under the *35S* promoter. In these trees, the number of both periclinal and anticlinal cell divisions of the meristematic cambial cells was reduced, resulting in compromised radial growth of the stem. As

was discussed in chapter 1.1.1., Schrader et al. (2004) had previously observed that in wild-type *Populus*, anticlinal divisions appeared to be restricted to the cambial region close to the phloem. In the *p35S::PttIAA3stabilized* overexpressing trees, anticlinal divisions were instead spread across a wider zone, also occurring in the middle of the cambium, and even occasionally close to xylem cells (Nilsson et al. 2008). As Schrader et al. (2004) have proposed that only the cambial initials undergo anticlinal cell divisions, this shift of the anticlinal divisions in the *p35S::PttIAA3stabilized* transgenic *Populus* indicates that auxin signalling may regulate the position of initials within the vascular cambium, or at least the domain where their anticlinal divisions take place.

Further evidence for the function of auxin as a positive regulator of cambial activity is provided by *INTERFASCICULAR FIBERLESS/REVOLUTA ifl Arabidopsis* mutants. In these mutants, down-regulation of auxin transporter expression results in dramatically reduced basipetal auxin flow and consequently reduced cambial activity in the basal parts of inflorescence stems (Zhong et al. 1997; Zhong and Ye 1999, 2001). Additionally, auxin has been shown to mediate the signalling of plant body weight to the cambium. Weight of the stem was shown to have some positive effect on cambial activity in Arabidopsis inflorescence stems in the work of Ko et al. (2004). In this study, it was reported that weight stimulus facilitates auxin transport in the inflorescence stem and subsequently promotes the development of secondary xylem.

However, the relationship between the status of auxin transport along the stem and the presence of an auxin gradient across the cambial zone appears to be complex. In *Populus*, during the transition to cambial dormancy, polar auxin transport is severely reduced (Schrader et al. 2003). This is reflected by the observation that the cambial activity cannot be reactivated when auxin is applied to decapitated stems which are in a dormant state (Little and Bonga 1974). Additionally, expression of the auxin inducible *PttIAA* genes is reduced during the transition of the active cambium into dormancy, indicating a down-regulated status of auxin signalling (Moyle et al. 2002). However, in contrast to the reduced auxin transport, the cessation of cambial growth upon the induction of dormancy does not decrease the actual cambial IAA concentration in *Pinus* (Uggla et al. 1996, 2001). Taken together, these results suggest the possibility that the level of cambial auxin transport, responsiveness and signalling, but not the level of the cambial auxin concentration itself, may regulate the cambial activity.

1.5.2. Gibberellin

Similar to auxin, application of gibberellin (GA) to decapitated *Populus* stems also stimulates cell divisions in the cambial zone (Digby and Wareing 1966; Wang et al. 1997; Björklund et al. 2007). However, when GA is applied the identity of the newly formed cells is somewhat obscure (Björklund et al. 2007). Instead of differentiating into xylem cells on the internal side of cambial zone, the new cells produced under GA-treatment appear to remain in a parenchymatous state. As a result, GA-treatment leads to the loss of an easily distinguishable vascular cambium

(Björklund et al. 2007). The loss of xylem differentiation in GA-treated stems is somewhat unexpected, as a tissue specific distribution pattern of GAs across the *Populus* stem shows that bioactive GAs peak in the expanding xylem cells (Israelsson et al. 2005). The concentration peak coincides with the expression of GA biosynthetic and signalling genes, which would indicate a role for GA in xylem differentiation (Israelsson et al. 2005). It is possible that hormone treatment may disturb the endogenous distribution of GA across the cambial zone (Björklund et al. 2007), and thus lead to slightly aberrant effects on xylem differentiation.

However, application of IAA together with GA to decapitated stems enhances cambial cell divisions more than either hormone alone; furthermore, xylem differentiation seems to proceed normally. This result indicates that these two hormones have a synergistic effect on cambial growth (Digby and Wareing 1966, Björklund et al. 2007). Further supporting this synergistic interaction, Björklund et al. (2007) observed that IAA concentration in stem tissues is higher when IAA is applied in combination with GA than when IAA is applied alone. This indicates that GA action promotes auxin transport. Furthermore, GA treatment induces the expression of a cambial abundant *Populus* auxin transport protein gene, *PttPIN1*. Auxin treatment also stimulates the expression of GA biosynthesis genes and inhibits expression of GA degradation genes; GA and auxin treatments induce similar changes in the transcriptome (Björklund et al. 2007).

Further support for the stimulating effect of GA on meristem activity was obtained when the shoot size of transgenic *Populus* trees was increased through ectopic overexpression of a GA biosynthetic enzyme (*AtGA20ox1*) (Eriksson et al. 2000). Future studies will further clarify the interplay between auxin and GA in the regulation of cambial cell divisions and xylem differentiation.

2. Aims of the study

The central question of my thesis work has been: Does cytokinin hormone signalling control cambial activity?

The specific aims have been to answer the following questions:

1) During evolution, has the cytokinin signal transduction pathway diversified between herbaceous plant and trees? Can similar cytokinin signalling and homeostasis related components be found in both Arabidopsis and *Populus*? (II, IV, V)

2) Are cytokinin signalling and homeostasis genes expressed in the *Populus* cambial zone? Does expression domain give any indication of their function in this meristem? (IV, V)

3) What is the role of the pseudo HPts in the development of Arabidopsis root vasculature? (III) Is there any indication that pseudo HPts function in cambial development? (V)

4) Is cytokinin required for cambial activity? If we reduce the level of cambial cytokinin signalling, do we see a reduction in cambial cell divisions? (IV)

3. MATERIALS AND METHODS

The materials and methods are described in detail in the respective publications. The methods used in this study are summarised in Table 1 with references to the publications in which they have been applied.

Table 1 Methods used in this study. Those in brackets were performed by my co-authors in the respective publications.

Method	Publication
Agrobacterium mediated transformation of Arabidopsis	III, IV
Agrobacterium mediated transformation of *Betula* and *Populus*	IV
Alignments of protein and DNA sequences	II, III, IV, V
Analyses of cambial cell numbers and xylem cell dimensions	IV
Confocal light microscopy	(III)
Cryo-sectioning of tree stems	IV, V
Cytokinin concentration analyses	(IV)
Ethylmethane sulfonate (EMS) mutagenesis and mutant screen	(III)
Fluorescent in situ hybridization (FISH)	(II)
Fuchsin staining	III
Gene identification through positional cloning	III
Grafting experiments	IV
Histological staining for GUS activity	III, IV
Identification and annotation of *Populus* genes	II, IV, V
In situ RNA hybridisation	(III), (IV)
In vitro Betula and *Populus* culture	IV
In vitro phosphotransfer assay	(III)
Light microscopy	III, IV, V
Plasmid construction	III, (IV)
Phylogenetic analyses	(II), (IV), (V)
Polymerase chain reaction (PCR) analysis	III, IV
Quantitative real-time PCR analysis	(III), IV, V
RNA extraction	(III), IV, (V)
Sectioning of plastic embedded samples	III, IV
Sequencing	(II), (III), (IV), (V)
Site-directed mutagenesis	(III)
Statistical analysis	(II), (III), IV, V
Tissue culture assays for cytokinin response	(III), IV

4. RESULTS AND DISCUSSION

4.1. The cytokinin receptor gene family is conserved between herbaceous and hardwood species

In order to determine whether herbaceous and hardwood plants share a similar mechanism of cytokinin perception, genes encoding cytokinin receptors were identified from two tree species, *Betula pendula* and *Populus trichocarpa.* mRNAs for three CRE family genes (*BpCRE1, BpHK2, BpHK3*) were isolated from *Betula*, and five CRE genes (*PtCRE1a, PtCRE1b, PtHK2, PtHK3a, PtHK3b*) were identified from the sequenced *P. trichocarpa* genome (II). These tree genes were orthologous to the three Arabidopsis CRE gene family members (*CRE1/WOL*, *AHK2* and *AHK3*) (IV, Fig. 1A), indicating a conserved receptor system in these species. Further evidence for conservation was obtained when it was verified that the *Betula* ortholog for *Arabidopsis CRE1*, *BpCRE1*, encodes a protein capable of functioning as a cytokinin receptor in Arabidopsis. When expressed under the Arabidopsis *CRE1* promoter, BpCRE1 was able to complement the root phenotype of an Arabidopsis mutant lacking all three CRE family genes (IV, Fig S1). As CRE cytokinin receptor genes have previously been identified from the monocotyledon species rice and maize (Yonekura-Sakakibara et al. 2004; Ito and Kurata 2006), it seems that all flowering plants perceive cytokinin through members of the CRE receptor family.

4.2. Representatives of the cytokinin signalling and homeostasis gene families are present in the *Populus* genome

Based on the receptor study, it was expected that other components of cytokinin signal transduction pathway would also be conserved between *Populus* and Arabidopsis. Indeed, genes representing all stages of the phosphorelay were identified from the *Populus* genome (V, Table 1). In addition to the five receptor genes, the *Populus* genome contains 16 *HPt*s and 44 *RR* genes (V, Fig. 5, 7, Table 1). RRs belonging to the subfamilies of type-A, type-B, pseudo and extra RRs are present in the *Populus* genome (V, Fig. 7; Table 1). Gene families coding for key cytokinin biosynthetic enzymes, IPTs, and key catabolic enzymes, CKXs, both had nine genes in *Populus*, as compared to nine and seven, respectively, in Arabidopsis (V, Fig. 1, 3, Table 1).

Thus, in general, the gene families related to cytokinin homeostasis and signalling are the same size or larger in *Populus* than in Arabidopsis. The larger gene number is expected since, due to a more recent genome duplication, the *Populus* genome in general has an average of 1.5 putative homologs for each Arabidopsis gene (II, Fig. 3A). In addition to the general gene family expansion rate, some two-component signalling related gene families have undergone a significant expansion in *Populus*. Both the *CKI1*-like two-component receptor family and the *RR* subfamily of atypical extra *RRs* consist of four genes in *Populus*, whereas Arabidopsis contains only one gene (V, Fig. 7, Table 1). Additionally the *Populus* HPt gene family is

significantly expanded: the *Populus* genome encodes 16 HPt genes, as compared to seven in Arabidopsis (V, Fig. 5).

4.3. The *CKI1*-like two-component gene family is expanded in *Populus* as compared to Arabidopsis

As discussed above, the size of the CRE cytokinin receptor family in *Populus* is 1.7-fold larger than in Arabidopsis, which is close to the general rate of gene family expansion (1.5-fold) since the divergence from a common ancestor. By contrast, one of the other two-component subfamilies, the CKI1-like histidine kinase family (Kakimoto 1996), is expanded 4-fold in *Populus*, from one Arabidopsis gene (*CKI1*) to four *Populus* genes (*PtCKI1a-d*) (V, Fig. 4). Alternatively, this gene family may have lost members in the Arabidopsis genome.

Little is currently known about the function of CKI1 (Kakimoto 2003). It has been seen to activate the cytokinin phosphorelay *in vivo*, but independently of cytokinin; thus it does not seem to represent a proper cytokinin receptor (Yamada et al. 2001). The gene is known to be essential for gametophyte development, as its knock-out mutant is female gametophyte lethal (Pischke et al. 2002). This lethal phenotype has hindered studies of its function in Arabidopsis development. It might be the case that activity of this receptor-like molecule is one reason why the triple CRE receptor Arabidopsis mutant can form a viable plant. CKI1, rather than the CRE cytokinin receptors, might activate the cytokinin phosphorelay response during some stages of early embryo development. However, this hypothesis has not yet been experimentally tested.

It would be interesting to study whether the expression of the *Populus* CKI1 orthologs is restricted to reproductive organs or if they also have expression elsewhere in the plant.

The second significantly expanded *Populus* gene family related to cytokinin signalling is the *HPts*, which is 2.3 times larger in *Populus* than in Arabidopsis (V, Fig. 5). To understand the possible role of members of the expanded HPt family in cambial growth, we will next take a look at function of pseudo HPts in Arabidopsis development.

4.4. The Arabidopsis pseudo HPt AHP6 represents an inhibitor of the cytokinin signalling phosphorelay

The function of pseudo HPts in cytokinin signalling was studied based on the isolation of *ahp6*, a mutant form of an Arabidopsis pseudo HPt, from a suppressor screen of the *wol* mutant (III). As discussed earlier, the *wol* mutant has fewer cell files in the primary root vasculature than wild-type plants and all of these files differentiate into protoxylem, resulting in aborted growth of the primary root (III, Fig. 1B). In the primary root of the *wol ahp6* double mutant, the number of cell files in the root vasculature is increased and undifferentiated cell files were present between the protoxylem files (III, Fig. 1B, S3C). This partial suppression of the *wol* vasculature phenotype also resulted in the rescue of the aborted root growth (III, Fig. 2A).

Through positional cloning, the *ahp6* mutation was identified to be in the At1g80100 gene, *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6* (III, Fig. 3A). Allelic mutations

were identified; in this text the *ahp6* refers to the *ahp6-1*, which probably corresponds to a null allele. The *ahp6* mutant displayed a subtle phenotype in the root vascular bundle: protoxylem differentiation occurred sporadically along the root, whereas in the wild-type plant the protoxylem cell file is continuous throughout the root (III, Fig. 1B, S3D, S4A). Thus, in the protoxylem poles of *ahp6*, there exist stretches of several undifferentiated cells between differentiated protoxylem cells (III, Fig. S4A).

The fate of the undifferentiated cells present in the protoxylem cell file in the *ahp6* mutant was studied further. Upon the activation of secondary development, a proportion of the procambial cell files between the xylem and phloem start to divide periclinally, thus forming the vascular cambium (III, Fig. 1A). Similar cell proliferation of the procambial cells could also be seen in the *ahp6* mutant. However, in addition to the cell divisions taking place in the developing cambium, the undifferentiated cells in the protoxylem position also underwent simultaneous periclinal cell division (III, Fig. 1B, S5). In wild-type plants, cells at the protoxylem positions at this longitudinal position in the root have already differentiated into protoxylem and therefore never divide. Taken together, the *ahp6* phenotype indicates that AHP6 has a role in promoting protoxylem differentiation in the root vascular bundle, and in the absence of AHP6 function the cell file maintains its procambial nature.

The most striking characteristic of the AHP6 protein sequence is that the conserved HPt motif of AHP6 differs from the five canonical AHP proteins; in AHP6 the conserved phospho-accepting histidine residue is replaced by an asparagine (residue number 83) (III, Fig. S6). The significance of this substitution for the phosphorelay was studied in an *in vitro* phosphotransfer assay. In this assay, the AHP6 protein could not be phosphorylated by a histidine kinase (III, Fig. 3C), whereas a mutant version of AHP6, in which the asparagine number 83 is replaced by a histidine, was able to accept a phosphoryl group (III, Fig. 3C). Furthermore, AHP6 was not only unable to accept a phosphoryl group, but it was also able to inhibit the phosphotranfer from canonical HPts to a type-B RR, ARR1 (III, Fig. 3C). These results indicate that AHP6 is unable to function as a phosphotransfer protein, due to the substitution of the conserved histidine, and that, instead, it has an inhibitory role in the two-component phosphorelay.

AHP6 has a specific expression pattern in the Arabidopsis root; it expressed only in the protoxylem position and in the protoxylem associated pericycle cell files (III, Fig. 3D, 3E, 4A, S8A). Thus, the effect of AHP6 on cytokinin signalling at the protoxylem position was studied. The expression of a cytokinin primary response gene, the type-A RR ARR15, was used as an indicator of the level of cytokinin signalling. The expression of type-A RRs is induced by cytokinin signalling; thus their expression level reflects the status of cytokinin signalling phosphorelay. In the wild-type plants ARR15 is expressed in the procambial cells between xylem and phloem; in the *ahp6* mutant its expression spreads to cover the protoxylem cell position as well, from which it is normally absent in the wild-type (III, Fig. 2C). This result indicates that the level of cytokinin signalling was increased at the protoxylem position in the *ahp6* mutant.

It can be concluded that AHP6 has a role in locally inhibiting cytokinin signalling in the protoxylem position in the Arabidopsis root, which subsequently enables differentiation of the protoxylem cell file.

We will next take a look at the structure of HPt gene family in *Populus*. The possible function of *Populus* pseudo HPts in cambial development will be discussed in chapter 4.7.

4.5. The *HPt* gene family is expanded in *Populus* as compared to Arabidopsis

It is interesting that *Populus* genome has a considerably higher number of genes coding for representatives of both canonical and pseudo HPt classes than Arabidopsis. Altogether, 16 HPt-encoding genes were identified in the *Populus* genome, compared to seven HPts present in Arabidopsis (V, Fig. 5). Eleven of the *Populus* HPts have a canonical HPt motif, whereas five lack the conserved phospho-accepting histidine residue, thus belonging to the class of pseudo HPts (V, Fig. 6).

The most extended group of the *Populus HPt*s is orthologous to one Arabidopsis gene, *AHP4*, and includes seven members (*PtHPt1*, *PtHPt3*, *PtHPt5*, *PtHPt7*, *PtHPt9*, *PtHPt13* and *PtHPt16*) (V, Fig. 5). Three of them (*PtHPt3*, *PtHPt9* and *PtHPt16*) contain a non-canonical HPt consensus motif; they lack the same conserved histidine as Arabidopsis AHP6, thus representing pseudo HPts (V, Fig. 6).

In contrast to the positive role in the phosphorelay associated with the other canonical HPts in Arabidopsis, the role of AHP4 is somewhat controversial. When Arabidopsis mutants lacking several AHPs were studied, inclusion of the *ahp4* null mutation appeared to moderately increase cytokinin sensitivity for some responses, whereas it appears to have either no role or a slightly positive role in some other responses (Hutchison et al. 2006). Thus it is not clear whether AHP4 has a negative or positive role in cytokinin signalling (Hutchison et al. 2006). It remains to be seen what the role of AHP4 orthologous *Populus* HPts is, and whether they code for negative or positive components of the phosphorelay.

4.6. The extra RRs subfamily is significantly expanded in *Populus*

Representatives of all subgroups of RRs are present in the *Populus* genome.
Type-A RRs represent negative feedback regulators of cytokinin signalling (D'Agostino et al. 2000; To et al. 2004), whereas type-B RRs are DNA-binding transcriptional activators that positively mediate cytokinin responses (Hwang and Sheen, 2001; Sakai et al. 2001). The pseudo RRs are known to participate in the regulation of light responses in Arabidopsis (Mizuno and Nakamichi, 2005) and are not known to have a role in cytokinin signalling.

The number of type-A, type-B and pseudo RR encoding genes is quite similar between *Populus* and Arabidopsis. The *Populus* genome encodes eleven type-A RRs as compared to ten representatives present in Arabidopsis (V, Table S1; Fig. 7); thirteen genes coding for type-B RR genes as compared to twelve in Arabidopsis (V, Table S1; Fig. 7); and twelve genes coding for pseudo RRs as compared to nine in Arabidopsis (V, Table S1; Fig. 7).

However, one *Populus RR* gene class is dramatically larger than in Arabidopsis:

the subfamily of atypical extra RRs includes eight members, as compared to two Arabidopsis genes *ARR22* and *ARR24* (V, Table S1; Fig. 7).

In Arabidopsis, the extra RRs are assumed to function in reproductive development, based on that their (potentially exclusive) expression in floral organs (Kiba et al. 2004; Gattolin et al. 2006). However, no effect on reproductive growth, seed development or vegetative growth could be detected in the double knockout mutant for *ARR22* and *ARR24* (Gattolin et al. 2006). Thus, the function of extra RRs is currently unknown. It would be interesting to study expression pattern of the extra RR genes in *Populus* to see if they are expressed in the reproductive organs or have more diverse expression patterns than their Arabidopsis orthologs.

4.7. Cytokinin signalling and biosynthetic genes are expressed in the cambial zone of *Populus*

Representatives of all stages of the cytokinin signalling phosphorelay, including receptors (IV, Fig. 1B, S3C), *HPt*s (V, Fig. 2C) and *RR*s (IV, Fig. 1B; V, Fig. 2D, 2E), are expressed in the *Populus* stem. Additionally, the cytokinin biosynthetic *IPT*s were expressed in the cambial zone, with *PtIPT5* showing the highest expression (IV, Fig. 3B). Expression of the receptors and the type-A *PtRR7*, clearly peaked in the cambial zone, compared to other parts of stem (IV, Fig. 1B). Presumably, expression of other signalling components, including *HP*ts, and the rest of the type-A and the type-B *RR*s, is also at its highest in this meristem.

In the cambial zone, expression of many of those genes that gave the strongest signal in qRT-PCR (receptors *PtHK3a* and *PtHK3b*, HPts *PtHPT2* and *PtHPt8*, type-A RRs *PtRR1* and *PtRR2*, type-B RR *PtRR14*) was slightly more abundant on the phloem side than on the xylem side (IV, Fig. S3C; V, Fig. 2B-E). This observed expression pattern appears to be quite reliable, as the genes with the highest expression give the most reliable signal in the qRT-PCR. It is correspondingly harder to make conclusions about expression patterns of genes with a low expression.

It is interesting that several *Populus* pseudo HPts (*PtHPt2, PtHPt3* and *PtHPt9*) are expressed in the cambial zone (V, Fig. 2C). As their expression is not restricted to the xylem side of this zone, it seems unlikely that they could promote xylem differentiation in this meristem. Supporting this observation, in the *ahp6* Arabidopsis mutant, only development of the protoxylem cell files was defected, whereas differentiation of the later emerging metaxylem cell files appeared normal (III, Fig. 1B, S4A, S5). It is thus possible that pseudo HPts have a specific function in promoting protoxylem differentiation, and their function is not related to xylem formation later in development.

Furthermore, as at least the start of cambial activity appeared normal in the *ahp6* mutant root (III, Fig. S5), this mutant does not indicate what the possible role of pseudo HPts could be during secondary development. On the other hand, this lack of a cambial development related phenotype is quite expected, as *AHP6* did not even show any obvious expression in the cambial zone or in the upper part of the root, where the cambium first develops (III, Fig. S8A). It is thus possible that Arabidopsis has some other HPts than AHP6, possibly the AHP-

like protein At4g04402 (V, Fig. 6) or AHP4, which negatively regulate cytokinin signalling in the cambial zone. It would be interesting to study if an Arabidopsis triple mutant lacking AHP6, the AHP-like gene and AHP4 would show any secondary development related phenotype.

4.8. Does reduced cytokinin signalling affect cambial activity in the *BpCRE1::CKX2* transgenic *Populus* trees?

The expression peak of cytokinin signalling genes in the tree cambial zone strongly indicates that cytokinin has a role in the regulation of the function of this meristem. To test this hypothesis through functional studies, transgenic *Populus tremula* × *tremuloides* trees with modified cambial cytokinin content were engineered (IV, Fig. 2A). These trees expressed a gene encoding a cytokinin degrading enzyme from Arabidopsis, CYTOKININ OXIDASE 2 (CKX2) (Werner et al. 2006), under a strong cambial promoter. The promoter of a *Betula* cytokinin receptor gene, *BpCRE1*, was used, as this promoter was assumed to be expressed at the same position and developmental stage where cytokinin signalling was taking place. Accordingly, a signal was seen in the cambial zone when the GUS reporter gene was cloned under this promoter (IV, Fig. S2G). A *Betula* promoter was chosen because the original aim was to produce transgenic *Betula* trees. However, no transgenic *Betula* lines with any evident *in vitro* phenotype could be produced (data not shown); therefore *Populus* was chosen to replace *Betula* as the model organism for these studies. According to the *BpCRE1::GUS* data (IV, Fig. 1F), the promoter seems to also direct strong expression in *Populus* cambium. The *pBpCRE1::CKX2* construct was thus directly transformed into *Populus tremula* × *tremuloides*. Several *pBpCRE1::CKX2* transgenic *Populus* lines were subsequently obtained. Some of the lines appeared normal during *in vitro* cultivation, but a significant number had an interesting phenotype: the shoot grew very slowly, whereas root growth was enhanced (data not shown). Propagation of some of the lines with stunted *in vitro* growth failed and these lines were subsequently lost. The rest of the lines were transferred to the greenhouse where they were able to grow into full-sized trees.

4.9. Stunted phenotype is connected to the level of *AtCKX2* expression

Those lines, which showed stunted shoot growth during *in vitro* propagation, also had a prominent phenotype under greenhouse conditions: they were unable to grow upright by themselves; their stems had to be supported from an early age to prevent them from falling onto the ground (data not shown). The lines with normal *in vitro* growth continued to look normal under greenhouse conditions. In order to determine whether these different phenotypes could be connected to the expression level of the *AtCKX2*, qRT-PCR was carried out. From the results it was clear that the wild-type - like lines had a very low level of the transgene expression, whereas the support-requiring trees were showing high *AtCKX2* expression (IV, Fig. 2D).

4.10. High *AtCKX2* expression is connected to reduced cytokinin responsiveness

Furthermore, the lines which had high levels of *AtCKX2* expression were less responsive to cytokinin. This was studied by a classical *in vitro* cytokinin response assay, where a high auxin to cytokinin ratio promotes root formation from stem segments, and a high cytokinin to auxin ratio induces shoot formation (Skoog and Miller, 1957). Segments form the lines with high *AtCKX2* expression required more cytokinin in the growth medium than the wild-type stem segments before they started to produce shoots (IV, Fig. 2G). This strongly indicates that the AtCKX2 was indeed degrading cytokinins in the transgenic lines with high *AtCKX2* expression, and thus reducing their cytokinin responsiveness.

4.11. High *AtCKX2* expression results in reduced cytokinin content

The cytokinin content of the *pBpCRE1::CKX2* trees was measured to determine whether the ectopically expressed AtCKX2 had been able to reduce it. For technical reasons, it was not possible to measure the cytokinin levels directly from the cambial zone of the *pBpCRE1::CKX2* trees. Instead, cytokinin levels of whole stem samples were analysed.

The results show that the concentration of the bioactive free base cytokinin, trans-zeatin, and its storage form, zeatin-*O*-glucoside (ZOG), were reduced in the *pBpCRE1::CKX2* trees. No reduction was seen in the concentration of the other cytokinin species analysed, including the bioactive free base (iP) and the measured cytokinin precursor or storage forms (IV, Fig. S4). However, some of the precursor forms, at least isopentenyladenosine-5′-monophosphate (iPMP) and isopentenyladenosine (iPA) and possibly also trans-zeatinriboside (tZR), may have been slightly elevated in the *pBpCRE1::CKX2* trees as compared to wild-type (IV, Fig. S4). This result could not be confirmed, however, since the data were quite variable between the three analysed individual trees (IV, Fig. S4). If some of the species really were elevated, it could reflect a feedback regulation of cytokinin synthesis; depletion of one cytokinin species could possibly lead to increased synthesis of its precursors. However, further studies would be needed to confirm this.

It is possible that more differences in the cytokinin content between the *pBpCRE1::CKX2* and wild-type trees might have been observed if the cytokinin concentration could have been measured directly from the cambial zone, where the transgenic *AtCKX2* had its highest expression.

4.12 Reduced cytokinin content results in impaired growth

Analysis of the stem diameter of the line with high *AtCKX2* expression confirmed that these trees indeed were thinner than wild-type trees of similar height, as suggested by their inability to stay upright, whereas the lines with low *AtCKX2* expression were as thick as the wild-type trees (IV, Fig. 2C). From now on, in this text, the term *pBpCRE1::CKX2* trees will refer to the transgenic trees with high *AtCKX2* expression levels and a thin-stem phenotype.

However, the apical growth of the *pBpCRE1::CKX2* trees was also compromised; they were always shorter than wild-type trees of similar age (IV, Fig. 2B). This was most probably caused by the fact that the *BpCRE1* promoter, which was used to direct the *AtCKX2* expression, is not strictly cambial specific. From both *pBpCRE1::GUS* and *in situ* analyses, it was evident that the promoter of *BpCRE1* gene was driving expression in the shoot and root apical meristems in addition to the cambial zone (IV, Fig S2A-G). This is a logical expression pattern for a cytokinin receptor, since cytokinin is well know to regulate both shoot and root apical meristem activity, as discussed above. The expression of *AtCKX2* under the *BpCRE1* promoter in the apical meristems also explained both the increased root growth and the inhibited shoot growth during *in vitro* propagation, as moderate reduction in cytokinin content is known to stimulate root growth, whereas it inhibits shoot growth (Werner et al. 2003).

The lack of specificity of the promoter presented a problem: could the reduced radial growth in these transgenic lines simply be a secondary effect caused by impaired apical growth, not a direct effect of down-regulated cambial cytokinin signalling?

4.13. Dissecting apical and radial growth of the *pBpCRE1::CKX2 Populus* trees through grafting

To separate the apical and radial growth of the *pBpCRE1::CKX2* trees, a grafting experiment was performed. The results of the grafting were clear: in the control grafts, wild-type scion grafted to wild type stock and *pBpCRE1::CKX2* scion grafted to the *pBpCRE1::CKX2* stock, the scion (the upper part of the shoot) and stock (the basal part of the shoot) were able to reach the same diameter (IV, Fig. 3). For technical reasons, grafting always resulted in the scion being somewhat thinner then the stock.

In grafts of *pBpCRE1::CKX2* scions onto wild-type stocks, it can be seen that the *pBpCRE1::CKX2* scion did not reduce the radial growth of wild-type stock down to the *pBpCRE1::CKX2* tree level; the scion and the stock did not reach the same diameter (IV, Fig. 3A, 3B). Reciprocally, the wild-type scion outgrew the *pBpCRE1::CKX2* stock, even though the scion was originally thinner than the stock part (IV, Fig. 3A, 3B). These latter grafts, wild-type scion to *pBpCRE1::CKX2* stock, eventually demanded strong external support, as the upper part of the tree was much heavier than what the stock part could support (data not shown). These results strongly indicate that the reduction in cambial activity was not a secondary consequence of the reduced apical growth rate, but instead a direct result of reduced cytokinin content.

This conclusion is further supported by the study of an Arabidopsis mutant lacking one of cytokinin biosynthetic IPT enzymes (*atipt3*) by Matsumoto-Kitano et al. (2008). In this mutant, radial growth of the inflorescence stem was reduced, whereas the apical growth, and subsequently the length of the stem, was not (Matsumoto-Kitano et al. 2008).

Nevertheless, it is interesting to note that radial growth of one part of the graft was in some degree dependent on the other part. The radial growth of the *pBpCRE1::CKX2* stock did improve when the wild-type scion was grafted onto it, as compared to the *pBpCRE1::CKX2* to

pBpCRE1::CKX2 graft (IV, Fig 3A); reciprocally, the radial growth of the wild-type stock was slightly reduced when the *pBpCRE1::CKX2* scion was grafted on it (IV, Fig. 3A). The reason for this adjustment is not known. However, as cytokinins have been shown to be readily transported between the shoot and root in Arabidopsis (Matsumoto-Kitano et al. 2008) and to be present in phloem and xylem sap in Arabidopsis (Hirose et al. 2008), their transport between the scion and stock could be an explanation for this observation.

In the grafting of a wild-type scion to a *pBpCRE1::CKX2* stock, cytokinins transported from the scion to the stock may have increased cytokinin concentration of the stock. The increased concentration may have partly overcome the degradation capacity of AtCKX2 and thus improved the radial growth. It was already seen from the cytokinin responsiveness assay that shoot development could eventually be induced even in the *pBpCRE1::CKX2* stem segments (IV, Fig. 2G), thus indicating that a high enough cytokinin concentration could overcome degradation by AtCKX2. It would be interesting to measure the cytokinin levels of the grafted stock and scion to see if this is the case.

On the other hand, it must be considered that the size of the stem probably has some correlation with the amount of secondary tissues it produces. In Arabidopsis, the weight of the inflorescence stem has been shown to affect the cambial activity in this organ to some extent (Ko et al. 2004). It is thus possible that the lighter weight of the *pBpCRE1::CKX2* scion, as compared to the wild-type scion, abolished some of the weight induced cambial activity in the wild-type stock. Correspondingly, the heavier wild-type scion may have induced weight related cambial activity in the *pBpCRE1::CKX2* stock. However, it is important to note that this weight-related cambial activity does not explain the difference between the *pBpCRE1::CKX2* and wild-type trees, as the radial growth of the *pBpCRE1::CKX2* stock was not returned to wild-type level through its grafting to the wild-type scion. Further supporting this, cambial activity in the inflorescence stem was reduced in the Arabidopsis *atipt3* mutant, even though the length of this organ, and presumably also its weight, was the same as in a wild-type plant (Matsumoto-Kitano et al. 2008). It remains to be seen whether the secondary xylem produced through the weight-stimulated cambial activity resembles the reaction wood produced in trees in response to physical stress.

4.14. Reduced cytokinin content correlates with a decreased number of meristematic cells in the cambial zone

It was next studied whether the reduction in radial growth would be reflected in the anatomy of the cambial zone of the *pBpCRE1::CKX2* trees. The meristematic cells in the cambial zone, defined as flat, thin-walled cells which did not yet show any cell expansion (IV, Fig. 4A), were counted. The number of meristematic cells was determined in cross-sections of the same internode, counting down from the shoot tip. In this case, as the *pBpCRE1::CKX2* trees were thinner, their internode had a smaller diameter (20th internode, diameter 5.5 mm) than the wild-type internode of the same number (20th internode, diameter 7.3 mm) (IV, Fig. 4A and B).

However, in wild-type trees it seemed that the number of meristematic cells in the cambial zone increased with the internode diameter. Thus, at least for some area in the upper part of the shoot, the cambial zone was wider when the internode number increased (data not shown). Thus, it could be possible that the cambial zone of the *pBpCRE1::CKX2* trees would have less meristematic cells than the wild-type simply because internodes of different diameter were compared. To ensure that this was not the case, the number of meristematic cambial cells was also analysed in the *pBpCRE1::CKX2* internodes (30th internode, diameter 7.3 mm) that had a similar diameter than the wild-type internode (20th internode, diameter 7.3 mm) (IV, Fig. A and B). In this case, the internodes of the *pBpCRE1::CKX2* trees had a higher ordinal number, as they were from lower position of the shoot than their wild-type counterparts.

It is evident from the data that the *pBpCRE1::CKX2* trees had fewer meristematic cells in the cambial zone than wild-type trees, regardless of whether cross-sections from internodes with the same ordinal number or the same diameter were compared (IV, Fig. 4A and B). This result shows that fewer periclinal cell divisions are taking place in the cambium of the *pBpCRE1::CKX2* trees. Presumably, the number of meristematic cells reflects the radial growth rate of the stem. The more frequently periclinal cell divisions are taking place, the faster new phloem and xylem cells are produced, and the faster the stem grows radially.

Reflecting the observation that in wild-type trees there was a correlation between the thickness of the internode and the number of meristematic cells in the cambial zone (data not shown), the *pBpCRE1::CKX2* trees also seemed to have slightly more meristematic cells in the samples representing thicker (7.3 mm) internodes than in the thinner ones (5.5 mm) (IV, Fig. 4B). It would be interesting to study whether the correlation between the number of meristematic cambial cells and the internode diameter continues along the whole stem length. However, presumably there is some position along the shoot where the increase in meristematic cell number stops; otherwise, if the number of meristematic cells correlates with the radial growth rate, one could expect that radial growth rate would be accelerated from the tip to the base of the stem. If this was the case, tree stems would presumably have a more cone-like, rather than cylindrical, shape.

4.15. Reduced cambial cytokinin signalling results in compromised radial growth

To confirm that the level of cambial cytokinin signalling was down-regulated in the *pBpCRE1::CKX2* trees, the status of cambial cytokinin signalling was further studied. Due to the thinness of these trees, a direct analysis of cytokinin gene expression in their cambial zone was not possible, since it was difficult to isolate enough tissues from the cambial zone.

The status of cambial cytokinin signalling was instead assessed by comparing the expression levels of a cytokinin primary response gene, the type-A RR *PttRR7*, to the level of the cytokinin receptor gene (*PttHK3a*) in the stem., The expression of *PttRR7* was quickly up-regulated in cytokinin treated *Populus* stem

segments, supporting its status as a primary response gene (data not shown).

The bulk of both *PttHK3a* and *PttRR7* expression originates from the cambial region of the stem (IV, Fig. 1B). *PttHK3a* expression peaks in the area where the cambial meristem marker gene *PtANT* has high expression (IV, Fig. 1C and E), confirming the cambial identity of these cells. Expression of the receptor was therefore assumed to reflect the capacity of cambial zone for cytokinin signalling, whereas the expression level of the *PttRR7* would indicate the level of cytokinin signalling actually taking place in these trees.

An essentially similar expression level of *PttHK3a* was seen in the wild-type and *pBpCRE1::CKX2* trees (IV, Fig. 1G), which indicates that the capacity of the transgenic trees for cytokinin perception in the cambial zone was close to the wild-type level. By contrast, the expression of *PttRR7* was dramatically reduced in the stem of the *pBpCRE1::CKX2* lines (IV, Fig. 1G). Together these results indicate that in the *pBpCRE1::CKX2* trees, the level of cytokinin signalling downstream of the receptors is severely reduced, presumably due to their reduced cytokinin content.

4.16. Connection of cytokinin signalling to cambial stem cell function?

Based on the results from the *pBpCRE1::CKX2* trees, it seems clear that cytokinin signalling modifies cambial activity by regulating the rate of periclinal cell divisions in the cambial zone of the *Populus* stem. Cytokinin signalling may regulate the rate of divisions in either the cambial stem cells or in the xylem and phloem mother cells derived from them, or in both. It remains to be determined whether cytokinin also has a similar role in the regulation of the anticlinal divisions of cambial cells.

Through cambial expression profiling, it was observed that many of the cytokinin signalling genes were slightly more abundant on the phloem side of cambial zone than on the xylem side. As discussed above, the cambial stem cells have also been proposed to be located in proximity to the phloem cells (Schrader et al. 2004). Thus, it is possible that the peak of cytokinin signalling could have some connection to the regulation of cambial stem cell function. However, it remains to be seen whether cambial cytokinin concentration levels correlate with the observed cytokinin signalling gene expression pattern. As the function of cytokinin activating LOG enzymes has been recently shown to be required to maintain shoot apical meristem activity in rice (Kurakawa et al. 2007), it would be interesting to study whether the *Populus LOG* orthologs are expressed in the cambial zone. This would indicate whether the shoot apical and cambial meristems share a similar mechanism for the activation of cytokinins, and respectively the cytokinin signalling phosphorelay, at specific meristematic domains.

Supporting a possible role of cytokinin signalling in cambial stem cell function, cytokinins are known to participate in stem cell differentiation and maintenance in apical meristems. In Arabidopsis, two transcription factors, SHOOTMERISTEM-LESS (STM), and WUSCHEL (WUS), which are known to be involved in the maintenance of stem cells in the shoot apical meristem (Long et al. 1996; Mayer et al. 1998), function in pathways related to

cytokinin and gibberellin signalling. *STM* has been shown to be a positive regulator of cytokinin biosynthesis (Jasinski et al. 2005; Yanai et al. 2005) and a negative regulator of GA biosynthesis (Sakamoto et al. 2001; Jasinski et al. 2005). Several type-A RRs, which act in a negative feedback loop on cytokinin signalling, have been shown to be negatively regulated by WUS and positively regulated by its feedback regulator, the signal peptide CLAVATA 3 (CLV3) (Leibfried et al. 2005). As discussed in chapter 1.4.2., a recent study by Müller and Sheen (2008) showed that cytokinin phosphorelay signalling is also essential for root stem cell specification during early embryogenesis. Further research will clarify the possible role of cytokinin signalling in the regulation of cambial stem cell function.

4.17. Interplay between cytokinin and other hormones in the regulation of cambial activity?

It is intriguing to note that cytokinin, auxin and gibberellin functions show differential patterns across the cambial zone (Fig. 2). The cytokinin pattern is based on my own studies and the patterns of auxin and gibberellin on literature. Cytokinin

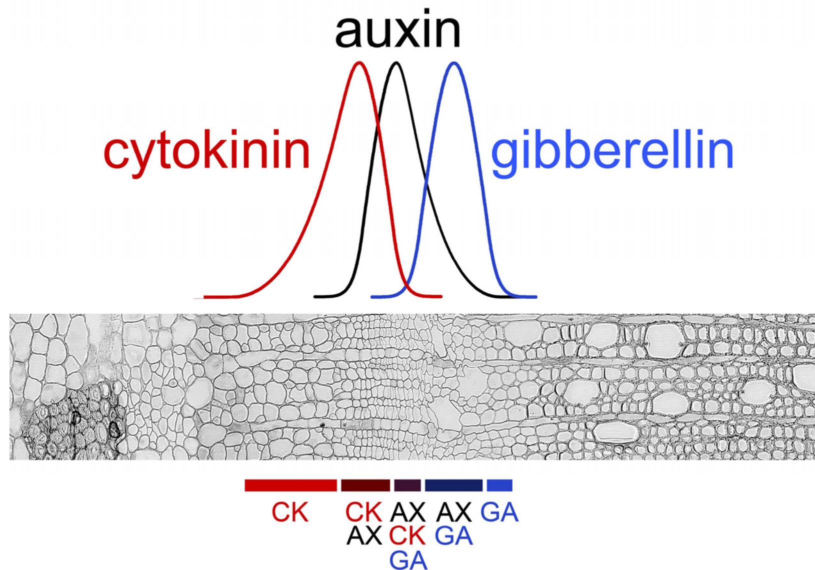

Fig. 2 Schematic overview of the cytokinin, auxin and gibberellin function domains across the *Populus* cambial zone. Auxin and gibberellin curves reflect both the concentration of the respective bioactive hormones (IAA and GA_4 and GA_1) (Tuominen et al. 1997; Israelsson et al. 2005) and general expression pattern of the auxin and gibberellin signalling genes across the cambial zone (Moyle et al. 2002; Israelsson et al. 2005). The cambial distribution of cytokinin is currently unknown, thus the cytokinin curve reflects only the expression peak of cytokinin signalling genes (III, IV). Cytokinin signalling genes peak in the phloem side of the cambial zone, whereas auxin concentration is highest in the middle of the cambial zone, and the bioactive gibberellins peak in the developing xylem cells. Based on these patterns, there are potentially five different domains of these three hormone functions across the cambial zone: 1) cytokinin (CK) function; 2) cytokinin and auxin (CK, AX); 3) auxin, cytokinin and gibberellin (AX, CK, GA); 4) auxin and gibberellin (AX, GA); 5) gibberellin (GA).

signalling genes seem to peak in the developing phloem cells of the cambial zone (IV, V), whereas auxin concentration and signalling genes are known to be highest in the middle of the cambium (Tuominen et al. 1997; Moyle et al. 2002), and bioactive gibberellins and gibberellin signalling genes peak in the developing xylem cells (Israelsson et al. 2005) (Fig. 2).

With respect to cytokinin, auxin and gibberellin patterns, there are potentially five different domains of these three hormone functions across the cambial zone: 1) cytokinin function 2) cytokinin and auxin 3) cytokinin, auxin and gibberellin 4) auxin and gibberellin, and 5) gibberellin (Fig. 2). It must however be taken into account, that these domains remain to be confirmed through studies where all three hormone concentrations (and preferably also the signalling gene expression patterns) are analysed from a same tree individual.

It is intriguing to consider that these domains of overlapping hormone activities could have specific functions in the regulation of different developmental processes taking place across the cambial zone. This would resemble a scenario proposed for the shoot apical meristem (Shani et al. 2006). In this model, a high cytokinin to auxin ratio and low GA concentration in the apex of the meristem promotes indeterminate growth. Contrariwise, high gibberellin and low cytokinin to auxin ratio promotes the initiation of lateral organs in the flanks of this meristem (Shani et al. 2006). Concerning the model for overlapping hormone functions in the cambium (Fig. 2), potential interplay between cytokinin and auxin has a role in the regulation of phloem development, and also, as discussed above, in the control of cambial stem cell maintenance and division, while auxin and gibberellin might act synergistically in the regulation of xylem development.

It would be interesting to study whether the levels of auxin and gibberellin are affected in the cambial zone of the *pBpCRE1::CKX2* trees. This analysis would help us understand the relationship between cytokinin and other hormones in the control of the cambial meristem. Furthermore, studies of cambial development in transgenic trees deficient for all different combinations of these three hormones in the cambial zone would clarify the significance of their interplay. However, it should be taken into account that other hormones, including ABA, ethylene and brassinosteroids, most probably also participate in the regulation of cambial meristem function.

5. CONCLUDING REMARKS

In my thesis work, I have shown that cytokinin signalling is a rate-limiting regulator of cambial activity. This conclusion is further supported by a study, published back-to-back with my paper IV, showing that cytokinins are required for cambial development in Arabidopsis (Matsumoto-Kitano et al. 2008).

However, many questions about cambial development remain unanswered. Little is currently known about the interplay between the cytokinin and other hormones in the regulation of the function of this meristem. It remains to be seen whether hormonal regulation of the cambial meristem shares several features with the apical meristems or if cambium displays some novel mechanisms to integrate the function of different hormones.

In the future, it will be especially interesting to study which gene functions are active downstream of cytokinin and other hormone signal transduction pathway. These genes must encode components regulating the maintenance and division rate of the cambial meristem stem cells.

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