JUHA IMMANEN

Cytokinin Signaling in Hybrid Aspen Cambial Development and Growth

INSTITUTE OF BIOTECHNOLOGY AND
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UNIVERSITY OF HELSINKI

Doctoral Thesis

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AUTHOR'S CONTRIBUTIONS:

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II) A.E., **J.I.**, K.N. and Y.H. wrote the manuscript.

III) **J.I.** and K.N. participated in the design of the study, in the gene identification from *Populus* and *Prunus* genomes and in the sequence alignment. H.D.S. and F.R.R. participated in the gene identification from *Prunus* genome and in the sequence alignment. V.A.A. performed the phylogenetic analyses. T.R.H. participated in the design of the study. L.A.M., H.S. and Y.H. conceived the study, and participated in its design and coordination.

IV) **J.I.**, K.N., L.P., P.A., H.S., and Y.H. designed the study. **J.I.**, K.N., and R.P.B. contributed to the tree transformations. **J.I.**, K.N., O.-P.S., M.K., J.A.S., P.K., J.Z., A.E., and N.S. carried out the experiments. **J.I.**, K.N., O.-P.S., J.A.S., and H.S. analysed the data. **J.I.**, K.N., O.-P.S., J.A.S., A.P.M., P.A., H.S., and Y.H. wrote the manuscript.

ABBREVIATIONS

ABA	abscisic acid
ABCG14	ATP-binding cassette transporter subfamily G14
ARF	auxin response factor
ATP/ADP	adenosine triphosphate/adenosine diphosphate
CK	cytokinin
CKI	cytokinin insensitive
CKX	cytokinin oxidase
CRE	cytokinin receptor
cZ	trans-zeatin
ENT	equilibrate nucleoside transporter
ETR	ethylene receptor
GA	gibberellic acid
HK	histidine kinase
HP	histidine containing phosphotransmitters
IAA	indole acetic acid, auxin
I-IV	Thesis publications I-IV.
iP	isopentenyladenine
ipt	isopentenyladenine transferase
LOG	lonely guy
MP	monopteros
My	million years
PCD	programmed cell death
PUP	purine permease
RAM	root apical meristem
RR	response regulator
SAM	shoot apical meristem
TCS	two-component system
TE	tracheary elements
tZ	trans-zeatin
WOL	wooden leg/AHK4/Arabidopsis Histidine Kinase 4

ABSTRACT

Life on Earth is carbon based and is largely enabled by plants. As autotrophic (self-sufficient) life-forms, they fix atmospheric carbon dioxide to sugar through photosynthesis, and atmospheric oxygen is a by-product of this process. Heterotrophic life-forms such as humans are fully dependent on energy and oxygen provided by plants and cyanobacteria.

Plants are thus very important for the human economy. Agriculture and forestry are large business sectors, with a global annual worth of over 3 trillion US dollars.

Vascular tissues develop from the vascular cambium and serve two main functions: they give physical structure and support and distribute the water, nutrients and other substances needed for growth. Phloem transports the nutrients, and xylem is mainly responsible for conducting water and providing structural support for the plant.

Trees are crucially important organisms for various ecosystems: about 31% of global terrain, over 4 billion hectares, is covered by forests. Trees contribute to carbon fixation, oxygen production, the global water cycle and soil erosion prevention. Woody biomass represents a valuable renewable source of energy and raw material for pulping and for the construction industry. Trees provide an excellent system for studying secondary vascular development in high resolution due to their massive size, which results from a wide cambial meristem. Understanding the hormonal regulation of radial growth that underlies wood development is of great importance for improving the use of tree products as a renewable resource. Detailed knowledge of these regulatory mechanisms could provide powerful tools to help tree breeders boost lignocellulosic biomass production. In the future, improved forestry and agriculture could produce the required energy, food, feed and raw biological materials using much less land, enabling the preservation of large areas of natural forest.

Cytokinins are important growth regulators, but there are many other important plant hormones and growth regulators. In the work included in this thesis, we provided the first comprehensive description of the gene families for cytokinin signaling and homeostasis in black cotton wood (*Populus trichocarpa*). We also analyzed the hormone distribution and genome-wide expression profiles across the *Populus* cambial zone at an unprecedentedly high resolution. We observed increased cambial auxin concentration and auxin-responsive gene expression in cytokinin over-producing transgenic hybrid aspen (*Populus*) trees. Our results indicate that a graded distribution of cytokinin signaling specifies meristematic activity by influencing the amplitude of the cambial auxin gradient. It seems that cytokinins and auxin together regulate cambial developmental fate and growth rate. The two major phytohormones clearly interact in a manner which calls for continuing research. Here, we show that cytokinin and auxin display different yet partially overlapping distribution profiles across the cambium. In contrast to auxin, which is most concentrated in the actively dividing cambial cells, the greatest concentration of cytokinins is in the developing phloem. By manipulating the cytokinin concentration in the cambial zone, we could increase and decrease cambial cell division activity. Stimulation of cambial growth dramatically increased the production of lignocellulosic trunk biomass in *Populus* trees under greenhouse conditions.

TIIVISTELMÄ

Hiilenkierto ja kasvit ovat mahdollistaneet nykymuotoisen elämän maapallolla. Kasvit ovat autotrofisia (omavaraisia) elämänmuotoja, jotka pystyvät yhteyttämisen avulla muodostamaan ilmakehän hiilidioksidista sokeria ja happea. Heterotrofiset (toisenvaraiset) elämänmuodot, kuten ihmiset, ovat täysin riippuvaisia kasvien ja syanobakteerien tuottamasta energiasta ja hapesta.

Kasvit ovat myös erittäin tärkeitä ihmiskunnan taloudelle. Maa- ja metsätalous ovat suurta maailmanlaajuista liiketoimintaa, jonka arvo on yli 3 biljoonaa Yhdysvaltain dollaria.

Kasvin johtosolukot kehittyvät jällestä. Johtosolukot antavat kasville sen fyysisen muodon ja tukirakenteen sekä huolehtivat kasvuun tarvittavan veden, ravinteiden ja muiden aineiden kuljetuksesta erilaisten solukoiden välillä. Nila kuljettaa lehdistä ravinteet, kuten sokerit ja aminohapot erilaisiin solukoihin, kuten esimerkiksi juuriin. Puuaines (ksyleemi) kuljettaa vettä juurista muihin solukoihin, kuten lehtiin ja antaa kasville tukirangan.

Puut ovat erityisen tärkeitä eliöitä erilaisissa ekosysteemeissä. Noin 31% maapinta-alasta eli yli 4 miljardia hehtaaria on erilaisten metsien peitossa. Metsillä on merkitystä hiilen sitomisessa, hapen muodostuksessa, maailman laajuisessa veden kierrossa ja maaperän eroosion ehkäisyssä. Puubiomassa on arvokas ja uudistuva luonnonvara niin energiaksi kuin raaka-aineeksi erilaisiin tarkoituksiin. Puut ovat oiva kohde jällen yksityiskohtaiseen tutkimukseen suuren kokonsa vuoksi. Puun kehityksen ja kasvun hormonaalisen säätelyn tunteminen olisi hyvin tärkeää, jotta voitaisiin tehostaa metsien kasvua. Tehostettu metsä- ja maatalous pystyisi tulevaisuudessa tuottamaan tarvittavan määrän energiaa, ravintoa ja raaka-aineita paljon pienemmällä käytettävällä maapinta-alalla, jotta luonnontilaiset metsät voisivat olla rauhassa.

Sytokiniinit ovat tärkeitä kasvun säätelijöitä, joskaan eivät ainoita. Olemme ensimmäisinä tutkijoina raportoineet sytokiniini-viestintäreitin ja kyseisen hormonin homeostasiaan vaikuttavat geenit haavan sukuisessa puussa (*Populus trichocarpa*). Analysoimme sytokiniini-kasvihormonin jakaumaa ja koko genomin laajuista geenien ilmentymistä aikaisempia tutkimuksia tarkemmalla erottelukyvyllä koko jälsivyöhykkeen läpi. Mielenkiintoisena, jonkinlaisena yllätyksenä havaitsimme, että lisäämällä geeniperäisesti sytokiniinien määrää myös jällen auksiinin määrä lisääntyy huomattavasti. Tulimme siihen johtopäätökseen, että ainakin sytokiniinit ja auksiini yhdessä vaikuttavat jällen kehitykseen ja puun paksuuskasvun nopeuteen ja eri hormonien yhteisvaikutuksien jatkotutkimus olisi erityisen tärkeää. Pystyimme myös osoittamaan, että sytokiniinilla ja auksiinilla on hieman toisistaan poikkeavat toiminnalliset vyöhykkeet. Sytokiniinit (ja sytokiniiniviestintä) ovat runsainta nilan puolella jälttä, kun taas auksiinivyöhyke on ksyleemin (puun) puolella jälttä (molemmat hormonit ovat voimakaasti läsnä jällessä). Olemme myös pystyneet muuttamaan (lisäämään ja vähentämään) jällen aktiivisuutta (muuttamalla sytokiniin määrää jällessä) ja siten vaikuttaneet tutkittavien puiden kasvuun. Lisäämällä geeniperäisesti jälsivyöhykkeen sytokiniinien määrää, havaitsimme huomattavaa puun paksuuskasvun lisääntymistä kasvihuoneolosuhteissa.

INTRODUCTION

The importance of plants

Planet Earth is a very special planet even at the interstellar or galactic level: Earth has life and complicated life-forms. Life on Earth is carbon based and largely enabled by plants. Plants are truly spectacular; it is hard to imagine life on Earth without them. As autotrophic (self-sufficient) life-forms, they fix atmospheric carbon dioxide to sugar through photosynthesis; atmospheric oxygen is a by-product of this process. Heterotrophic life-forms such as humans are fully dependent on energy and oxygen provided by plants and cyanobacteria. Accordingly, plants are very important for the human economy: agriculture and forestry are large businesses, with a global annual worth of over 3 trillion US dollars [FAO, Global Forest Resources Assessment 2010 – FRA 2010].

Plant growth

Plant species have extremely versatile body types, varying from tiny herbs to majestic trees. This diversity of dicot land plants was enabled through the evolution of vasculature, the conductive tissues consisting of xylem and phloem. Plant vascular tissues serve two main functions: they give physical structure and support and they distribute water, nutrients, and other substances needed for growth. The phloem tissue transports the nutrients, while the xylem tissue is mainly responsible for conducting water and providing structural support to the plant [reviewed by Evert 2006, Elo et al. 2009, **II**, Beck 2010, Nieminen et al. 2012, Kucukoglu 2015]. The vasculature interconnects all of the parts of the plant, from the roots through the stem(s) into the leaves. Both xylem and phloem consist of various cell types, most of them elongated, hollow cells. Angiosperm xylem consists of water-conducting tracheary elements (TEs), fibers, and parenchyma cells, whereas phloem is made up of conductive sieve elements and their adjacent companion cells.

In angio- and gymnosperm plants that increase their width by growing radially, two different phases of vascular development are recognized: primary and secondary. The shoot apical meristem (SAM) and root apical meristem (RAM) are responsible for the apical (primary) growth of shoots and roots; this is complemented by the activity of the vascular cambium, enabling (secondary) radial growth [reviewed by Elo et al. 2009, **II**, Nieminen et al. 2012, Kucukoglu 2015]. The evolution of vascular cambium during the Middle Devonian (386 to 377 Mya) [Rowe & Speck 2005, Gerrienne et al. 2011] enabled an increase in the amount of vascular tissue and a subsequent increase in plant girth, leading to the development of woody plants. The emergence of the perpendicular parenchymatic cells known as vascular rays provided enhanced transport and storage across the axial vascular cell files in the xylem and phloem and further enabled the increase in plant size and the subsequent evolution of tree species.

Inspirational trees

Trees are crucially important organisms for various ecosystems: about 31% of the global terrain, over 4 billion hectares, is covered by forests [FAO, Global Forest Resources Assessment 2010 – FRA 2010]. There are approximately 3.04 trillion trees in the world, with 1.30 trillion in tropical and subtropical forests, 0.74 trillion in boreal regions, and 0.66 trillion in temperate regions [Crowther et al. 2015]. Trees contribute enormously to global carbon fixation, oxygen production, and water cycling, as well as preventing soil erosion. Trees and wood were required for the development of human civilization; the number of trees in the world has been estimated to have fallen by approximately 46% since the start of human civilization, and an estimated 15 billion trees are cut down each year [Crowther et al. 2015]. Woody biomass represents a valuable renewable source of energy and raw material for pulping and construction [reviewed in Nieminen et al. 2012]. Furthermore, due to the massive size enabled by their wide cambial meristem, trees provide an excellent system for studying secondary vascular development in high resolution (as compared to, e.g., *Arabidopsis thaliana*) [Immanen et al. 2016, **IV**].

For my entire life, I have been interested in trees, the largest of all plants, for several reasons: I am a forest owner and have seen trees throughout my life and studied them in my day job for quite many years. I therefore wanted to prepare a thesis about trees even though "easier" model species options were available. I hope that my thesis will teach you something new about these fascinating organisms.

Populus as a model organism

I used two very similar *Populus* species (*Populus trichocarpa* [black cotton wood] and *Populus tremula x tremuloides*, referred to as hybrid aspen) as the main model organisms for my thesis research due to their many beneficial traits. The genus *Populus* is distributed throughout the northern hemisphere from subtropical to boreal forests [Rodgers-Melnick et al. 2012, reviewed in Nieminen et al. 2012] and represents one of the most economically and ecologically important genera of forest trees. It is well known for its rapid growth, straightforward vegetative propagation, tolerance of environmental stress, and the numerous uses of its wood [reviewed by Cronk 2005 and Nieminen et al. 2012]. Furthermore, the genus has become a popular research model due to its small genome size and the availability of a complete *P. trichocarpa* genome [Tuskan et al. 2006].

The dynamic nature of plant genomes has influenced the evolution of all gene families in vascular plants. All angiosperm lineages have undergone recurring genome duplications, indicating that polyploidization confers a fitness advantage. Each whole-genome duplication is followed by gradual gene loss; this rediploidization ultimately evokes a new duplication, resulting in a cyclical repeat of the process [Chapman et al. 2006, Rodgers-Melnick et al. 2012]. *Populus* has undergone a more recent genome duplication than *Arabidopsis* and therefore has more genes in every gene family [Tuskan et al. 2006].

Vascular cambium

The vascular tissues emerge very early during plant development. Through the activity of the two apical meristems, a subset of provascular cells differentiate into two conductive tissue types, xylem and phloem. Some meristematic cells (called procambial cells) persist between the vascular tissues throughout primary development. During the initiation of secondary development, these procambial cells start to divide and become the fascicular part of the vascular cambium [reviewed by Elo et al. 2009, **II Fig. 1B**]. The cambial cylinder is completed through the activation of cell divisions in the interfascicular regions between the vascular strands in shoots and at the xylem pole pericycle cells in roots. Secondary xylem and phloem are subsequently produced via both anti- and peri-clinal cell divisions in the cambium. These cell divisions contribute to both the self-maintenance of the cambium and the tangential growth of plant organs; the anticlinal divisions increase the number of cell files formed by the cambium, whereas the periclinal divisions add to the number of cells in each file.

Depending on their life strategy, seed plants display great developmental variability in the extent of their radial growth. Most monocotyledons lack the secondary developmental phase altogether, whereas dicots and gymnosperms undergo secondary development to varying extents. Herbaceous plants (like *Arabidopsis*) are characterized by diminutive secondary growth (and thus a short and slender stature), whereas woody plants undergo an extensive amount of secondary development, most evident in the expansive girth of tree trunks. Secondary development can also take place in roots and, to a much lesser extent, in leaf veins.

Tree-like phenotypes have emerged, disappeared, and reappeared several times during plant evolution, implying that relatively few genetic differences may separate the woody and herbaceous growth habits [reviewed by Spicer & Groover 2010]. Although plants have evolved several different cambial organizations [reviewed by Spicer & Groover 2010], the most common vasculature arrangement is the bifacial concentric pattern in which xylem tissues are produced inwards and phloem tissues outwards [reviewed by Elo et al. 2009, **II Fig. 1B**]. In the region flanking the cambium, there exist two radial developmental gradients (one for xylem and one for phloem) consisting of regions of cell division, cell expansion, cell differentiation and cell death [reviewed by Elo et al. 2009, **II Fig. 1B**].

Differentiation and cell death are required for plant life

Wood cells grow by a unique combination of intrusive growth, in which differential growth of the cell wall results in the projection of newly formed parts between adjacent cells or into intercellular spaces, and symplastic growth, in which a group of cells grow without moving or forming new contacts while undergoing mutual adjustments [Siedlecka et al. 2008].

Plant development requires specific cells to die in a genetically controlled manner. Dying cells undergo autolysis to degrade their cellular organs but also gain new functions [reviewed by Escamez & Tuominen 2017]. Xylem cell differentiation culminates in programmed cell death (PCD) [Bollhöner et al. 2018, reviewed by Minina et al. 2017]. The

development of lignification, PCD, secondary cell wall formation, and xylem tracheary elements (TEs), were evolutionary breakthroughs that enabled long-distance transport of nutrients and upright growth in vascular land plants [Ewbank et al. 1996, Serk et al. 2015]. TE cell walls are reinforced with the phenolic polymer lignin, which is deposited after TE cell death by the cooperative supply of monomers and other substrates from the surrounding living cells. Lignification of TEs is thus non-cell autonomous [Courtois-Moreau et al. 2009, Serk et al. 2015]. The composition of lignin varies in different wood tissues [Fagerstedt et al. 2015]. The appearance of lignification in the Lower Devonian (409 to 386 Mya) [Ewbank et al. 1996] allowed plants to adapt both their size and their form to the environmental conditions on land.

Hormonal regulation of cambial development

The development of plant organs is orchestrated by their hormones. Thus far, at least auxin, cytokinins, gibberellins, abscisic acid (ABA), ethylene, strigolactones, and several peptides have been identified to contribute to the regulation of various developmental processes [reviewed by Elo et al. 2009, **II**, El-Showk et al. 2013, Schaller et al. 2015].

Cytokinins

Cytokinins are chemicals that induce cell proliferation and, when applied together with auxin at the correct ratio, induce shoot differentiation in callus tissue [Skoog & Miller 1957]. Cytokinins are known to also play key roles in many other aspects of plant growth and development, including meristem maintenance and activity, vascular patterning, flower and seed development, nutrient uptake, chloroplast differentiation, light perception, and production of endodermal symmetry and passage cells [Sakakibara 2006, Durbak et al. 2012, Hwang et al. 2001, Hwang et al. 2012, reviewed by Elo et al. 2009, **II**, Nieminen et al. 2012, El-Showk et al. 2013, Kieber & Schaller 2014, 2018, Osugi & Sakakibara 2015, Schaller et al. 2015, Andersen et al. 2018]. Cytokinins also positively regulate several commercially important agricultural traits, such as grain size and biomass accumulation [reviewed in Kieber & Schaller 2014, 2018]. In addition, this hormone regulates several key developmental programs in perennial woody plants, including the activity of the vascular cambium [Nieminen et al. 2008, **I**; Immanen et al. 2016, **IV**], the branching pattern of the shoot, and the leaf senescence. Overall, cytokinins are essential signaling molecules in regulating growth and development throughout a plant's life.

With the help of various enzymes, cytokinins can shift between different forms (**FIGURES 1 & 2**), like the vampire in the novel "Dracula" [Stoker 1897]. Structurally, most cytokinins are adenine derivatives; during biosynthesis they can exist in several different nucleotide-conjugated precursor forms (triphosphate, diphosphate and monophosphate) (**FIGURE 2**). The bioactive cytokinins are the free-base forms and differ from one another in their side chain identity. Bioactive cytokinins can be inactivated through reversible glucosylation. Several different bioactive cytokinin species, including isopentenyl-adenine

(iP), trans-zeatin (tZ), cis-zeatin (cZ), dihydrozeatin (DZ), and ortho-topolin (oT), have been identified in plants [reviewed by Sakakibara 2006, Osugi & Sakakibara 2015] (**Figure 1**), but only iP and tZ play a major physiological role in most studied plant species, including *Populus* [Immanen et al. 2016, **IV**]. This is reflected in their relatively high abundance and receptor affinity [Sakakibara 2006, Osugi & Sakakibara 2015]. Artificial cytokinins, including kinetin and thidiazuron (TDZ), may activate cytokinin receptors when administered to plants, but they are not physiological regulators of plant growth [Osugi & Sakakibara 2015].
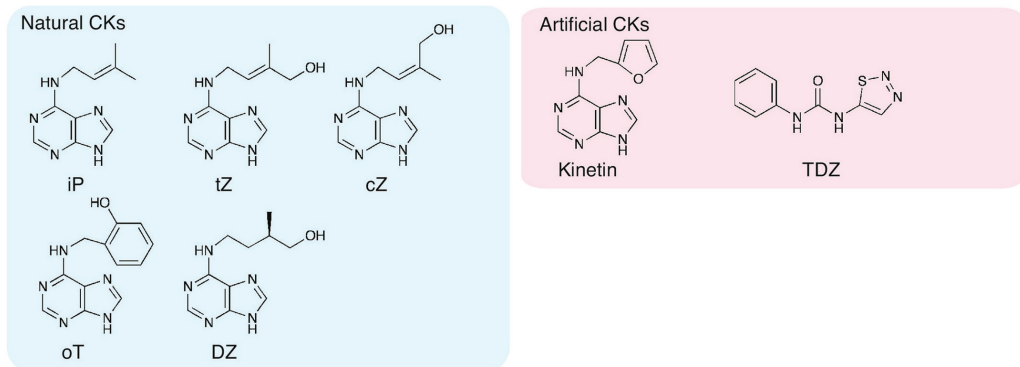

Figure 1: Structure of bioactive cytokinins (free-base forms) and some artificial cytokinins. [reprinted with permission from Osugi & Sakakibara 2015].

Cytokinin homeostasis

Natural cytokinin concentrations are estimated to vary between 1–40 nM in various tissues and developmental stages [Lomin et al. 2015]. Cytokinins are readily transportable hormones [reviewed in Kudo et al. 2010] which have been shown to move between plant organs (shoot to root and vice versa) by tracer experiments using isotope-labeled cytokinins [Kiba et al. 2013, Bishopp et al. 2011, Sasaki et al. 2014, Zhang et al. 2014]. Furthermore, reciprocal grafting of cytokinin-deficient mutants with wild-type seedlings can rescue their phenotypes [Matsumoto-Kitano et al. 2008], confirming that root-synthesized cytokinins can be transported to the shoot and vice versa.

Cytokinin biosynthesis is catalyzed by the ATP/ADP isopentenyltransferase (IPT) enzymes [Takei et al. 2004a, reviewed by Sakakibara 2006, Osugi & Sakakibara 2015, Kieber & Schaller 2018] (**Figure 2**) [Kakimoto 2001, Takei et al. 2001, Miyawaki et al. 2004, Miyawaki et al. 2006, Matsumoto-Kitano et al. 2008], and the CYP735A enzymes can convert the iP forms into tZ species (**Figure 2**) [Takei et al. 2004b]. There are seven IPT genes in *Arabidopsis* [Kieber & Schaller 2014] and in *Populus* [Immanen et al. 2013, **III**].

The active cytokinin concentration is locally regulated through the activity of the LONELY GUY (LOG) enzymes, which convert conjugated cytokinin nucleotides into
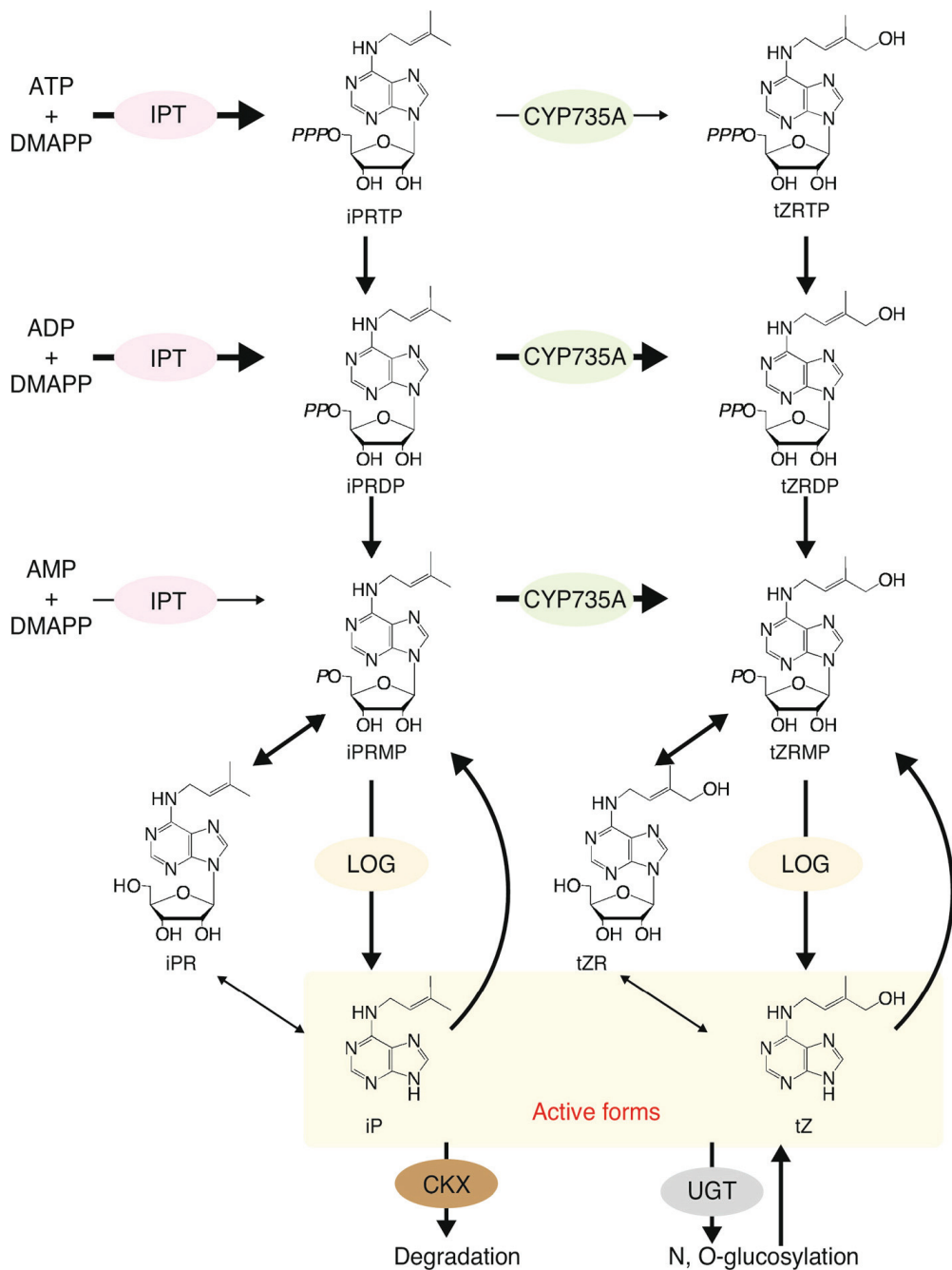
Figure 2: Current model of iP and tZ biosynthesis and metabolic pathways in *Arabidopsis*. IPT preferentially uses ATP and ADP, and CYP735A preferentially uses isopentenyladenosine-monophosphate (iPRMP) and isopentenyladenosine-diphosphate (iPRDP) as substrates. LOG exclusively reacts with the monophosphate forms. Active cytokinins are degraded by CKX, glucosylated by UGT, or reverted to their precursors by the purine salvage pathway [reprinted with permission from Osugi & Sakakibara 2015].

their bioactive nucleobase forms (**Figure 2**) [Kuroha et al. 2009, Tokunaga et al. 2012]. The LOGs are important regulators of shoot and root apical meristem activity [Kuroha et al. 2009, Tokunaga et al. 2012, Kurakawa et al. 2007, Chickarmane et al. 2012]. Their action enables plants to separate and refine the location and timing of cytokinin activation and perception [Kuroha et al. 2009, Tokunaga et al. 2012]. Seven LOG genes have been identified in *Arabidopsis* [Kieber & Schaller 2014] and 13 in *Populus* [Immanen et al. 2013, **III**].

Cytokinin oxidase/dehydrogenases (CKXs) function as irreversible cytokinin degradation/catabolism enzymes (**Figure 2**). Like the LOGs, the CKX genes function as local repressors of cytokinin signaling whose expression is regulated in a site-specific manner [Werner et al. 2001, Werner et al. 2003]. Seven CKX genes are present in *Arabidopsis* [Kieber & Schaller 2014] and eight in *Populus* [Immanen et al. 2013, **III**]. Expression of the CKX genes in *Populus* is induced by cytokinin, indicating the existence of feedback regulation [Immanen et al. 2016, **IV**].

Cytokinin transport

Recent studies have identified the *ATP-BINDING CASSETTE TRANSPORTER SUBFAMILY G14* (*ABCG14*) as a key gene for root-to-shoot translocation of cytokinin [Zhang et al. 2014, Ko et al. 2014]. In addition to *ABCG14*, *PURINE PERMEASE 1* and *2* (*PUP1* and *PUP2*) and *EQUILIBRATE NUCLEOSIDE TRANSPORTER* (*ENT*) have been shown to be involved in the transport of cytokinins [Burkle et al. 2003, Hirose et al. 2005]. The mechanisms of cytokinin transport *in planta*, especially those involved in long-distance translocation, are still poorly understood [reviewed by Kang et al. 2017, Kieber & Schaller 2018, Romanov et al. 2018].

Cytokinin signal transduction pathway

Cytokinin signaling is an ancient hormone signaling pathway. All the components are found in the genome of moss *Physcomitrella patens*, which diverged from vascular plants hundreds of millions of years ago [Pils & Heyl 2009, Ishida et al. 2010]. The cytokinin signaling pathway has become much more diverse during the evolution of land plants, leading to many more genes in cytokinin signaling families in vascular plants than in *Physcomitrella* [Pils & Heyl 2009, Ishida et al. 2010]. Cytokinin signaling has been argued to be happen in membrane of endoplasmic reticulum and/or at plasma membrane [Romanov et al. 2018].

Cytokinins trigger physiological responses by regulating gene expression. A multistep, two-component histidine-aspartate phosphorelay system (TCS) transduces the cytokinin signal to the target genes (**Figure 3**). The phosphorelay is initiated when a cytokinin ligand binds to a histidine kinase receptor, triggering its autophosphorylation. After an intramolecular move of the phosphoryl, it is transferred to a cytosolic histidine phosphotransfer (HPt) protein. The HPts can cycle between the cytosol and nucleus, providing a connection between these two compartments. In the nucleus, the HPts transfer

the phosphoryl onto phospho-accepting response regulators (RRs) [Hwang & Sheen 2001, Inoue et al. 2001, Mähönen et al. 2006b, reviewed by Hwang et al. 2012; El-Showk et al. 2013, Osugi & Sakakibara 2015, Zürcher & Müller 2016, Kieber & Schaller 2018, Romanov et al. 2018]. Phosphorylated type-B RRs bind to target DNA and induce the expression of genes involved in the primary response to cytokinin. Among them are the type-A RRs, which are involved in a negative feedback mechanism that helps fine-tune the function of the cytokinin signaling pathway. Type-A RRs repress the activity of type-B RRs [Hwang & Sheen 2001, Imamura et al. 1998] and are regulated by HPt-mediated phosphorylation [To et al. 2007].

Adding further flexibility to the signaling pathway, many of its components can form both homo- and heterodimers [Dortay et al. 2006, 2008, Caesar et al. 2011, Punwani et al. 2010, Veerabagu et al. 2012, reviewed by Hwang et al. 2012, El-Showk et al. 2013, Osugi & Sakakibara 2015, Zürcher & Müller 2016, Kieber & Schaller 2018, Romanov et al. 2018]. Different combinations of the two-component elements presumably add diversity to the signal transduction process and modify its outcome.
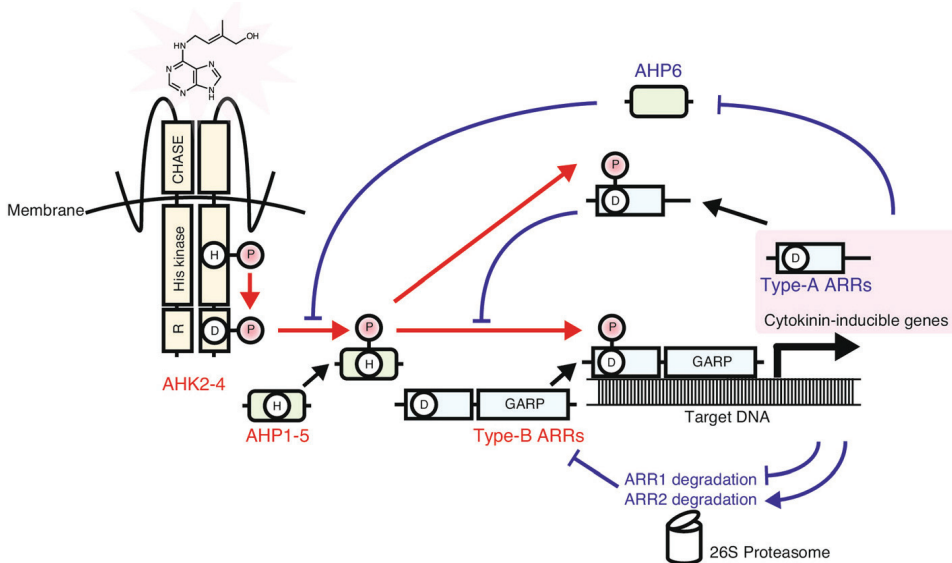

Figure 3: Diagram of the cytokinin two-component system (TCS). AHKs are autophosphorylated in response to cytokinins. The phosphoryl group is transferred to type-B ARRs through AHPs. Phosphorylated type-B ARRs bind to target DNA and induce the expression of genes involved in primary cytokinin response. The stability of type-A ARRs, which repress cytokinin TCS signaling, is controlled through proteolysis by the 26S proteasome in a feedback loop. Expression of *AHP6*, which inhibits phosphotransfer between AHKs and canonical AHPs, is repressed by cytokinin. Red arrows indicate phosphotransfer. Solid blue arrows and bar-ended lines represent positive and negative regulation, respectively [reprinted with permission from Osugi & Sakakibara 2015].

Cytokinin receptors

The initial perception of bioactive cytokinins takes place through CRE1-like two-component histidine kinase receptors (**Figure 3**), which belong to the superfamily of two-component regulators [Yamada et al. 2001, Inoue et al. 2001, Higuchi et al. 2004, Mähönen et al. 2000]. *Arabidopsis* has three cytokinin receptor genes, *CRE1/WOL/AHK4, AHK2, and AHK3* [Inoue et al. 2001, Higuchi et al. 2004, Mähönen et al. 2000], whereas *Populus* has five, *PtHK2*, *PtHK3a*, *PtHK3b*, *PtCRE1a*, and *PtCRE1b* [Nieminen et al. 2008, **I**, Immanen et al. 2013, **III**]. The three *Arabidopsis* receptors share overlapping functions; single null mutants have no notable phenotypes, whereas the triple mutant is a severely dwarfed and practically infertile plant [Higuchi et al. 2004]. One of the three *Arabidopsis* receptors, CRE1, has both kinase and phosphatase activity; upon binding cytokinin, it phosphorylates histidine-containing phosphotransmitters (HPts), whereas in the absence of the hormone, CRE1 instead dephosphorylates them [Mähönen et al. 2006a]. Its phosphatase activity helps to inactivate the phosphorelay when cytokinin levels decrease.

In addition to the three canonical receptors, *Arabidopsis* has a fourth two-component histidine kinase, *CYTOKININ-INDEPENDENT 1* (*CKI1*), which is capable of activating the cytokinin signal transduction pathway [Kakimoto 1996, Hwang et al. 2001, Kakimoto 2003, Iwama et al. 2007]. Since this kinase can initiate the phosphorelay independently, even in the absence of cytokinin, it does not represent an actual cytokinin receptor [Mähönen et al. 2006a, Yamada et al. 2001, Deng et al. 2010, Pekárová et al. 2011]. *Populus* has 3 orthologues of *CKI1* [Immanen et al. 2013, **III**].

Histidine containing phosphotransmitters

The CRE1-like receptors initiate phosphorylation of the histidine-containing phosphotransmitters (HPts), which continuously cycle between the cytosol and nucleus [Punwani et al. 2010]. This movement enables the transfer of phosphoryl groups from the membrane-localized receptors to the nuclear-localized response regulators (**Figure 3**). Six HPt genes have been identified in *Arabidopsis* [Suzuki et al. 1998, Suzuki et al. 2000, Mähönen et al. 2006a] and 14 in the *Populus* genome [Immanen et al. 2013, **III**].

In contrast to the five canonical *Arabidopsis* HPts, the pseudo HPt *AHP6* lacks the phospho-accepting His residue and has an inhibitory role on the cytokinin phosphorelay [Mähönen et al. 2006a]. The negative function of *AHP6* contributes to the generation of distinct and well-defined domains of low cytokinin signaling. Two *Populus* genes (*PtHP6a*, *PtHP6b*) are orthologous to *AHP6* [Immanen et al. 2013, **III**].

Response regulators

The response regulators (RRs) are the final components of the cytokinin signaling phosphorelay. Based on their protein domain structure and function, they can be classified into four different subfamilies: A-, B-, C-, and pseudo-RRs. Members of the type-A, -B, and -C RRs are known to participate in the cytokinin signaling phosphorelay (**Figure 3**)

[Hwang et al. 2002, Kakimoto 2003, Kiba et al. 2004], whereas the pseudo-RRs instead function in the regulation of light responses [Makino et al. 2000, Mizuno 2004, Murakami et al. 2004].

Type-A RRs

The phosphorylated type-B RRs activate the expression of type-A RR genes, which function as negative regulators of cytokinin signaling [To et al. 2004 and 2007, Buechel et al. 2010, Zhang et al. 2011]. Since the ARR-A genes are primary response genes of the cytokinin signaling pathway, their expression levels and patterns are considered good indicators of cytokinin signaling and concentration [D'Agostino et al. 2000, Nieminen et al. 2008, **I**, Immanen et al. 2016, **IV**]. Like the type-B RRs, individual type-A RRs are generally redundant in *Arabidopsis*; sensitivity to cytokinin increases progressively in higher order mutants [Ishida et al. 2008, Argyros et al. 2008, To et al. 2004]). The *Populus* genome contains eleven types-A RRs (*PtRR1-11*) [Ramírez-Carvajal et al. 2008, Pils & Heyl 2009, Immanen et al. 2013, **III**], compared to the ten genes present in *Arabidopsis*.

Type-B RRs

Type-B RRs are DNA-binding transcriptional regulators that positively mediate cytokinin responses [Hwang & Sheen 2001, Sakai et al. 2001, Imamura et al. 2003, Mason et al. 2005, Sakai et al. 1998, Sakai et al. 2000, Sakai et al. 2001]. They activate transcription of cytokinin primary response genes, among them the type-A RRs. The expression of type-B RRs is not induced through cytokinin signaling; their activity is regulated through HPt-mediated phosphorylation. In *Arabidopsis*, the type-B RRs has partially redundant functions; higher order null mutants show a progressively decreasing sensitivity to cytokinin [Mason et al. 2005, Ishida et al. 2008, Argyros et al. 2008]. There are eleven type-B RRs in *Arabidopsis* [Tajima et al. 2004, Kieber & Schaller 2014], while *Populus* has thirteen (*PtRR13-25*) [Ramírez-Carvajal et al. 2008, Pils & Heyl 2009, Immanen et al. 2013, **III**].

Type-C RRs

Type-C RRs (also known as extra RRs) are a response regulator subfamily characterized by an atypical amino acid sequence in the receiver domain [Mizuno 2004]. *Arabidopsis* has two, *ARR22* and *ARR24*. They display very restricted expression patterns: *ARR22* is expressed in developing seeds [Horak et al. 2008] and *ARR24* in developing and mature pollen grains [Gattolin et al. 2006]. ARR22 can dephosphorylate HPt proteins *in vitro*; it thus appears to be a negative regulator of the cytokinin signaling phosphorelay [Kiba et al. 2004, Horak et al. 2008]. The function of type-C RRs remains unknown [Horak et al. 2008, Gattolin et al. 2006], although inducible over-expression of *ARR22* enhances freezing and drought tolerance in *Arabidopsis* [Kang et al. 2013]. While *Arabidopsis* has two type-C RR genes, *Populus* has eight (*PtARR26-33*) [Immanen et al. 2013, **III**].

Auxin

The application of auxin has been shown to reactivate cambium in decapitated shoots in classic hormone treatment experiments [Snow 1935, Digby & Wareing 1966, Little & Bonga 1974, reviewed by Savidge 1988]. Auxin has long been considered one of the key regulators of cambial activity [reviewed by Sundberg et al. 2000, Bhalerao & Bennett 2003, Elo et al. 2009, **II**, El-Showk et al. 2013, Bhalerao & Fisher 2014, Schaller et al. 2015]. Further evidence for the role of auxin in secondary development was obtained when a radial gradient of auxin (IAA) was detected across the cambial zone of both *Populus* and *Pinus* trees [Uggla et al. 1996, 1998, Tuominen et al. 1997]. Since these publications, auxin concentration has been shown to be highest in the dividing cambial cells and to decrease steeply toward the differentiating phloem and more gradually towards the differentiating xylem [Elo et al. 2009, **II Figure 3**, Immanen et al. 2016, **IV Figures 1B & 3B**].

The auxin concentration gradient is assumed to form via auxin transport both downwards and across the stem. The shoot apex is a major source of auxin [Sundberg & Uggla 1998] from which it is transported basipetally down the stem [Little & Savidge 1987, Björklund et al. 2007]; auxin is then distributed differentially across the cambial zone. Supporting this idea, several genes encoding auxin transporters (auxin influx and efflux carriers) are expressed in the cambial zone in *Populus* [Schrader et al. 2003, Immanen et al. 2016, **IV**]. The auxin maximum in dividing cambial cells correlates with strong expression of auxin signaling genes [Moyle et al. 2002]. The expression of many auxin-responsive genes is higher in the differentiating xylem cells than in the dividing phloem cells [Nilsson et al. 2008]. This result may indicate a potentially non-linear correlation between hormone concentration and the induction of auxin-responsive gene expression. Furthermore, it suggests that auxin is a major regulator of xylem development [reviewed by Sundberg et al. 2000, Bhalerao & Bennett 2003, Elo et al. 2009, **II**, El-Showk et al. 2013, Bhalerao & Fisher 2014, Schaller et al. 2015].

Functional studies using transgenic *Populus* trees have provided further evidence for the role of auxin in regulating secondary development [Nilsson et al. 2008]. Transgenic *Populus* trees were engineered to express a transcriptional repressor of auxin signaling (*PttIAA3m*) under the 35S promoter, resulting in reduced auxin responsiveness [Nilsson et al. 2008]. In these trees, there were fewer periclinal and anticlinal cell divisions in the vascular cambium, compromising the radial growth of the stems [reviewed by Elo et al. 2009, **II Figure 3**, Bhalerao & Fisher 2014]. Weight-induced cambial activity may occur via enhanced auxin signaling [Ko et al. 2004]. During the transition to cambial dormancy, both polar auxin transport and the expression of genes encoding auxin transporters are significantly reduced in *Populus* [Schrader et al. 2003]. Furthermore, the expression of the auxin-inducible *PttIAA* genes, which encode auxin signaling repressors, is reduced during the transition to dormancy [Moyle et al. 2002], indicating that cambial auxin signaling is down-regulated during dormancy. Thus, cambial growth cessation and dormancy appear to involve modulation of auxin responsiveness rather than auxin levels in the vascular cambium [Baba et al. 2011, reviewed by Kucukoglu 2015].

Gibberellin

Gibberellic acid (GA) (also known as gibberellin) has been implicated in regulating the cambium based on its stimulatory effect on cambial activity in hormone treatments on tree stems, particularly when applied together with auxin [Digby & Wareing 1966, Wang et al. 1997, Björklund et al. 2007, reviewed by Elo et al. 2009, **II Figure 3**]. The application of gibberellin to decapitated and auxin-depleted *Populus* stems stimulates cell division in the cambial zone. The identity of the newly formed cells was reported to be relatively obscure [Björklund et al. 2007]. First, the morphology of the GA-induced cells in the cambial zone was compromised; in a cross-section they appeared more spherical than the flat, thin-walled cells of the untreated cambium. Second, the GA-induced cells did not appear to differentiate into xylem cells on the xylem side of the cambial zone; instead, they seemed to remain in a parenchymatic state. Thus, GA treatment resulted in the loss of an easily distinguishable vascular cambium, which indicates that GA alone is not sufficient to maintain and stimulate cambial activity [Björklund et al. 2007]. The application of IAA together with GA enhanced cambial cell divisions more than either hormone alone, indicating that these two hormones function synergistically in the regulation of cambial activity [Digby & Wareing 1966, Björklund et al. 2007]. Additionally, GA treatment induces the expression of a *Populus* auxin transport protein gene which is abundant in the cambium, *PttPIN1*. Together, these results indicate that GA action promotes auxin transport [reviewed by Elo et al. 2009, **II**]. Reciprocally, auxin treatment stimulates the expression of GA biosynthesis genes and inhibits the expression of genes encoding GA degrading enzymes. Furthermore, both GA and auxin treatment induce similar transcriptional changes [Björklund et al. 2007]. It is possible that GA stimulates cambial activity by promoting polar auxin transport into the cambial cells.

Transient induction of a GA biosynthetic enzyme gene (*PttGA20ox*) was observed in the spring during cambial reactivation [Druart et al. 2007], indicating a possible function of GA signaling in regulating the termination of dormancy. Only trace amounts of GAs have been detected in dividing cells in active cambium, whereas the highest levels have been detected in differentiating xylem cells in *Populus* [Israelsson et al. 2005]. Consistent with this, GA biosynthesis and signaling genes have low expression in dividing cambial cells and higher expression in both differentiating phloem and xylem cells [Israelsson et al. 2005]. In contrast, a study by Mauriat & Moritz [2009] showed that GA receptors are most strongly expressed in the phloem and dividing cambial cells of the *Populus* stem. Thus, the expression pattern of GA signaling genes across the cambial zone remains to be verified. The tissue-specific distribution pattern of GAs across wood-forming tissues in *Populus* shows that bioactive GAs are most concentrated in the expanding xylem cells [Israelsson et al. 2005, Immanen et al. 2016, **IV**]. Both analysis of transgenic trees overproducing GAs and hormone application experiments have shown that GAs can stimulate xylem fiber elongation [Digby & Wareing 1966, Eriksson et al. 2000], indicating a role for GA signaling in the regulation of xylem differentiation.

Cytokinin-auxin plant hormone interaction

The interactions between the auxin and cytokinin hormonal pathways have increasingly been shown to be highly complex and heavily dependent on context (tissue and developmental stage), fueling an immense research interest in their interaction during plant development [reviewed by El-Showk et al. 2013, Schaller et al. 2015, Chandler et al. 2015].

Auxin is known to both boost [Müller & Sheen 2008] and repress [Zhao et al. 2010] the activity of cytokinin response repressors (type-A RRs regulators and *AHP6*), whereas cytokinin signaling can activate negative regulators of auxin signaling (Aux/IAA). In addition, cytokinin is known to induce auxin biosynthesis [Moubayidin et al. 2013, Müller et al. 2017], whereas auxin can promote cytokinin synthesis [Miyawaki et al. 2004; Dello Ioio et al. 2008] and degradation [Werner et al. 2006].

Cytokinins are further known to modulate and direct auxin transport; they can both up- and down-regulate expression of the PINs and modify their polar localization and expression [Ruzicka et al. 2009, Bishopp et al. 2011a, 2011b, Marhavý et al. 2011]. Increased cytokinin concentration in the cambial region of *Populus* also increases the auxin concentration in the cambium [Immanen et al. 2016, **IV**]. In *Arabidopsis*, the homeostasis of these two hormones is known to be connected; cytokinin has been shown to contribute to the regulation of both auxin homeostasis [Jones et al. 2010] and transport [Björklund et al. 2007, Simaskova et al. 2015].

All of these interactions create highly complex feedback loops in which the activity of these two hormones in adjacent and interconnected domains is essential for the regulation of plant development. Several recent studies have focused on computational modeling of these processes [El-Showk et al. 2015, Di Mambro et al. 2017, Mellor et al. 2017].

AIMS OF THE STUDY

My thesis work is focused on the role of cytokinins in cambial development and secondary growth. I think that building a profound understanding of tree growth through research is important for multiple reasons; it affects climate change, deforestation, and the availability of food and energy for humankind.

My aims:

- Characterize cytokinin signaling and homeostasis genes in *Populus*.
- Study the role of cytokinins as growth regulators and their importance for radial growth and biomass production by manipulating cytokinin to affect cambial cell divisions.
- Analyze the cytokinin distribution and make genome-wide gene expression profiles at unprecedentedly high resolution across the cambial zone.

MATERIALS AND METHODS

Materials and methods are listed in [**Table 1: Methods**] and described in (more) detail in publications (**I, III**, and **IV**). Those in brackets were used by my co-authors in the respective publications.

Table 1: Methods

Method	Publication
Agrobacterium-mediated transformation	I, IV
Anatomical analyses	I, IV
Cryo-sections	I, IV
Cytokinin hormone induction/assays	I, IV
DNA extraction	I, IV
Gene cloning/Plasmid construction	I, IV
Gene expression profiling	I, IV
Grafting experiments	I, IV
Histological techniques and physiological assays	I, IV
In situ experiments	(I)
Light microscopy	I, IV
Phylogenetic analyses	I, III
Polymerase Chain Reaction (PCR) analyses	I, IV
Quantification of hormones	(I), (IV)
Quantitative real time PCR	I, IV
RNA extraction	I, IV
RNA sequencing	(IV)
Sequence analysis	I, III, IV
Statistical analysis	I, III, IV

RESULTS AND DISCUSSION

Hormonal domains in the cambial zone of *Populus*

We studied hormone concentrations in black cotton wood (*P. trichocarpa*) in six cryofractions across the developmental gradient in the stem cambial zone: old phloem, conducting phloem, developing phloem, cambium, developing xylem, and xylem [Immanen et al. 2016, **IV Figure 1**]. Our findings supported our earlier hypothesis that the cytokinin domain is on the phloem side of cambium [Elo et al. 2009, **II Figure 3**]. We found that cytokinins and auxin have distinct yet partially overlapping distributions across the vascular cambium. Cytokinin concentration [Immanen et al. 2016, **IV Figures 1 & 3**] and the expression of cytokinin signaling genes [Immanen et al. 2016, **IV Figure 4**]) were highest on the phloem side of the cambial zone. The concentration of the major bioactive auxin (IAA) was highest in the middle of the cambial zone, where cambial cells are dividing. This result confirms the cambial auxin gradient and response domain previously reported in tree species [Uggla et al. 1996, Uggla et al. 1998, Tuominen et al. 1997, Moyle et al. 2002].

The importance of auxin for cambial development has been verified by functional studies. Fewer cell divisions were observed in the vascular cambium of transgenic hybrid aspen trees with reduced auxin responsiveness (due to expression of the auxin signaling repressor *PttIAA3m* under the *35S* promoter), resulting in compromised radial stem growth [Nilsson et al. 2008]. In contrast to auxin, the two major bioactive cytokinin species, isopentenyladenine (iP) and trans-zeatin (tZ), were both concentrated in developing phloem cells [Immanen et al. 2016, **IV Figure 3**] , and iP riboside (iPR), a precursor of bioactive iP, had a similar distribution profile.

The third plant hormone under investigation, bioactive gibberellin (GA4), had its highest concentration in developing xylem. A gibberellin distribution profile similar to that reported in Immanen et al. [2016] (**IV, Figure 1)** has been previously reported by Israelsson et al. [2005]; in their study, bioactive GA4 and GA1 were most concentrated in the expanding xylem cells in *Populus*. The observed distribution coincides with the activity of gibberellin signaling in the regulation of xylem development; the rate of secondary xylem production was higher in transgenic plants with enhanced gibberellin signaling [Mauriat & Moritz 2009] or biosynthesis [Eriksson et al. 2000, Biemelt et al. 2004, Dayan et al. 2010, Gou et al. 2011]. Thus, our findings confirm the results of earlier auxin and gibberellic acid experiments [Uggla et al. 1996, Uggla et al. 1998, Tuominen et al. 1997, Eriksson et al. 2000, Moyle et al. 2002, Biemelt et al. 2004, Israelsson et al. 2005, Björklund et al. 2007, Dayan et al. 2010, Gou et al. 2011].

Characterization of cytokinin signaling and homeostasis genes in *Populus*

In Immanen et al. [2013] (**III**), we reported the first comprehensive description of cytokinin signaling and homeostasis genes in black cotton wood (*Populus trichocarpa*) based on sequence homology with *Arabidopsis thaliana*. Previous studies [Pils & Heyl 2009,

Nieminen et al. 2008, Ramírez-Carvajal et al. 2008] only presented and discussed a smaller subset of cytokinin signaling genes.

We identified genes belonging to six families: cytokinin oxidases (CKXs), isopentenyl transferases (IPTs), LONELY GUY genes (LOGs), two-component receptors, histidine containing phosphotransmitters (HPts), and response regulators (RRs). Altogether, 85 *Populus* cytokinin signaling and homeostasis genes were identified and compared with their *Arabidopsis* orthologs through phylogenetic analyses [Immanen et al. 2013, **III**]. Publications by the Sakakibara lab [Takei et al. 2004b, Kiba et al. 2013] have reported that *CYP735A1* and *CYP735A2* encode cytokinin hydroxylases that catalyze the biosynthesis of trans-zeatin in *Arabidopsis*. These genes were not studied in my thesis. Due to recent revelations about their importance [Osugi & Sakakibara 2015], they may be included in my continuing research beyond the scope of this thesis. Cytokinin transport (influx/efflux) was also not included in the scope of my thesis, although our genome-wide RNA sequencing analysis [Immanen et al. 2016, **IV**] may reveal new cytokinin transporters in the future.

In general, the structure of the gene families was very similar in *Arabidopsis* and *Populus*. Some cytokinin signaling and homeostasis gene families were distinctly expanded in *Populus* compared to *Arabidopsis* [Immanen et al. 2013, **III**]. One of the expanded clades is the *CKI1*-like subfamily of the two-component histidine kinases, which has three members in *Populus* compared a single gene in *Arabidopsis* [Immanen et al. 2013, **III Figure 4**]. In *Arabidopsis CKI1* has been shown to participate in the regulation of both reproductive development and secondary vascular development [Kakimoto 1996, Hejatko et al. 2009]. *Populus* also has four orthologs of *Arabidopsis AtLOG5* [Immanen et al. 2013, **III Figure 3**].

Another difference was seen in the phosphotransmitter (HPt) gene family, where *Populus* has four homologs of the single *Arabidopsis AHP4* gene [Immanen et al. 2013, **III Figure 5**]. The structure of this family also differs between the two species. Two of the *Populus* clades (one consisting of *PtHP8a*, *PtHP8b* and another of *PtHP1a* and *PtHP1b*) had no evident *Arabidopsis* orthologs [Immanen et al. 2013, **III**]. Furthermore, one of the *Populus* genes contains non-canonical consensus motifs lacking the conserved histidines and is therefore considered *PtHP-like*; it is not known whether this gene/protein participates in the phosphorelay [Immanen et al. 2013, **III**].

The type-C *RR*s, has multiplied four-fold in the *Populus* lineage as compared with *Arabidopsis* [Immanen et al. 2013, **III Figure 6**]. The expansion of the type-C subfamily appears to be specific to the evolution of *Populus*, as there are fewer genes in several other species. Since the function of the type-C RRs has remained elusive in *Arabidopsis*, *Populus* could potentially be a better model in which to study their activity.

Cytokinin signaling and homeostasis genes are expressed in the cambial zone of *Populus*

We studied the expression of cytokinin signal transduction components and cytokinin homeostasis genes across the cambial zone using high-resolution cryosections (each containing only a few cell files) for RNA sequencing and quantitative real-time polymerase

chain reaction (qPCR) analyses [Nieminen et al. 2008, **I Figures 1B-1F** and Immanen et al. 2016, **IV Figure 4** and **Supplementary Figure 1**]. Our genome-wide RNA sequence analysis of the *Populus* cambial zone was the first of its kind conducted in any tree species. Later, a more detailed analysis of the xylem was done by Sundell et al. [2017] to complement our work. We hope our data will be combined with the AspWood database [Sundell et al. 2017] to serve the cambial research community.

The expression of cytokinin signaling and homeostasis genes in the vascular cambium suggests that cytokinin signaling participates in the regulation of cell divisions in this meristem. Signaling and biosynthesis genes were expressed on the phloem side of the cambium, where concentrations of active cytokinin are highest [Immanen et al. 2016, **IV Figures 3 and 4**]. The identified cytokinin and auxin response domains coincide well with measured hormone gradients. Almost all of the cytokinin signaling and biosynthesis genes were most strongly expressed in the developing phloem cells, which also have the maximum cytokinin concentration. In contrast, most of the auxin response genes had maximal expression in the middle of the cambial zone, coinciding with the greatest auxin concentration and cell division activity. The high expression of cytokinin catabolic genes in the same domain might possibly contribute to shaping the cambial cytokinin distribution.

We used cytokinin induction experiments to test the responsiveness of the selected genes, and we conducted RNA-sequencing and qPCR analyses of cytokinin-treated wild type (WT) *P. tremula × tremuloides* (hybrid aspen) stem discs [Immanen et al. 2016, **IV Supplementary Figure 3**]. As expected, almost all type-A RRs were upregulated following one hour of cytokinin treatment, whereas most other genes were unaffected. Since the expression of some IAAs is known to be directly upregulated by the type-B RRs [Dello Ioio et al. 2007, Moubayidin et al. 2010], our focus was to identify PttIAA genes that are not cytokinin induced. The expression of most IAAs was unaffected, indicating that they represent true auxin response marker genes, but two out of 33 were potentially upregulated [Immanen et al. 2016, **IV Supplementary Figure 3**] and were omitted from further profiling.

We also noticed that expression of the *Populus Aintegumenta* (*PttANT*) gene was elevated in the cambium of cytokinin-overproducing (*LMX5::AtIPT7*) transgenic trees [Immanen et al. 2016, **IV Figure 4**]. This gene is known to be upregulated by prolonged cytokinin treatment and to act as a positive regulator of cambial activity in *Arabidopsis* and *Populus* [Randall et al. 2015], leading us to hypothesize that it may be involved in increasing cell division rate in the *pLMX5::AtIPT7* transgenic *Populus* trees [Immanen et al. 2016, **IV**].

The role of cytokinin signaling in *Populus*

We engineered transgenic hybrid aspen trees (*P. tremula × tremuloides*) [Nieminen et al. 2008, **I Figures 2A and 2D**] with reduced cytokinin concentrations [Nieminen et al. 2008, **I Figure 2E and Supplementary Figure 4**] and compromised cytokinin signaling [Nieminen et al. 2008, **I Figure 1G**] during cambial development. These trees expressed a cytokinin catabolic gene from *Arabidopsis*, *CYTOKININ OXIDASE 2*, under the cambial promoter of a birch cytokinin receptor, *BpCRE1* [Nieminen et al. 2008, **I Supplentary Figures 1 and**

2A-H]. CKX enzymes irreversibly degrade active cytokinin species [Werner et al. 2006, Sakakibara 2006], and they have been used to reduce overall cytokinin levels in several plant species when expressed under the systemic *35S* promoter [Werner et al. 2001, Werner et al. 2003, Yang et al. 2003, Galuszka et al. 2007]. The *BpCRE1* promoter was chosen for its strong cambial expression [Nieminen et al. 2008, **I Figure 1F**]; as a promoter driving the expression of a cytokinin receptor gene, it would presumably direct the expression of the cytokinin-degrading enzyme to the location of cytokinin perception.

The outcome of down-regulating cytokinin signaling in the transgenic aspen trees was quite similar to previous reports in different plant species [Werner et al. 2001, Werner et al. 2003]. Furthermore, these transgenic hybrid aspen lines displayed various symptoms indicative of reduced cytokinin action, such as impaired shoot regeneration in tissue culture (the *CKX2* overexpressor lines needed more cytokinin than wild type trees to produce shoots) [Nieminen et al. 2008, **I Figure 2G**], enhanced apical dominance (or reduced branching) [Nieminen et al. 2008, **I Figure 2A**], enhanced leaf senescence [Nieminen et al. 2008, **I Figure 2A**], and impaired apical growth of the shoot.

The transgenic trees with high levels of *AtCKX2* expression [Nieminen et al. 2008, **I Figures 1G and 2D**] had impaired radial growth. Radial growth appears to be more affected in these lines than apical growth, since the trees were thinner than the wild type [Nieminen et al. 2008, **I Figures 2B and 2C**]. Furthermore, we have shown through grafting experiments that the defects in apical growth cannot explain the reduced radial growth [Nieminen et al. 2008, **I Figure 3**]. The trees also had longer internodes and enhanced leaf senescence, differing from earlier reports of short internodes and unaccelerated leaf senescence in p35S::CKX tobacco and *Arabidopsis* lines [Werner et al. 2001, Werner et al. 2003].

We showed that transgenic hybrid aspen lines strongly expressing the transgene [Nieminen et al. 2008, **I Figures 1G and 2D**] produced less of the cytokinin tZ and its storage form ZOG [Nieminen et al. 2008, **I Figures 2E and 2F**] . A detailed anatomical characterization of the transgenic lines revealed that the number of undifferentiated cell files in the cambial zone was significantly reduced in transgenic plants with high *AtCKX2* expression [Nieminen et al. 2008, **I Figures 4A and 4B**]. We also observed slight differences in wood anatomy: fiber lengths and vessel widths were slightly reduced [Nieminen et al. 2008, **I Supplementary Figures 6A and 6B**].

We have shown that a reduction in cytokinin concentration leads to compromised radial growth in transgenic *Populus* trees [Nieminen et al. 2008, **I**]. Similarly, cambial activity was abolished in the root of the *Arabidopsis ipt1,3,5,7* quadruple mutant, which lacks the four major cytokinin biosynthesis enzymes [Matsumoto-Kitano et al. 2008].

Based on our cytokinin profiling data and previous results, we next studied the effect of elevated cambial cytokinin signaling on trunk growth. In order to stimulate cytokinin biosynthesis in transgenic *P. tremula × tremuloides* trees, we overexpressed the *AtIPT7* (AT3G23630) gene from *Arabidopsis*. This gene encodes one of the key enzymes in the biosynthesis of major bioactive cytokinins [Miyawaki et al. 2004, Miyawaki et al. 2006, Kakimoto 2001]. The *AtIPT7* transgene was cloned under the *PttLMX5* promoter [Love et

al. 2009], which drives strong expression in the cambial zone and developing xylem cells. To evaluate the effect of *AtIPT7* activity on tree development, we followed the growth of the transgenic trees under greenhouse conditions [Immanen et al. 2016, **IV Figures 2A and B**]. The apical growth rate of *pLMX5:AtIPT7* lines was similar to wild-type (WT) plants; transgenic plants had the same height as the controls [Immanen et al. 2016, **IV Figure 2A**]. In contrast, the stem diameter was greater in the transgenic trees than in WT trees [Immanen et al. 2016, **IV Supplementary Figure 2A**]; the stimulatory effect of cytokinin on radial growth is therefore independent of the apical growth rate. Accordingly, the stem volume, which was measured as the additive volume of internodes, was greater in *pLMX5:AtIPT7* trees [Immanen et al. 2016, **IV Figure 2B**]. The stem biomass (after the branches were removed from the trunk) was measured in three WT trees and three cytokinin over-producing trees at the age of 13 weeks; the average dry weight was up to 80% greater in the cytokinin-overproducing trees (under greenhouse conditions). This result was better than we were able get with *pLMX5:AtCKI1* and *p35S:AtCKI1* lines in previous experiments [Immanen et al. 2011] (the data is not included in my thesis).

We also analyzed the anatomy of the transgenic trees. No differences were observed between the WT and *pLMX5:AtIPT7* trees in the dimensions of xylem cells. In contrast, a difference was observed in cambial anatomy. The vascular cambium of the cytokinin over-producer lines contained more meristematic cells than the cambial cell files in WT trees [Immanen et al. 2016, **IV Figure 3A and Supplementary figure 2B**]. The increased cell number indicates that the cambial cell files underwent additional cell divisions compared with WT. Our results confirm that cytokinins act as major positive regulators of cambial activity in trees.

The hormonal responsiveness of two *pLMX5:AtIPT7* lines was tested in an *in vitro* assay [Immanen et al. 2016, **IV Figure 2C**] where a lower cytokinin:auxin ratio in the growth medium induces root regeneration and a higher ratio promotes shoot regeneration [Skoog & Miller 1957]. We observed enhanced cytokinin responsiveness in the transgenic trees. Several internodes produced shoots and, unexpectedly, also roots even with no added cytokinin, whereas the wild type internodes produced neither. As high auxin concentration promotes root formation, these results indicate that the transgenic lines may have had higher concentrations of both cytokinin and auxin than the WT trees.

Cytokinin-Auxin interaction in *Populus*

We compared the hormonal profiles across the cambial zone of WT and *pLMX5:AtIPT7* trees [Immanen et al. 2016, **IV Figure 3B**]. The hormonal distributions in WT *P. tremula* × *tremuloides* and WT *P. trichocarpa* were similar; cytokinin concentrations were highest in the developing phloem tissue, and auxin (IAA) concentration was highest in the middle of the cambium. Several differences in hormonal levels were observed between the transgenic trees and the WT: concentrations of bioactive isopentenyladenine (iP) and trans-zeatin (tZ) were elevated, and dramatic increases were seen in the concentrations of IAA and the iP precursor iPR [Immanen et al. 2016, **IV Figure 3B**]. These results confirm increased

cambial cytokinin content in the *pLMX5::AtIPT7* trees and further show that this increase also leads to an elevation in the auxin concentration. In *Arabidopsis*, homeostasis of these two hormones is known to be connected — cytokinin has been shown to contribute to the regulation of both auxin homeostasis [Jones et al. 2010] and transport [Björklund et al. 2007, Simaskova et al. 2015] — and similar processes may be at work in *Populus*.

CONCLUSIONS

Hormonal control of cambium in *Populus*

Understanding the hormonal regulation of the radial growth that underlies wood development is of great importance for the future use of tree products as a renewable resource. Detailed knowledge about the regulatory mechanisms controlling cambial development could provide powerful tools for tree breeders (using a marker-based approach) to boost lignocellulosic biomass production in forest trees. The better we understand the molecular mechanisms controlling wood formation, the easier it will be to translate this knowledge into improvements in forest biotechnology and tree breeding. In the work reported in this thesis, **we were able to stimulate cambial cell division by cytokinin over-production, resulting in dramatically increased production of lignocellulosic trunk biomass** (by up to 80% dry weight under greenhouse conditions) [Immanen et al. 2016, **IV**].

This thesis does not resolve the complete picture of hormonal control of the cambium. Studies in this thesis shows that **there is no single "master" regulator of cambial growth**. Cytokinins are important growth regulators, but there are many other important plant hormones and growth regulators. For example, abscisic acid (ABA) is an important regulator of the growth season. Dormancy is regulated by ABA-mediated blockage of symplastic communication (shown in hybrid aspen [Tylewicz et al. 2018]). This communication blockage prevents growth signals from reaching the meristem, thus blocking meristem activation in winter (so trees can survive winter conditions) [Tylewicz et al. 2018].

Based on one of the studies in this thesis (Immanen et al. [2016], **IV),** it seems that **cytokinins and auxin together regulate cambial development** fate and growth rate. It is clear that plant hormones interact with each other and those interactions should be the subject of continuing research. We were able to show here that two major phytohormones, cytokinin and auxin, display different yet partially overlapping distribution profiles across the cambium. In contrast to auxin, which is most concentrated in actively dividing cambial cells, cytokinins are most concentrated in the developing phloem. By connecting these hormonal domains with the developmental zonation of the vascular cambium and addressing the interconnected nature of the hormone distributions, our work complements recent studies of cell-specific auxin and cytokinin profiles in the *Arabidopsis* root apical meristem [Dello Ioio et al. 2008, Petersson et al. 2009, Antoniadi et al. 2015]. The cytokinin and auxin signaling profiles we observed across the cambial zone differed from those identified in *Arabidopsis* primary root development, where auxin response was high in developing xylem cells and cytokinin was highest in the dividing procambial cells between the xylem and phloem [Bishopp et al. 2011b, De Rybel et al. 2014]. These differences reflect the adaptability of hormonal regulation during plant development; the outcome of hormonal signaling is highly dependent on its context, enabling the same hormones to play versatile role during different developmental processes.

Characterization of cytokinin signaling and homeostasis genes in *Populus*

We reported in Immanen et al. 2013, **III**, **the first comprehensive description of cytokinin signaling and homeostasis gene families in black cotton wood (*Populus trichocarpa*).** There is lot of activity in the cytokinin research field, and in the future, we could expand this gene characterization to also include cytokinin transport and modification genes. The identification of the cytokinin signaling and homeostasis genes in *Populus* may serve as a reference upon which functional analyses can be developed to determine the role that cytokinin plays in vegetative and reproductive tree development. These genes may serve as potential candidates for marker-assisted breeding to increase wood production. The methods used in our gene characterization work, Immanen et al. 2013, **III,** have also been used in several other projects [Laitinen et al. 2005, Toikkanen et al. 2007, Salojärvi et al. 2017] and could serve as a reference for similar studies.

We analyzed the hormone distribution and genome-wide gene expression profiles across the cambial zone at an unprecedentedly high resolution [Immanen et al. 2016, **IV**]. We observed increased cambial auxin concentration and auxin-responsive gene expression in cytokinin over-producing transgenic hybrid aspen (*Populus*) trees. Our results indicate that cytokinin signaling specifies meristematic activity through a graded distribution that influences the amplitude of the cambial auxin gradient. I hope to combine our *Populus* cambial data with recently available databases such as AspWood [Sundell et al. 2017] **to better serve the research community** with our combined strength. We have also taken the first steps towards studying cytokinin target genes; so far, we have investigated the interaction of *Aintegumenta* (*ANT*) and *CYCD3* genes in *Arabidopsis* and *Populus* [Randall et al. 2015].

Cytokinins modulate radial growth of tree

During primary vascular development, cytokinins appear to be required for both cell proliferation and cell specification [Dello Ioio et al. 2007, Mähönen et al. 2000, Mähönen et al 2006a, Mähönen et al 2006b]. Our experimental focus here was whether cytokinins are required for the secondary phase of vascular development characterized by the activity of the vascular cambium, a stem cell population that orchestrates plant radial growth. We have shown [Nieminen et al. 2008, **I**] that **reduced cytokinin concentrations** and signaling reduced cell proliferation, **making thinner stems**. We have also shown [Immanen et al. 2016, **IV**] that **increased cytokinin concentrations** and signaling enhance cambial zone cell proliferation, **making thicker stems**, confirming that cytokinins are major regulators of cambial activity. Our transgenic hybrid aspen trees had increased cytokinin concentrations, leading to elevated cytokinin signaling. Furthermore, **elevation of the cytokinin concentration led to an increase in cambial auxin concentration**, **highlighting**

the interconnected nature of these two hormonal gradients. The stimulation of cambial activity may occur through the elevation of both hormonal signaling responses. The contribution of vertical and lateral transport (relative to biosynthesis) to the cambial hormone distributions remains to be determined by future studies.

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