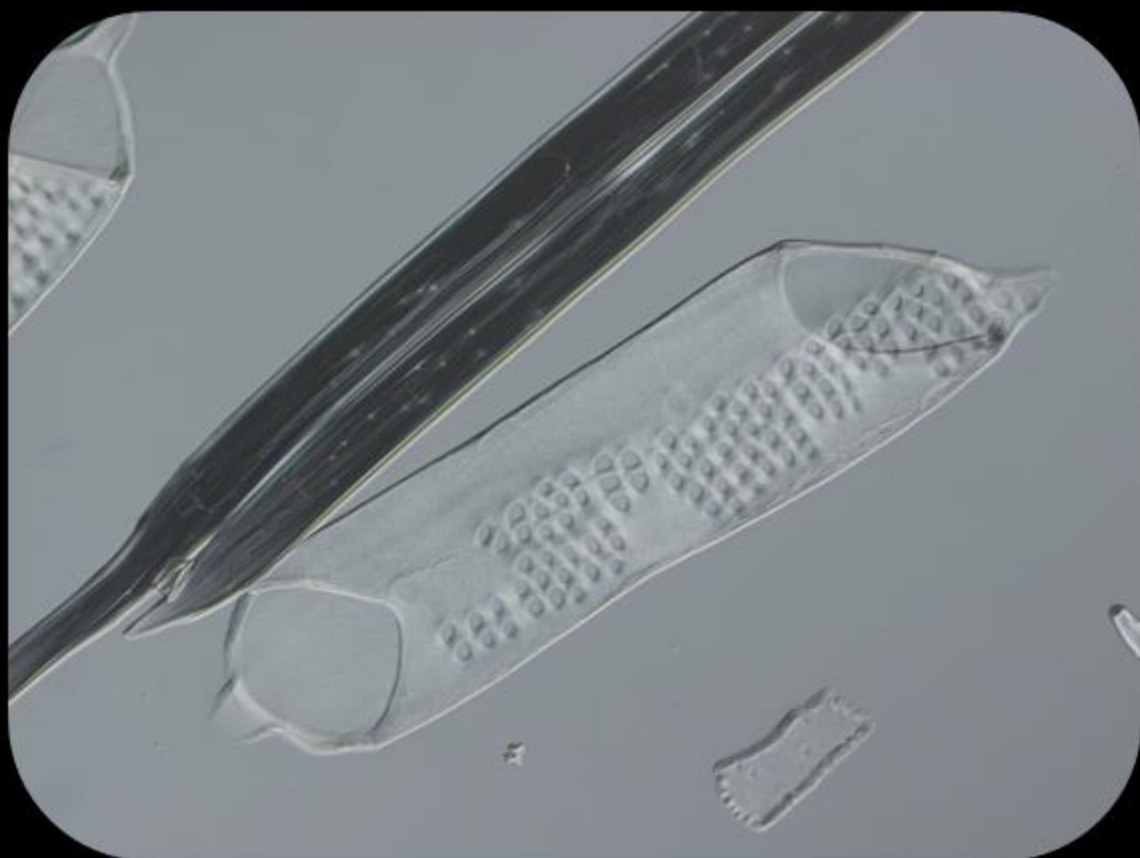


Novel insights into plant vascular development:
disclosing a mechanism to maintain
thermospermine homeostasis in the xylem

Ana Filipa Gonçalves Milhinhos



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
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*Aos meus pais e ao Pedro,
pelo vosso amor e apoio.*

*The most exciting phrase to hear in science, the one that heralds
new discoveries, is not 'Eureka!' (I found it!)
but 'That's funny ...'*

Isaac Asimov

(1920-1992)

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Ana Milhinhos participated in the experimental design, performing the laboratory experiments, and writing the paper (Chapter IV).

LIST OF ABBREVIATIONS

ACL5	ACAULIS5
APL	ALTERED PHLOEM DEVELOPMENT
ARF	AUXIN RESPONSE FACTOR
ARR	ARABIDOPSIS RESPONSE REGULATOR
AUX/IAA	AUXIN RESISTANT1/INDOLE ACETIC ACID
ATP	Adenosine triphosphate
BAP	6-benzylaminopurine
BCIP	5-bromo-4-chloro-3'-indolylphosphate
bp	Base pair
BR	Brassinosteroid
CaMV 35S	Cauliflower mosaic virus 35S promoter
cDNA	Complementary DNA
CDS	Coding sequence
CK	Cytokinin
CNA	CORONA/ATHB15
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
CG-MS	Gas chromatography mass spectrometry
GUS	β -Glucuronidase
GFP	Green fluorescent protein
h	Hour
HD-Zip III	Homeodomain leucine zipper Class III
IAA	Indole-3-acetic acid
IBA	Indole butyric acid
KAN	KANADI
Mbp	Mega base pair
μg	Microgram
μl	Microlitre
μm	Micrometer
μM	Micromolar
mg	Milligram
ml	Millilitre
mM	Millimolar

min	Minute
M	Molar
mm	Millimetre
m	Meter
mRNA	Messenger RNA
miRNA	MicroRNA
MS	Murashige and Skoog medium
nm	Nanometer
NBT	Nitro blue tetrazolium
NPTII	Neomycin phosphotransferase II
PAO	Polyamine oxidase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PHB	PHABULOSA
PHV	PHAVOLUTA
Ptt	<i>Populus tremula</i> × <i>Populus tremuloides</i>
Ptr	<i>Populus trichocarpa</i>
REV	REVOLUTA
RT-qPCR	Real-time quantitative reverse transcription PCR
SAM	S-adenosyl-methionine
SAM	Shoot apical meristem
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SPDS	Spermidine synthase E.C.2.5.1.16
Spd	Spermidine
Spm	Spermine
SPMS	Spermine synthase E.C.2.5.1.22
SSPE	Saline-sodium phosphate-EDTA buffer
TDZ	Thidiazuron
Tspm	Thermospermine
tSPMS	Thermospermine synthase E.C.2.5.1.79
WT	Wild-type

SUMMARY

Wood is one of the most important natural renewable resources. The developmental processes that underlie wood formation follow a well defined sequence of events that start at the core of the vascular cambium - the stem cell niche - that perpetually nourishes cells to the inside of the stem that ultimately become xylem/wood cells. The processes of specification and differentiation of cells into the xylem cell-types are far from being fully understood. It is a known fact that cells fated to become xylem have to commit suicide in order to serve this purpose. The correct timing of the cell death program is essential and relies on the action of thermospermine, a polyamine that it is thought to prevent the premature cell death of differentiating xylem in *Arabidopsis*. However, the molecular mechanisms underlying thermospermine action in xylem are poorly understood. In order to contribute towards understanding the role of thermospermine in xylem development in a woody plant species, *Populus* plants were genetically manipulated to increase thermospermine accumulation. Herein we describe the isolation and overexpression of *POPACAULIS5*, the gene that we found encoding for thermospermine synthase in *Populus*. Given that the thermospermine synthase gene in *Arabidopsis* is xylem-specific we performed expression and thermospermine quantification in xylem cells scraped from the woody stem. Evaluation of several growth parameters in the transgenic trees and detailed anatomical and ultrastructural analyses of the woody stems were carried out. We observed that high levels of thermospermine accumulated in leaf tissues of both *in vitro* and two month-old greenhouse grown transgenic trees. Intriguingly, despite being under the regulation of a constitutive promoter, *POPACAULIS5* transcript and thermospermine levels consistently failed to increase in stem and secondary xylem tissues of transgenic *Populus*. However, *in vitro* transgenic plants grown in the presence of auxin accumulated high levels of thermospermine in

all tissues and exhibited a dwarf phenotype that partially recovered once transferred to an auxin-free medium, with concomitant restoration to normal or slightly suppressed thermospermine levels in the stem. To understand the reason behind this effect, we quantified indole-3-acetic acid (IAA) in the transgenic trees, which revealed that *POPACAULIS5* had a negative effect on IAA levels. Conversely, exogenous auxin had a stimulatory effect on *POPACAULIS5*, as shown by auxin treatment time-course experiments. These results strongly indicate that excessive accumulation of thermospermine in xylem is prevented by a negative feedback control mechanism that maintains steady-state levels of thermospermine and that auxin is a mediator of this feedback control. To further understand the molecular mechanism underlying such control of thermospermine homeostasis, we searched for possible transcriptional regulators of *POPACAULIS5* and found *PttHB8*, a class III HD-Zip transcription factor family member, to be a good candidate. We demonstrated that upregulation of *POPACAULIS5* expression negatively affects *PttHB8* expression while upregulation of *PttHB8* induced *POPACAULIS5* expression. Therefore, we propose that a tissue-specific negative feedback loop controls *POPACAULIS5* transcript levels through suppression of IAA levels, while *PttHB8* is involved in the transcriptional control of *POPACAULIS5* expression. Moreover, by using a heterologous expression system we demonstrated that this mechanism is conserved in *Arabidopsis* where *POPACAULIS5* and *PttHB8* overexpression affected the endogenous levels of their homologs in *Arabidopsis* in a similar manner as in *Populus*. Additionally, the results obtained from overexpression of *PttHB8* and its paralog gene, *PttHB7*, in *Arabidopsis* suggest that novel roles in organ polarity may have evolved for this transcription factor in *Populus* genus. Furthermore, we provide a detailed analysis to the thermospermine-induced changes on the microscopic structure and transcriptome of *Populus* stems. The results demonstrated that increased thermospermine affected the cambial

zone, as vascular cambium failed in differentiating xylem. At the transcriptome level, *POPACAULIS5* overexpression had a positive effect on cytokinin levels, perception and signalling, suggesting that thermospermine and cytokinin may crosstalk in preventing xylem differentiation and a broader role at provascular stages of development may be attributed to thermospermine. Thermospermine limiting effect on auxin-induced xylem differentiation is here proposed to result from reduced auxin levels, distribution and responsiveness. Furthermore, the dwarfism imposed by increased *POPACAULIS5* expression and simultaneous auxin supply were found to be correlated with an increase in ethylene perception and response. Thus, we provide a framework to the detailed genetic dissection of thermospermine molecular mode of action in xylem differentiation in higher plants. Altogether, this work provides evidence that thermospermine is a novel plant growth regulator with specific functions in xylem differentiation and contributes to a better understanding of the transcriptional networks activated in response to thermospermine. We propose that a safeguard mechanism operates in secondary xylem tissues to ensure thermospermine homeostasis, which facilitates its fundamental role in xylem differentiation.

SUMÁRIO

A madeira é um dos nossos recursos naturais renováveis mais relevantes. Os processos de desenvolvimento que estão na base da formação da madeira seguem uma sequência de eventos bem definida que se inicia no câmbio vascular – um nicho de células estaminais – que perpetuamente fornece células para o interior do caule e que se vão diferenciando em células xilémicas. Os processos de especificação e diferenciação nos diferentes tipos de células xilémicas estão longe, contudo, de estar completamente compreendidos. No entanto, é conhecido que nas células destinadas a assumir a identidade de xilema se desencadeia um processo de morte celular para que se possa cumprir a sua função. É essencial que a morte celular programada destas células decorra num espaço temporal adequado, o que depende da acção da termospermina, uma poliamina, cujo papel em *Arabidopsis* se pensa ser o da prevenção da morte celular prematura do xilema em diferenciação. No entanto, os mecanismos moleculares subjacentes à acção da termospermina no xilema são ainda pouco compreendidos. De forma a contribuir para a compreensão do papel da termospermina no desenvolvimento do xilema numa planta lenhosa, manipulámos geneticamente plantas de *Populus* para aumentar a acumulação de termospermina. Neste trabalho, descrevemos como foi isolado e sobreexpresso o gene *POPACAULIS5*, que descobrimos codificar a termospermina sintase em *Populus*. Dado que o gene homólogo em *Arabidopsis* se expressa especificamente no xilema, quantificámos a expressão e a produção de termospermina em células do xilema das árvores transformadas. Foram também avaliados vários parâmetros de crescimento e efectuadas análises anatómicas e ultra-estruturais aos caules das árvores transgénicas. Quantificámos uma elevada acumulação de termospermina em folhas de plantas transgénicas que cresceram *in vitro* bem como em folhas de árvores com dois meses de idade. Curiosamente, e apesar de estar sob a

regulação de um promotor constitutivo, os níveis do transcrito *POPACAULIS5* e de termospermina no caule e xilema secundário das plantas transgênicas não foram superiores aos níveis detectados nas plantas controlo. Contudo, em plantas transgênicas cultivadas *in vitro* na presença de auxina houve uma elevada acumulação de termospermina em todos os tecidos, inclusivé no caule. Estas plantas exibem um fenótipo anão, e uma vez transferidas para um meio isento de auxina, recuperam parcialmente o fenótipo com o concomitante retorno dos níveis de termospermina no caule para níveis controlo ou até para níveis ligeiramente inferiores. Para compreender a razão subjacente a este efeito da auxina, quantificou-se o ácido indole-3-acético (IAA) nas árvores transgênicas, que revelou que o *POPACAULIS5* teve um efeito negativo sobre os níveis de IAA. Por outro lado, a auxina teve um efeito estimulador na expressão do *POPACAULIS5*, tal como evidenciado através de tratamentos com auxina. Estes resultados indicam que a acumulação excessiva de termospermina no xilema é evitada por um mecanismo de controlo de *feedback* negativo que mantém estáveis os níveis de termospermina bem como evidencia que a auxina é um mediador deste controlo. Para compreender melhor o mecanismo molecular subjacente a este controlo da homeostase da termospermina procuraram-se possíveis reguladores da transcrição de *POPACAULIS5* e identificou-se um possível candidato, o factor de transcrição PttHB8, membro da família classe III HD-Zip. Demonstrou-se que a sobreexpressão de *POPACAULIS5* afecta negativamente a expressão de *PttHB8* e por outro lado, a sobreexpressão de *PttHB8* induz a expressão de *POPACAULIS5*. Desta forma, propomos que um mecanismo de *feedback* negativo controla os níveis de transcritos do gene *POPACAULIS5* através da supressão dos níveis de IAA e que, por outro lado o factor de transcrição PttHB8 está envolvido no controlo transcricional do gene *POPACAULIS5*. Adicionalmente, usando um sistema de expressão heteróloga foi demonstrado que este mecanismo é conservado em *Arabidopsis* uma vez que a sobreexpressão de *POPACAULIS5* e *PttHB8*

afectou os níveis endógenos dos transcritos homólogos em *Arabidopsis* de uma forma semelhante ao observado em *Populus*. Os resultados obtidos a partir da sobreexpressão do gene *PttHB8* bem como do seu parálogo *PttHB7*, em *Arabidopsis*, sugerem que estes factores de transcrição evoluíram no género *Populus* adquirindo novas funções na polaridade dos órgãos. São também apresentadas análises detalhadas ao nível da estrutura microscópica e do transcriptoma nos caules de *Populus* com alterações induzidas pela acumulação de termospermina. Os resultados demonstraram que a termospermina afectou a zona cambial, na medida em que o câmbio vascular não diferenciou xilema. Ao nível do transcriptoma, a sobreexpressão de *POPACAULIS5* teve um efeito positivo nos níveis, na percepção e nas vias de sinalização das citocininas, o que sugere a interacção entre termospermina e citocinina na prevenção da diferenciação do xilema e que a termospermina pode ter um papel mais amplo numa fase provascular do desenvolvimento. Propomos que o efeito restritivo que a termospermina exerce na diferenciação de xilema induzida por auxina seja o resultado de uma redução nos níveis de auxina, na distribuição e na capacidade de resposta à auxina. Além disso, o nanismo imposto pela sobreexpressão de *POPACAULIS5* na presença de auxina foi correlacionado com um aumento na percepção e na resposta ao etileno. Contribuímos assim com um enquadramento geral para uma futura dissecção genética detalhada do modo de acção da termospermina na diferenciação do xilema em plantas superiores. Globalmente, este trabalho evidencia que a termospermina é um regulador do crescimento das plantas com funções específicas na diferenciação do xilema e contribui para uma melhor compreensão das redes de transcrição activadas em resposta à termospermina. Propomos que existe um mecanismo de salvaguarda da homeostase da termospermina nos tecidos do xilema secundário de modo a assegurar o papel fundamental da termospermina na diferenciação do xilema.

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CHAPTER I

GENERAL INTRODUCTION[†]

[†] **Milhinhos A. and Miguel C. (2013)** Hormone interactions in xylem development: a matter of signals. *Plant Cell Reports*, (doi: 10.1007/s00299-013-1420-7).

Plant vascular development

Early plants were simple and small unicellular or filamentous organisms highly dependent on being submersed in the aquatic environment. It was about 440 million years ago that plants evolved adaptations to land life. One of such remarkable adaptations was the evolution of vascular tissues that allowed vascular plants to colonize vast areas of the earth's terrestrial surface. The appearance of vascular tissues not only made it possible to transport nutrients and water as it granted the plant with tools for increased organismal complexity, allowing growth in height away from the shadow of other plants, for instance. Xylem and phloem thus evolved as a network throughout the plant that connected the plant body and formed the vascular system. While phloem functions to transport and distribute the photoassimilates produced in the shoot, xylem transports water and minerals taken up by the roots and provides structure and girth to the plants. The ability for growth in height was accompanied by lateral growth, which afforded the plant kingdom to evolve the largest organisms on earth. The lateral growth largely originates from the activity of the vascular cambium, a secondary meristem that drives the formation of secondary xylem (commonly named wood). The formation of the wood is modulated by several internal signals that control the vascular cambium activity. However, little is known on the hormonal and genetic background that is behind this secondary growth. Fair to say that in the last decades the bloom of genetic and genomic tools has led to increased understanding of the molecular mechanisms underlying secondary growth, mainly due to this great landmark that was the sequencing of the *Populus* tree genome (Tuskan *et al.*, 2006).

The aim of this work was to characterize the function of a novel plant growth regulator, the polyamine thermospermine, in secondary growth. The *Populus* tree was therefore chosen as the main research model system, complemented by studies carried out in *Arabidopsis* annual herbaceous

model plant. This thesis starts by introducing general concepts, with a description of the processes involved in vascular development, with emphasis on xylem specification and differentiation. The organization and establishment of the vascular tissues are also discussed. The current understanding on hormone signaling interactions in xylem development and their role in the regulation of plant meristem activity will be introduced. In addition, a broad view on structure and metabolism of polyamines, as well as their role in plant development are presented, with emphasis on thermospermine role in vascular development. The PhD studies own results will be discussed within this context.

The vascular system

Vascular cells are produced from the apical meristems located at the shoot and root apices. The apical meristem contains the stem cell niche, composed of a few undifferentiated cells, called pre-procambial or provascular cells that give rise to the different cell types present in the plant body, such as procambial and cambial cells, from which xylem and phloem precursor cells develop. Xylem cells differentiate from xylem precursor cells into tracheary elements (tracheary and vessel elements, the later only present in angiosperms), xylem parenchyma cells and xylem fibers. Phloem precursor cells differentiate into sieve elements, companion cells, phloem parenchyma cells and phloem fibers.

Procambium and cambium

During primary growth, procambium promotes growth of vascular tissues in the apical directions. Procambial cells which are vascular stem cells derive from pre-procambial/provascular cells in the apical meristem and form within the leaves primordia and root primary tissues early during embryogenesis

(Figure 1; Esau, 1977; Clay and Nelson, 2002). Procambial cells are cytoplasm-dense cells that are organized in continuous strands, the vascular bundles. Coordinated, oriented divisions, parallel to the direction of the vascular bundle axis confer the narrow and elongated shape to procambial cells (Scarpella *et al.*, 2004).

Vascular cambium derives mainly from the procambium within the vascular bundle, but also from the parenchyma between the vascular bundles. Cambial cells divide anticlinally (perpendicularly to the surface of the stem) to produce the derivative initials. These derivative initials then divide periclinally (parallel to the surface of the stem) to produce specialized cells (phloem and xylem) thus promoting growth in the lateral directions (Esau, 1977). These divisions give rise to the secondary meristem, the cylindrical vascular cambium, and mark the start of secondary development (Figure 1; Baucher *et al.*, 2007). The vascular cambium term is used to refer to the cambial initials file of cells, however because it is often difficult to distinguish between the initials and their immediate derivatives, the term is usually applied to the group of cambial initials and their immediate derivatives, and instead some authors prefer to name this region as the cambial zone (Raven *et al.*, 2005).

Xylem

Primary xylem differentiates from procambium and secondary xylem from the vascular cambium activity. Protoxylem cells are the first xylem cells to differentiate within the primary vascular bundles, whereas metaxylem cells differentiate later in development. In the shoot, the protoxylem cells differentiate in the innermost position of the vascular bundles. Protoxylem and metaxylem can be distinguished based on the patterns of secondary cell wall depositions. Protoxylem has less complex ring-like (annular) or helical (spiral) secondary cell wall thickenings, that can be stretched, making it

possible to elongate in the direction of growth, whereas metaxylem cells have net-like (reticulate) or porous (pitted) wall thickenings and are structurally stronger and cannot be stretched (Chapter II).

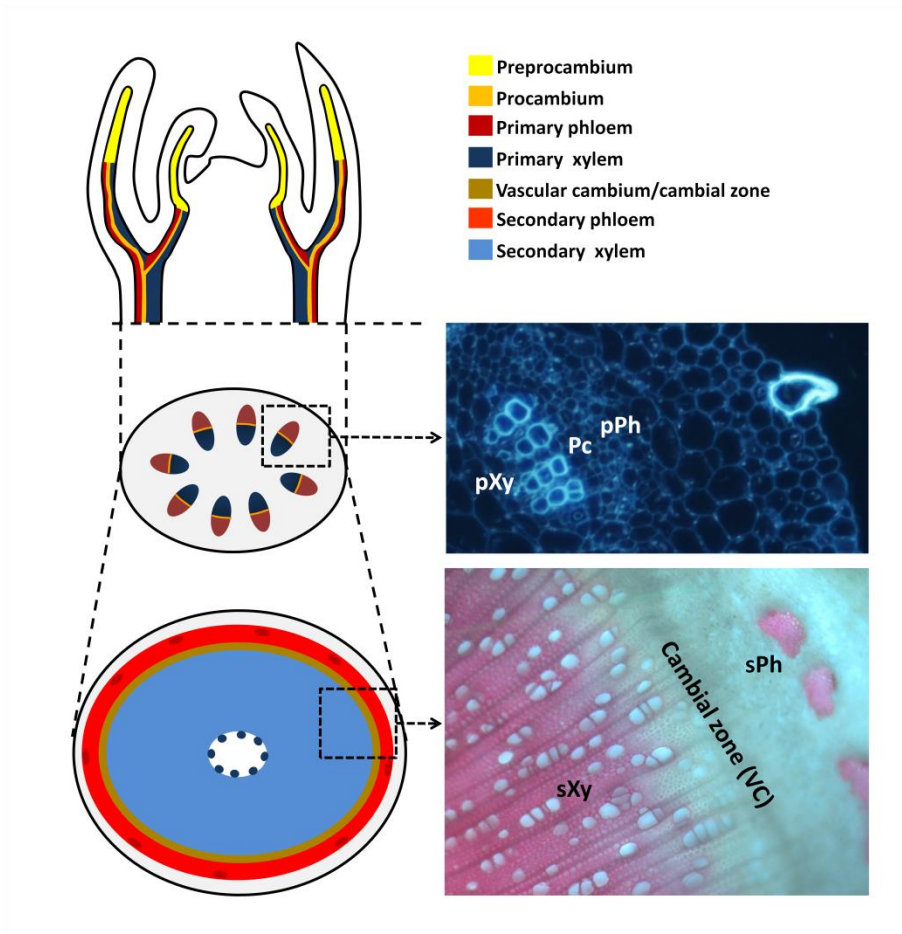


Figure 1. Schematic representation of vascular tissues organization during primary and secondary growth in higher plants. (From the top) sections represent the shoot apical meristem, where preprocambial/provascular cells precede vascular development. At the cellular level, the formation of the venation pattern begins in the leaf primordium with the siting of procambial cells among equivalent provascular cells, which are cells in an uncommitted meristematic state with vascular potential (Clay and Nelson, 2002). Procambium strands result from longitudinal divisions that give rise to aligned elongated cells. The vascular strands include primary xylem and phloem tissues that differentiate from the procambium cells in opposite directions. The ►

◀transition from primary to secondary growth involves the formation of fascicular cambium, that arises within vascular bundles and interfascicular cambium that arises between vascular bundles (not shown, reviewed in Sanchez *et al.*, 2012). Secondary growth of vascular tissues, shows secondary phloem and xylem that derive from the vascular cambium, a layer of meristematic/cambial cells. The cambial cells at the vascular cambium undergo cell divisions that originate xylem precursor cells (towards the interior of the stem) and phloem precursor cells (towards the exterior of the stem). Xylem precursor cells then differentiate into different xylem cell types (such as vessels, fibers and ray cells). Primary and secondary development is also shown in the corresponding *Populus tremula* × *Populus tremuloides* stem cross-sections (on the right). Pc, procambium; VC, vascular cambium; pPh and sPh, primary and secondary phloem; pXy and sXy, primary and secondary xylem.

Vascular cambium activity gives rise to secondary xylem (wood). Wood comprises three general types of cells: xylem vessels, xylem fibers and xylem parenchyma (Figure 2). The interconnected xylem vessels consist of lignified secondary cell walled and hollow lumen cells that, joined together at their perforation plates, form the finite conduits. The sap flowing through the lumens in these conduits typically includes nutrient ions, amino acids, hormones, proteins, and traces of carbohydrates (Biles and Abeles, 1991; Davies and Zhang, 1991; Gollan *et al.*, 1992; Satoh *et al.*, 1992; Goodger *et al.*, 2005). Between the adjacent xylem conduits the sap transport occurs via the numerous pits concentrated in the lignified secondary cell walls (Figure 2a). The xylem fibers, which are thick secondary cell walled cells, provide structural support (Figure 2b); and xylem parenchyma cells. The vascular ray cells are mostly parenchymatic cells that transport photosynthesis products and water across the stem between secondary xylem and phloem cells (Esau, 1977).

Xylem development is a process of terminal cell differentiation that includes initial cell division, followed by rapid cell expansion, secondary cell wall formation and a programmed cell death (PCD) (Fukuda, 1996; Déjardin *et al.*, 2010; Figure 3). Not all xylem cell types undergo the exact same cell death program and neither the same lifespan (Bollhöner *et al.*, 2012). Vessel

elements die within two days after their specification, fibers stay alive for approximately one month in *Populus* and ray parenchyma cells stay alive for decades (Bollhöner *et al.*, 2012; Nakaba *et al.*, 2006; 2011). Still, the main events of tracheary elements differentiation include: early differentiation in the cambial zone, cell expansion, secondary cell wall formation, changes in tonoplast permeability and vacuolar rupture, DNA degradation, final autolysis and hydrolysis of the non-lignified primary cell walls (Bollhöner *et al.*, 2012). Maturation of xylem, including lignification of xylem cell wall coincides normally with cell death in time and place (Bollhöner *et al.*, 2012). This is, as lignin accumulation increases, the xylem fiber elements death program is maybe triggered but it is not currently known whether the two processes are coordinated (Figure 3).

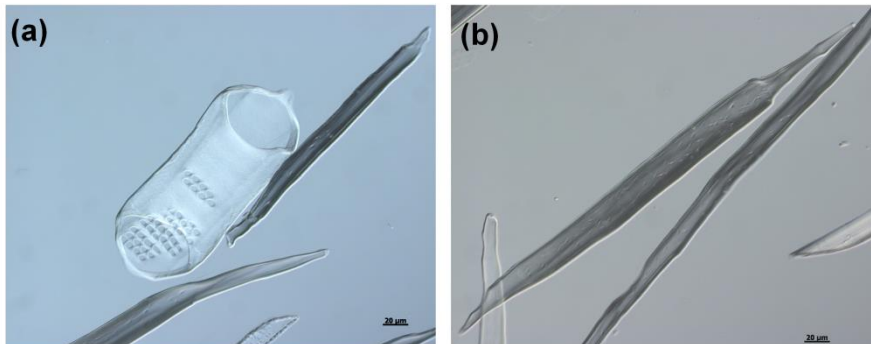


Figure 2. Secondary xylem cell types present in *Populus* wood. (a) secondary xylem vessel and (b) xylem fiber. Examples of primary xylem vessels are illustrated in Chapter II.

Phloem

Like xylem cells, phloem (bark) originates from the vascular cambium activity, and is differentiated towards the opposite side to xylem. The phloem is the principal conductor of photosynthetic products, proteins, hormones and mRNAs involved in plant development. At maturity, sieve elements lack nuclei, tonoplast and have a degenerate endoplasmic reticulum, some plastids

and mitochondria close to the wall (Raven *et al.*, 2005). It may be primary or secondary in origin as xylem, but contrary to xylem, phloem is living at maturity. Phloem comprises the conducting sieve elements (sieve tubes and sieve cells) and the non-conducting fibers and parenchyma cells. The interconnected sieve elements that are metabolically sustained by adjacent companion cells in source organs, such as leaves, load the photoassimilates and unload to sink organs such as roots and storage tissues (Raven *et al.*, 2005).

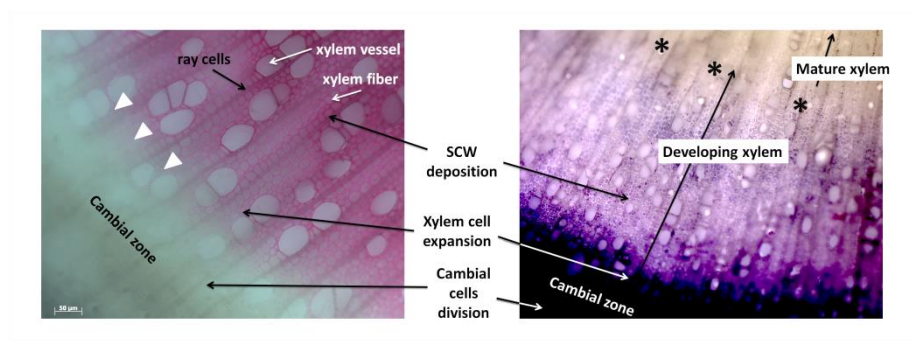


Figure 3. Cross section of hybrid-aspen (*Populus tremula* × *Populus tremuloides*) stem, showing progression of xylem lignification and cell death. (on the left) lignification of xylem as indicated by phloroglucinol staining and (on the right) cell viability test by nitroblue tetrazolium staining observed in developing xylem (methods detailed in Chapter II). The stages of xylem differentiation are indicated. Arrows depict beginning of lignification and asterisks mature xylem.

Populus and Arabidopsis as model systems for vascular development studies

In the past years, our knowledge on how vascular tissues are formed has significantly increased; in great part due to the use of *Arabidopsis* root model system as well as *Zinnia elegans* xylogenic cultures. Several studies have also reported the use of more complex systems such as tree stems, for which

Populus has been the model system of choice. The natural choice of a tree for xylem development studies comes from the utter amount of secondary growth it presents. *Populus* has become largely accepted in the research community as the ‘model’ woody plant arguing that such a ‘model’ tree is needed to complement the genetic resource developed in *Arabidopsis*. The genus *Populus* (poplars, cottonwoods and aspens) comprises approximately 30 species of woody plant, all found in the Northern hemisphere and characterized by the fastest growth rates in temperate trees (Taylor, 2002). There are several reasons that elected *Populus* as a model tree, but the most important is based on the fact that the *Populus* genome was the first tree genome to be sequenced and made available (Tuskan *et al.*, 2006). The relatively small genome size (450–550 Mbp), the large number of molecular genetic maps and the ease of genetic transformation of *Populus* are additional reasons for the choice. *Populus* is also easily propagated vegetatively allowing the mass production of clonal material for experiments. In addition, and contrary to *Arabidopsis*, *Populus* trees also have true commercial value for timber, plywood, pulp and paper (Taylor, 2002).

However, there are challenges to working with trees when compared to the *Arabidopsis* simple model plant. One of the main constraints is related to the difficulty in establishing genetic methods on trees (Groover, 2005). *Populus* does not reach maturity for several years and grows to a quite large size, many times far beyond convenience for genetic studies. It is also dioecious, which makes selfing and back-cross manipulations impossible. Furthermore, compared to *Arabidopsis* it is rather impossible to perform mutagenesis-based genetic screens as well as producing any homozygous loss-of-function mutants. Whilst, producing gain-of-function mutants is possible, and the function of individual genes can be performed to detail by the use of the transgenic approach that produces a dominant phenotype in the transformed plants (Chapters II, IV). Tools such as activation-tagging for forward gene discovery (Busov *et al.*, 2011), along with other genomic and

transgenic technologies have also been applied to *Populus* with success.

In vascular development studies, the large size and radial organization of *Populus* tree stems allows to harvest significant amounts of specialized cells from the cambial zone at different stages of specification. The cambial zone can therefore be divided into separate fractions that correspond to meristematic cambial cells, developing xylem cells, and functional phloem cells. This method is not as easily attained in *Arabidopsis* stem due to its small scale and the need for specialized technology such as laser microdissection to gather a much reduced amount of cells; while it has been employed successfully in numerous studies using trees (Uggla *et al.*, 1998; Hertzberg *et al.*, 2001; Schrader *et al.*, 2003; Schrader *et al.*, 2004; Nieminen *et al.*, 2008; Chapter II).

Arabidopsis has gained a supreme role as a model plant for dicotyledons. Not only was it the first plant genome to be sequenced (Arabidopsis Genome Initiative, 2000) as it has a small size, small genome, rapid life-cycle (that allows up to 8 generations per year), easy transformation procedures, knock-out mutants collections, making it a central resource for plant science. Although not to a large extent, *Arabidopsis* also develops secondary growth in the hypocotyls and roots (Chaffey *et al.*, 2002) resembling the secondary growth in trees but to a smaller scale. Thus, it is possible that parallel mechanisms exist in the control of *Arabidopsis* and *Populus* vascular development. However, for instance, *Arabidopsis* plants lack many characteristics of trees, such as perennial, annual cambial activity and dormancy and also do not have ray cells in secondary xylem (Chaffey *et al.*, 2002). Therefore, it is becoming evident that to study the formation of wood, researchers can take advantage of both systems complementing their research to fully understand vascular development processes in trees.

Hormone interactions in xylem development: a matter of signals

In the last decades the bloom of genetic and genomic tools has led to increased understanding of the molecular mechanisms underlying the function of the traditional plant hormones in xylem specification and differentiation. Critical functions have been assigned also to novel signalling molecules, such as thermospermine. It is evident that these signals do not function independent of each other but in close interaction in a manner that only now is beginning to be understood.

Previous studies demonstrated that the dynamics of shoot and root apical meristems is regulated by similar molecular mechanisms (Sarkar *et al.*, 2007; Stahl *et al.*, 2009). The apical meristem and the vascular cambium are also thought to be controlled by similar regulators (Sanchez *et al.*, 2012; Aichinger *et al.*, 2012). For instance, Yordanov *et al.* (2010) showed that the cambium zone is a boundary region that is likely under the same type of regulation as the one described in the shoot apical meristem (SAM) that separates the stem cell niche from the emerging lateral organ primordia. The authors reported that, in poplar, the LATERAL ORGAN BOUNDARIES1 (PtLBD1) transcription factor is expressed in the phloem side of the cambial zone and regulates secondary phloem production. The expression analysis of putative PtLBD1 targets suggested that this function is likely mediated through suppression of genes that promote meristem cell identity (such as *KNOX1*, class I *KNOTTEDLIKE HOMEODOMAIN*) and the activation of genes that trigger differentiation of phloem (such as *APL*, *ALTERED PHLOEM DEVELOPMENT*). LATERAL ORGAN BOUNDARY (LBD) target genes have similar expression patterns in the cells neighbouring the cambium (in *Populus*) and in the SAM (in *Arabidopsis*), supporting their similar function in both types of meristem (Yordanov *et al.*, 2010). The same kind of mechanistic resemblance between SAM and vascular cambium has been identified for the *ARBORKNOX* genes in *Populus* and

SHOOTMERISTEMLESS (STM)/ BREVIPEDICELLUS (BP) in *Arabidopsis* (Groover *et al.*, 2006; Du *et al.*, 2009), as well as in the monocotyledoneous maize and rice plants, suggesting that *KNOX* genes are conserved mediators of meristematic potential (Scofield and Murray, 2006). Due to such similarities, the following sections review the state of the art integrating data obtained from the study of procambium in the *Arabidopsis* shoot and root meristem model systems and in inflorescence stems but also data on lateral growth and the emerging knowledge from studies in *Arabidopsis*, *Zinnia elegans* xylogenic cultures and *Populus* trees.

New insights into how signaling molecules direct phloem and xylem differentiation are coming to light. Hormones, synthesised either locally or at a long distance, are important signals in this process, being recognised and integrated into responses during vascular development. Auxin, cytokinin, gibberellins and ethylene were early on identified as regulators of vascular development, but more recently also brassinosteroids, nitric oxide, jasmonic acid and strigolactones have been pointed out involved in the process (Sachs, 1981; Mähönen *et al.*, 2000; Eriksson *et al.*, 2000; Savidge, 1988; Choe *et al.*, 1999a; Gabaldón *et al.*, 2005; Sehr *et al.*, 2010; Agusti *et al.*, 2011a). Other important signals include thermospermine, H₂O₂ and small peptides (Vera-Sirera *et al.*, 2010; Ros Barceló *et al.*, 2002; Ito *et al.*, 2006). Even though signaling pathways for some of these compounds are quite well characterized (such as for auxin, cytokinin, ethylene, gibberellin and brassinosteroids) the exact molecular mechanism underlying control of vascular development is not fully understood for any of them.

In the following sections, the current knowledge on the hormone signaling pathways and their cross-talk in cambial cell initiation, maintenance and in xylem specification and differentiation is discussed. It begins by approaching how auxin promotes the transition of undifferentiated cells into procambial cells and which signals maintain procambial/cambial cell identity and prevent or promote their development into xylem cells. Figure 4

summarizes the hormones action in cambium cells formation, maintenance and in xylem specification and differentiation hereby described.

Auxin role in cambial cell identity: initiate and keeping it cambial

Auxin is a key regulator of almost any aspect of plant development and vascular development is no exception. It has long been known that the initiation of pre-procambial cells depends on auxin signaling and transport. According to the auxin canalisation theory, auxin creates its own transport channels as a result of an initial auxin flux from an auxin source to an auxin sink. Thus, canalization of auxin flow initiates vascular cells, which also promotes further canalization of auxin into these channels. The preferred channels inhibit further canalisation in the surroundings and thus differentiate as narrow vascular strands (Sachs, 1981). Evidence of auxin transport and signaling components functioning in vascular specification, together with pharmacological studies of auxin transport inhibitors, have supported the fundamental role of auxin in the induction of vascular bundles (Galweiler *et al.*, 1998; Mattsson *et al.*, 1999; Sieburth, 1999).

Earlier studies have also shown the involvement of auxin in initiating and promoting vascular cambium growth. Auxin supply from the shoot apical meristem is required for cambial cell proliferation (Aloni, 1987; Shiner, 1979). Increased auxin concentrations have been found in the cambial stem cells of *Pinus* (Uggla *et al.*, 1996). Also, in transgenic *Populus*, a decrease in auxin levels was shown to diminish cell division in the xylem (Tuominen *et al.*, 1997; Nilsson *et al.*, 2008). Baba *et al.* (2011) have demonstrated that, during dormancy, cambial cell division ceases due to a decrease in cambium responsiveness to auxin. The auxin gradient, with its maxima in the cambial zone, is thought to be essential for cambial proliferation, but only recently have the molecular mechanisms underlying this dependence of vascular development on auxin gradients started to be unveiled.

Auxin is perceived by the family of F-box domain receptors TRANSPORT INHIBITOR RESPONSE1 (TIR1) (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005). TIR1 is part of an ubiquitin ligase complex that targets the degradation of AUXIN/INDOLE ACETIC ACID (Aux/IAA) transcriptional regulators by the 26S proteasome in an auxin-dependent manner (Gray *et al.*, 1999, 2001). The *Aux/IAAs* are auxin-responsive genes that repress the transcriptional activities of the AUXIN RESPONSE FACTOR (ARF) family members (Figure 4; Kieffer *et al.*, 2010; Mockaitis and Estelle, 2008). Thus, the *Aux/IAA-ARF* system modulates the developmental responses to auxin, since ARFs are the elements that transcriptionally activate or repress the downstream developmental genes. In the early stages of *Arabidopsis* vascular development, initiation of procambium in the embryo relies on the auxin flow that promotes the degradation of *Aux/IAA* proteins, thus relieving MONOPTEROS/AUXIN RESPONSE FACTOR 5 (MP/ARF5) from repression. The *MP* gene is essential in establishing procambium cells, as shown by the lack of the central provascular cylinder in *mp* embryos (Berleth and Jurgens, 1993; Hardtke and Berleth, 1998) and the decreased auxin sensitivity in *mp* mutants (Mattsson *et al.*, 2003). It has been also shown that MP/ARF5 confers procambium identity, possibly by activating the *CLASS III HOMEODOMAIN LEUCINE ZIPPER 8* (HD-Zip III *AtHB8*) gene transcription in *Arabidopsis* leaf veins (Donner *et al.*, 2009). In response to increased auxin levels, the *MP* gene becomes transcriptionally activated (Wenzel *et al.*, 2007). Reports also show that MP may regulate the expression of *PIN-FORMED1* (*PIN1*), a major auxin efflux carrier protein encoding gene (Sauer *et al.*, 2006; Wenzel *et al.*, 2007; Schuetz *et al.*, 2008). PINs are polarly localized transmembrane proteins fundamental for directional cell-to-cell auxin transport, that is, for polar auxin transport (PAT) (Leyser, 2005). Prior to procambium identity definition there is an increase in the expression of *PIN1* (Scarpella *et al.*, 2006; Wenzel *et al.*, 2007). This

increase in auxin flow is what determines pre-procambial cell state acquisition. *ATHB8* is necessary to stabilize pre-procambial cell specification against auxin transport perturbations (Donner *et al.*, 2009) and probably acts by reducing the sensitivity to auxin, in terms of *PIN1* expression, and thereby confining procambium precursor cell state acquisition to narrow regions in the developing leaf (Scarpella *et al.*, 2006; Wenzel *et al.*, 2007; Donner *et al.*, 2009; Ohashi-Ito and Fukuda, 2010). Since procambial cell formation in the *athb8* null mutant does not deviate from the pattern observed in the wild-type, it is also possible that MP acts on several other key components to promote procambium identity (Baima *et al.*, 2001; Prigge *et al.*, 2005). Curiously, while *ATHB8* was found to be activated during interfascicular cambium formation, *PIN1* and MP were not detected, suggesting the existence of an alternative mechanism of *ATHB8* activation, as in the procambium (Agusti *et al.*, 2011b).

CLE peptides: keep it cambial and suppress xylem differentiation

The maintenance of cambial cell identity and activity has been shown to involve another signal: the tracheary element differentiation inhibitory factor (TDIF). TDIF is a CLAVATA3/ENDOSPERM SURROUNDING REGION (CLE)-family peptide produced by the activity of the *CLE41* or *CLE44* genes and is involved in short-range signaling and cell-to-cell communication (Ito *et al.*, 2006; Fukuda *et al.*, 2007). TDIF also inhibits the transdifferentiation of tracheary elements in *Zinnia* cell cultures and promotes proliferation of the procambium cells in *Arabidopsis* hypocotyls and leaves (Hirakawa *et al.*, 2008). The receptor of TDIF is a leucine-rich repeat receptor kinase (LRR-RLK) named TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR RECEPTOR (TDR). This receptor is also called PHLOEM INTERCALATED WITH XYLEM (PXY) because it was initially cloned from the *pxy* mutant that showed vascular patterning defects in the

positioning of xylem and phloem (Fisher and Turner, 2007).

Several studies have demonstrated that *WUSCHEL-RELATED HOMEODOMAIN* (*WOX*) gene family members cooperate with *CLAVATA* (*CLV*)/*CLE* genes to organize initial cell populations during development (Brand *et al.*, 2000; Schoof *et al.*, 2000; Ji *et al.*, 2010). For instance, it has recently been found that *WOX4* is transcribed in the procambium of developing vascular bundles in the root and shoot lateral organs of *Arabidopsis* and tomato, and that the downregulation of this gene reduces vascular development and increases accumulation of undifferentiated ground tissue (Ji *et al.*, 2010). This discloses an essential role for *WOX4* in promoting cambium activity in the lateral meristem. TDIF/PXY signaling targets *WOX4*, which maintains proliferation of the cambial cells in response to the TDIF signal (Figure 4; Hirakawa *et al.*, 2010; Ji *et al.*, 2010). Suer *et al.* (2011) further revealed that the auxin-dependent cambium stimulation requires *WOX4* and its upstream regulator, TDR/PXY (the receptor of TDIF). The application of an inhibitor of auxin transport, 1-*N*-naphthylphthalamic acid (NPA), to the bottom internodes of *Arabidopsis* inflorescences, stimulated cambial activity above the treated area, likely due to accumulation of the basipetally transported auxin in the wild type, but not in *wox4* mutants (Suer *et al.*, 2011). These observations suggest that *WOX4* is essential in the translation of the basipetal auxin transport into cambium activity. It has also been shown that a TDIF peptide signal is produced in the phloem cells and perceived in procambial cells by TDR/PXY, leading to the upregulation of *WOX4* in the vascular cambium cells (Hirakawa *et al.*, 2008; Matsubayashi, 2011). TDR/PXY is thus required for the correct polar patterning of xylem and phloem tissues. The disclosure of this TDIF-PXY system of communication is remarkable as it shows a crosstalk between phloem and xylem, whereby the peptide signals produced in the phloem cells activate a signaling cascade perceived by receptors in the plasma membrane of the procambial cells, which results in the stimulation of procambial cell

proliferation and the inhibition of xylem differentiation.

The mechanisms behind establishment and maintenance of stem cell populations in the SAM and root apical meristem (RAM) show many similarities (Sarkar *et al.*, 2007). This suggests that the same type of mechanisms that regulate stem cell homeostasis may well exist in the lateral meristem-vascular cambium. In fact, the molecular mechanism involved in the TDIF/CLE41/CLE44-TDR/PXY-WOX4 module closely resembles the signaling module comprising CLAVATA3 (CLV3) peptide and the receptor components CLAVATA1 (CLV1) and CLAVATA2 (CLV2) that operate in the control of stem cell maintenance in the SAM. Despite the differences, surprising parallels can be found in the molecular regulation of the apical and lateral meristems: both pathways comprise a CLE peptide, an LRR-RLK, and a WOX transcriptional regulator. However, the CLV pathway limits the expression of *WUS* or *WOX5*, whereas TDIF promotes the expression of the *WUS*-like gene *WOX4* (Hirakawa *et al.*, 2010).

A parallel action of TDR/PXY and ethylene signaling in the control of cambial cell division has also been proposed. *pxy* loss-of-function *Arabidopsis* mutant does not exhibit a drastic reduction in vascular cell number, indicating that a compensatory pathway may be activated in the absence of TDR/PXY. Blocking ethylene signaling aggravates the typical defects of the *pxy* mutant, suggesting that ethylene signaling, *WOX4*, and TDR/PXY work in parallel to regulate cell divisions during *Arabidopsis* vascular development (Etchells *et al.*, 2012). CLE peptides have been shown to inhibit protoxylem vessel formation in the *Arabidopsis* root via the cytokinin signaling pathway, suggesting crosstalk between CLE peptide signaling and cytokinin signaling in protoxylem vessel formation (Kondo *et al.*, 2011). It would be interesting to further explore the hormone signaling crosstalk to dissect this TDIF/PXY regulation module.

Cytokinin signaling: keep it cambial and suppress xylem differentiation II

Cytokinin (CK) signaling is important in the maintenance and proliferation of cambial cells and in cambial cell specification. CK perception involves a family of CK receptors CYTOKININ RESPONSE1/WOODEN LEG/ARABIDOPSIS HISTIDINE KINASE4 (CRE1/WOL/AHK4) and ARABIDOPSIS HISTIDINE KINASE2 (AHK2) and AHK3 that activate a phosphorylation cascade whereby histidine phosphotransfer proteins (AHPs), in the nucleus, activate type-B ARABIDOPSIS RESPONSE REGULATORS (type-B ARR), which in turn activate CK responses (Figure 4; reviewed in Bishopp *et al.*, 2006; Kieber and Schaller, 2010). Type-B ARR proteins are also known to activate transcription of type-A ARRs that in turn negatively regulate back CK signaling (Dello Ioio *et al.*, 2008a).

The expression of the key CK catabolic gene *CYTOKININ OXIDASE 2 (CKX2)*, in the *Populus* cambium, leads to a decrease in CK concentration and results in reduced radial growth (Nieminen *et al.*, 2008). In a similar manner, in *Arabidopsis*, the disruption of four genes encoding CK biosynthetic isopentenyltransferases results in plants unable to form cambium and with reduced stem and root thickenings (Matsumoto-Kitano *et al.*, 2008). These studies have confirmed that CKs are important cambial regulators. Furthermore, CK receptors are highly expressed in dividing cambial cells. Hejatko *et al.* (2009) have shown that the histidine kinase CYTOKININ-INDEPENDENT1 (CKI1), AHK2 and AHK3 are important for vascular development by regulating procambium proliferation and/or the maintenance of its identity in *Arabidopsis* shoots. Mutations in the CK-induced receptors AHK2 and AHK3 result in defects in vascular tissue formation in the inflorescence stem that are partially rescued by overexpression of CKI1 (Hejatko *et al.*, 2009). The *wol* mutants that are defective in *CRE1/WOL/AHK4* gene have reduced cell proliferation and cell files within the pericycle layer only differentiate into protoxylem (Scheres *et al.*, 1995;

Mähönen *et al.*, 2000; 2006). A concrete mechanism was unveiled when Mähönen *et al.* (2006) found that CK negatively regulates protoxylem specification and that AHP6 (a histidine phosphotransfer protein lacking the histidine residue necessary for phosphorelay) counteracts CK signaling, thus having a positive effect on protoxylem formation. On the other hand, CK signaling negatively regulates the spatial domain of *AHP6* expression. The hypothesis is that this balance between proliferation and differentiation of cell lineages directs vascular development in early embryogenesis (Mähönen *et al.*, 2006). Furthermore, auxin was also brought to this equation.

Auxin and cytokinin interplay in protoxylem differentiation

Recently, Bishopp *et al.* (2011) further elucidated the AHP6-mediated crosstalk between auxin and CK signaling in vascular patterning. The authors showed that, in the *Arabidopsis* root vasculature, the cells fated to be protoxylem exhibit high auxin and low CK signaling, whereas the procambial cells exhibit high CK but low auxin signaling. A mutually inhibitory mechanism was proposed wherein high CK signaling in the procambial cells promotes the expression of *PIN*s and the lateral localization of PIN proteins. This CK-dependent PIN activity forces a lateral flow of auxin from the procambial cells, to the meristematic cells from which protoxylem will form. The increase in auxin signalling in these cells promotes their specification into protoxylem and the transcription of *AHP6*, which in turn inhibits CK signaling (Bishopp *et al.*, 2011). Moreover, a piece of this story has been linked to the mobile GRAS family transcription factor SHORT-ROOT (*SHR*) required for cell specification and patterning of the *Arabidopsis* root (Helariutta *et al.*, 2000; Nakajima *et al.*, 2001). *SHR* directly regulates a cytokinin oxidase (*CKX3*), which is preferentially expressed in the protoxylem; *shr* mutants have elevated levels of CK. Therefore it has been proposed that *SHR* controls vascular patterning by controlling CK

homeostasis (Cui *et al.*, 2011): when SHR is functional it imposes low CK levels to the cell, which promotes xylem differentiation; on the other hand, when *SHR* is disrupted (*shr*) or in the presence of exogenous CK, high CK levels suppress xylem cell fate (Cui *et al.*, 2011).

Yet another mechanism of auxin-CK crosstalk is present in the *Arabidopsis* root meristem. On the one hand, type-B ARR_s (ARR1 and ARR12), which are the end points of CK signaling as mentioned above, inhibit the *Aux/IAA* gene *SHORT HYPOCOTYL 2* (*SHY2/IAA3*). On the other hand, *SHY2/IAA3* inhibits *PIN* gene expression but also feeds-back to repress CK biosynthetic genes. IAA has also been shown to repress back *SHY2/IAA* expression. These loops that feedback to hormone synthesis (Ruzicka *et al.*, 2009; Dello Ioio *et al.*, 2008b; Jones *et al.*, 2010), the time and space of action, as well as the involvement of other hormones, such as gibberellin and brassinosteroids (Depuydt and Hardtke, 2011), further increase the complexity of this crosstalk.

Jasmonates and strigolactones: new players in keeping it cambial?

Recently, jasmonates (JA) signaling pathway components revealed to be cambium regulators by triggering cell division. Moreover, cambium activity was positively affected by JA application (Sehr *et al.*, 2010). Strigolactones (SLs) are a group of hormones that also positively regulate cambial activity (Agusti *et al.*, 2011a). Plants with reduced SL signaling or biosynthesis show reduced cambium activity, and vice versa, treatment with a synthetic SL analog enhances cambial growth in the *Arabidopsis* inflorescence stem. Even though secondary growth is reduced in SL-deficient mutants, auxin concentration and signaling is enhanced in the vasculature of these plants. This suggests that in the SL biosynthesis mutant, the decrease in secondary growth is not due to reduced auxin levels, but instead to a direct role of SL signaling in the regulation of cambium activity independently or downstream

of auxin accumulation (Agusti *et al.*, 2011a). More recently, SL signaling was found to trigger PIN1 depletion from xylem parenchyma cells and inhibit bud outgrowth in the stems by counteracting the bud-activating auxin fluxes (Shinohara *et al.*, 2013), suggesting that molecular crosstalk between strigolactones and auxin could be taking place during secondary growth.

HD-Zip III family, KANADIs and hormonal interactions: keeping it cambial and triggering xylem cell fate

The onset of vascular tissue differentiation from cambial cells also involves the interaction between two other well characterized genes families, class III homeodomain-leucine-zipper transcription factors (HD-Zip III) and GARP transcription factors KANADI (KANs), as well as their effect on auxin flow. In *Arabidopsis*, the HD-Zip III protein family includes five members: PHABULOSA (PHB), INTERFASCICULAR FIBERLESS1/REVOLUTA (IFL/REV), PHAVOLUTA (PHV), CORONA (CNA/ATHB15) and ATHB8 (Baima *et al.*, 1995; McConnell *et al.*, 2001; Ohashi-Ito and Fukuda, 2003; Ohashi-Ito *et al.*, 2005; Otsuga *et al.*, 2001; Prigge *et al.*, 2005). The patterns of expression of *HD-Zip III*s have been intensively studied in *Arabidopsis*, in *Zinnia elegans* and, more recently, in *Populus*. In *Arabidopsis* and in *Zinnia*, *AtHB15/ZeHB13* is predominantly expressed in procambial cells and proposed to be a regulator of procambium formation (Ohashi-Ito and Fukuda, 2003). Kim *et al.* (2005) reported that repression of *AtHB15*, in the *Arabidopsis* inflorescence stems, actually accelerates xylem cell differentiation from the procambial cells. Therefore, *ATHB15* could have a role in maintenance of cambial cell identity. However, in *Populus*, the *AtHB15* ortholog *POPCORONA* was not found to be restricted to provascular cells or primary xylem, but was also found in secondary vascular tissues, namely ray xylem cells and secondary xylem tissue, which could imply that HD-Zip IIIs are involved in new roles in xylem formation in trees (Du *et al.*,

2011). The *AtHB8* domain of expression coincides with tracheary element precursors and *ATHB8/ZeHB10* gain-of-function plants have increased production of tracheary elements in the vascular bundles (Ohashi-Ito *et al.*, 2005; Baima *et al.*, 2001). In *Populus*, the *AtHB8* homolog *PttHB8* has increased expression that coincides with the auxin radial concentration gradient in the cambial cells of the stem, thus suggesting a conserved role in promoting xylem specification (Nilsson *et al.*, 2008). The *IFL/REV/ZeHB11/ZeHB12* was found expressed in procambium and xylem parenchyma cells, and increased *REV* expression led to increased production of xylem precursor cells, but not to increased differentiation into tracheary elements (Emery *et al.*, 2003; Ohashi-Ito *et al.*, 2005; Zhong and Ye, 2004). In *Populus*, the *REV* ortholog *POPREVOLUTA (PRE/PtaHB1)* was found localized in cambial cells and has been suggested to have a role in secondary growth, perhaps in the transition between primary to secondary growth, since transgenic poplar for *PRE* constitutive expression showed reverse polarity of xylem and phloem after abnormal cambial cells were produced from cortical parenchyma cells (Ko *et al.*, 2006; Robischon *et al.*, 2011). These observations mean that *REV* may function both in cambial maintenance and in xylem specification.

While *HD-Zip III*s are mainly expressed in procambial and xylem precursor cells and thought to promote xylem differentiation, *KAN*s are mainly expressed in phloem and seem to act antagonistically on vascular specification (Figure 4; Eshed *et al.*, 2001; Kerstetter *et al.*, 2001; Ilegems *et al.*, 2010). However, *KAN* loss-of-function *Arabidopsis* mutants develop phloem cells, indicating that these genes are not essential for phloem specification. *HD-Zip III*s and *KAN*s also control tissue polarity in the vascular bundles. In the *Arabidopsis* shoot, gain-of-function *HD-Zip III* mutations and lack of *KAN*s expression in the triple mutant *kan1 kan2 kan3* result in a shift from the typical collateral vascular bundle arrangement to an amphivasal one, wherein xylem surrounds the phloem. Vice versa, *HD-Zip*

III loss-of-function mutants result in an amphicribal arrangement, wherein phloem surrounds xylem (Emery *et al.*, 2003; McConnell and Barton, 1998; McConnell *et al.*, 2001; Zhong and Ye, 2004). The loss-of-function of all five *HD Zip III*, observed in the quintuple *phb phv rev cna athb8* mutant, and ectopic *KAN1* expression result in no vascular development at all. In fact, in the *phb phv rev cna athb8* mutant there is suppression of the differentiation of procambial cells into xylem cells and a subsequent increase in procambium cells proliferation (Carlsbecker *et al.*, 2010).

Expression of *KAN1*, driven by the *AtHB15* promoter, has a negative effect on expression and polar localization of PIN1 proteins resulting in the inhibition of procambium formation in early stages of *Arabidopsis* embryogenesis (Ilegems *et al.*, 2010). It is well known that *HD-Zip III* genes have an overlapping pattern of expression with the pattern of auxin distribution (Izhaki and Bowman, 2007), which likely indicates that auxin interplays with the *HD-Zip III* genes. In fact, the expression of *ATHB8*, *REV*, *PHV* and *CNA/ATHB15* is known to be induced by auxin, and auxin flux is modulated by *HD-Zip III* (Baima *et al.*, 1995; Zhou *et al.*, 2007; Izhaki and Bowman, 2007; Yoshida *et al.*, 2009; Ilegems *et al.*, 2010). This suggests that KAN proteins control cambial activity by negatively acting on auxin transport, whereas *HD-Zip III* promote xylem differentiation by having a role in the canalization of auxin flow (Ilegems *et al.*, 2010).

Mobile signals and HD-Zip IIIs: a matter of signal dosage?

HD-Zip III genes are suppressed by the expression of microRNA 165/166. miRNA 165/166 are known to target *HD-Zip III*, as mapped in the *HD-Zip III Arabidopsis* gain-of-function mutations (Emery *et al.*, 2003; Kim *et al.*, 2005; Zhong and Ye, 2004). A link to SHR and SCARECROW (SCR) proteins has been established showing that both are involved in root vascular patterning as transcriptional activators of miRNA 165/166 (Figure 4; Carlsbecker *et al.*,

2010). These authors described how crosstalk between the vascular cylinder and the surrounding endodermis is mediated by the cell-to-cell movement of SHR in one direction and miRNAs in the other. SHR, produced in the vascular cylinder, moves into the endodermis to activate SCR and together these transcription factors activate mir165/166. The miRNA 165/166, in turn, migrates from the cells where they are produced, in the endodermis of the *Arabidopsis* root, into the stele periphery where they act on *HD-Zip III PHB* levels (Carlsbecker *et al.*, 2010). Thus, this non cell autonomous, dose-dependent, action of miRNA165/166 modulates the PHB gradients in the stele, controlling xylem differentiation: where a high dosage of PHB specifies metaxylem, while a low dosage of PHB specifies protoxylem differentiation (Carlsbecker *et al.*, 2010; Miyashima *et al.*, 2011). Furthermore, SHR is thought to regulate miR165/166 through its effect on CK homeostasis, since high CK levels repress xylem specification in the *shr* mutant (Cui *et al.*, 2011). Another regulatory model for the interplay between PHB and miRNA165 that also involves CK has been proposed for the *Arabidopsis* root meristem, wherein PHB induces CK biosynthesis by activating the biosynthetic *ISOPENTENYL TRANSFERASE 7 (IPT7)* gene, thereby promoting cell differentiation, but CK feedback represses both *PHB* and miRNA165, thus negatively regulating both its activator and the activator repressor. This almost *non-sense* regulatory circuit is proposed to be a mechanism of balancing the division and differentiation of stem cells during root growth (Dello Ioio *et al.*, 2012). Miyashima *et al.* (2011) recently suggested that miRNA165/166 might act as morphogens, given that they are emitted from a local source, affecting the neighbouring tissues and their *HD-Zips III* targets dosage is read by each receiver cell for different cell fates. Morphogen-like action in plants may not follow the exact criteria as defined in animal systems, therefore it has been suggested that a *morphogenetic trigger is a factor or signal that induces, through unequal distribution of its activity, acquisition of a new developmental fate in a cell or a group of cells*

(Benková *et al.*, 2009; Dubrovsky *et al.*, 2008). IAA could, therefore, also be considered a morphogenetic trigger in several contexts, as its local maximum acts like an instructive signal for initiation of organ formation, such as lateral root initiation in *Arabidopsis* (Benková *et al.*, 2009; Dubrovsky *et al.*, 2008) or the developmental gradient of secondary xylem cell specification found in *Pinus* and *Populus* that coincides with the auxin concentration gradient (Uggla *et al.*, 1996; Tuominen *et al.*, 1997). *MP* (discussed above) is also a genetic switch triggered in response to auxin in a threshold-dependent manner (Lau *et al.*, 2011). *MP*, which likely regulates SAM (Zhao *et al.*, 2010), links auxin signaling and meristem function. Morphogenetic triggers or morphogen-like action is receiving increased attention, as RNAi-derived small RNAs and auxin embody the required mobile signals (Bhalerao and Bennet, 2003; Benková *et al.*, 2009; Skopelitis *et al.*, 2012). Vascular differentiation may involve yet more substances with morphogen type of action.

Brassinosteroids: are we closer to xylem identity?

Brassinosteroids (BRs) are also involved in *HD-Zip III* regulation. BR deficient *Arabidopsis* mutants produce extreme dwarf plants, with reduced amounts of xylem (Szekeres *et al.*, 1996; Choe *et al.*, 1999b). The BRs are synthesized in the procambial cells and are perceived by receptors in xylem precursor cells [LRR receptor kinases: BR INSENSITIVE 1 (BRI1)/BR RECEPTOR LIKE 1 and 3 (BRL1 and BRL3)], inactivating the negative regulator BR INSENSITIVE 2 (BIN2), thus allowing the un-phosphorylated forms bri1-EMS-SUPPRESSOR 1 (BES1) and BRASSINAZOLE RESISTANT 1 (BZR1) to promote xylem differentiation by increasing *HD-Zip III* genes expression (Figure 4; Caño-Delgado *et al.*, 2004; Ohashi-Ito and Fukuda, 2003; Ohashi-Ito *et al.*, 2002; Fukuda, 2004). Indeed, in *Zinnia* cell cultures, *AtHB8* and *REV* homologues are repressed by inhibitors of BR

biosynthesis, but restored by exogenous application of BR (Ohashi-Ito *et al.*, 2002). *Zinnia AtHB15* homolog expression is also induced by BR, which suggests that *HD-Zip III* genes function in vascular differentiation also in response to BR signaling (Ohashi-Ito and Fukuda, 2003). In addition, BR perception is promoted by HD-Zip III, as increases in *HD-Zip III* genes expression induce BRL3 and BRI1-associated receptor kinase-1 (BAK1) (Ohashi-Ito *et al.*, 2005).

Interestingly, BRs and auxin converge in a set of common target genes, suggesting coordinated signaling pathways. For instance, BIN2 kinase regulates the *AUXIN RESPONSE FACTOR 2 (ARF2)* that inhibits transcription of auxin responsive genes (Figure 4; Vert *et al.*, 2008), while auxin inhibits the binding of the transcriptional repressor BZR1 to the promoter of the BR biosynthesis gene *DWARF4 (DWF4)*, implicating auxin in BR biosynthesis (Chung *et al.*, 2011). The overexpression of *BR-RELATED ACYLTRANSFERASE1 (BAT1)*, a gene encoding a putative acyltransferase, renders a typical BR-deficient phenotype. Additionally, auxin also highly induces *BAT1*, suggesting that the conversion of brassinolide intermediates into acylated-BR conjugates is promoted by auxin (Choi *et al.*, 2012). Thus, auxin seems to be involved in the control of BRs homeostasis, while BRs repress the inhibition of auxin responsive genes transcription, acting synergistically in vascular development. Moreover, it has been suggested that polar auxin transport (PAT) is enhanced by BRs, possibly by modulation of *PIN* genes expression (Li *et al.*, 2005). The number of vascular bundles is also enhanced by BRs and BRs have been predicted to modulate the procambial cell number required to set the number of auxin maxima at the shoot vasculature, suggesting that PAT acts in coordination with BR signaling (Ibañez *et al.*, 2009). In sum, BRs and auxin overlap in their transcriptional control of common target genes, and both hormones exert effects on each other's signaling and perception. However, how these crosstalks are mechanistically integrated into xylem differentiation

is still largely unknown.

Xylogen: a mobile signal towards xylem

Xylogen, an extracellular arabinogalactan protein (AGP), is a mobile signal found in procambium and xylem cells that promotes xylem cell differentiation in the vascular tissues (Motosé *et al.*, 2004). It was first isolated from *Zinnia elegans* xylogenic culture medium and found to be secreted from differentiated xylem cells to promote differentiation of uncommitted cells into tracheary elements (Motosé *et al.*, 2004). Two *Arabidopsis* genes, *XYLOGEN PROTEIN 1* and 2 (*AtXYP1* and *AtXYP2*), and thirteen xylogen-type genes (*XYLP*) have been identified. *AtXYP2* is proposed to be the best candidate as the *Arabidopsis* counterpart to the *Zinnia* xylogen gene, *ZeXYP1*, responsible for the production of the xylogen peptide. *AtXYP1* and *AtXYP2* are both expressed in the vascular tissues and the *xyp1 xyp2* double mutant, disrupted in xylogen function, displays discontinuous xylem (Motosé *et al.*, 2004; Kobayashi *et al.*, 2011). However, the xylogen mode of action in xylem is not yet understood. It is possible that it is a coordinator molecule, secreted from differentiating vascular cells, that induces xylem differentiation in neighbouring uncommitted cells. As for hormonal interactions, it has been shown that the expression of *ZeXYP1* is induced by auxin, and that auxin and cytokinin induce the accumulation of xylogen, suggesting that both hormones act synergistically as positive regulators of xylogen, although by unknown mechanisms (Motosé *et al.*, 2004).

Ethylene has dual roles: keeping it cambial but also differentiating xylem

ETHYLENE RESPONSE 1 (*ETR1*) was the first hormone receptor to be identified, but other ethylene receptors have been identified since then

(Chang *et al.*, 1993; for detailed reviews on ethylene signaling see Alonso and Stepanova, 2004; Stepanova and Alonso, 2009). Downstream of the ethylene receptors is CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), a negative regulator of ethylene signaling, and downstream of CTR1 is the positive regulator ETHYLENE-INSENSITIVE2 (EIN2). EIN3, a transcription factor that mediates responses to ethylene, is downstream of EIN2 and is shunt to the proteasomal degradation pathway (Kieber *et al.*, 1993; Alonso *et al.*, 1999). Recent work in transgenic *Populus* trees with disrupted ethylene perception has demonstrated that ethylene has a stimulatory effect on cambial cell division, at least in trees that are mechanically stimulated (Love *et al.*, 2009). Previously, in the *Arabidopsis* root meristem, it had already been indirectly shown that the over accumulation of ethylene by the loss of ETHYLENE OVERPRODUCER1 (ETO1) function had a stimulatory effect on cell division at the core of the stem cell niche, the quiescent centre that during normal growth scarcely undergoes cell division (Ortega-Martinez *et al.*, 2007). However, Pesquet and Tuominen (2011) suggested that ethylene has a dual function in vascular development, one stimulating the rate of tracheary elements differentiation and another controlling stem cell pool size during secondary growth *in planta*.

Gibberellins: late players in xylem differentiation

The discovery of the gibberellin (GA) receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1) allowed further understanding of the molecular mechanisms involved in GA signaling (Ueguchi-Tanaka *et al.*, 2005). DELLA proteins are central repressors of the GA signaling pathway by acting immediately downstream of GID1 receptor. Binding of GA to GID1 causes binding of GID1-GA to DELLAs and leads to their degradation via the ubiquitin-proteasome pathway (for specific reviews on GA signaling

pathway see for instance Sun, 2011 and Schwechheimer, 2012). The effects of GAs in vascular differentiation suggest that GA is essential for xylem proliferation. Indeed, the overexpression of *GIBBERELLIN 20-OXIDASE1* (*GA20ox*), a GA biosynthetic gene, in *Populus* results in increased growth and xylem fiber length (Eriksson *et al.*, 2000). *GA20ox* mRNA and bioactive GAs have also been demonstrated to accumulate in the expansion zone of developing xylem (Israelsson *et al.*, 2005). Moreover, Mauriat *et al.* (2011) showed that decreased GA precursor levels and reduced bioactive GA levels result in reduced secondary growth. Altogether, these results suggest a role for GAs in xylogenesis.

Crosstalk between GA and auxin pathways has been demonstrated in *Populus* stems: Björklund *et al.* (2007) found that GA stimulates auxin transport, and exogenous application of GAs and auxin to decapitated trees had a positive synergistic effect on cambial growth. The application of gibberellin to decapitated *Populus* trees did not trigger xylogenesis, but instead disrupted the meristematic identity of the cambial cells, again showing that the auxin maxima in cambium cells is indispensable for cambial activity, whereas GA acts later in xylem formation. In this same study, GA treatment induced the expression of *PIN1*. This coincides with observations made by Willige *et al.* (2011) demonstrating that *Arabidopsis* GA biosynthesis and signaling deficient mutants have reduced polar auxin transport. The GA-deficient plants showed a reduced abundance of PIN1, PIN2 and PIN3, but all PIN protein levels recovered to wild-type levels after GA treatment. This suggests that for normal PIN protein accumulation GA promotes the degradation of DELLA proteins via the GID1 pathway (Willige *et al.*, 2011).

Applying gibberellic acid-3 (GA3) to *Zinnia* xylogenic cultures increased the differentiation of tracheary elements and their lignin content, whereas GA biosynthesis inhibitors decreased tracheary elements differentiation (Tokunaga *et al.*, 2006). Again, it seems that GA acts in

differentiation of developing xylem cells differentiation program rather than having a role on cambial activity. Ragni *et al.* (2011) recently proposed that GA is actually the mobile shoot-derived signal that triggers xylem expansion upon flowering initiation in *Arabidopsis*. This work however reports that auxin does not appear to be limiting for increased xylogenesis, evidencing discrepancies between *Arabidopsis* hypocotyls and inflorescence stems and *Populus* stems.

Xylem differentiation transcriptional network

The final steps of xylem development involve secondary cell wall formation and programmed cell death (PCD). An intricate regulatory network of transcriptional factors involved in differentiation of xylem cells has been identified (Kubo *et al.*, 2005). By analysis of transdifferentiating tracheary elements in *Arabidopsis* cell culture, Kubo *et al.* (2005) isolated several VASCULAR RELATED NAC DOMAIN (VND1-VND7) transcription factor genes. In particular, VND6 and VND7 were found to be key regulators of xylem cell specification, as ectopic expression of VND6 induced metaxylem cell type specification, whereas VND7 induced protoxylem differentiation (Kubo *et al.*, 2005; Yamaguchi *et al.*, 2010a). Investigation of hormonal control of such transcription factors is lacking. Nevertheless, the combined application of auxin, cytokinin and brassinosteroids to hypocotyls of wild-type and seedlings carrying transgenic promoter GUS fusions of VND6 and VND7 led to increased expression of VND6 and VND7, suggesting that these transcription factors act downstream of the hormone signaling pathways (Kubo *et al.*, 2005). Yoshida *et al.* (2009) also found that VND genes are overexpressed shortly after NAA application to differentiating *Zinnia* cell cultures. BRs also promote expression of VNDs and programmed cell death stages in *Populus* and *Arabidopsis* xylem tracheary elements by controlling other regulatory proteins, such as GTP-binding RabG3b protein (Kwon *et al.*,

2010; 2011). So far, no hormonal regulatory mechanism of this transcriptional network has been unveiled.

Several regulatory elements from another NAC family of transcription factors are involved in the terminal stages of xylem development. *NAC SECONDARY WALL THICKENING PROMOTING FACTOR1* (*NST1*), *NST2* and *SECONDARY WALL-ASSOCIATED NAC-DOMAIN 1* (*SND1/NST3*) are key regulators of secondary cell wall thickening, particularly in *Arabidopsis* fibers (Zhong *et al.*, 2006; 2007a; Mitsuda *et al.*, 2007). Examples of characterized transcription factors from *Arabidopsis*, poplar, pine and eucalyptus suggest that the NAC-mediated transcriptional regulation of secondary wall biosynthesis is a conserved mechanism throughout vascular plants (Zhong *et al.*, 2010a). A number of MYB transcription factors function downstream of *SND1* to upregulate the synthesis of secondary cell wall components, such as cellulose and lignin (Zhong *et al.*, 2007b, McCarthy *et al.*, 2009). Hussey *et al.* (2011) also observed that *Arabidopsis SND2* regulates genes involved in secondary cell wall development in *Arabidopsis* fibers, while overexpression of *AtSND2* in *Eucalyptus* increases fiber cell area.

A recent work by Yamaguchi *et al.* (2011) has dissected the possible direct targets of VND transcriptional action in xylem vessel differentiation. These authors showed that VND7 directly regulates both secondary cell wall thickening and programmed cell death by revealing that recombinant VND7 protein binds to the promoter sequences of downstream genes involved in both xylem developmental processes. The direct targets of VND7 include genes encoding cellulose synthase subunits *CesA4/IRX5* and *CesA8/IRX1*, but also encoding to their MYB regulators *MYB46*, *MYB83* and *MYB103*, as well as *XYLEM CYSTEINE PROTEASE1* (*XCP1*) and *XCP2* genes (Figure 4; Zhong *et al.*, 2007b, 2008; McCarthy *et al.*, 2009; Yamaguchi *et al.* 2011). The *XCP1* and *XCP2* function could be traced by analysis of *xcp1* and *xcp2* mutations in *Arabidopsis* roots showing delayed autolysis during xylogenesis

(Avci *et al.*, 2008).

The transcriptional network in control of secondary cell wall deposition and programmed cell death includes members of the LOB DOMAIN PROTEIN/ASYMMETRIC LEAVES LIKE (LBD/ASL) protein family. For example, the overexpression of *LBD30/ASL19* and *ASL20* genes induces transdifferentiation of *Arabidopsis* cells from nonvascular tissues into tracheary element-like cells, similar to those induced upon *VND6/7* overexpression (Soyano *et al.*, 2008). Moreover, *VND7* transcription factor has been shown to directly target *LBD30/ASL19* and *LBD15/ASL11* genes (Yamaguchi *et al.*, 2011), whereas the LBD proteins *ASL20/LBD18* and *ASL19/LBD30* are part of a positive feedback loop that amplifies the expression of *VND6* and *VND7* (Soyano *et al.*, 2008). These observations reveal an intricate transcriptional regulatory network, but also indicate that most of the regulatory factors involved in secondary cell wall deposition are also implicated in programmed cell death during development.

Two negative regulators of xylem formation have been described in *Arabidopsis*: *VND-INTERACTING2* (*VNI2*) that acts to suppress the *VND7* capacity to activate transcription; and a gene encoding *XYLEM NAC DOMAIN1* (*XND1*), whose overexpression causes the complete suppression of vessel secondary wall biosynthesis and programmed cell death, suggesting that it negatively regulates xylem vessel differentiation (Figure 4; Yamaguchi *et al.*, 2010b; Zhao *et al.*, 2008). Curiously, both *VNI2* and *XND1* are suggested to be targeted for proteasomal degradation by 20S proteasome (20SP). The 20SP is thought to be part of the ubiquitin/26SP proteolytic system, and it possesses caspase-3-like activity, characteristic of animal cell apoptosis. 20SP may degrade *VNI2* and *XND1* to induce tracheary elements differentiation in *Arabidopsis* and *Populus* (Han *et al.*, 2012). Yet another signal, thermospermine, acts as a negative regulator of xylem differentiation, as discussed below.

Polyamines: small polycations with major roles

Polyamines are low molecular weight, aliphatic polycations, having two or more primary amine groups and are present in almost all living organisms. Polyamines are involved in a variety of fundamental biological processes, including RNA modification, protein synthesis and the modulation of enzyme activities (Tabor and Tabor, 1999). The most abundant polyamines in flowering plants are the diamine putrescine, the triamine spermidine and the tetramines spermine and thermospermine (Figure 5), each with specific biological functions (reviewed in Takahashi and Kakehi, 2010). The following sections will briefly review the current knowledge on polyamine biosynthesis and catabolism; a special emphasis will be given to the current knowledge on polyamines role in plant vascular development.

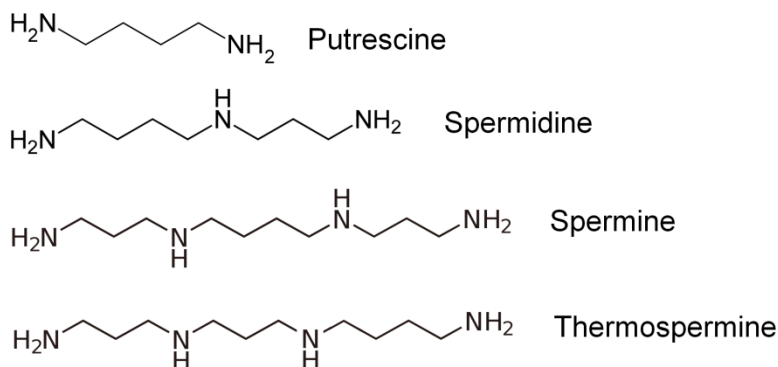


Figure 5. Chemical structure of the main polyamines found in plants.

Polyamines biosynthesis and catabolism: a tight control of polyamine homeostasis

The polyamine biosynthesis pathway initiates with the biosynthesis of putrescine, either by the decarboxylation of L-ornithine by ornithine

decarboxylase (ODC) or from L-arginine in a series of steps catalysed by arginine decarboxylase (ADC), agmatine iminohydrolase (AIH) and *N*-carbamoylputrescine amidohydrolase (CPA) (Figure 6). The ADC pathway seems specific to bacteria, archaea, and plants, contrary to animal and fungi, in which ODC is the first and rate-limiting enzyme in the synthesis of

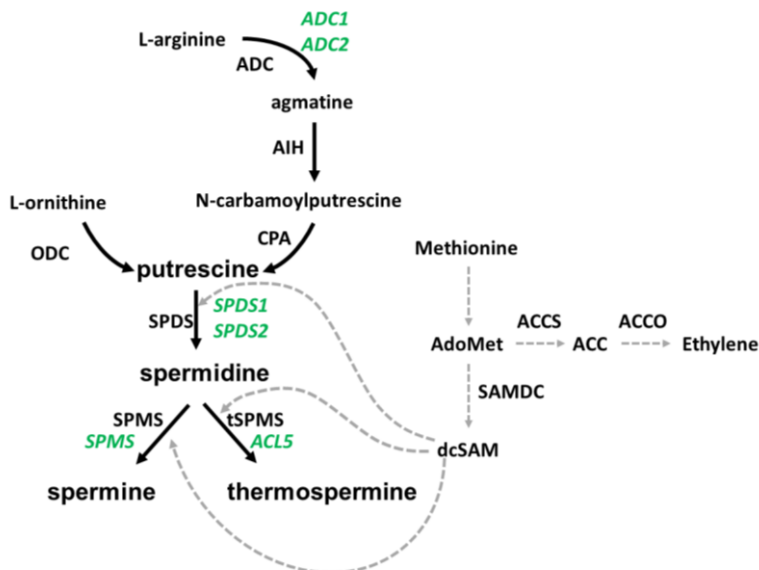


Figure 6. Biosynthesis pathway of polyamines. Depicted in green are the genes identified in *Arabidopsis thaliana* to be involved in polyamine biosynthesis. Depicted in black are the enzymes responsible for the pathway steps. The cross-point to ethylene biosynthesis is indicated, since both pathways share SAM as common substrate. ADC: arginine decarboxylase; AIH: agmatine iminohydrolase; CPA: *N*-carbamoylputrescine amidohydrolase; ODC: ornithine decarboxylase; SPDS: spermidine synthase; SPMS: spermine synthase; tSPMS: thermospermine synthase; dcSAM: decarboxylated S-adenosylmethionine; SAMDC: SAM decarboxylase; ACCS: 1-amino-cyclopropane-1-carboxylic-acid synthase; ACCO: ACC oxidase.

polyamines (Burrell *et al.*, 2010; Takahashi and Kakehi, 2010). Sequences encoding for ODC are absent from the *Arabidopsis thaliana* genome, though

some ODC activity has been detected in this species, suggesting that another gene is responsible for ODC activity (Hanfrey *et al.*, 2001; Tassoni *et al.*, 2003). Putrescine is subsequently converted to the triamine spermidine by spermidine synthase (SPDS), an aminopropyltransferase that transfers an aminopropyl group from the decarboxylated S-adenosylmethionine (dcSAM) donor to the diamine putrescine. Similarly, spermidine is converted to spermine by the activity of spermine synthase (SPMS) or to thermospermine by the activity of thermospermine synthase (tSPMS). The aminopropyl moieties used by SPDS and SPMS are derived from SAM, which also serves as a common substrate for ethylene biosynthesis (Figure 6). In *Arabidopsis*, several genes encoding the enzymes in polyamines biosynthetic pathway have been described. Two genes have been found to encode for ADC activity (*ADC1* and *ADC2*; Galloway *et al.*, 1998), one that encodes AIH (*AIH*; Janowitz *et al.*, 2003) and one that encodes CPA (*NLP1*; Piotrowski *et al.*, 2003). Two genes encode for SPDS (*SPDS1* and *SPDS2*; Hashimoto *et al.*, 1998), one for SPMS (*SPMS*; Panicot *et al.*, 2002) and one for tSPMS (*ACL5*; Knott *et al.*, 2007).

Polyamines can be metabolized by oxidation and conjugation with other molecules. The de-amination of polyamines is catalysed by amine oxidases. Amine oxidases include the copper-containing amine oxidases (CuAO) and the flavin-containing polyamine oxidases (PAO) (Cona *et al.*, 2006, and references therein). Plant CuAOs generally oxidise the diamine putrescine (by diamine oxidase, DAO) and cadaverine, producing aminoaldehydes (4-aminobutanal) and hydrogen peroxide (Figure 7). Plant PAOs catalyse the oxidation of spermine and spermidine, and/or their acetylated derivatives at the secondary amino groups, producing N-(3-aminopropyl)-4-aminobutanal and 4-aminobutanal, respectively, in addition to 1,3-diaminopropane and hydrogen peroxide. This is considered the terminal catabolism (degradation) of polyamines pathway and it was the only polyamine catabolic route attributed to plants until recently. Indeed in 2006,

PAOs from *Arabidopsis* have been shown to oxidise spermine and spermidine in a similar mode as in animals and yeasts (Tavladoraki *et al.*, 2006). Later back-conversion of spermine and thermospermine and its acetylated forms to spermidine, and spermidine to putrescine were suggested to take place in plants (Takahashi *et al.*, 2010; Fincato *et al.*, 2011). These discoveries changed the established idea that plant and animals have distinct catabolic pathways and showed plants also share a polyamine back-conversion pathway. In addition to their free forms, conjugation of polyamines with small molecules, such as amides and hydroxycinnamic acids occurs in plants (Alcázar *et al.*, 2010; and references therein). Thus, it is clear that several mechanisms contribute to polyamine homeostasis, at the same time as polyamines and their catabolic products (e.g. hydrogen peroxide) play important roles during some developmental processes, such as stress responses and plant vascular development.

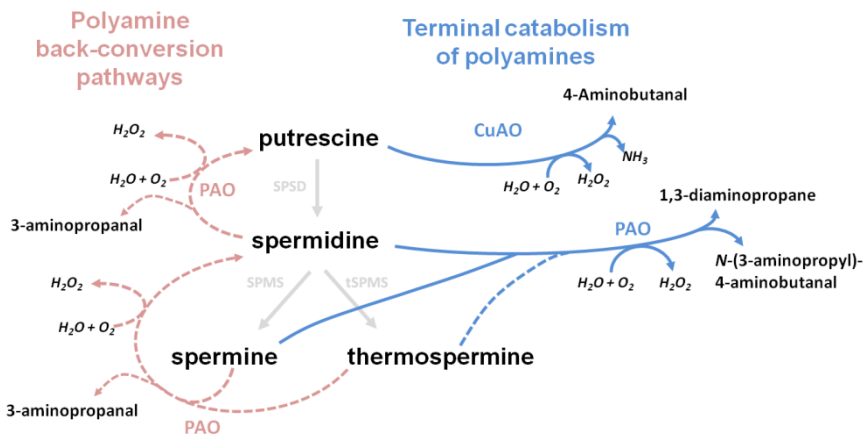


Figure 7. Polyamines catabolism pathway. The polyamine terminal degradation pathway is highlighted in blue and the back-conversion pathway in pink (after Cona *et al.*, 2006; Fincato *et al.*, 2011).

Polyamines in plant vascular development

Polyamines have been implicated in several aspects of plant growth and development, such as embryogenesis, fruit development, senescence, and responses to stress (Kusano *et al.*, 2008; Alcázar *et al.*, 2010; Handa and Mattoo, 2010 and references therein). We focus here on the current knowledge concerning polyamines involvement in plant vascular development. Some clues that polyamines were implicated in the formation of vasculature originated from the observation of stem growth and elongation defects reported after manipulation of polyamine metabolism. A link to vascular development was demonstrated when one polyamine biosynthesis mutant, *acaulis5* (*acl5*), producing dwarf plants, impaired in stem elongation, was shown to develop vascular bundles surrounded by a great amount of differentiated cells with thickened walls (Hanzawa *et al.*, 1997; 2000; Clay and Nelson, 2005). At that time, this phenotype was proposed to be attributed to the disruption of *spermine synthase* encoding gene (Hanzawa *et al.*, 1997; 2000; Clay and Nelson, 2005; Muñiz *et al.*, 2008; Kakehi *et al.*, 2008). However, the identification of another spermine synthase encoding gene (*SPMS*) for which the matching knockout mutant did not show any stem elongation defects, raised the doubt to whether *ACL5* encoded spermine synthase (Panicot *et al.*, 2002; Imai *et al.*, 2004). Knott *et al.* (2007) last solved the question, showing that *ACL5* in fact catalyses the formation of thermospermine and not spermine. The problem resided in that both were indistinguishable by the usual high performance liquid chromatography (HPLC) methods employed for separating polyamines, and were later separated by gas chromatography-mass spectrometry (GC-MS) (Knott *et al.*, 2007; Rambla *et al.*, 2010; Naka *et al.*, 2010). Finally, it was also shown that contrary to spermine, thermospermine is required for stem elongation and *acl5* mutants partially restore the wild-type phenotype when exogenously supplied with thermospermine (Kakehi *et al.*, 2008; 2010).

Thermospermine, preventing a troubled premature death

Thermospermine has a role in xylem development: *ACL5* is found specifically expressed in procambial and xylem vessels of *Arabidopsis*; and the *acl5* mutant shows a complete lack of xylem fibers, overproliferation of immature vessels and disrupted secondary growth (Muñiz *et al.*, 2008). This suggests that the timing of xylem differentiation in *acl5* mutants is inappropriate, leading to stunted plants, where xylem differentiation proceeds too fast. Therefore, thermospermine is thought to prevent the premature cell death of the xylem elements (Muñiz *et al.*, 2008). The precise mechanism by which this happens is still unknown, but some regulatory factors have been described. The disruption of a basic-helix-loop-helix (bHLH) transcription factor, named SUPPRESSOR OF ACAULIS5 1 (*SAC51*) restores the wild-type phenotype in the absence of thermospermine (Imai *et al.*, 2006). The *SAC51* mRNA contains five upstream open reading frames (uORFs) and the *sac51-d* allele has a mutation in one of these uORFs, creating a stop codon leading to the production of a truncated *SAC51* polypeptide (Imai *et al.*, 2006). In the *acl5* mutant, the translation of the *SAC51* main ORF is suppressed. In the double mutant *sac51-d acl5-1* that shows restored stem elongation, the inhibition of the *SAC51* translation is suppressed, and a functional *SAC51* is overproduced. This suggests that the *ACL5* or thermospermine activates the translation of *SAC51* by inhibiting this uORF and preventing it from negatively regulating *SAC51* main ORF translation (Imai *et al.*, 2006). In this way, the effect of thermospermine would be to bypass the inhibition of the *SAC51* uORF (Yoshimoto *et al.*, 2012; Takano *et al.*, 2012). Furthermore, *VND7* is thought to induce *SAC51*. Therefore, a more complex network could be functioning to balance the repression and induction of xylem differentiation, in a timely manner, involving the action of NAC domain transcription factors (Zhong *et al.*, 2010b; Bollhöner *et al.*, 2012).

Thermospermine is increasingly viewed as a novel plant growth regulator (Takechi *et al.*, 2010; Yoshimoto *et al.*, 2012; Takano *et al.*, 2012; Chapter II). Although it was first suggested that the defects in *acl5* mutant result from deficient auxin transport (Clay and Nelson, 2005) some evidences show that IAA concentrations are actually increased in *acl5* seedlings (Vera-Sirera *et al.*, 2010). Similarly, the increased expression of the IAA marker line *DR5::GUS* in the hypocotyls of *acl5* seedlings suggests that thermospermine interacts with auxin in differentiating xylem (Vera-Sirera *et al.*, 2010). The relationship between auxin and thermospermine was elucidated by Yoshimoto *et al.* (2012), showing that thermospermine is required to suppress the auxin-inducible xylem differentiation. 2,4-dichlorophenoxy acid (2,4-D) and other auxin synthetic analogs were shown to induce excessive xylem vessels differentiation in cotyledons of *acl5* mutant, but not in wild-type, suggesting a rather high threshold for auxin inducible xylem formation. Furthermore, in the double mutant *sac51-d acl5-1* the application of 2,4-D did not induce xylem differentiation (Yoshimoto *et al.*, 2012). This means that while auxin exerts a positive effect on xylem differentiation, thermospermine acts as a limiting factor to differentiation and the auxin effect on xylem differentiation may be mediated through SAC51 (Yoshimoto *et al.*, 2012). Interestingly, we have found *ACL5* to negatively affect endogenous auxin levels while endogenous auxin positively affects *ACL5* expression, in a mechanism that maintains steady-state levels of thermospermine in *Populus* xylem tissues (Chapter II). These results are in line with recent findings by Cui *et al.* (2010) showing that the expression of auxin inducible genes is reduced in the *bushy and dwarf 2 (bud2)* mutant, which is disrupted in S-adenosylmethionine decarboxylase 4 (SAMDC4) enzyme function (Ge *et al.*, 2006). SAMDC is an example of a cross-point between polyamine biosynthesis and ethylene biosynthetic pathway, in the sense that both pathways need S-adenosylmethionine (SAM) as substrate (Figure 7). Curiously, the *bud2* mutant phenotype, which has decreased

thermospermine synthesis ability, closely resembles the *acl5* mutant, and displays enlarged vascular tissues and high lignin content (Ge *et al.*, 2006). The *BUD2* gene is also induced by auxin (Cui *et al.*, 2010), suggesting that polyamine levels or encoding transcripts might play a role in regulating auxin levels or responses to auxin.

Polyamines, ethylene and NO signals for death

Other polyamine catabolism and biosynthesis products have been shown to influence xylem differentiation. Tisi *et al.* (2011) demonstrated that after spermidine supply and PAO overexpression, the H₂O₂ derived from polyamine catabolism behaves as a signal for secondary wall deposition and for induction of developmental programmed cell death. Waduwara-Jayabahu *et al.* (2012) also observed that the recycling of 5'-methylthioadenosine (MTA), a by-product of polyamine and ethylene biosynthesis, is essential to maintain normal vascular development. Since thermospermine and other polyamines share common substrates with the biosynthetic pathway of ethylene, it will be interesting to know if there is cross talk between them in xylem development. Some hints are emerging. For instance, polyamines were shown to modulate genes that are involved in ethylene biosynthesis and signaling pathways during olive mature fruit abscission (Parra-Lobato and Gomez-Jimenez, 2011). This work describes that polyamines play a positive role in NO production and discovered an inverse correlation between nitric oxide (NO) and ethylene presence in the abscission tissue. Also, the presence of NO in the xylem, proximal to the abscission zone of olive fruit, is indicative of the involvement of NO in xylem cell wall lignification and differentiation (Parra-Lobato and Gomez-Jimenez, 2011). In other contexts, NO could be a linking signal molecule between polyamines, ethylene and xylem cell death. Gabaldón *et al.* (2005) have shown that NO production is assigned to the vascular tissues of *Zinnia elegans* and that the spatial NO

gradient was inversely related to the degree of xylem differentiation. The authors observed a NO burst associated to the single cell layer of pro-differentiating thin-walled xylem cells. The scavenging of NO inhibited the tracheary element differentiation but increased cell viability suggesting that NO production is sustained during secondary cell wall synthesis and cell autolysis (Gabaldón *et al.*, 2005). The crosstalk among these signals and signaling pathways will be crucial to understand the trigger mechanism of cell death in xylem differentiation.

In summary, hormone signaling pathways have been identified in the last 15 years and increasing knowledge on the crosstalks that involve hormone signaling was gained. The developmental context of these cross-talks is also being elucidated. Many mechanisms of cambial state maintenance have been proposed, and new players, like thermospermine, are being brought to an already complex scenario. Figure 8 integrates the place of action of hormones during xylem differentiation cellular events in *Populus*.

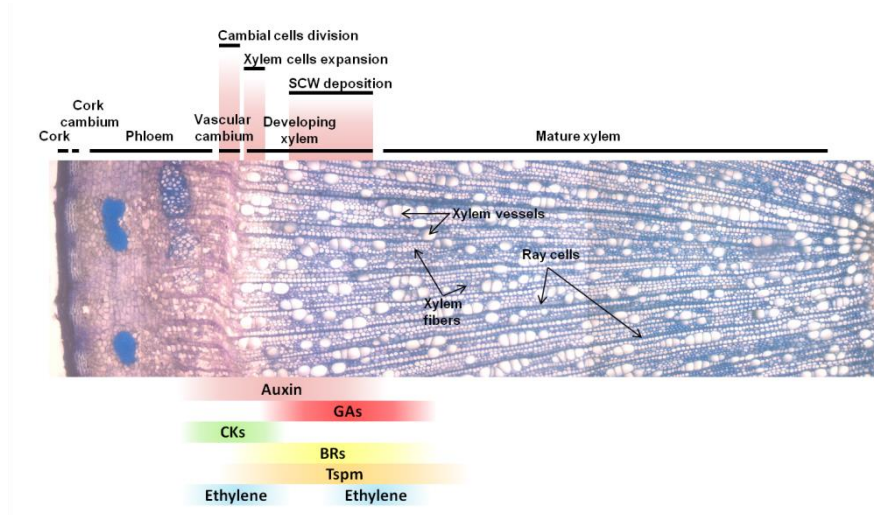


Figure 8. Cross section of hybrid-aspen (*Populus tremula* × *Populus tremuloides*) stem, showing the stages of xylem differentiation and the spatial context of hormones action. The vascular cambium, a secondary lateral meristem in tree stems, produces secondary phloem towards the outside and secondary xylem (wood) towards the inside. In the middle of the ►

◀vascular cambium reside the stem cells that are the meristematic cells from which the phloem and xylem precursor cells originate. Since the exact location of the stem cells is not known, this region is also commonly named cambial zone. Xylem development initiates with active cambial cell division, followed by rapid cell expansion and deposition of secondary cell wall and programmed cell death. The xylem cell types in *Populus* wood (ray parenchymatic living cells, xylem vessel elements and xylem fibers) are indicated. The known or possible function domains of auxin, gibberellin (GAs), cytokinin (CK), brassinosteroids (BRs), thermospermine (Tspm) and ethylene across the *Populus* cambial zone are shown. Auxin (Tuominen *et al.*, 1997) and GA (Israelsson *et al.*, 2005) labels reflect the concentration of the bioactive hormones and expression pattern of auxin and GAs signaling genes (Moyle *et al.*, 2002; Israelsson *et al.*, 2005). CK label depicts the peak of expression of cytokinin signaling genes in the phloem side of the cambial zone (Nieminen *et al.*, 2008). Ethylene label depicts its stimulatory effect on cambial cell division (Love *et al.*, 2009; Pesquet and Tuominen 2011). Tspm label depicts place of action on developing xylem, delaying xylem cell death (Muñiz *et al.*, 2008). BRs are currently not studied in *Populus*, thus the BR label depicts current knowledge from *Arabidopsis* and *Zinnia* showing that BRs are produced in procambial cells, perceived in the xylem precursor cells to induce xylem differentiation (see main text). The cork cell layers cover the surface of the stem, which are produced by the activity of the cork cambium, the other secondary lateral meristem in tree stems.

Research objectives and thesis layout

The present work aims at contributing to the understanding of wood formation, one of the most important and ultimately the defining biological process happening in a tree. Forestry production for wood and bioenergy products is increasingly attractive. Therefore, it is essential to deepen our knowledge of the molecular mechanisms governing wood formation.

Strong evidences point to the fact that thermospermine plays a pivotal role in xylem development in *Arabidopsis*. Yet, how this polyamine affects xylem development in trees, where extensive secondary growth occurs, has not been addressed before. Moreover, how the regulation of thermospermine levels is accomplished and its interaction with other

signaling pathways in the cell is largely unknown. To help narrow this gap, we proposed to investigate the effects of altering thermospermine metabolism in *Populus* trees to provide some clues to these fundamental questions. Specifically, this work aims at:

1. Understanding how thermospermine affects xylem formation in woody stems of *Populus* by altering the thermospermine metabolism through genetic modification of *Populus* to overproduce thermospermine.
2. Providing a broad view of other hormone crosstalk to thermospermine using a transcriptomic approach to screen for the main genes whose expression is affected by thermospermine metabolic de-regulation, especially those related to hormones and to xylem differentiation.
3. Contributing to the understanding of the regulatory mechanisms of thermospermine homeostasis in the *Populus* xylem, by studying how the manipulation of the transcript levels of putative upstream regulators affects the dynamics of thermospermine production.

This work followed the outline described in Figure 9, the methods summarized in Table I and it is presented in Chapters II to IV in the form of articles. Final conclusions from this work and future perspectives on the theme are discussed in Chapter V.

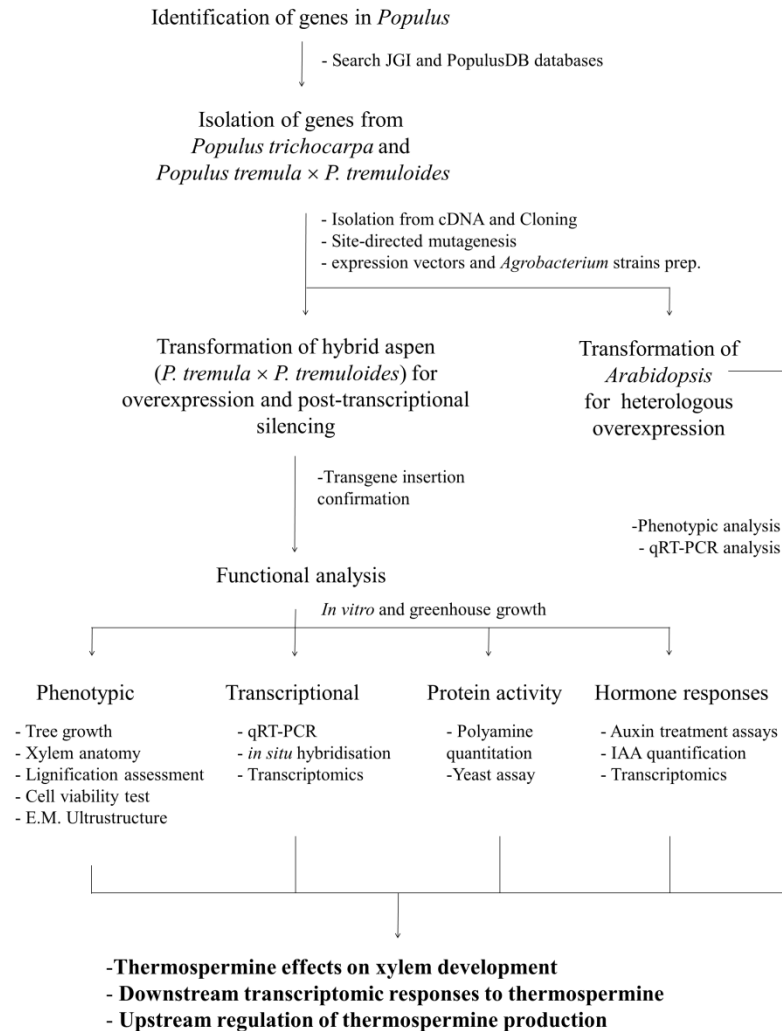


Figure 9. General organization of the research and thesis, highlighting the main techniques used during thesis studies.

Table I. Methods used in this work.

Method	Chapter
Auxin response assays	II
Electron microscopy for xylem analysis	(II)
Gene identification by database search	II, III, IV
Genetic transformation of <i>Arabidopsis</i> using floral dip method	IV
Genetic transformation of <i>Populus</i>	II, IV
Histological staining for lignin analysis (Phloroglucinol-HCl)	II
Histological staining for cell viability (NBT)	(II)
Histological general staining (Toluidine blue-O, Hematoxylin-Eosin)	I, II, III, IV
Histological GUS staining	(II)
IAA quantification by GC-MS	(II)
<i>In situ</i> RNA hybridisation	IV
Light and fluorescence microscopy	I, II, III, IV
Microarray analysis	III
Phylogenetic analysis	II, IV
Plasmid construction	II, IV
Polyamine extraction, purification, derivatization and quantification by GC-MS	II, IV
Polymerase chain reaction (PCR) analysis	II, IV
Promoter <i>in silico</i> analysis in databases	IV
Quantitative real-time PCR analysis	II, IV
Sectioning of plastic embedded samples	II, III, IV
Site-directed mutagenesis	II, IV
Tree growth measurements for growth analysis	II, IV
Xylem chemical maceration for cell characterization	II
Yeast assays for protein activity assessment	(II)

Methods performed by collaborators are indicated in brackets.

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CHAPTER II

THERMOSPERMINE HOMEOSTASIS IN *POPULUS* XYLEM[†]

[†] **Milhinhos A., Prestele J., Bollhöner B., Matos A., Vera-Sirera F., Rambla J.L., Ljung K., Carbonell J., Blázquez M.A., Tuominen H. and Miguel C.M.** Thermospermine levels are controlled by an auxin-dependent feedback-loop mechanism in *Populus* xylem. (*revised manuscript submitted*).

Thermospermine levels are controlled by an auxin-dependent feedback-loop mechanism in *Populus* xylem

Summary

Polyamines are small polycationic amines widespread in living organisms. Thermospermine, synthesized by thermospermine synthase *ACAULIS5* (*ACL5*), was recently shown to be an endogenous plant polyamine. Thermospermine is critical for proper vascular development and xylem cell specification but it is not known how thermospermine homeostasis is controlled in the xylem. We present data in the *Populus* model system supporting presence of a negative feedback control of thermospermine levels in stem xylem tissues, the main site of thermospermine biosynthesis. While overexpression of the *ACL5* homolog in *Populus*, *POPACAULIS5*, resulted in strong upregulation of *ACL5* expression and thermospermine accumulation in leaves, the corresponding levels in the secondary xylem tissues of the stem were similar or lower than those in the wild-type. *POPACAULIS5* overexpression had a negative effect on accumulation of indole-3-acetic acid (IAA), while exogenous auxin had a positive effect on *POPACAULIS5* expression, thus promoting thermospermine accumulation. Further, overexpression of *POPACAULIS5* negatively affected Class III homeodomain-leucine zipper (HD-Zip III) transcription factor *PttHB8*, a homolog of *AtHB8*, while upregulation of *PttHB8* positively affected *POPACAULIS5* expression. These results support that excessive accumulation of thermospermine is prevented by a negative feedback control of *POPACAULIS5* transcript levels through suppression of IAA levels, and that *PttHB8* is involved in the control of *POPACAULIS5* expression. We propose that this negative feedback loop functions to maintain steady state levels of thermospermine, required for proper xylem development, and that it is dependent on the presence of high concentrations of endogenous IAA, such as those present in the secondary xylem tissues.

Keywords

POPACAULIS5, *ACAULIS5* (*ACL5*), Class III homeodomain-leucine zipper transcription factors (HD-Zip III), wood development, polyamine, *Populus tremula* × *Populus tremuloides*, *Populus trichocarpa*.

Introduction

Polyamines are essential organic polycationic amines implicated in several processes in plants such as biotic and abiotic stress responses (Yamaguchi *et al.*, 2006; Kusano *et al.*, 2007; Naka *et al.*, 2010; Alcázar *et al.*, 2006; Wang *et al.*, 2011; Gonzalez *et al.*, 2011; Sagor *et al.*, 2012) wound-responses (Perez-Amador *et al.*, 2002), nitric oxide signaling (Flores *et al.*, 2008), fruit development (Nambeesan *et al.*, 2010; Trénor *et al.*, 2010) and stem growth and elongation (Hanzawa *et al.*, 2000; Alcázar *et al.*, 2005). The most common polyamines are the diamine putrescine, the triamine spermidine and the tetramines spermine and thermospermine. Putrescine is produced from ornithine by ornithine decarboxylase or from arginine by arginine decarboxylase. Spermidine and spermine production is catalysed by aminopropyltransferases, which transfer an aminopropyl residue from the decarboxylated S-adenosylmethionine to an amine acceptor on putrescine or spermidine to produce, respectively, the triamines and the tetraamines. Thermospermine is a structural isomer of spermine that only recently was identified in plants (Knott *et al.*, 2007; Rambla *et al.*, 2010; Naka *et al.*, 2010). It is synthesized by thermospermine synthase ACAULIS5 (ACL5) (Knott *et al.*, 2007) that is expressed specifically in xylem vessel elements (Muñiz *et al.*, 2008). Disruption in the function of ACL5 in *Arabidopsis* leads to plants with impaired stem elongation, thinner veins in leaves as well as lack of secondary growth (Hanzawa *et al.*, 1997; 2000; Clay and Nelson, 2005; Muñiz *et al.*, 2008; Takechi *et al.*, 2008).

The downstream events of ACL5 expression have been subject of intensive study in recent years. At least two extragenic suppressors of the *acl5* mutation have been described. One of them disrupts an upstream open reading frame and enhances the translation of a basic helix-loop-helix (bHLH) transcription factor encoded by *SUPPRESSOR OF ACAULIS51* (SAC51); and the second one (*sac52-1d*) affects RPL10a, an important

component of the large ribosomal subunit (Imai *et al.*, 2006; 2008). However, the upstream events that regulate *ACL5* expression are largely unknown. Class III homeodomain-leucine zipper transcription factors (HD-Zip III TFs) have been hypothesized to have a role in transcriptional control of *ACL5*, since both the HD-Zip III TFs as well as *ACL5* have been implicated in the control of metaxylem development (Muñiz *et al.*, 2008; Carlsbecker *et al.*, 2010). Especially *AtHB8* is a good candidate for control of *ACL5* since it is the HD-Zip III family member that shows highest transcriptional alterations in the *acl5* mutant background (Imai *et al.*, 2006). Another important factor is auxin that is well known for its role in xylem development (Uggla *et al.*, 1996; Tuominen *et al.*, 1997; Nilsson *et al.*, 2008) as well as in transcriptional activation of both *ACL5* (Hanzawa *et al.*, 2000; Imai *et al.*, 2006; Rambla *et al.*, 2010) and *AtHB8* (Baima *et al.*, 1995). A model for thermospermine regulation of xylem differentiation involving auxin has been proposed, where it is suggested that HD-Zip III TFs mark the procambial cells that are destined to xylem specification in an auxin-dependent manner, leading to up-regulation of *ACL5* and concomitant differentiation of xylem vessel elements (Vera-Sirera *et al.*, 2010; Takano *et al.*, 2012). However, the role of the HD-Zip III TFs in the control of *ACL5* expression has never been studied in detail.

In the current work, we wanted to elucidate the relationship between *ACL5*, auxin and HD-Zip III TFs. *Populus* trees were selected as the model system due to extensive development of xylem that is the site of thermospermine production as well as auxin transport in plants. Tree stems therefore allow isolation of large amounts of tissues that are enriched in xylem elements transporting auxin and expressing *ACL5*. *ACL5* ortholog, *POPACAULIS5*, was cloned from hybrid aspen (*Populus tremula* × *Populus tremuloides*) and black cottonwood (*Populus trichocarpa*) and thermospermine levels were altered in trees by manipulating the expression levels of *POPACAULIS5*. Also, *PttHB8*, a hybrid aspen homolog of *AtHB8*,

was overexpressed in hybrid aspen to investigate the effect on *POPACAULIS5* transcript levels. The results on expression levels of *POPACAULIS5* and *PttHB8*, thermospermine accumulation and IAA measurements in the transgenic trees support a novel regulatory mechanism, mediated through auxin and *PttHB8*, in maintenance of thermospermine homeostasis in secondary xylem tissues of the stem.

Experimental procedures

Plant material, growth conditions and sampling

Hybrid aspen (*Populus tremula* L $\times$ *P. tremuloides* MICHX.; clone T89) was subcultured on MS basal salt medium at half-strength (Murashige and Skoog, 1962), termed auxin-depleted medium. *Populus trichocarpa* Nisqually-1 clone was maintained in the greenhouse. Plants were grown in growth chambers at 21°C and 16 h light/8 h dark photoperiod. Transgenic and wild-type plants were transferred to soil and trees grown for 2 months in the greenhouse at 21°C and 18 h light/6 h dark photoperiod. The greenhouse growth experiment was repeated twice.

Sampled tissues were directly frozen in liquid nitrogen when collected and stored at -80°C. Leaves, first internode (apical stem) and internode closest to the base (basal stem) of plants grown on auxin-containing medium were collected and pooled in groups of ten from each line for gene expression analysis. Leaves, stem between the third and the seventh internode from the top (young stem), stem between the seventh and the basal internode (older stem) and root apices from *in vitro* grown material (on auxin-depleted medium) were ground to powder and portioned for gene expression, polyamine and IAA quantification analyses. For greenhouse-grown trees, the five youngest fully-expanded leaves were collected.

Secondary xylem tissues were obtained between stem internodes 40 and 45 (from the top) by peeling off the bark and scraping from the surface of the frozen woody core until emergence of fully mature wood. Since no fully mature wood was present in lines B4 and B13, the whole xylem part of the stem was used. The tissues were ground to powder and portioned for gene expression, polyamine and IAA quantifications.

Sequence analysis

To identify *P. trichocarpa* and *P. tremula* × *P. tremuloides* putative ACL5 coding regions, *POPACAULIS5* and *PttACL5*, respectively, we carried BLAST/browse searches in different databases [JGI *Populus trichocarpa* v.1.1 (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html; Tuskan *et al.*, 2006), Phytozome *Populus* v.2 (Goodstein *et al.*, 2012), and *Populus* DB (<http://www.populus.db.umu.se/>; Sterky *et al.*, 2004)] using the *Arabidopsis* ACL5 sequence (GI:145358223; AT5G19530) as query. Predicted aminoacid sequence alignments were performed using ClustalX or MUSCLE. For the phylogenetic analysis, putative *POPACAULIS5* (POPTR_0006s23880; GI:224088768), ACL5-like (POPTR_0008s15120, GI:224102051; POPTR_0010s09940, GI:224108055) and *P. tremula* × *P. tremuloides* (*PttACL5*; GenBank accession JX444689) sequences were used together with predicted sequences from genomes within the rosoid clade, comprising representative orders of angiosperms. Evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). Phylogenetic analyses were conducted in MUSCLE and MEGA4 (Edgar, 2004; Tamura *et al.*, 2007). The alignment is available in Supporting information Figure S1.

Isolation of *POPACAULIS5* and *PttHB8* coding regions

One µg of total RNA, extracted with RNeasy Plant Mini Kit (Qiagen) from

shoot apices of *P. tremula* × *P. tremuloides* and *P. trichocarpa* were used for cDNA synthesis using 1st Strand cDNA synthesis kit for RT-PCR (Roche) and oligo-dT, following the manufacturer's instructions. A 1028 bp sequence downstream of the start codon of *PttACL5* and *POPACAULIS5* was amplified from the cDNA of *P. tremula* × *P. tremuloides* and *P. trichocarpa*, respectively, and cloned into pCR2.1 vector (Invitrogen). Primers used were: *POPACAULIS5*-forward, 5'-ATGGGTACTGAGGCAGTTGAG-3' and reverse 5'-CCCACCAGCAAAGGTATGAG-3'. Similarly, a 2817 bp sequence downstream of the start codon of *PttHB8* was isolated from hybrid-aspen with the primers, *PttHB8* forward, 5'-ATCTCTAATCCGATCTACG-CCAGG-3' and reverse 5'-GCTCCCAAAGGTTTTTAGGC-3' by amplification from cDNA and cloned into pCR2.1 vector. Sequence identity was confirmed by sequencing.

Site-directed mutagenesis of PttHB8 miRNA 165/166 binding site

A site-directed mutagenesis approach was followed to block miRNA165/166 from cleaving the *PttHB8* transcript (Emery *et al.*, 2003; Zhong and Ye, 2004; Kim *et al.*, 2005). In the miRNA binding site of the isolated *PttHB8* cDNA sequence, two nucleotides (T and G) were substituted for A nucleotides, by PCR amplification. The complete pCR2.1 target plasmid bearing the *HD-Zip III* isolated sequence was amplified using the Phusion Hot-Start DNA polymerase (Finnzymes). The mutations were introduced by using the mutated forward primer, 5'-CTGGGATGAAGCCTGGACCAGA-TTCCATTGG-3' (underlined are the point mutations) and reverse, 5'-GCATTTGGACCCACTCCACAGCAGTTCCAGT-3'. The mutated PCR product (named *PttHB8*-miRNAd) was then re-circularized by ligation with T4 Quick DNA ligase (New England Biolabs). The point synonymous mutations were confirmed by sequencing and the *PttHB8*-miRNAd cDNA sequence was used to construct the overexpression vector under the

constitutive CaMV 35S promoter.

Hybrid aspen transformation

Cloned sequences were subcloned into pDONOR221 and recombined with gateway vectors pK7GW2.0 for *POPACAULIS5*, *PttACL5* and *PttHB8* overexpression (Karimi *et al.*, 2002; Gateway Technology, Invitrogen) and introduced into *Agrobacterium tumefaciens* strain GV3101pMP90 (Koncz and Schell, 1986). Hybrid aspen was transformed as previously described (Nilsson *et al.*, 1992). Transformant selection and shoot elongation were achieved in MS medium, with 20 g l⁻¹ sucrose, 0.1 µg ml⁻¹ indole-butyric acid (IBA), 0.2 µg ml⁻¹ 6-benzyl-aminopurine (BAP), 500 µg ml⁻¹ cefotaxime and 80 µg ml⁻¹ kanamycin-monosulphate, termed auxin-containing medium. For confirming insertions, PCR was performed using the primers: *POPACAULIS5* forward 5'-ATGGGTACTGAGGCAGTTGAG-3', and reverse 5'-TCAATTTTTGTTAGCCACCCCATG-3'; *PttHB8* forward, 5'-ATCTCTAATCCGATCTACGCCAGG-3' and reverse 5'-GAAAGACA-GTGTAAGGAG-3'; 35S forward, 5'-CTCATCAAGACGATCTACC-CGAG-3' and reverse, 5'-TGGGCAATGGAATCCGAGGAGGT-3'; *NPTII* forward 5'-GAATCGGGAGCGGCGATACCGTAAA-3', and reverse, 5'-CAAGATGGATTACACGCAGGTTCTC-3'; and *virBG* forward 5'-GCGGTGAGACAATAGGCG-3', and the reverse 5'-GAACTGCTTGCTGTCGGC-3' for false positives screening. After shoot elongation, plants were transferred to half-strength MS medium for rooting.

Anatomical and ultrastructural analysis

Tree height, internode length, leaf dimensions and stem diameter at internode 35 and stem base (15 cm above soil) were measured. The maturation internode was determined as the youngest internode where xylem showed

signs of complete maturation by the presence of fully lignified, highly autofluorescing xylem fibers as described by Bollhöner *et al.* (2012). The 45th internode (from the apex), found below maturation internode in wild-type, B2 and B14 lines was selected as reference internode from which growth and stem anatomy were analysed. Viability of the xylary cells was accessed by staining 0.5-1 mm stem sections with 10 mg ml⁻¹ nitroblue tetrazolium (NBT) in buffered succinate (Berlyn and Miksche, 1976; Gahan, 1984). Lignin staining was performed with phloroglucinol (Sigma) saturated solution in 20% HCl (Jensen, 1962). Sections were observed with a Zeiss Axioplan light microscope and images captured by Axioplan digital camera and Axiovision 4.5 software (Zeiss). Measurements were taken at four positions around the circumference of the stem using Axiovision 4.8 software. Stem segments were FAA fixed overnight, dehydrated in an ethanol series and gradually infiltrated, and embedded in LR White (TAAB). For sectioning a Leica RM2155 microtome (Leica Microsystems) was used. Sections were heat-fixed to slides, Toluidine Blue O-stained and mounted in mounting medium for observations as described. Tree growth, growth parameters and microscopy analyses were performed twice.

Electron microscopy

Electron microscopy images of fiber and vessel elements were taken from stem segments from the reference internode fixed in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, embedded in Spurr resin (Sigma) according to Rensing (2002), and examined with a Hitachi H-7000 transmission electron microscope (TEM).

Fiber and Vessel elements measurements

One centimetre-long stem segments were collected below the reference

internode. Pieces of wood were cut to exclude inner pith, outer bark and vascular cambium. The wood samples were immersed in a maceration alkaline solution as described by Berlyn and Miksche (1976). The wooden blocks were mechanically disaggregated. Xylem cell suspensions were observed with a light microscope as described above. Measurements of length and width of at least 200 fiber and 50 vessel elements were done manually for at least four individual trees and classified according to the secondary wall thickening patterns (Esau, 1977).

Histochemical GUS staining

Hand sections of stem segments were placed in 90% acetone for 30 min at -20°C, washed twice in distilled water and incubated in X-Gluc staining solution (1 mM X-Gluc, 1% Triton-X-100, 10 mM EDTA, in phosphate-buffer) at 37°C in darkness until staining was visible. Stem segments were washed with distilled water, dehydrated in an ethanol series to 50%, fixed for 10 min in formaldehyde/acetic acid/ethanol (5%/5%/50%), washed with 50% ethanol for 2 min, cleared in 100% ethanol, incubated o/n in 70% ethanol at 4°C, mounted in 50% glycerol and documented with a Zeiss Axioplan microscope.

Quantitative real-time RT-qPCR

Total RNA was extracted from 100 mg of frozen powdered tissues from *in vitro* grown material with RNeasy Plant Mini kit (Qiagen) as described in the sampling section and extracted from the trees tissues following Chang *et al.* (1993). cDNA synthesis was performed on 1 µg of DNase-treated total RNA using Transcriptor HF cDNA synthesis kit (Roche) with oligo-dT primers. qPCR was performed in LightCycler 480 PCR system with LightCycler480 SYBR Green I Master (Roche Applied Science), to monitor double stranded

DNA products. Specific primer pairs were designed to generate amplicons of *POPACAULIS5* and *PttHB8* (POPTR_0006s25390) used in detection. *Pt1* (POPTR_0002s12910) or *CYP2* (POPTR_0009s13270) were used as reference genes (Czechowski *et al.*, 2005; Gutierrez *et al.*, 2008). *PNAC058* (POPTR_0013s11740) primers used were described by Hu *et al.* (2010). The amount of target transcripts was normalized by the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). For all experiments, the mean of triplicate qPCR reactions was determined and at least three biological replicates or pooled biological samples were used. The experiments were repeated at least twice. Primers used were: *POPACAULIS5* forward, 5'-AAGATGCAGAGTGCC-GAAGT-3', and reverse, 5'-GACTTGTGCTTGAGGGCTTC-3', *PttHB8* forward, 5'-ATCTCTAATCCGATCTACGCCAGG-3', and reverse, 5'-CGCATAGAGCTTGGCTTAGG-3'; *Pt1* forward, 5'-GCGGAAAGAA-AAACTGCAAG-3', and reverse, 5'-TGACAGCACAGCCCAATAAG-3'; *CYP2* forward 5'-TAAGACCGAATGGCTTGACG-3' and reverse, 5'-AGAACGCACCCCAAACTACTA-3'.

Quantification of polyamines

Polyamines were extracted from about 100 mg of frozen tissues collected as described in the sampling section and purified (Rambla *et al.*, 2010), derivatized (Fernandes and Ferreira, 2000), identified and quantitated as described by Rambla *et al.* (2010). Representative mass spectra for the heptafluorobutyric derivatives of thermospermine (tspm), spermine (spm), spermidine (spd) and putrescine (put) are shown in Figure S10.

POPACAULIS5 thermospermine activity assays in yeast

The *POPACAULIS* coding sequence was extracted from pCR2.1-*POPACAULIS5* plasmid as an *Eag I* fragment and cloned into the yeast

expression vector pCM190 (Gari *et al.*, 1997). The pCM190-*POPACAULIS5* vector and control empty vector were introduced into yeast, with Yeastmaker-Yeast Transformation System 2 (Clontech). After lyses by intense vortex with 100 μ l of 0.5 mm diameter glass beads, polyamines levels in yeast extracts were determined by gas chromatography–mass spectrometry as above described.

Quantification of IAA

Tissues from trees and from *in vitro* grown plants were collected as described in the sampling section and 10-20 mg were used for quantification of free IAA content. Sample extraction and purification was performed according to Andersen *et al.* (2008), with 500 pg $^{13}\text{C}_6$ -IAA internal-standard added to each sample before extraction. After derivatization, the samples were analysed by gas chromatography-selected reaction monitoring-mass spectrometry as described (Edlund *et al.*, 1995).

Auxin treatments for expression analysis

Three centimeter-long stem segments from five week-old *in vitro* grown wild-type, 35S::*POPACAULIS5* transgenic lines were cut between internodes. Six segments from six individual plants were immediately frozen after cutting, representing the pooled control (0 h). All remaining stem segments were placed on an auxin-free half-strength MS medium to deplete them from auxin for 16 h, after which pools of six segments derived from six individual plants were grouped and sampled (16 h). Half of the remaining segments were then placed in fresh half-strength MS medium (mock) and the other half in the same medium with 20 μ M IBA, from which further sets of six stem segments, derived initially from six individual plants, were pooled after mock/IBA treatments in a time-course experiment for 4 h (point at 20

h), 24 h (point at 40 h) and 32 h (point at 48 h). As negative controls to the auxin treatment experiments, stems from two 35S::*GUS:GFP Populus* lines were used to monitor response to 2 μ M and 20 μ M IBA. Total RNA, cDNA synthesis and RT-qPCR was performed as above described. The experiment was repeated twice.

Statistical analysis

Non parametric Mann-Whitney *U*-test was employed to assess significant differences in gene expression, polyamine and IAA contents and tree growth parameters. A significance level of $\alpha=0.05$ was considered. Statistics were performed using the software Statistica (Statsoft), after Zar (1998).

Results

POPACAULIS5 is the Populus ACAULIS5 ortholog

In *Arabidopsis*, *ACAULIS5* (*ACL5*) gene encodes thermospermine synthase (Knott *et al.*, 2007). Our search in *Populus* genome retrieved a putative *ACL5* sequence from *P. trichocarpa*, POPTR_0006s23880 (Phytozome *Populus* v.2) with 86.1% identity to *ACL5*. Two other sequences were found in the *P. trichocarpa* genome, POPTR_0008s15120 and POPTR_0010s09940, showing higher similarity amongst them than to *ACL5* or to POPTR_0006s23880 (Figure 1a). BLAST searches of POPTR_0006s23880 against *Populus* DB, retrieved a 496 bp EST (GI:3853671) from *P. tremula* $\times$ *P. tremuloides*, with 95% identity over 99% of the sequence (Figure 1b). Alignment of *ACL5* amino acid sequences from *Arabidopsis*, *Populus* and other land plants showed that POPTR_0006s23880, called hereafter as *POPACAULIS5*, is the most similar sequence to *ACL5*.

The relationship of POPACAULIS5 with other known and predicted ACL5 sequences was inferred from a phylogenetic analysis that generated a single tree (Figure 1a), in accordance with previously reported relationships amongst plant aminopropyltransferases (Minguet *et al.*, 2008; Rodriguez-Kessler *et al.*, 2010). The tree shows two major groups of clusters, one identified as thermospermine synthase-predicted proteins and the other as thermospermine-synthase-like proteins (Figure 1a, see also Figure S1 from Supporting information). POPACAULIS5 putative sequence from *P. trichocarpa* and *P. tremula* × *P. tremuloides* (PttACL5) group within the former cluster where ACL5 sequence is also included, suggesting that this is the most conspicuous candidate to being thermospermine synthase. We confirmed that POPACAULIS5 is a true ortholog of ACL5 by demonstrating its thermospermine synthase activity in yeast cells (Figure S2).

35S::POPACAULIS5 trees show reduced overall growth and slight defects in xylem development

We isolated and cloned POPACAULIS5 cDNA from *P. trichocarpa* and *P. tremula* × *P. tremuloides* (PttACL5) for overexpression under the control of 35S CaMV constitutive promoter. Transformation of hybrid aspen allowed recovery of 90 kanamycin-resistant transgenic lines for 35S::POPACAULIS5, and 39 for 35S::PttACL5. The ectopic expression of POPACAULIS5 from both constructs resulted in the same dramatic changes in shoot and root development and the transgenic lines obtained were grouped according to the severity of observed phenotypes (Figure 2a, see also Figure S3a-f). Dwarf transgenic plants from group B (Figure 2a) were able to develop a rooting system and partially restored the wild-type phenotype once transferred to a plant growth regulator(PGR)-free medium (Figure 2b). For this reason, we selected from group B the transgenic lines 35S::POPACAULIS5-B2, B4, B13, B14 and B15 for further analysis of the effects of POPACAULIS5

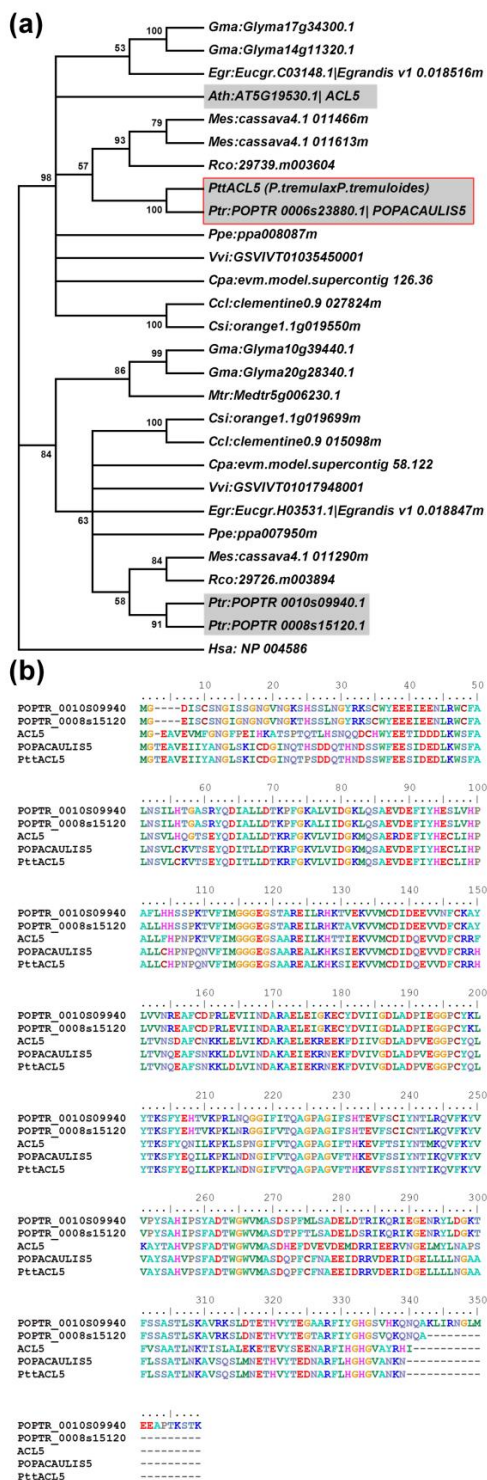
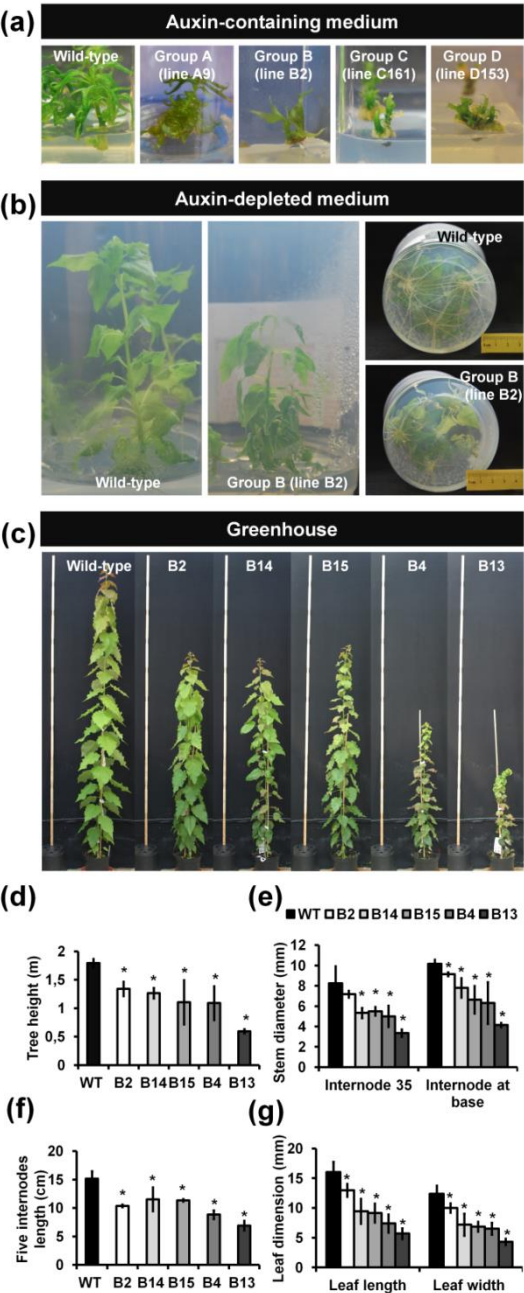


Figure 1. Phylogenetic and sequence analysis of ACAULIS5 from *Populus*, *Arabidopsis* and other taxa. (a) Phylogenetic tree with relationships amongst ACL5-predicted or ACL5-like amino acid sequences. ACAULIS5 from *Arabidopsis thaliana* (Ath; ACL5), *Populus trichocarpa* (Ptr; POPACAULIS5), *P. tremula* × *P. tremuloides* (Ptt; PttACL5) were used together with putative thermospermine synthase predicted proteins from other land flowering plants including Mes (*Manihot esculenta*), Rco (*Ricinus communis*), Vvi (*Vitis vinifera*), Csi (*Citrus sinensis*), Ccl (*Citrus clementina*), Ppe (*Prunus persica*), Cpa (*Carica papaya*), Egr (*Eucalyptus grandis*), Mtr (*Medicago truncatula*) and Gma (*Glycine max*). Spermine synthase sequence from Hsa (*Homo sapiens*) was used as outgroup. Sequence and references from taxa are detailed in Figure S1. Numbers at branches represent bootstrap values (Felsenstein, 1985). (b) Alignment of amino acid sequences from *Arabidopsis*-*sis* ACL5 and POPACAULIS5 from *Populus trichocarpa* (POPTR_0006s-23880 and ACL5-like proteins POPTR_0008s15120 and POPTR_00-10s09940) and *P. tremula* × *P. tremuloides* (PttACL5, GenBank accession JX444689).

overexpression in trees (Figure 2c).

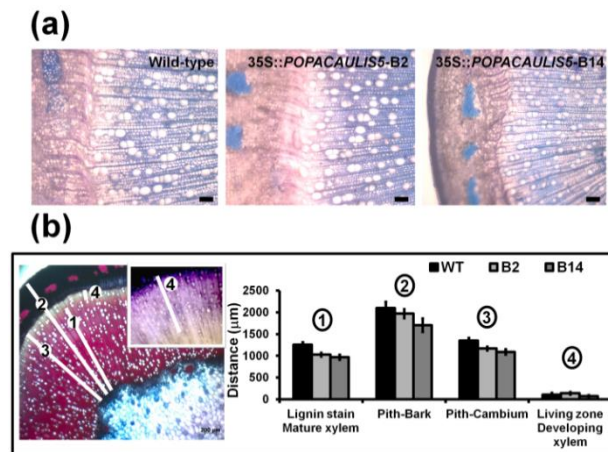
Tree height was significantly reduced in the 35S::*POPACAULIS5* trees grown in the greenhouse for a period of two months (Figure 2c,d). Also, diameter and internode length of mature stem as well as leaf size were smaller in the transgenics than in the wild-type (Figure 2e-g). Strong phenotypic variation was observed between independent transgenic lines (Figure 2c, Figure S4a) and even in individual trees from the same transgenic line. Maturation of secondary xylem was screened along the stem by searching for the youngest internode with fully lignified xylem fibers, detected by the appearance of highly autofluorescing tissues in the secondary xylem. In wild-type, fully lignified secondary xylem fibers were found in the 25th internode. Xylem maturation was delayed in the transgenic lines as fully lignified secondary xylem fibers were only observed in the 32nd internode in B2, the 39th internode in B14 and absent from the stem in lines B4 and B13. We performed further analyses at the internode 45 from the top (so-called reference internode; see Experimental procedures). Xylem anatomy and cell morphology were analyzed at the reference internode from the transgenic lines B2 and B14, which showed intermediate phenotype in xylem maturation as well as in the various growth parameters (Figure 3a, Figure S4a,b). The absence of ACL5 function in the *acl5* mutants of *Arabidopsis* has a profound effect on xylem maturation, resulting in premature cell death of xylem elements and reduction in complexity of the secondary cell wall patterning (Muñiz *et al.*, 2008). We observed by nitroblue tetrazolium (NBT) staining of stem transverse sections that the width of the living xylem zone in the reference internode of the transgenic lines was similar to that of the wild-type (Figure 3b). Shorter distance from pith to cambium together with equal width of the xylem living zone observed in the transgenic lines indicate that cell death in xylem fibers might be slightly delayed in the transgenic lines when compared to the wild-type (Figure 3b). No major alterations were observed in size (Figure S5a-f) or cell wall patterning in xylem elements of

Figure 2. Phenotypic characterization of transgenic lines expressing 35S::*POPACAULIS5* in *Populus*. (a) *POPACAULIS5* overexpression effect on hybrid aspen grown *in vitro* on auxin-containing medium. A9 is representative of transgenic lines with mild phenotype closer to the wild-type (group A), B2 represents transgenic lines with dwarf phenotype that have elongation and rooting defects when grown on auxin-containing medium, but that partially recover once transferred to an auxin-free medium (group B). C161 and D153 represent transgenic lines with dwarf phenotype that totally lack roots, and are lethal when transferred to an auxin-free medium (groups C and D). Increasing severity in abnormal phenotypes found in C161 and D153 correlated well with increased *POPACAULIS5* transcript level (Figure 4c). The appearance of dwarf plants was observed in transgenic lines recovered from the six independent transformation assays, with *ACL5* homolog cDNA from both *P. trichocarpa* (35S::*POPACAULIS5*) and *P. tremula* × *P. tremuloides* (35S::*PttACL5*). (b) Five week-old *in vitro* grown 35S::*POPACAULIS5*-B2 line showing partially restored wild-type phenotype. (c) Two-month old transgenic trees (from lines 35S::*POPACAULIS5*-B2, B14, B15, B4 and B13). (d) Comparison of height, (e) stem diameter at internode 35 and at the stem base, (f) mean length of five internodes and (g) mean dimensions of five fully expanded leaves, around internode 35 (two above and three below). Values are means ± SD of at least three biological replicates (each replicate sampled from one individual tree). Asterisks indicate significant differences from the wild-type ($p < 0.05$, Mann-Whitney *U* test). The greenhouse growth experiment was performed twice.



the transgenic tree stem samples collected from the reference internode (Figure S6a, Figure S7). However, a slight increase in proportion of the primary xylem vessel types was observed (Figure S6b,c). Altogether, the lack of major defects in secondary xylem development as a consequence of manipulating *POPACAULIS5* raised the question on what was the level of *POPACAULIS5* overexpression and whether thermospermine was overproduced in the secondary xylem tissues of the 35S::*POPACAULIS5* woody stems.

Figure 3. Anatomy of stem tissues and xylem development in transgenic *Populus* trees. (a) Transverse sections taken from the reference internode from stems of wild-type and transgenic 35S::*POPACAULIS5*-B2 and B14 trees stained with Toluidine Blue-O. (b) Schematic representation of measurements



taken from stems transverse sections. 1, represents width of mature xylem (as observed by phloroglucinol staining); 2, distance outer bark to pith; 3, distance vascular cambium to pith and 4, width of living zone of the xylem on the basis of the NBT viability stain (smaller inset). Scale bars: 100 μm. Values are means ± SD from six (wild-type) and four (B2, B14) biological replicates. For each tree cross-section the measurements were taken at four approximately equidistant positions around the circumference of the stem, in six or four individual trees.

Thermospermine accumulation is suppressed in 35S::*POPACAULIS5* *Populus* woody stem, but not in leaves

Contents of the main polyamines putrescine, spermidine, spermine and thermospermine were measured in samples that were collected from leaves

and scrapings of the living zone of the secondary xylem in the stem. Spermidine and spermine were highly abundant in all plant tissues while putrescine and thermospermine were less abundant but still easily detectable (Figure 4a,b). As expected, thermospermine content was higher in leaves of the transgenic lines when compared to the wild-type. However, similar or even lower levels of thermospermine were observed in the secondary xylem tissues collected from the same tree stems (Figure 4a). Similar results with higher levels of thermospermine in the leaves but not in the stem were obtained in plants grown *in vitro* on auxin-free medium (Figure 4b). It was also interesting to note that the levels of the other polyamines were not increased in the stem or xylem samples of the transgenic lines (Figure 4a,b), making it unlikely that the lack of thermospermine overaccumulation in these samples is due to the back-conversion of thermospermine to spermidine or putrescine. A decrease in spermidine and spermine levels was observed in several samples (Figure 4b), which could be related to increased overall polyamine catabolism similar to what was recently proposed in *Arabidopsis* 35S::*ACL5* plants (Marina *et al.*, 2013).

Expression of *POPACAULIS5* followed the changes in the thermospermine levels of the transgenic trees. RT-qPCR analysis to the same samples analysed for polyamine content showed about 40-fold and higher increases in *POPACAULIS5* expression in leaves of the transgenic trees, while in xylem tissues the expression was unaltered in lines B2, B13 and B4 and even reduced in lines B14 and B15, when compared to the wild-type (Figure 4a). To exclude the possibility that this result was not due to inactivity of the 35S promoter in the secondary xylem, we analysed transgenic trees carrying a 35S::*GUS:GFP* construct and demonstrated by histochemical GUS assay that the 35S promoter is active in the secondary xylem tissues (Figure S8). The lack of *POPACAULIS5* overexpression in the secondary xylem explains the lack of major phenotypic changes in the xylem tissues of the transgenic trees but also raises a question on the mechanism

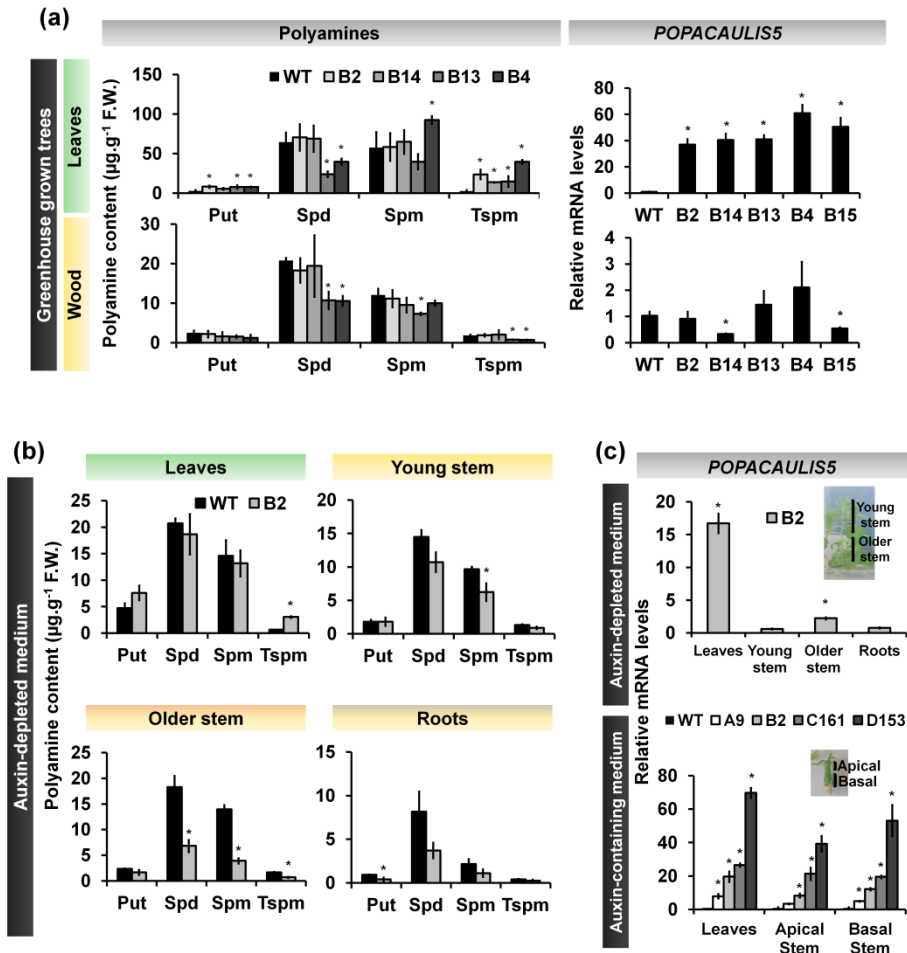


Figure 4. Thermospermine content and *POPACAULIS5* transcript levels of *in vitro* and greenhouse grown transgenic *Populus* trees. (a) Polyamine levels (left panel) and *POPACAULIS5* transcript levels, analysed by RT-qPCR (right panel), in leaves and secondary xylem. (b) Polyamine levels in organs of five week-old *in vitro* grown B2 transgenic plants, in an auxin-depleted medium. (c) Relative *POPACAULIS5* transcript levels in organs of B2 transgenic plants *in vitro* grown on an auxin-depleted medium (upper panel) and in transgenic lines grown on auxin-containing medium (lower panel). Values are means $\pm$ SD of three biological replicates (sampled as three pools of six to ten individual plants from *in vitro* grown material; and for greenhouse-grown trees, each replicate sampled from one individual tree) and three technical replicates. Transcript levels are given relatively to the wild-type level in each tissue. Asterisks indicate statistically significant differences from the wild-type ($p < 0.05$, Mann-Whitney *U* test). Putrescine (Put), spermidine (Spd), spermine (Spm), thermospermine (Tspm). The experiments for gene expression analysis were performed twice.

leading to suppression of the *POPACAULIS5* transgene expression in xylem tissues but not in leaves. Interestingly, increased expression of *POPACAULIS5* was observed in the stem of plants grown *in vitro* on auxin-containing medium (Figure 2a, Figure 4c), which led us to hypothesize that auxin levels somehow control accumulation of *POPACAULIS5* transcripts and question whether the transgenic 35S::*POPACAULIS5* trees had lower levels of auxin.

***POPACAULIS5* overexpression suppresses endogenous auxin levels in the secondary xylem**

IAA levels were measured in leaves and stems of *in vitro* grown plants and in leaves and secondary xylem tissues from greenhouse-grown trees. *In vitro* grown plants showed similar IAA levels in leaves of wild-type and transgenic lines, but a decrease was found in the young stems when compared to the wild-type (Figure 5a). In greenhouse-grown trees, IAA levels were strongly reduced in the leaves and in the secondary xylem tissues of the transgenic trees (Figure 5b). This was even more pronounced in lines with more severe reduction in growth. The low levels of auxin and the lack of *POPACAULIS5*

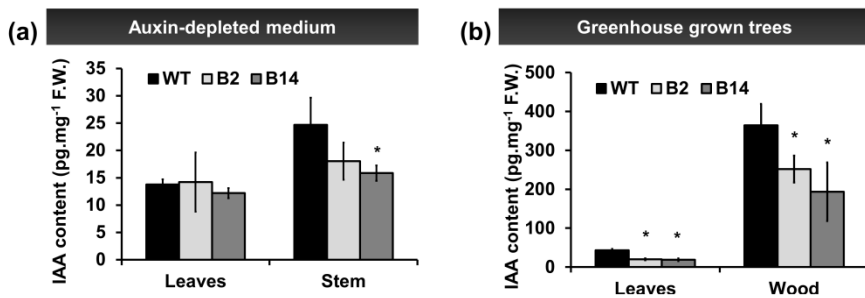


Figure 5. IAA endogenous levels analysis in (a) leaves and stem tissues of *in vitro* grown and (b) leaves and scrapped living xylem tissues from greenhouse-grown trees. Values are means $\pm$ SD of three biological replicates, each representing three technical replicates. Asterisks indicate statistically significant differences from the wild-type ($p < 0.05$, Mann-Whitney *U* test). The experiment was performed twice.

overexpression in xylem tissues of the transgenic 35S::*POPACAULIS5* is suggestive of a negative feedback mechanism where increased *POPACAULIS5* expression functions to reduce IAA levels, which in turn prevents further expression of *POPACAULIS5*. The reduction in the IAA levels in the 35S::*POPACAULIS5* leaves (Figure 5b) most probably reflects functioning of the feedback mechanism in the leaf vasculature, which is the main site of auxin accumulation in this organ (Ljung *et al.*, 2001; Teichmann *et al.*, 2008; Petrásek and Friml, 2009).

We tested our hypothesis by studying whether exogenous auxin could affect expression of *POPACAULIS5*. Auxin levels were modulated in young stem tissues of wild-type and of two transgenic lines, B2 and B15, by exogenous application of indole-butyric acid (IBA) (Figure 6a-c). Stems pieces were first depleted of auxin for 16 h. As expected, exogenous supply of auxin resulted in a strong increase in *POPACAULIS5* transcript levels after 4 h in both transgenic lines but not in the wild-type. Twenty-four hours after auxin treatment, the transcript levels decreased in the transgenic stems (point 40h; Figure 6b,c). To exclude the possibility that the auxin-induced increase in *POPACAULIS5* expression was due to induction of the 35S promoter itself, transgenic *Populus* trees carrying 35S::*GUS:GFP* construct were analysed and shown to be non-responsive to exogenous IBA on the basis of expression analyses of the *GUS* and *GFP* genes by qPCR (Figure S9). Altogether, these findings suggest that auxin can stimulate *POPACAULIS5* expression on a post-transcriptional level.

Class III HD-Zip PttHB8 overexpression stimulates POPACAULIS5 expression

HD-Zip III family member AtHB8 is a good candidate in control of *ACL5* expression in *Arabidopsis* (Baima *et al.*, 2001; Imai *et al.*, 2006; Carlsbecker *et al.*, 2010). We therefore tested if the autoregulatory feedback mechanism

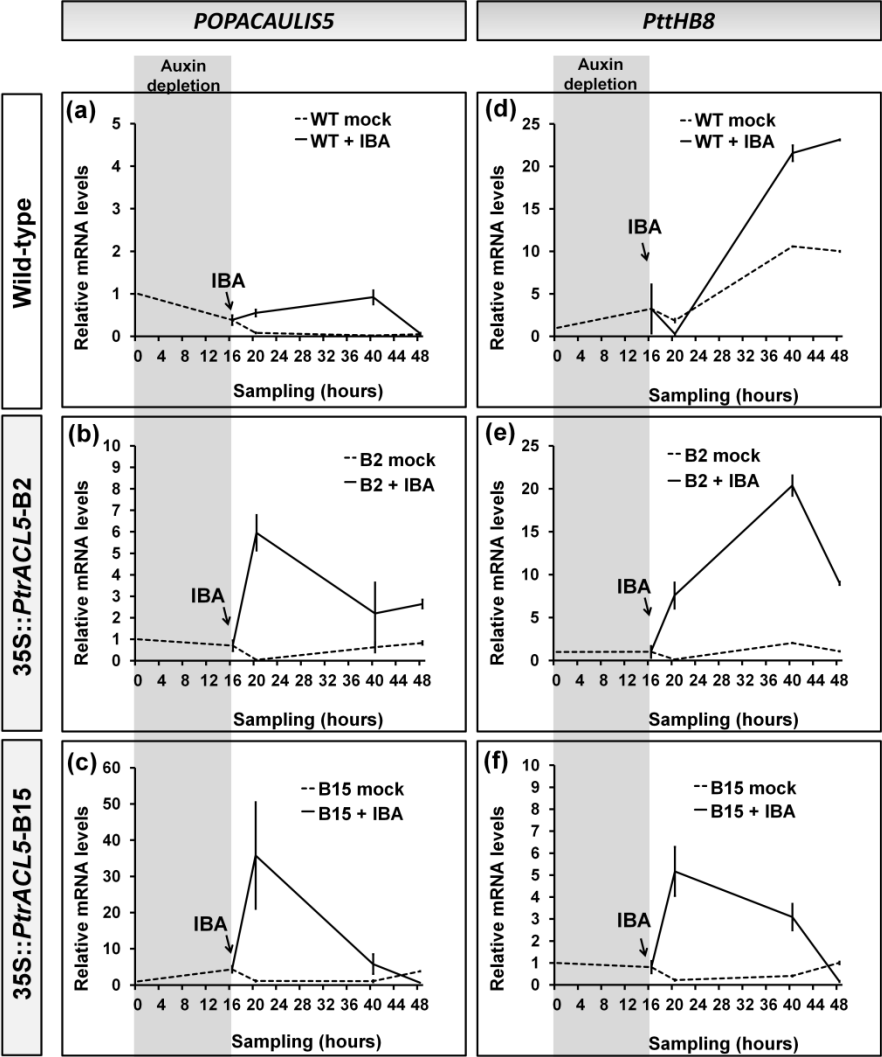
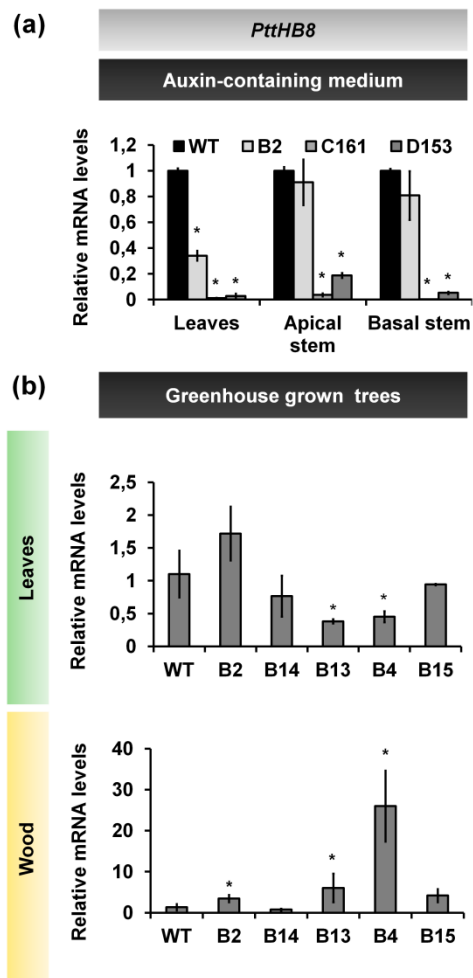


Figure 6. Time-course analysis of *POPACAULIS5* and *PttHB8* expression in response to exogenous auxin. Five week-old *in vitro* grown stem segments of wild-type, 35S::*POPACAULIS5*-B2 and B15 transgenic plants were depleted of their auxin levels, by decapitation and incubation on half-strength MS medium without auxin for 16 h, after which stems were transferred to IBA-containing or kept in the auxin-free medium (mock). Samples were taken at: 0 h (point 0), after 16 h depletion (point 16), and 4 h (point 20), 24 h (point 40) and 32 h (point 48) after transfer to IBA or mock medium. *POPACAULIS5* (a-c) and *PttHB8* (d-f) expression was assayed by RT-qPCR. Values represent relative transcript levels normalized to expression values at the beginning of the experiment (point 0). Values are means $\pm$ SD of three replicate samples, consisting of six stems from six individual plants pooled at each time point. The experiment was carried out twice.

of *ACL5* expression proceeds through *AtHB8*. In *Populus*, the closest homolog to *AtHB8* is *PttHB8* (Ko *et al.*, 2006). In the *in vitro* transgenic 35S::*POPACAULIS5* lines, grown on auxin-containing medium, *PttHB8* expression was significantly suppressed when compared to the wild-type (Figure 7a). In the greenhouse grown trees, *PttHB8* expression was suppressed in the leaves from most of the lines but unaltered or slightly upregulated in xylem tissue in comparison to the wild-type (Figure 7b).

Figure 7. *PttHB8* expression in transgenic 35S::*POPACAULIS5* trees. Relative expression of *PttHB8* is shown in the dwarf transgenic plants from lines B2, C161 and D153, grown on auxin-containing medium (a) and in leaves and woody tissues (b). Values are means $\pm$ SD of three replicate samples (from a pool of ten) in *in vitro* grown plants and of three biological replicates each consisting of three technical replicates in the greenhouse grown trees. The asterisks indicate statistically significant differences from the wild-type ($p < 0.05$, Mann-Whitney *U* test). The experiment was performed twice.



These results demonstrate that *POPACAULIS5* overexpression that is present in the *in vitro* plants and in the leaves of the greenhouse grown trees (Figure 4a,c) functions to suppress *PttHB8* expression (Figure 7a,b). We could also show that expression of *PttHB8* was rapidly induced by exogenous auxin (Figure 6d-f), and it is therefore possible that the suppression of *PttHB8* expression by *POPACAULIS5* overexpression is mediated through IAA.

To further understand *PttHB8* involvement in *POPACAULIS5* regulation we expressed a miRNA165/166 misregulated form of *PttHB8* under the control of the 35S promoter in hybrid aspen. Three 35S::*PttHB8*-miRNA transgenic lines, L175, L176 and L179, were obtained. Similar to what was earlier observed for 35S::*POPACAULIS5* trees, upregulation of *PttHB8* was observed only in leaves but not in the stem (Figure 8). This suggests that *POPACAULIS5* and *PttHB8* expression levels are controlled by one and same mechanism. Most importantly, overexpression of *PttHB8* in the leaves resulted in increased levels of *POPACAULIS5*, suggesting that *PttHB8* activates expression of *POPACAULIS5* either directly or indirectly.

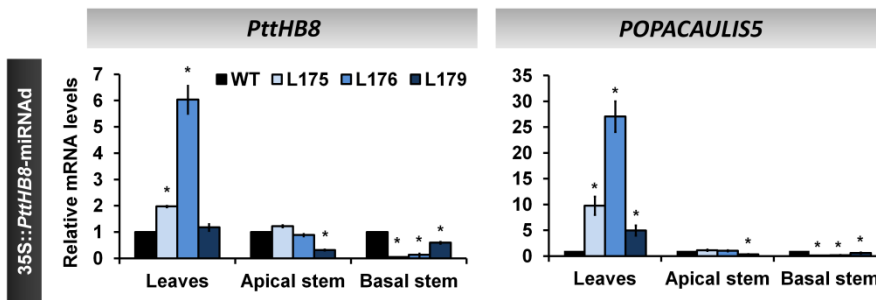


Figure 8. *PttHB8* and *POPACAULIS5* relative transcript levels in transgenic *Populus* plants expressing dominant, gain-of-function *PttHB8*. Relative expression of *PttHB8* is shown in leaf, apical and basal stem tissues of the transgenic 35S::*PttHB8*-miRNA lines L175, L176 and L179. Values are means $\pm$ SD of three replicate samples (from pools of tissues from six *in vitro* grown plants for each genotype). Asterisks indicate statistically significant differences from the wild-type ($p < 0.05$, Mann-Whitney *U* test). The experiment was performed twice.

Discussion

This work focused on *POPACAULIS5* that was shown to be the true ortholog of the *Arabidopsis* thermospermine synthase *ACL5*. Our results provide evidence that thermospermine levels are strictly controlled by a negative feedback mechanism involving *POPACAULIS5*, auxin and HD-Zip III transcription factor *PttHB8*. The evidence for the feedback mechanism is based on the surprising inability to raise the levels of *POPACAULIS5* transcript or thermospermine in xylem tissues by ectopic expression of *POPACAULIS5* under the control of CaMV 35S promoter in transgenic *Populus* trees (Figure 4a). This inability correlated with reduced accumulation of auxin (Figure 5b), suggesting that overproduction of thermospermine suppresses biosynthesis of auxin. This is in accordance with the opposite situation observed in *Arabidopsis acl5* mutant where increased auxin levels were present in young seedlings (Vera-Sirera *et al.*, 2010). Auxin on the other hand, is known to induce expression of *ACL5* (Hanzawa *et al.*, 2000; Rambla *et al.*, 2010) and this was also shown for *POPACAULIS5* in the *Populus* stem (Figure 4c, Figure 6b,c). Therefore, thermospermine and auxin are part of a feedback loop that involves the negative effect of thermospermine on auxin and positive effect of auxin on thermospermine. Presence of a negative feedback loop was noticed early on when it was observed that *acl5* mutants had increased expression levels of *ACL5* (Hanzawa *et al.*, 2000; Imai *et al.*, 2006; Muñiz *et al.*, 2008). In this work, we have identified auxin as a mediator of this feedback control.

The negative feedback loop functions to suppress thermospermine levels specifically in secondary xylem tissues as high expression levels of *POPACAULIS5* from the 35S promoter were easily observed in the leaves of the transgenic *Populus* trees (Figure 4a,b). It is probable that the feedback mechanism operates specifically in the xylem vessel elements due to the fact that both *ACL5* and *HB8* are specifically expressed in the xylem vessel

elements (Baima *et al.*, 2001; Muñiz *et al.*, 2008; Zhang *et al.*, 2011). The absence of the feedback loop in other cell types leads to high levels of thermospermine (Figure 4) that seems to be detrimental to overall growth of the plants (Figure 2, Figure S3). A decrease in height growth of the 35S::POPACAULIS5 trees could be a direct effect on apical growth but also secondary caused, by, for instance, the smaller leaf size, impaired photosynthetic capacity or impaired water transport capacity of the transgenic trees. This decrease is somewhat surprising considering that suppression of *ACL5* expression in *Arabidopsis* causes dwarfism of the inflorescence stem (Hanzawa *et al.*, 2000; Clay and Nelson, 2005; Muñiz *et al.*, 2008). Hence, one would expect an increase rather than decrease in height growth of the stem in an *ACL5* overexpressor. We can only speculate about the reasons for this, but it is a general phenomenon that plant hormones function is dose-dependent until a threshold level after which increases in hormonal concentrations become inhibitory (Srivastava, 2002). Thermospermine levels resulting from 35S::POPACAULIS5 expression clearly seem to exceed the threshold level for thermospermine action in control of height growth. It is actually quite probable that the threshold for optimal thermospermine levels is very low or maybe even very close to zero in other cells than xylem vessel elements since they normally do not synthesize any thermospermine. In conclusion, we propose that any increase in thermospermine levels of non-vessel cells is detrimental for growth, while a decrease in the thermospermine concentration in the xylem vessel elements, such as in the *acl5* mutant, would also lead to reduced growth of the inflorescence stem due to problems in xylem specification.

Several previous reports have demonstrated the importance of polyamines for cambial development and xylem differentiation (Vera-Sirera *et al.*, 2010; Tisi *et al.*, 2011; Waduware-Jayabahu *et al.*, 2012). Disruption of *ACL5* in *Arabidopsis* results in overproliferation of xylem vessel elements with spiral or reticulate secondary wall thickenings, smaller size of the vessel

elements and lack of xylem fibers (Muñiz *et al.*, 2008). However, only modest defects in xylem development were observed in the transgenic 35S::*POPACAULIS5* *Populus* trees. Expansion of the secondary xylem was reduced in the 35S::*POPACAULIS5* trees but this was not surprising considering the severe reduction in the overall height growth of these trees. In spite of this, morphology of xylem elements was scarcely altered as the size and abundance of the different xylem cell types as well as the type of secondary cell wall thickenings of vessel elements were similar in wild-type and transgenic trees. Unaltered xylem morphology correlates well with the fact that thermospermine levels were not increased in the secondary xylem tissues of the transgenic trees. It is also possible that the slight defects observed in xylem development are due to increased thermospermine levels in the early stages of plant growth in the auxin-rich environment *in vitro* (Figure S3). Alternatively, thermospermine levels may fluctuate slightly during the growth of the trees, and measurement of the thermospermine levels in the pool of xylem tissues did not maybe reveal these fluctuations. Fluctuations are expected to occur as a result of the presumable strong overexpression of *POPACAULIS5* from the 35S promoter that needs to be counteracted by the negative feedback loop. We observed high variation in growth within transgenic lines that could reflect these fluctuations. Similar variation was earlier observed within transgenic lines in *Populus* trees where expression levels of HD-Zip III TFs family member *POPREVOLUTA* were increased (Robischon *et al.*, 2011).

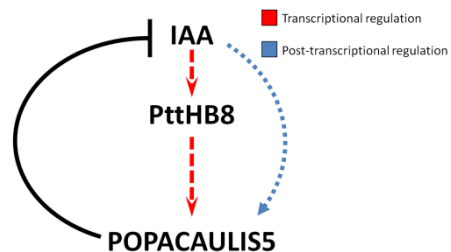
An interesting question is whether decrease in secondary growth of the stem of the transgenic lines could be due to the low IAA levels found. IAA is known to be a central regulator of cambial growth and xylem specification (Ohashi-Ito and Fukuda, 2010; Ursache *et al.*, 2012) and alterations in levels of auxin have long been known to have severe effects on xylem development (Gälweiler *et al.*, 1998; Hardtke and Berleth, 1998). It was also recently reported that 2,4-dichlorophenoxy acid and other auxin

synthetic analogues induce ectopic xylem vessel differentiation in *acl5* mutant but not in wild-type *Arabidopsis* (Yoshimoto *et al.*, 2012a, 2012b). The authors suggested that xylem differentiation is controlled by auxin and that thermospermine acts to suppress IAA synthesis and/or sensitivity. Our data provides the evidence for suppression of IAA levels by thermospermine. But we also showed that inclusion of auxin in the growth medium *in vitro* did not alleviate growth defects but instead further reduced xylem differentiation in the transgenic 35S::*POPACAULIS5* trees (Figure S3c,e), supporting function of thermospermine rather than auxin as the central regulator of xylem differentiation during secondary growth of the stem.

On the basis of our data, we propose operation of a mechanism in secondary xylem tissues to maintain thermospermine at safe levels in order to facilitate its fundamental role in xylem differentiation. In this proposed mechanism, IAA mediates *POPACAULIS5* expression through *PttHB8*, and thermospermine levels feedback control *PttHB8* and consequently *POPACAULIS5* transcript levels through repression of IAA in a loop (Figure 9). How *POPACAULIS5* or thermospermine functions to suppress IAA biosynthesis is not clear currently. Another open question is how interaction between *PttHB8* and *POPACAULIS5* takes place. Our results on upregulation of *POPACAULIS5* expression as a result of overexpression of *PttHB8* suggests that *POPACAULIS5* expression could be under direct transcriptional regulation by *PttHB8*. Transcriptional control of *POPACAULIS5* levels by auxin and *PttHB8* does not explain the lower levels of *POPACAULIS5* transcript and thermospermine in the secondary xylem tissues of the 35S::*POPACAULIS5* trees. We propose therefore that *POPACAULIS5* is regulated in the transgenic 35S::*POPACAULIS5* trees by auxin through post-transcriptional control of the mRNA stability. Hence, *POPACAULIS5* overexpression in the xylem elements of the 35S::*POPACAULIS5* trees reduces auxin levels which in turn results in destabilisation of the *POPACAULIS5* transcripts. The feedback mechanism cannot cope with

excessive amounts of auxin, as inclusion of auxin in the *in vitro* medium resulted in high levels of *POPACAULIS5* expression and damaging levels of thermospermine in the 35S::*POPACAULIS5* stems. Whether post-transcriptional regulation of *POPACAULIS5* occurs also for the endogenous transcript in the wild-type plants is not known, but feasible since it would allow rapid alterations in thermospermine homeostasis. It is therefore possible that auxin mediates transcriptional activation of *ACAULIS5* through HB8, and that the post-transcriptional control allows rapid alterations in thermospermine signalling especially in situations where IAA concentrations are excessive. Similar kind of mechanism involving both transcriptional and post-transcriptional control of gene expression by IAA has been shown in the context of other genes as well, such as the *AUX/IAA* genes (for a review see Benamins and Scheres, 2008). In any case, it is clear from our data that thermospermine levels must be tightly controlled in the secondary xylem tissues of the stem as insurance to proper xylem differentiation and that auxin is a central component in this control.

Figure 9. Proposed model for the feedback control mechanism of thermospermine homeostasis in *Populus* secondary xylem tissues. *POPACAULIS5* expression is induced by auxin through PttHB8. *POPACAULIS5* suppresses biosynthesis of IAA, which in turn results in reduced activation of *PttHB8* transcription and therefore *POPACAULIS5* expression.



Auxin is also proposed to mediate post-transcriptional regulation of *POPACAULIS5* mRNA stability. The feedback loop mechanism operates specifically in the xylem as a safeguard mechanism against damaging effects of increased thermospermine levels. Black line depicts negative effect of *POPACAULIS5* on IAA. Red dotted lines depict transcriptional regulation. Blue dotted lines depict post-transcriptional regulation.

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Supporting information

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Gma:Glyma20g28340.1	MG-----KIAL-PNGISNGNI-----NG-----
Cpa:evm_model.supercontig_58.1	MG-----ETEC-SHG-----NG-----
Ppe:ppa007950m	MG-----DIET-SHGTNNNGGNGNGNGNGNG-----NG-----
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Col:clementine0.9_015098m	MG-----ETEC-SHGISQA-----NG-----
Mes:cassava4.1_011290m	MG-----DIAC-SHGISNG-----NG-----
Rco:29726.m003894	MG-----DIAC-SHGISNGT-----NG-----
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Gma:Glyma17g34300.1	MG-----KAPKVFY-INGFSKFKK-----HK-----
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Rco:29739.m003604	MG-----KAVEIYY-TNGFSKLC-----TQ-----
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Egr:Eucgr.C03148.1 Egrandis_v1	NRGRNVREVPADGRV-WYEEKIEDDLK-----
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Gma:Glyma17g34300.1	TQONNH-VMDQNDSS-WYEEKIEDDLK-----
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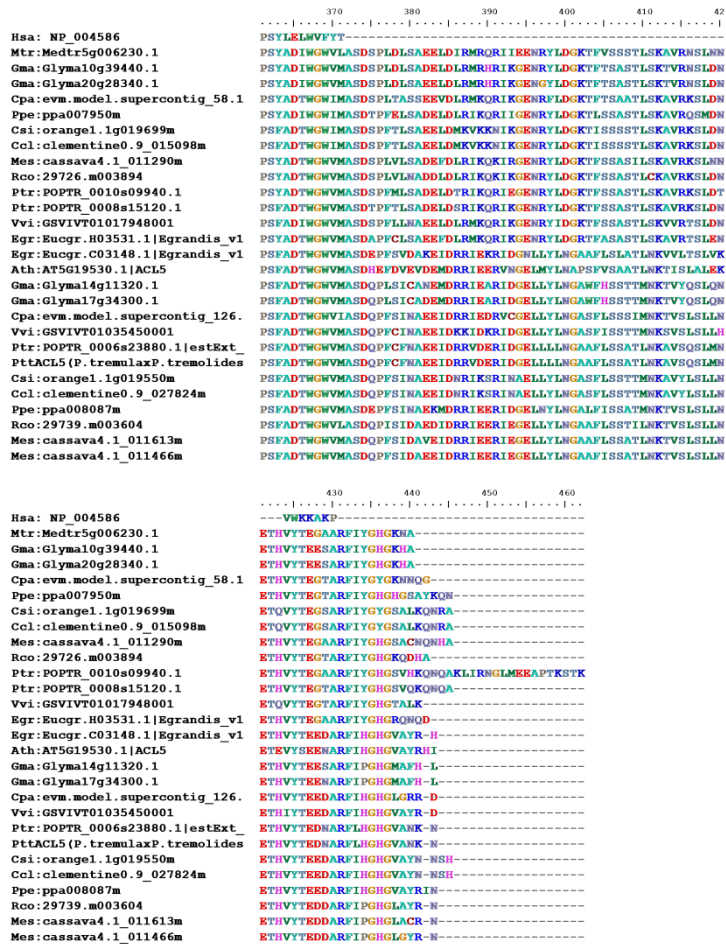


Figure S1. Aminoacid sequence alignment from taxa used in the phylogenetic analysis. Protein sequences predicted and annotated in Phytozome (v.7). Designations shown are in accordance to JGI Populus v1.1, Phytozome Populus v2 and GenBank accession number given when available for Ptr (*Populus trichocarpa*; POPTR_0006s23880, gene model estExt_Genewise1_v1.C_LG_VI2502, GI:224088768; POPTR_0008s15120.1, gw1.VIII.1889.1, GI:224102051; POPTR_0010s09940.1, gw1.X.5941.1, GI:224108055), Ptt (*Populus tremula* × *P. tremuloides*; GenBank accession JX444689) Ath (*Arabidopsis thaliana*; AT5G19530.1|ACL5, GI:18419941), Mes (*Manihot esculenta*; cassava4.1_011290m; cassava4.1_011466m; cassava4.1_011613m), Rco (*Ricinus communis*; 29739.m003604, GI:255550143; 29726.m003894, GI:255551457), Vvi (*Vitis vinifera*; GSVIVT01035450001, GI:225429646; GSVIVT01017948001, GI:225432624), Csi (*Citrus sinensis*; ►

◀orange1.1g019699m; orange1.1g019550m), Ccl (*Citrus clementina*; clementine0.9_015098m; clementine0.9_027824m), Ppe (*Prunus persica*, ppa007950m; ppa008087m), Cpa (*Carica papaya*; evm.model.supercontig_126.36; evm.model.supercontig_58.122), Egr (*Eucalyptus grandis*; Egrandis_v1_0.018516m; Egrandis_v1_0.018847m), Mtr (*Medicago truncatula*; Medtr5g006230.1, GI:357480843), Gma (*Glycine max*; Glyma14g11320.1, GI:356552394; Glyma17g34300.1, GI:356564025; Glyma10g39440.1, GI:356536017; Glyma20g28340.1, GI:359807232) and Hsa (*Homo sapiens*; NP_004586, GI:21264341). All positions containing gaps and missing data were eliminated from the dataset. Sequence alignment and phylogenetic analysis conducted in ClustalX, MUSCLE and MEGA4.

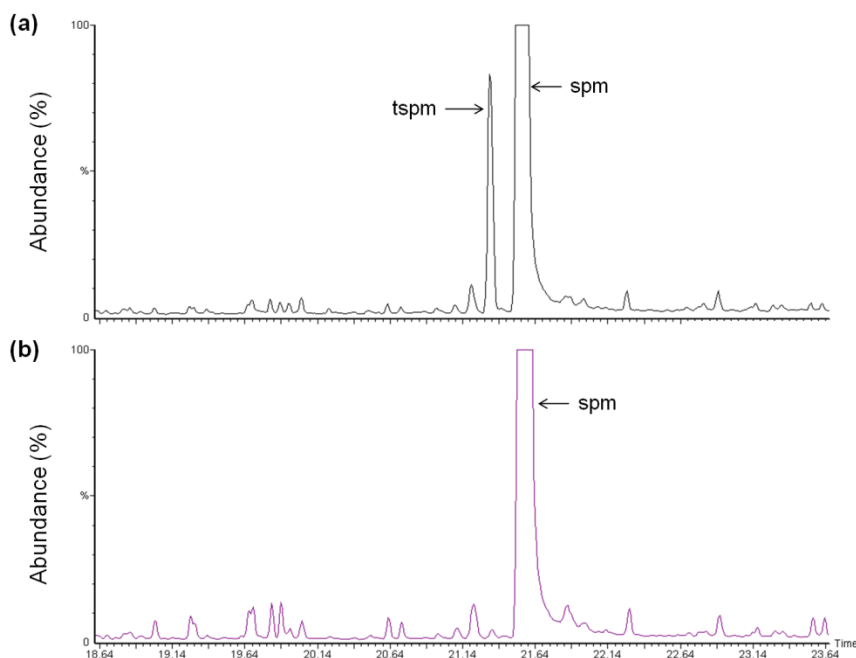


Figure S2. Thermospermine synthase activity validation assays in yeast. Extracted ion chromatograms of the ions m/z 226 and m/z 254 of extracts from (a) yeast strain expressing *POPACAULIS5*, showing the peak for thermospermine (tspm) and (b) yeast control empty expression vector.

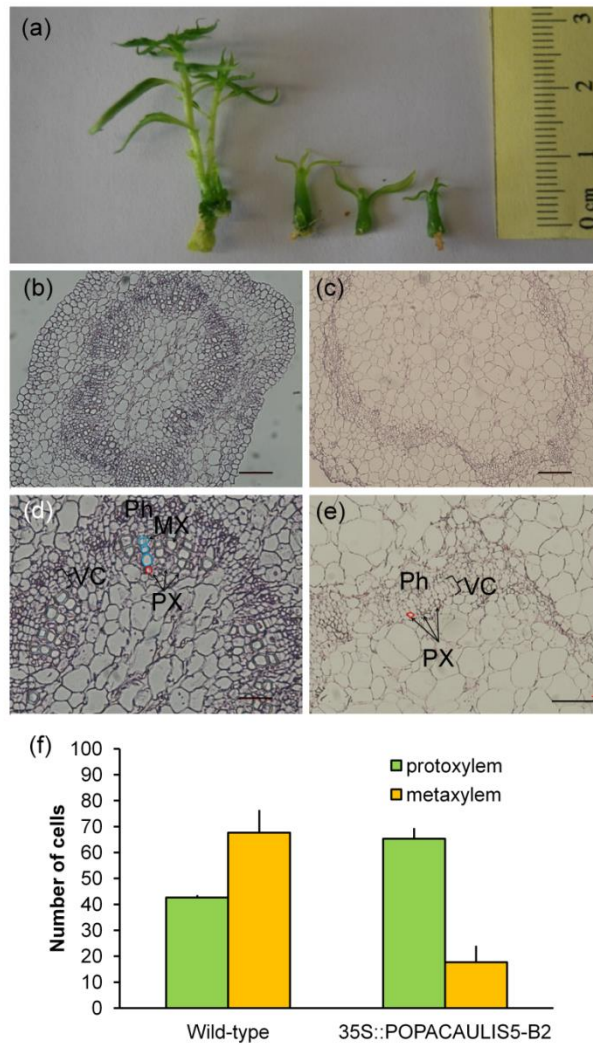


Figure S3. Phenotypic characterization of 35S::POPACAULIS5 transgenic lines grown on auxin-containing medium. (a) Severe dwarfism as an effect of POPACAULIS5 overexpression. Left to right: wild-type and 35S::POPACAULIS5 plants from three independent transgenic lines from group B. Stem transverse sections from wild-type (b) and 35S::POPACAULIS5-B2 dwarf plants (c). The correct collateral display of the phloem and xylem strands observed in the wild-type (d) is not altered in the transgenic lines (e). A decrease in the number of metaxylem cells in the transgenic lines was observed (f). Plant phenotype and transverse stem sections are representative of wild-type and of both 35S::POPACAULIS5 and 35S::PttACL5 transgenic stems. Values are means $\pm$ SD of three biological replicates. MX-metaxylem; Ph-phloem; PX-protoxylem; VC-vascular cambium Scale bars: (b), (c), 200 μ m; (d), (e), 50 μ m.

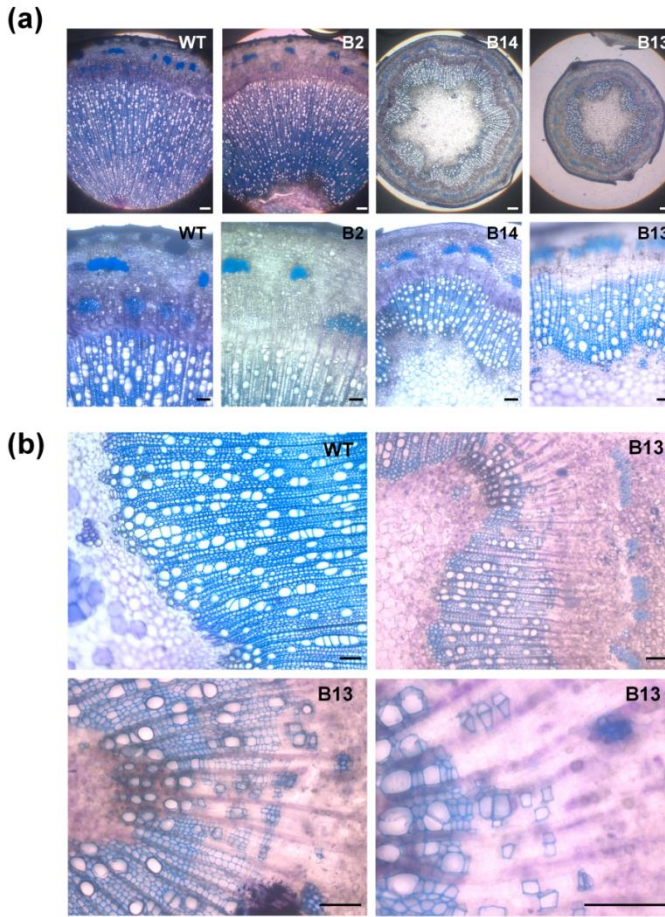


Figure S4. Light microscopy and xylem anatomical analysis to stem sections of greenhouse grown trees, where strong phenotype variation between transgenic lines was observed. (a) Toluidine blue-O stained stem sections evidencing the overall reduced growth in B4 and B13 transgenic trees and width of mature xylem. (b) Detail of secondary xylem cells from B13 transgenic stems, where areas of no secondary cell wall deposition in secondary xylem appear together with well differentiated xylem (lower right). Scale bars: (a) top panel: 200 μm , bottom panel: 100 μm ; (b) 100 μm .

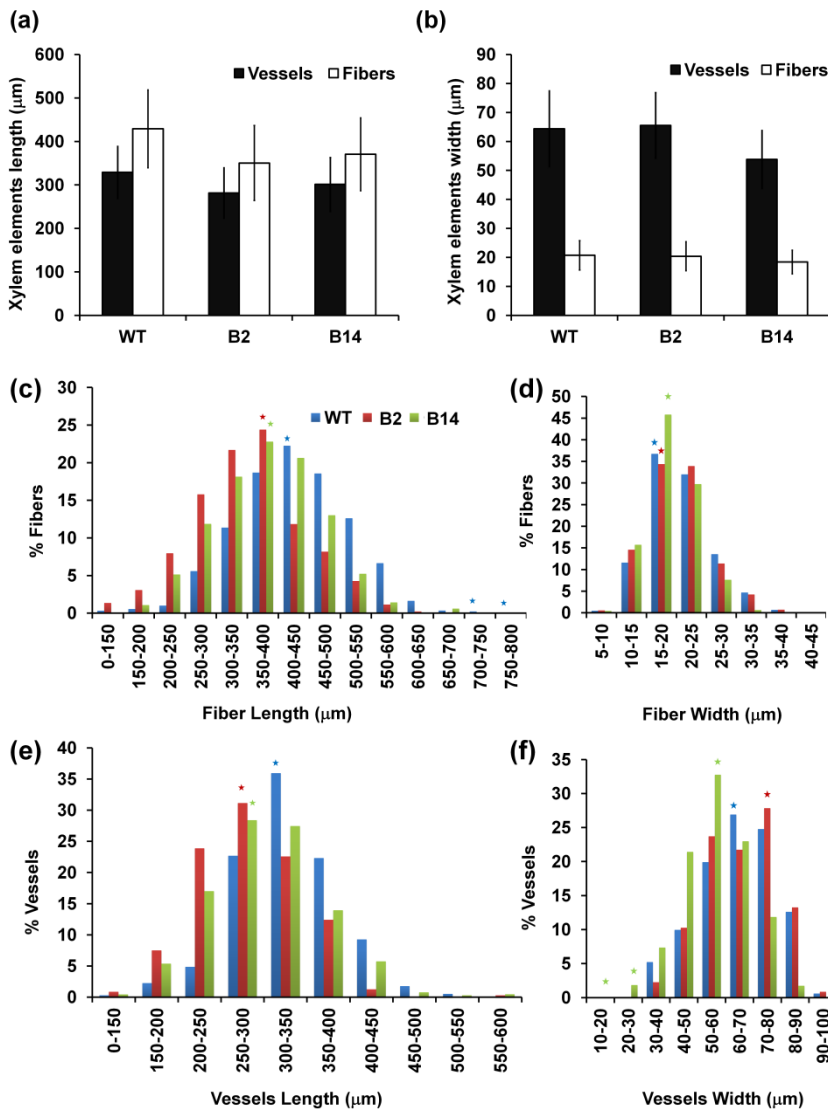


Figure S5. Length and width of fibers and secondary xylem vessels. Measurements were taken from alkaline macerates of the woody stem taken from the reference internode of 2-month old greenhouse grown wild-type and 35S::POPACAULIS5-B2 and B14 transgenic trees. The length of both fibers and vessel elements was not found to be significantly reduced in the transgenic lines. Additionally, fiber and vessel widths were similar amongst the B2 transgenic line and wild-type and smaller in B14. (a) Mean xylem elements length. (b) Mean xylem elements width. (c) Frequency distribution of fibers lengths. (d) Frequency distribution of fibers width. (e) Frequency distribution of vessels length. (f) Frequency distribution of secondary xylem vessels width. Stars represent the peak size interval or the size interval ►

◀ found only in the transgenic lines or only in the wild-type. Over 200 fibers and 50 vessels from minimum four different trees of each genotype were measured. Values represent the percentage of cells that fall into the size interval indicated, obtained from the mean values from at least four different trees analysed.

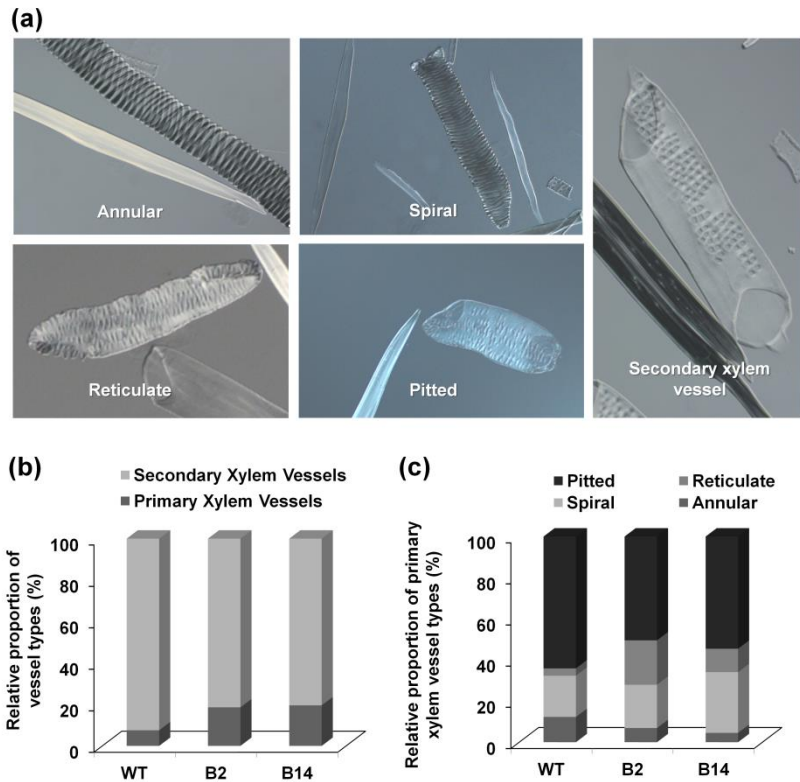


Figure S6. Xylem vessels types distribution in the woody stem of 2-month old greenhouse-grown wild-type (WT) and 35S::*POPACAULIS5* trees (B2 and B14) taken from the reference internode. (a) Primary xylem vessels types found, classified according to the secondary cell wall thickening patterns complexity. Annular is the less elaborate primary xylem vessel cell type found, followed by spiral, then reticulate and pitted, in increasing order of complexity (Esau, 1977). A representative secondary xylem vessel is also shown in the right side of the panel. (b) In B2 and B14 wood, relative proportion of primary xylem vessels to secondary xylem vessels was found to be 2.5- and 2.6-fold that of the wild-type. (c) Primary xylem vessel types relative proportion. Less primary xylem vessels with pitted secondary cell wall thickenings were observed in the transgenic stems. More reticulate and less annular secondary cell wall thickenings amongst the primary xylem vessels were also found. Values presented are percentages of mean values of total primary and secondary xylem vessel cell types found in sampling from at least four woody stems per genotype.

Stage of secondary cell wall deposition

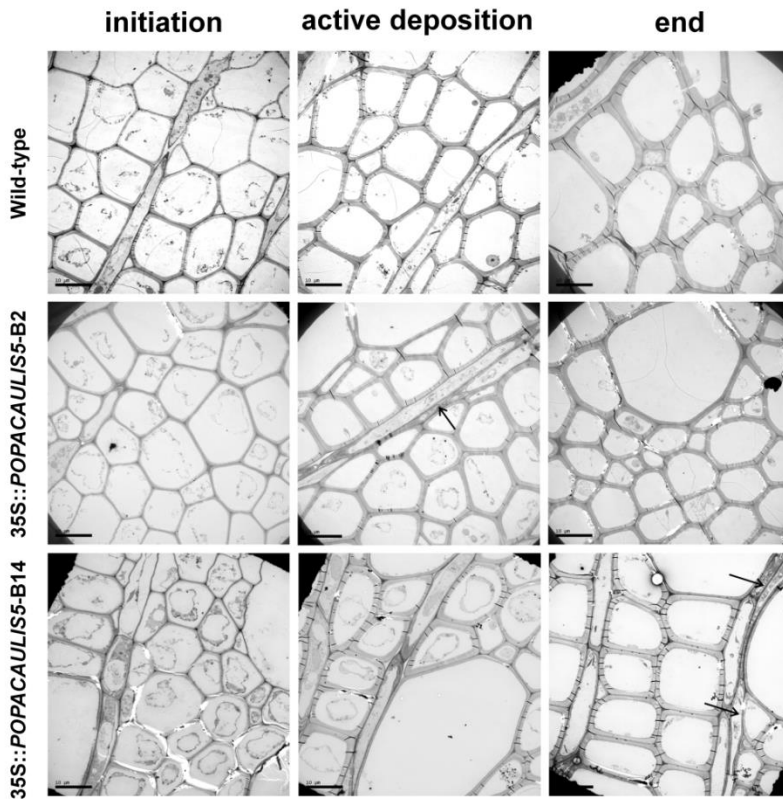


Figure S7. Stages of secondary cell wall deposition as observed by xylem cells ultrastructure analysis in wild-type and transgenic stems examined using transmission electron microscopy (TEM). Secondary cell wall deposition initiates in the transformants and in wild-type at earlier stages of maturation of the xylem cell fibers and vessels when vacuole contents are being released (first row). When secondary cell wall active deposition occurs in B2 and B14 transgenic xylem cells no major differences were observed, at the time of post-central vacuole collapse and post-autolysis of the released vacuole contents. Double rows of ray parenchymatic cells were often observed in the transgenic but not in wild-type secondary xylem (arrows). No differences were observed in the size of intracellular spaces. Secondary cell wall deposition was similar in wild-type, B2 and B14 xylem cells and when the characteristic cell death program is terminated no differences are observed in the ultrastructure of secondary cell walls. Scale bars: 10 μ m.

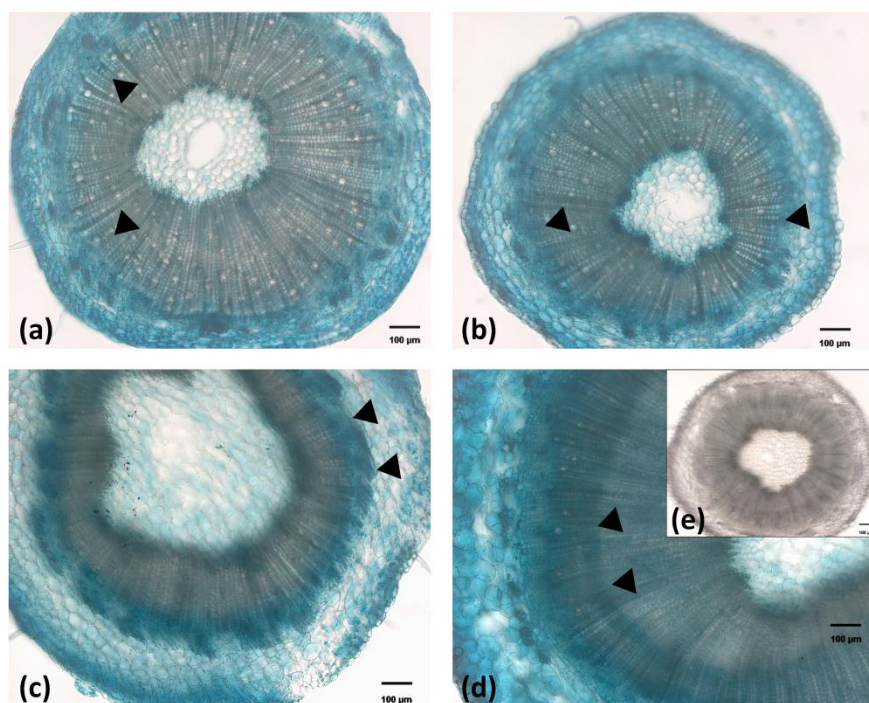


Figure S8. 35S promoter activity in xylem tissues. Histochemical GUS staining pattern in stem transverse sections of three transgenic 35S::*GUS:GFP* lines: (a) L1, (b) L3 and (c) L4. (d) higher magnification of GUS staining in L3. (e) negative control, GUS staining in wild-type stem. Arrows depict strong staining in xylem cells, visible also around the circumference of the stems. Scale bars: 100 μm.

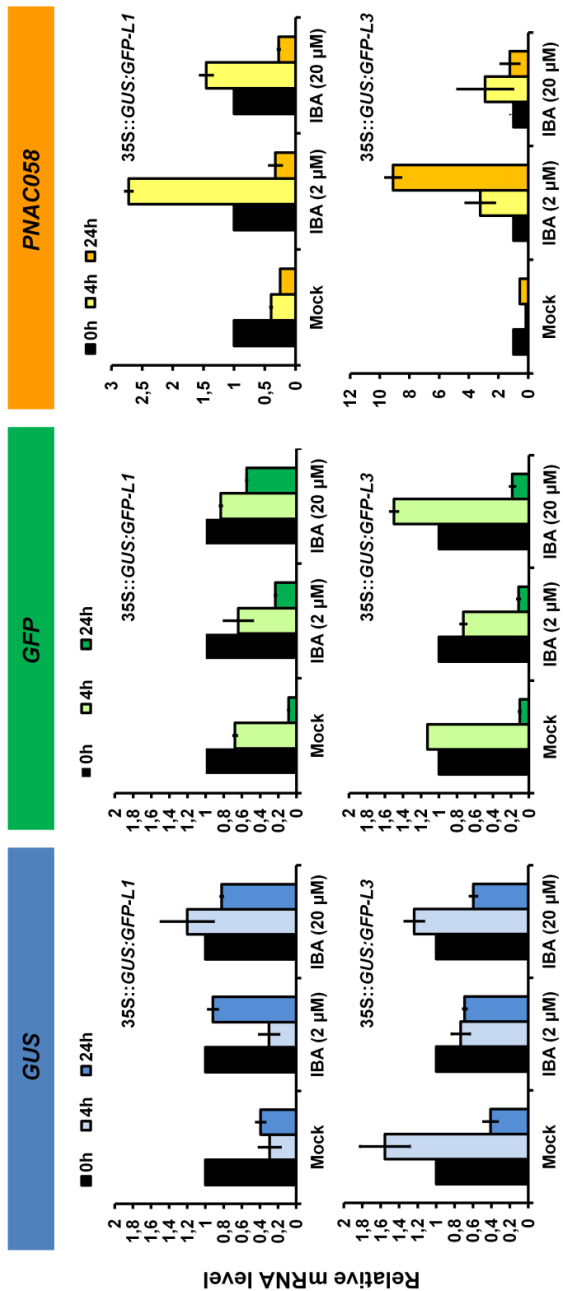


Figure S9. Time course analysis of 35S response to auxin in 35S::GUS:GFP plants, by monitoring GUS and GFP transcript levels. Modulation of auxin in the four week-old *in vitro* grown stem segments of 35S::GUS:GFP-L1 and L3 transgenic plants, started by depleting the samples of their auxin levels, by decapitation, during 16 h, after which stems were transferred to IBA-containing or to the same medium depleted of IBA (mock). Samples were taken at points shown: 0 h, 4 h and 24 h after auxin supply (2 µM and 20 µM IBA) or continuous auxin depletion (mock). GUS and GFP transcript levels were quantified by RT-qPCR. In the stems of L1 and L3 lines, GUS and GFP transcript levels were similar or lower than those of the wild-type, suggesting 35S is not auxin-inducible. As positive control, the transcript levels of *PNAC058*, a homolog to *Arabidopsis VND7*, reported as a xylem-specific and auxin-inducible gene in *Arabidopsis* (Kubo *et al.*, 2005; Hu *et al.*, 2010) were monitored. Increased transcript levels of *PNAC058* could be observed after auxin induction in both lines. Values represent relative transcript levels normalized to 0 h expression values of each genotype. Values are means $\pm$ SD of three technical replicates (consisting of three stems from individual plants pooled at each point).

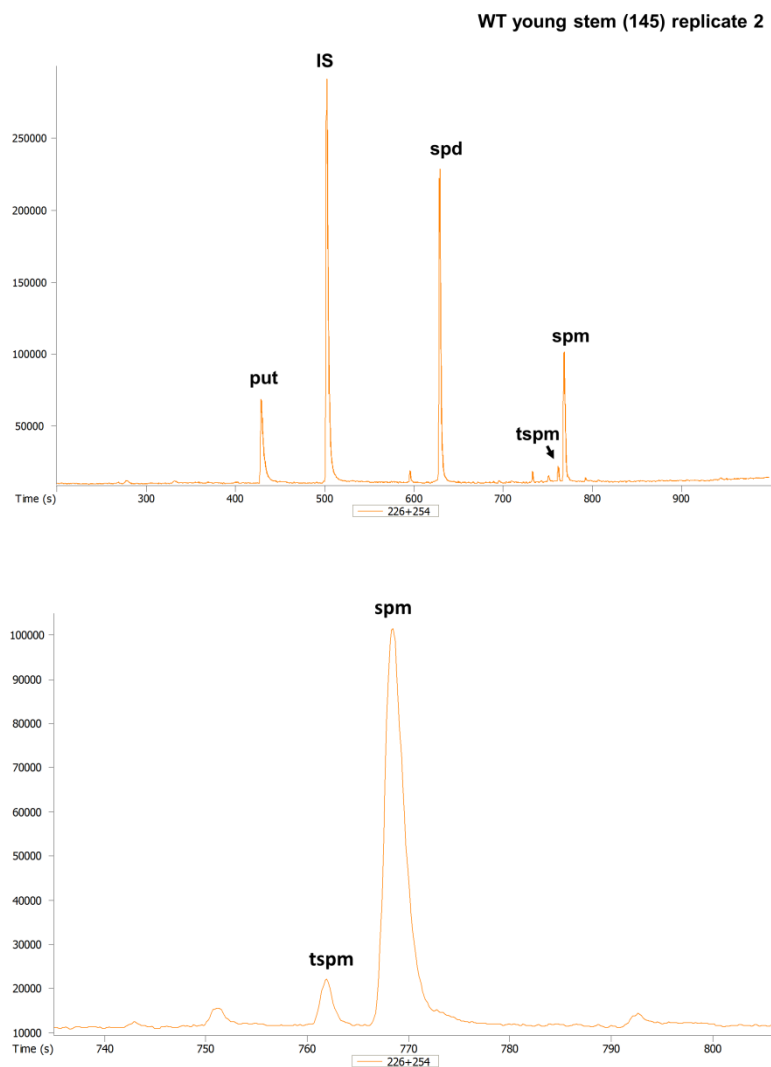


Figure S10. Extracted ion chromatogram of the ions m/z 226 and m/z 254 of an extract of wild-type hybrid aspen young stem by the combined method of ion-pair extraction and GC-MS. Previous analytical methods for polyamines did not allow separation between thermospermine (tspm) and spermine (spm). Polyamine quantitation was performed as described in Rambla *et al.* (2010).

CHAPTER III

THERMOSPERMINE-INDUCED TRANSCRIPTOMIC CHANGES IN *POPULUS* STEMS[†]

[†] **Milhinhos A., Matos A., Miguel C.M.** Thermospermine-induced transcriptomic responses reveal hormone crosstalk in *Populus* stems (*submitted*)

Thermospermine-induced transcriptomic responses reveal hormone crosstalk in *Populus* stems

Summary

Polyamines are small organic cations essential for life. One plant polyamine, thermospermine, has proved essential for proper vascular development and xylem differentiation. We have previously identified *POPACAULIS5* gene that controls the thermospermine levels in *Populus* xylem and proposed a feedback regulatory mechanism that maintains thermospermine homeostasis. In the present study, using microarray and microscopic structure analysis we investigated the molecular and cellular effects of increasing thermospermine production in the *Populus* stems. At the cellular level, increased levels of thermospermine affect the cambial zone, where the atypical vascular cambium fails in differentiating xylem. At the transcriptome level, we present data suggesting that *POPACAULIS5* overexpression has a positive effect on cytokinin levels, perception and signalling, while auxin levels, distribution and responsiveness are negatively affected. The plant developmental and architectural defects imposed by increased *POPACAULIS5* expression and simultaneous auxin supply were correlated to an increase in ethylene perception and response. Well known cambial and xylem regulators were also found altered in our data. Overall, these results provide a framework to detailed genetic dissection of thermospermine molecular mode of action in xylem differentiation in higher plants.

Keywords

Polyamine, thermospermine, *ACAULIS5* (*ACL5*), *POPACAULIS5*, vascular development, xylem, auxin, cytokinin, ethylene

Introduction

Polyamines are ubiquitous biogenic amines that are essential for animal and plant life. In plants, putrescine, spermidine, spermine and thermospermine are the most common polyamines found and have been proposed to function in responses to environmental stresses (Perez-Amador *et al.*, 2002; Yamaguchi *et al.*, 2006; Kusano *et al.*, 2007; Naka *et al.*, 2010; Alcázar *et al.*, 2006; Wang *et al.*, 2011; Gonzalez *et al.*, 2011; Sagor *et al.*, 2012) and in growth and development (Flores *et al.*, 2008; Nambeesan *et al.*, 2010; Trénor *et al.*, 2010; Hanzawa *et al.*, 2000; Clay and Nelson, 2005; Alcázar *et al.*, 2005; Muñiz *et al.*, 2008); among other roles (see for review Kusano *et al.*, 2008; Alcázar *et al.*, 2010; Handa and Mattoo, 2010, and references therein). Defects in stem growth and elongation have been reported after manipulation of polyamines metabolism (Alcázar *et al.*, 2005; Clay and Nelson, 2005; Muñiz *et al.*, 2008). One polyamine biosynthesis gene, *ACAULIS5* (*ACL5*) codes for thermospermine synthase, that produces thermospermine by transferring an aminopropyl group from the decarboxylated S-adenosylmethionine to an amine acceptor on spermidine (Knott *et al.*, 2007). *ACL5* has been found specifically expressed in procambium and xylem elements in *Arabidopsis* inflorescence stems and its loss-of-function mutant lacked stem elongation and secondary growth (Hanzawa *et al.*, 2000; Clay and Nelson, 2005; Muñiz *et al.*, 2008; Kakehi *et al.*, 2008). Thermospermine seems to be the first polyamine described to have a specific role in xylem differentiation.

The increased auxin responsiveness at the vascular cambium and its close vicinity is known to be essential for xylem development in trees (Uggla *et al.*, 1996; Tuominen *et al.*, 1997; Nilsson *et al.*, 2008; Baba *et al.*, 2011). In *Arabidopsis*, several studies have also shown that auxin transcriptionally activates *ACL5* (Hanzawa *et al.*, 2000; Imai *et al.*, 2006; Rambla *et al.*, 2010; Vera-Sirera *et al.*, 2010) and although the underlying mechanisms linking

ACL5 to auxin are unknown, some hints are emerging. In *Populus*, we have previously found that *POPACAULIS5* (the *Populus ACL5* ortholog) stable overexpression is dependent on auxin presence in the growth medium. On the other hand, *POPACAULIS5* expression suppresses the endogenous auxin levels in *Populus* leaves and xylem tissues (Chapter II). The opposite roles of auxin and thermospermine in xylem development have recently been further clarified by Yoshimoto *et al.* (2012a). The application of auxin analogs to *acl5* loss-of-function mutant seedlings revealed that the lack of thermospermine potentiated the increase in xylem differentiation, but xylem differentiation was suppressed when auxin inhibitors or exogenous thermospermine were added to the medium (Yoshimoto *et al.*, 2012a). This suggests thermospermine plays a role in channelling the auxin effect on xylem differentiation, but it has an opposite effect to auxin in xylem differentiation.

The auxin-induced *POPACAULIS5* expression resulted in the stunted growth of the 35S::*POPACAULIS5* plants. However, depletion of auxin from the growth medium allowed almost full recovery of the normal phenotype (Chapter II). Although several reports have identified downstream events to *ACL5* expression (Imai *et al.*, 2006; 2008) no reports currently exist on the transcriptome response to changes in *ACL5* expression. We wanted to elucidate the surprising effect auxin-containing growth medium has on the phenotype of the 35S::*POPACAULIS5* stems and find cues to possible hormone crosstalks with thermospermine at the molecular level. Therefore, we report here on the transcriptome changes in stems of *Populus* plants having altered thermospermine production. An overview of the effects on known cambial and xylem regulators is provided which may prove valuable to increase our understanding of the molecular functions of thermospermine.

Experimental procedures

Plant material, growth conditions and sampling

Wild-type hybrid aspen (*Populus tremula* L × *P. tremuloides* MICHX) clone T89 and 35S::*POPACAULIS5* plants were maintained by subculture every five weeks, on MS basal salt medium at half-strength (Murashige and Skoog, 1962), termed MS6 or PGRs-depleted medium. Transformation of petioles with the *POPACAULIS5* cDNA under the influence of 35S CaMV constitutive promoter was performed as previously reported (Chapter II). Regenerated transgenic shoot were elongated on MS medium, with 20 g l⁻¹ sucrose, 0.1 µg ml⁻¹ indole-butyric acid (IBA), 0.2 µg ml⁻¹ 6-benzyl-aminopurine (BAP), 500 µg ml⁻¹ cefotaxime and 80 µg ml⁻¹ kanamycin-monosulphate, termed MS2 or PGRs-containing medium, with every two-weeks subculture (Nilsson *et al.*, 1992). After elongation, shoots were transferred to MS6 and maintained by regular subcultures. *In vitro* plants were grown in growth chambers at 21°C and 16 h light/8 h dark photoperiod.

Five-week-old, *in vitro* grown stem segments were collected from wild-type and transgenic line 35S::*POPACAULIS5*-B2, grown on MS6 medium. Two week-old stem segments were collected from wild-type and 35S::*POPACAULIS5*-B2 transgenic plants, grown on MS2 medium. Sampled tissues were directly frozen in liquid nitrogen when collected (all at the same day and time) and stored at -80°C. Three pools of stem segments from three individual plants (nine in total) from each line (wild-type and transgenic) in both growth conditions (MS6 and MS2) were used, constituting three biological replicates for each pair genotype/growth condition.

RNA extraction and quality assessment

Total RNA was extracted and isolated from each pool of stem tissues using

the RNeasy Plant Mini Kit (Qiagen). Concentration and purity were determined by spectrophotometer measurements and gel electrophoresis. RNA samples A260/A280 ratios ranged from 1.86 to 2.17. Integrity was confirmed using an Agilent 2100 Bioanalyser with an RNA 6000 Nano assay (Agilent Technologies).

Array hybridizations and quality control

The Affymetrix Poplar Genome Arrays were labeled and hybridized at the Gene Expression Unit (Affymetrix Core Lab, Instituto Gulbenkian de Ciência), according to Affymetrix protocols. Arrays were scanned in Affymetrix GeneChip scanner 2500. The quality of data was first assessed by probe array image inspection, average background and noise values calculation, poly-A controls (lys, phe, thr, dap), hybridization controls (bioB, bioC, bioD, and cre), internal control genes (3' to 5' ratios of β -actin), percentage of absent/present calls, scaling and normalization factors. Relative log expression signal, relative log probe cell intensity and Pearson correlations of expression signal were assessed with Affymetrix MAS 5.0 software. To ensure reliability and reproducibility of the results each GeneChip experiment was performed in three biological replicates.

GeneChip Data analysis

The GeneChip Poplar Genome Array has 61,251 probe sets representing 56,055 transcripts and gene predicted transcripts that were used to obtain the transcriptional profiles of *Populus* stems from wild-type (WT) and 35S:POPACAULIS5-B2 transgenic line (B2) grown on MS2 and MS6 growth media. Analysis was pursued using dChip (<http://www.dchip.org>, Wong Lab, Harvard, Li and Wong, 2001a). Normalization parameters applied were those widely described (Pina *et al.*, 2005; Boavida *et al.*, 2011;

Costa *et al.*, 2013). We took a sample wise normalisation, where the array with median overall intensity was used as the baseline array against which other arrays were normalized at the probe intensity level. Thus, the median probe cell intensity (CEL) of one of the arrays (the median array) was scaled to the median CEL intensity of all arrays (12 arrays with a median CEL intensity of 79). The remaining replicates were normalized to this baseline array applying an Invariant Set Normalization Method (dchip, Li and Wong, 2001a). Normalized CEL intensities of the 12 arrays were used to obtain model-based gene expression indices based on a Perfect Match-only model (Li and Wong, 2001b). Genes called present in at least 20% of the arrays and within a variation across samples of $0.5 < (SD/mean) < 10$ were used for global gene expression profile analysis (6343 probesets). Thus, genes called absent in all arrays and genes with inconsistent presence were excluded. Non-logged mean signal intensity (MSI) values for the probesets discussed herein are presented in Supporting Information (Tables S1-S6). Global clustering analysis was performed using dchip. Finally, all genes used in pair wise comparisons were considered differentially expressed if the 90% lower confidence bound of the fold change between experiment and baseline was above 1.2 (Tables S1-S6). The lower confidence bound criterion defines that one can be 90% confident that the fold-change values are between the lower and the upper confidence bound values. The lower confidence bound is a conservative estimate of the fold change and consequently more reliable as a ranking statistic for changes in gene expression (Li and Wong 2001a,b). This criterion has been used in other gene expression studies (Becker *et al.*, 2003; Pina *et al.*, 2005; Boavida *et al.*, 2011; Zhang *et al.*, 2011; Costa *et al.*, 2013). GO terms enrichment used in the pairwise comparisons was employed by performing the cross comparison of singular enrichment analysis with *AgriGO* tools, using the statistical Fisher test, with a significance level of 0.05 and a minimum number of five mapping entries (Zhou *et al.*, 2010). Annotations for the approximately 56 000 genes represented on the Poplar

genome array were obtained from the NetAffx database (www.affymetrix.com) as of November 2010 and when possible were further completed with resource to the PopArray probe annotation tool (Tsai *et al.*, 2011) and with resource to literature. All GeneChip datasets are available in a MIAME-compliant format through NCBI GEO (GSE45879).

Several authors have repeatedly reported that consistency in results obtained from Affymetrix platform is very high (see Bao *et al.*, 2009; Hu *et al.*, 2010; Gou *et al.*, 2010; Zhang *et al.*, 2011). Because several quality control studies report that results obtained from RT-PCR sustain the gene expression from Affymetrix microarrays, and that RT-PCR is not substantially more precise than the array platform employed itself (Bao *et al.*, 2009; Hu *et al.*, 2010; Gou *et al.*, 2010; Zhang *et al.*, 2011; Costa *et al.*, 2013), RT-PCR validation was not performed.

Stem microscopic structure analysis

To analyse the microscopic structure of the plant stems, 10 µm thick transverse and longitudinal sections of paraffin embedded stem tissues from WT or lines B2, D153 and D155 were stained with eosin/hematoxylin. For sectioning a Leica RM2155 microtome was used. Fresh plant tissues were hand-sectioned and stained with toluidine blue-O general stain. Detection of callose in fresh hand-made sections of plant stems was performed using the aniline blue staining method. Briefly, sections were incubated for 5 min in a sodium phosphate buffer solution. Incubation of stem sections in a 0.05% aniline blue in phosphate buffer solution for 60 min was followed by rinsing with distilled water until complete clearance of the aniline blue solution. Samples were immediately observed under a microscope with bright-field and fluorescence. All observations were performed with a Nikon Eclipse TE 300 microscope and images registered using a Nikon Digital Sight DS-Fi1 video camera.

Results and Discussion

Populus growth defects in vitro as a result of *POPACAULIS5* overexpression

Severe disruption of growth was observed in 35S::*POPACAULIS5* transgenic *Populus* grown on a PGRs-containing medium (MS2) routinely used to promote elongation of transformed shoots (Figure 1). MS2 medium is

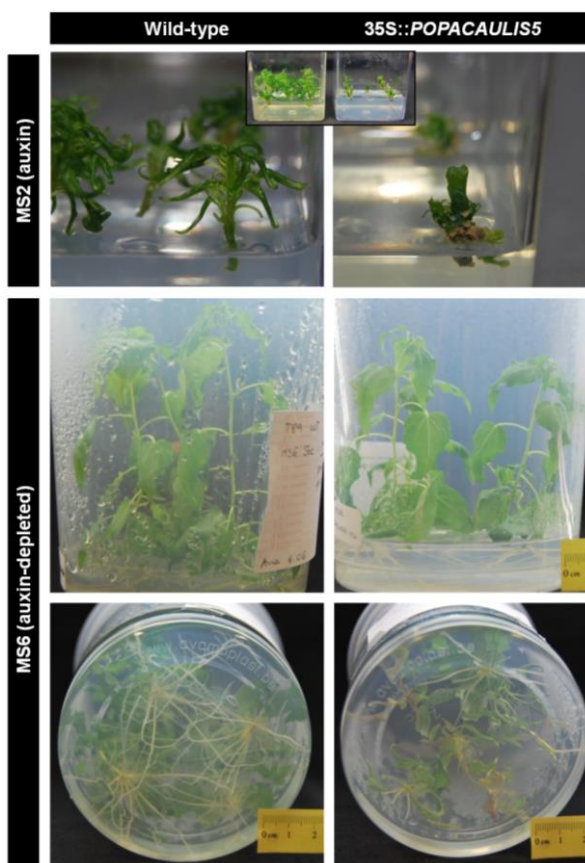


Figure 1. Morphology of wild-type and 35S::*POPACAULIS5* stems. The *in vitro* growth on auxin-containing medium MS2 imposed a specific dwarfing effect on the transformed plants, not observed in the wild-type plants. The transition of transgenic plants to the auxin-depleted MS6 medium was accompanied of recovery the transformed plants (Chapter II).

supplemented with indole-3-butyric acid (IBA), an auxin; 6-benzylaminopurine (BAP), a cytokinin; and sucrose as a source of carbon (Nilsson *et al.*, 1992). We have previously described that expression of

POPACAULIS5 under CaMV 35S constitutive promoter is induced by auxin presence in the medium but repressed by a feedback loop mechanism that controls thermospermine levels in the stems of plants growing free from auxin (Chapter II). Increased *POPACAULIS5* auxin-induced expression was accompanied by arrestment of shoot development, evidenced by the lack of internode elongation and inhibition of root development, resulting in dwarf phenotype plants in several 35S::*POPACAULIS5* independent lines (Figure 1). More importantly, the defects were overcome when the dwarf transgenic plants from line B2 were transferred from MS2 to a PGRs-depleted medium (MS6) as previously described (Chapter II). Microscopic analysis of wild-type stems revealed that wild-type plants grown on MS2 have normal vascular development (Figure 2a). Transverse sections of 35S::*POPACAULIS5*-B2 dwarf stems grown on MS2 medium, on the other hand, revealed the presence of lower number of xylem cells than in the wild-type as well as a wider stem, with larger pith parenchymatic cells (Figure 2b). Longitudinal sections of the B2 transgenic stems further revealed that xylem in the vasculature was immature, where protoxylem with annular cell wall thickenings extended throughout the plant stem but more elaborate vessel cell types and fibers were not present (Figure 2c-f). Therefore, the increased *POPACAULIS5* expression in the transgenic plants grown under auxin influence prevents the normal xylem progression, which supports that thermospermine has a relevant role in preventing the auxin-induced xylem differentiation (Muñiz *et al.*, 2008; Vera-Sirera *et al.*, 2010; Yoshimoto *et al.*, 2012a).

Several transgenic lines failed in reversing the dwarf phenotype once transferred to the rooting medium which correlated well with increased levels of thermospermine accumulation (Chapter II). Two of those transgenic lines, 35S::*POPACAULIS5*-D153 and D155, were further analysed by microscopy to elucidate on the vascular defects caused by the strong thermospermine increase. Contrary to B2 stems, both D153 and D155 showed an atypical

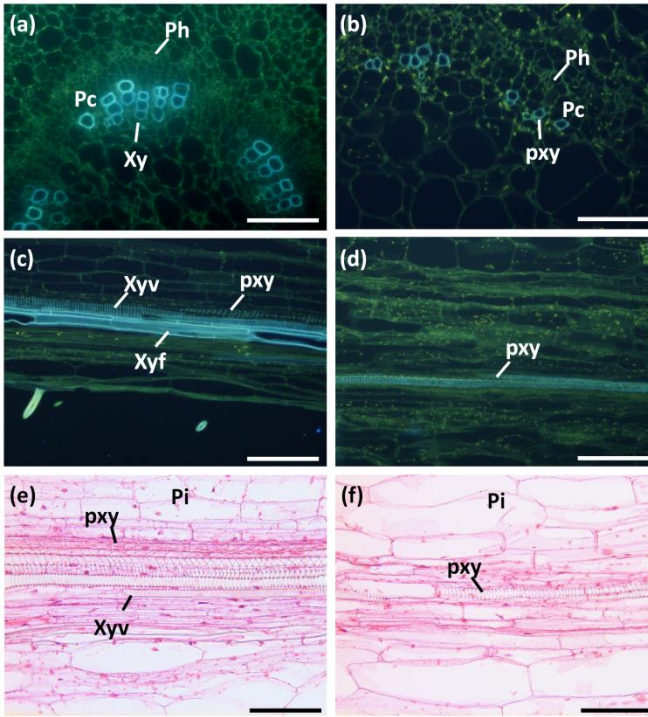


Figure 2. Anatomical features of vascular tissues from wild-type and 35S::POPACAULIS5-B2 stems. Growth on the auxin-containing MS2 medium revealed a regular vascular pattern formation as shown in the wild-type hybrid aspen stem cross section (a). The 35S::POPACAULIS5-B2 stem cross section grown in the MS2 medium showed arrested metaxylem formation and larger parenchymatic cells (b).

Longitudinal sections of the wild-type stems showed the presence of protoxylem, annular secondary cell wall thickenings in xylem, and xylem fibers and vessels beginning to be formed in (c) and (e). The generalized stunting in growth of 35S::POPACAULIS5-B2 plants reveals the arrestment in xylem development as shown in longitudinal stem sections, where xylem present seems immature, where mostly protoxylem is observed in (d) and (f). Hematoxylin-eosin staining was used to highlight xylem cells observed under the UV fluorescence microscope (a-d) and bright field (e,f). Pc, procambium; Xy, xylem; Ph, phloem; pXy, primary xylem; Xyv, xylem vessel; Xyf, xylem fiber; Pi, pith. Scale bars: 50μm.

cambial zone where the vascular cambium failed to be completely formed (refer to Figures S1 and S2 in Supporting Information). Moreover, in the transgenic stems the cambial cells were found to differentiate only into one specialized cell type. To understand if cambial cells were exclusively specified into phloem cells we used aniline blue staining to detect callose that is preferentially formed in phloem sieve cells. No xylem cells could be observed, whereas phloem cells within the cambial zone were visible in transgenic D153 stems grown on MS2 medium (Figure S3). According to our

observations of microscopic structure, this is the major differential aspect between lines that are able to recover the phenotype and those that maintain dwarf. Altogether, the lack of xylem cells as a consequence of increasing thermospermine production in the stem of the transgenic plants suggests that thermospermine has a role in preventing xylem specification in *Populus* stem, raising the question to whether thermospermine is somehow decisive prior to xylem cell fate acquisition during vascular cambium formation.

Identification of thermospermine-induced global transcriptome responses

To identify genes involved in the disruption of xylem differentiation in the dwarf transgenic plants through a global transcriptome profiling analysis we obtained expression profiles in four conditions: stems of wild-type (WT) and transgenic 35S::*POPACAULIS5* (B2) plants propagated on normal standard conditions *in vitro*, depleted from auxin or other PGRs (MS6); and grown on elongation promoting (auxin-containing) medium (MS2). A total of 6343 probes were identified as differentially expressed within all samples, representing 5611 unique genes. We clustered the genes for analysis of the major biological processes represented in the *Populus* stems under each experimental condition. Twelve clusters were obtained representing the different patterns of expression that were further grouped according to the more general pattern of up or downregulated genes in the samples (Figure 3). A first glance global analysis of the biological processes represented in the *Populus* stems revealed that in wild-type stems grown on MS6 medium (WT MS6) there was a clear investment in building up plant body by committing to cell wall biogenesis processes with low metabolic effort. Based on the GO annotation, we observed that genes related to cell wall, cellulose synthase activity, cellular glucan metabolic process, amongst others, were up-regulated in wild-type stems grown under regular hormone-depleted growth conditions (clusters 8-12). However, analysis of wild-type stems grown on

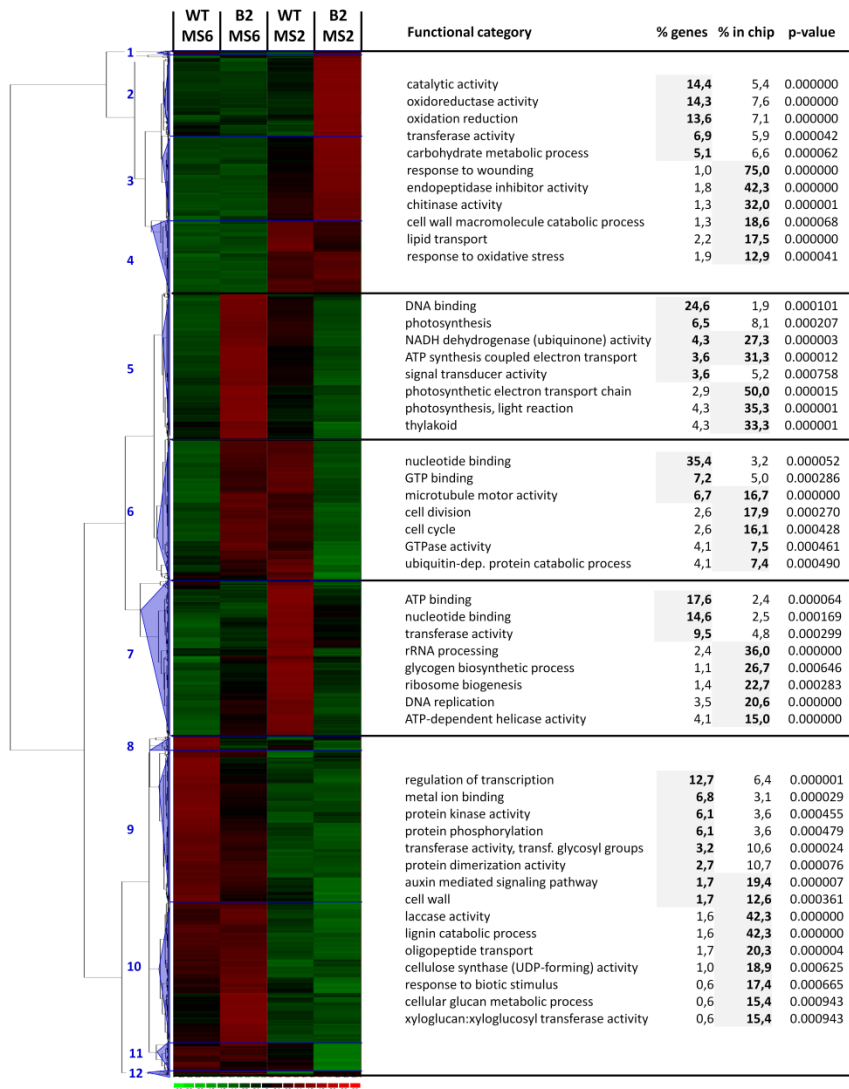


Figure 3. Hierarchical clustering of all differentially expressed genes in stems of wild-type and 35S::POPACAULIS5 transgenic line B2. Mean expression values are represented where green denotes downregulation and red denotes upregulation. After filtering the genes, 5611 unique genes were clustered (after each probe set expression value was standardized to have mean 0 and standard deviation 1). Redundant probe sets were masked (732 redundant probe sets). Twelve sub-clusters reflecting different patterns of expression were identified and the corresponding branches were analysed for significant functional enrichment. The most represented functions are listed. The enrichment in a function in all the clusters was considered relevant at p-value threshold of 0.001. The relative amount of genes that have a given annotation term identified in each group of sub-clusters is indicated as “% genes”, relative to the total number of genes in that same group of sub-clusters. The number of genes present in the sub-cluster relatively to all genes on array that have that same annotation term is also given as “% in chip”.

auxin-containing medium (WT MS2) revealed an opposite profile where metabolism was highly active, with processes such as cell division, ribosome biogenesis, protein metabolic processes being induced, but less investment was channelled to cell wall related processes (clusters 6 and 7), which is not surprising since this is a multiplication-promoting medium. Analysis of transgenic stems grown on MS6 medium (B2 MS6), revealed the investment in cell wall metabolism related genes (similarly to what happens in wild-type) but interestingly there was upregulation of photosynthesis related genes (cluster 5) and cell-cycle related genes (cluster 6), the latter also activated in WT MS2. B2 MS6 plants also showed upregulation of auxin signalling related genes (Cluster 10). On the other hand, B2 MS2 plants seem to avoid efforts on metabolic or cell wall related processes to invest on catabolic and stress response processes (clusters 1-4). The phenotype observed may well be a consequence of this shut down of molecular functions that seem to be active in WT MS2.

Four pair wise comparisons: (a) WT MS6 / B2 MS6, (b) WT MS2 / B2 MS2, (c) WT MS6 / WT MS2 and (d) B2 MS6 / B2 MS2 were performed using a stringent cut off (90% lower confident bound (LCBs) of fold-change > 1.2 , $P < 0.01$) to highlight specific mechanisms (Figure S4, Table I). The ectopic expression of *POPACAULIS5* (WT MS6/B2 MS6) upregulated genes involved in primary metabolic processes, biosynthetic processes, RNA processing, photosynthesis, response to hormone stimulus, cell cycle, among others; and downregulated genes involved in cellular components vesicle and cell wall. However, introducing auxin in the medium (WT MS2/ B2 MS2) downregulated most of the genes involved in the biological processes that were induced in B2 MS6 stems, such as RNA processing, regulation of hormone levels, cell cycle and cell wall biogenesis. As expected from the histological observations, the cell wall macromolecular catabolic process-related genes were found up-regulated in B2 MS2 stems (Table I).

Table I. Gene ontology (GO) functional classification of differentially expressed genes in stems of 35S::POPACAULIS5 transgenic *Populus*.

GO cat.	GO Term	Function category	(a) WT MS6 / B2 MS6		(b) WT MS2 / B2 MS2		(c) WT MS6 / WT MS2		(d) B2 MS6 / B2 MS2	
			Up	Down	Up	Down	Up	Down	Up	Down
Biological Process	GO:0044238	primary metabolic process	368	73	111	492	522	222	235	264
	GO:0006807	nitrogen compound metabolic process	227	36	68	282	310	126	134	160
	GO:0009058	biosynthetic process	197	34	51	243	269	101	116	135
	GO:0006139	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	154	24	36	197	208	78	71	103
	GO:0010467	gene expression	137	17	17	159	159	61	15	88
	GO:0006950	response to stress	72	24	42	97	129	59	100	46
	GO:0005975	carbohydrate metabolic process	66	20	35	84	114	53	79	40
	GO:0009056	catabolic process	50	9	28	55	86	34	55	45
	GO:0007165	signal transduction	38	15	12	61	49	38	25	29
	GO:0006952	defense response	28	13	15	35	47	16	41	18
	GO:0022613	ribonucleoprotein complex biogenesis	27	*	*	34	37	*	6	11
	GO:0009314	response to radiation	23	20	5	20	15	23	8	21
	GO:0006396	RNA processing	23	*	*	35	36	*	*	14
	GO:0009725	response to hormone stimulus	21	14	12	34	33	35	26	23
	GO:0006979	response to oxidative stress	16	5	10	20	32	20	25	16
	GO:0009607	response to biotic stimulus	15	6	14	22	34	17	34	11
	GO:0015979	photosynthesis	15	*	*	7	134	*	*	13
	GO:0006955	immune response	13	*	13	13	7	6	12	10
	GO:0007049	cell cycle	6	*	*	14	13	*	*	8
	GO:0042546	cell wall biogenesis	6	*	*	9	6	8	*	5
	GO:0010817	regulation of hormone levels	5	*	*	7	9	5	6	5
	GO:0051726	regulation of cell cycle	*	*	*	*	*	*	*	5
	GO:0016998	cell wall macromolecule catabolic process	*	*	7	*	6	*	12	*
	GO:0006869	lipid transport	*	*	12	*	7	7	*	*

Table I. (continued)

GO cat.	GO Term	Function category	(a) WT MS6 / B2 MS6		(b) WT MS2 / B2 MS2		(c) WT MS6 / WT MS2		(d) B2 MS6 / B2 MS2	
			Up	Down	Up	Down	Up	Down	Up	Down
Molecular Function	GO:0005488	binding	481	98	136	578	632	279	284	348
	GO:0016798	hydrolase activity, acting on glycosyl bonds	33	*	26	30	47	18	39	15
	GO:0016491	oxidoreductase activity	14	21	40	77	115	59	86	50
	GO:0004857	enzyme inhibitor activity	*	12	21	8	10	9	21	*
	GO:0004674	protein serine/threonine kinase activity	*	11	13	*	*	*	20	*
	GO:0004867	serine-type endopeptidase inhibitor activity	*	*	8	*	10	*	8	*
	GO:0004568	chitinase activity	*	*	7	*	8	*	13	*
Cellular Component	GO:0005737	cytoplasm	436	88	150	505	559	248	278	298
	GO:0016020	membrane	143	66	88	205	197	173	141	130
	GO:0005634	nucleus	118	19	18	150	152	68	31	102
	GO:0005739	mitochondria	114	25	45	*	*	60	79	70
	GO:0009579	thylakoid	41	*	6	20	24	*	5	29
	GO:0005856	cytoskeleton	22	*	*	23	19	7	*	16
	GO:0005783	endoplasmic reticulum	19	*	5	28	38	7	12	14
	GO:0031975	envelope	15	*	11	16	21	*	19	11
	GO:0031982	vesicle	*	36	45	*	*	*	*	*
	GO:0005618	cell wall	*	19	10	20	17	29	17	13

WT MS6 and B2 MS6, wild-type and 35S::POPACAU¹IS5-line B2 grown on auxin-depleted MS6 medium; WT MS2 and B2 MS2, wild type and 35S::POPACAU¹IS5-line B2 grown on auxin containing MS2 medium. The number of up- or down- regulated genes under each GO category for each pairwise comparison is indicated. *Up* indicates that the higher signal was from the second member of the pair-wise sample comparison, while *Down* indicates that the higher signal was from the first member of the comparison. *p* < 0.05 Fischer test and FDR <0.05 are highlighted in bold. Asterisks indicate less than five genes or no gene represented in the category. A given gene may be present in more than one functional category.

Increasing POPACAULIS5 expression does not affect other polyamine biosynthetic enzymes

Investigation of the related genes indicated that the polyamine biosynthesis was not affected by the increase in thermospermine synthase activity. From all the genes known to belong to the polyamine biosynthetic pathway present in the array (arginine decarboxylase, agmatine iminohydrolase, ornithine decarboxylase, S-adenosylmethionine decarboxylase, thermospermine/ spermine/ spermidine synthase), none with the exception of probesets for the thermospermine synthase and putative S-adenosylmethionine decarboxylase proenzyme encoding genes were found differentially expressed in our dataset (Table S1). The manipulated thermospermine synthase gene was found induced and two other probesets representing putative *POPACAULIS5* paralogs were found highly repressed in B2 MS2 stems. This may indicate that the feedback repression we have previously found for *POPACAULIS5* also affects the other paralogs transcript levels. On the other hand, two putative polyamine oxidase genes (PAOs), which are involved in the maintenance of polyamine homeostasis (reviewed in Cona *et al.*, 2006; Moschou *et al.*, 2008) were found differentially expressed in the samples. Both predicted proteins show similarities to *Arabidopsis* PAO4 that catalyzes the oxidative conversion of spermine to spermidine (Kamada-Nobusada *et al.*, 2008; Takahashi *et al.*, 2010; Fincato *et al.*, 2011). The putative *Populus* homolog to PAO4 was found upregulated in transgenic B2 MS6 stems but especially induced by auxin in WT MS2 and to a lesser extent in B2 MS2 (Table S1). AtPAO4 has been shown to oxidise thermospermine although with lower affinity to thermospermine when compared to other PAOs (such as AtPAO1 or AtPAO2) (Kamada-Nobusada *et al.*, 2008; Takahashi *et al.*, 2010; Fincato *et al.*, 2011). These results are in agreement with our previous work showing no augment in the other polyamines in the 35S::*POPACAULIS5* plants (Chapter II). It seems, therefore, that affecting

the thermospermine content in stems does not lead to a significantly higher back-conversion effect in the transgenic plants, suggesting thermospermine homeostasis is mainly mediated by other mechanisms.

Thermospermine affects cell cycle-related genes

Polyamines are important molecules for proper cell cycle progression (Alm and Oredsson, 2009). Since less differentiated xylem cells were present in B2 MS2 stems (Figure 2) we questioned whether cell cycle genes were affected in transgenic plant stems. Indeed, cell cycle genes were downregulated in B2 MS2 stems, but more importantly induction of cell cycle genes occurred in B2 MS6 stems (Figure 4 and Table S2). This leads to the conclusion that cell cycle related genes may be actually induced by thermospermine. Paschalidis and Roubelakis-Angelakis (2005) have reported before that spermidine synthesis and abundance positively correlated with cell division and negatively with cell expansion and cell size. Therefore, this may be the case where the dosage of thermospermine has a preponderant role, given that in the dwarf stems the increased *POPACAULIS5* expression/thermospermine production is accompanied by the downregulation of cell cycle related genes but the opposite is observed in B2 stems grown on MS6 medium.

Type A cyclins (CYCAs; CYCA1-1, CYCA2-2, CYCA2-4) and type B cyclins (CYCBs; CYCB2-4) revealed a similar pattern of expression showing upregulation in WT MS2 and B2 MS6 stems. In *Populus*, reports show that increased CYCA2 expression is maintained during xylem development, suggesting xylem cells maintain the capacity to divide until late in development (Schrader *et al.*, 2004). Furthermore, in *Arabidopsis* CYCA2 is associated with cell division competence (Burssens *et al.*, 2000). Additional genes related to cell cycle have been found altered in our study, such as D-type cyclins, which are involved in secondary xylem proliferation in the vascular cambium (Fujii *et al.*, 2012); also CELL DIVISION CYCLE

Cell cycle and MAPK related genes

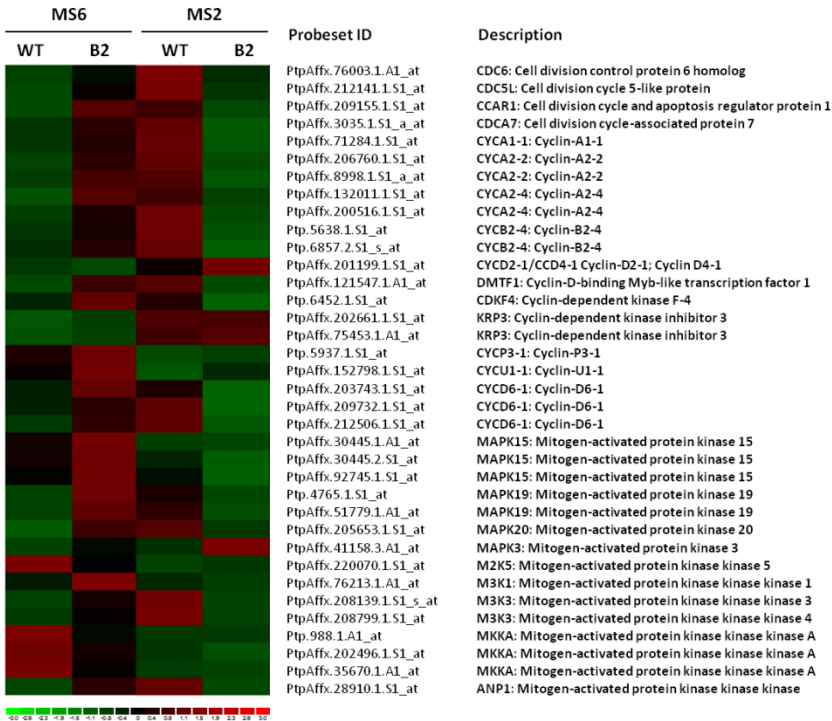


Figure 4. Transcript expression patterns of cell cycle-related genes and MAPK genes in wild-type and 35S::POPACAULIS5-B2 *Populus* stems. Cyclins (CYC) and cyclin-dependent kinase (CDK) genes with differential expression levels across all samples were identified. Expression values for each probeset across all samples were standardized (linearly scaled) to have mean 0 and standard deviation 1 as indicated by red/green coloured squares. Complete information for each probeset in the gene lists can be found in the Supporting Information, Table S2. WT, wild-type hybrid aspen; B2, 35S::POPACAULIS5; MS6, PGRs-depleted growth medium; MS2, PGRs-containing medium.

AND APOPTOSIS REGULATOR PROTEIN 1 (CCAR1), which functions to diminish cell cycle regulatory proteins such as cyclin B and to regulate apoptosis (Rishi *et al.*, 2006; Kim *et al.*, 2008); and several MITOGEN-ACTIVATED PROTEIN KINASES (MAPKs), involved in responses to various biotic and abiotic stresses, hormones, cell division and developmental

processes (Ichimura *et al.*, 2002), exhibiting a peak of expression in B2 stems. The upregulation of cell-cycle and cell division related genes in 35S::POPACAULIS5 B2 stems grown on MS6 suggests that thermospermine may act upstream of these genes.

Auxin transport is disrupted in 35S::POPACAULIS5 stems

Since auxin is known to promote *ACL5* expression and loss of *ACL5* function disrupts polar auxin transport in *Arabidopsis* (Clay and Nelson, 2005; Vera-Sirera *et al.*, 2010), we examined the effects of altering *Populus* *ACL5* expression on auxin transport related genes. In *Arabidopsis*, auxin short-distance transport out of the cell is accomplished by PIN-FORMED membrane proteins (PIN) while uptake by other cells is performed by the AUXIN RESISTANT1/LIKE-AUX1 (AUX/LAX) family of influx carriers (Grunewald and Friml, 2010; Swarup and Péret, 2012). Several members of the PIN and AUX/LAX families are present in the *Populus* genome (Carraro *et al.*, 2012). We found four *PIN* genes differentially expressed in our dataset (*PtrPIN2*, *PtrPIN3*, *PtrPIN7* and the putative *Populus* *PIN8*) (Figure 5 and Table S3). *PtrPIN3* and *PtrPIN7* were upregulated in B2 MS6 stems and suppressed in B2 MS2 stems. Although *PIN3* and *PIN7* are induced by auxin in *Arabidopsis* seedlings (Paponov *et al.*, 2008) no significant increase in expression in WT MS2 compared to WT MS6 stems could be observed. This shows that *PIN* genes expression is diverse in different species and in a tissue-dependent manner; and that highly available auxin and prolonged exposure to auxin (such as that of the MS2 media) may be counterbalanced by a decrease in auxin transport and in auxin responsiveness. Another hypothesis is the uncoupling of the PAT machinery from its regulation by auxin at the transcriptional level, suggested by the loss of auxin responsiveness both in wild-type and B2 stems on MS2, similarly to what happens in *Populus* stems during winter dormancy (Baba *et al.*, 2011). Since

the endogenous IAA levels are lower in B2 MS6 when compared to the wild-type stems (Chapter II) the upregulation of *PtrPIN3* and *PtrPIN7* may also represent a compensatory way of channelling auxin where it is needed for stem growth.

Analysis of the *Populus* auxin influx transporters AUX/LAX genes represented in the dataset unveiled a reduction in auxin cell uptake in B2 transgenic stems when compared to the wild-type. This is yet another demonstration that polar auxin transport is disrupted in the 35S::*POPACAULIS5* stems. Like above mentioned, the *acl5* mutant also exhibits reduced auxin transport in the inflorescence stem (Clay and Nelson, 2005) and thermospermine is known to suppress the auxin-inducible xylem differentiation acting as a limiting factor while auxin exerts a positive effect on xylem differentiation (Yoshimoto *et al.*, 2012a,b). Together with our results, this indicates that thermospermine or encoding transcripts not only control the endogenous auxin levels but may as well affect the auxin distribution. If increasing *POPACAULIS5* expression results in disruption of auxin distribution as it seems the case, the auxin maxima required for vascular cells formation (Sachs, 1981) may be perturbed by thermospermine effect on auxin channelling and explain its limiting effect on auxin-induced xylem differentiation.

Thermospermine decreases auxin sensitivity/responsiveness

Among the differentially expressed auxin-related genes we could find members of the three families of auxin-early response induced by auxin/indole-3-acetic acid: Aux/IAA, GH3 and small auxin-up RNA (SAUR). Aux/IAA genes code for nuclear localized proteins that control the auxin transcriptional response by binding to the auxin response factors (ARFs) and thus prevent ARFs from inducing or repressing transcription of auxin responsive genes (Santner and Estelle, 2009). In the *Populus* genome,

35 genes encoding Aux/IAA proteins and 39 *ARF* genes have been identified (Kalluri *et al.*, 2007). In our study, fourteen *Populus* Aux/IAA genes were found differentially expressed in response to *POPACAULIS5* increased levels (Figure 5 and Table S3). *PtrIAA16.1/IAA16*, *PtrIAA19.1/AUX22* and *PtrIAA28.1/IAA26* were found upregulated in WT MS2 stems and downregulated in normal growth conditions (WT MS6), suggesting these genes maintain auxin-inducibility after prolonged exposure, and may require

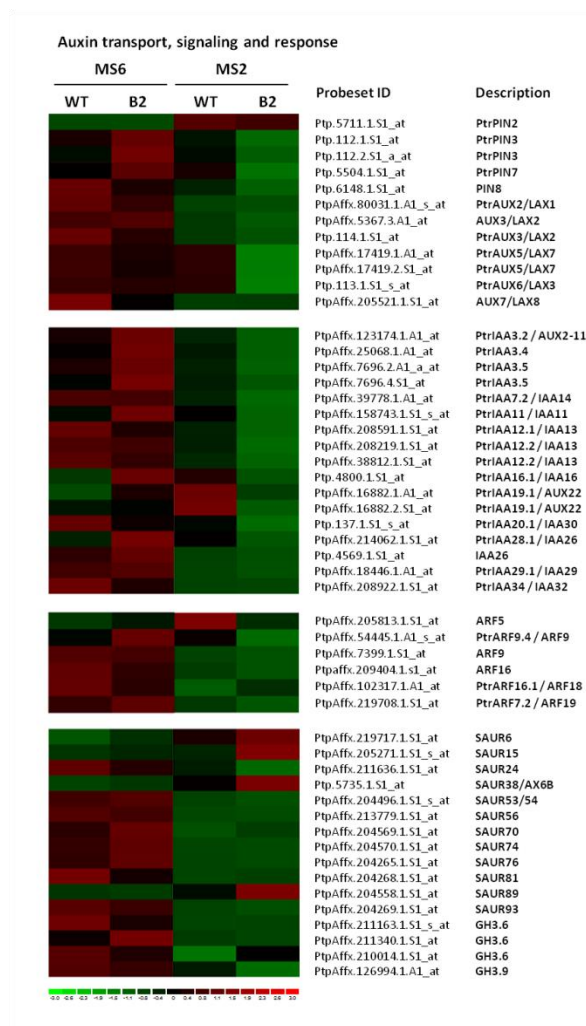


Figure 5. Transcript expression patterns of auxin carriers *PIN* and *AUX/LAX* genes, auxin signalling (*AUX/IAA*) and auxin responsive-related (*ARF*, *SAUR*) genes in wild-type and 35S::*POPACAULIS5*-B2 *Populus* stems. Genes with differential expression levels across all samples were identified. Complete information for each probeset in the genes lists can be found in the Supporting Information, Table S3. WT, wild-type hybrid aspen; B2, 35S::*POPACAULIS5*; MS6, PGRs-depleted growth medium; MS2, PGRs-containing medium.

high auxin concentrations to perform their function. However, other factors than auxin have to be involved because not all *Aux/IAA*s have an auxin-responsive element in their promoter (Paponov *et al.*, 2008). Most *Aux/IAA* found were upregulated in B2 MS6 stems, but also strongly down-regulated in the presence of thermospermine and auxin (B2 MS2). Currently, it is not known if thermospermine or other hormones could be a triggering signal to induce *Aux/IAA*, but given that many *Aux/IAA* genes were upregulated in the B2 MS6 stems this may be the case. On the other hand, it is likely that the increased expression of some *Aux/IAA* genes in B2 MS6 stems reflects the low endogenous IAA levels, which are known to promote the binding of *Aux/IAA* to ARFs and inactivate auxin-response (Ulmasov *et al.*, 1999; reviewed in Santner and Estelle, 2009). We hypothesize that thermospermine may induce *Aux/IAA*, that (in the presence of low intracellular IAA) on their turn bind to ARF and repress the auxin-response. This would also help explain the bypass of the auxin effect on xylem differentiation in the presence of thermospermine (Yoshimoto *et al.*, 2012a,b) that we here further attribute to a decrease in auxin sensitivity/responsiveness.

It is known that a rapid auxin response is mediated by the *Aux/IAA* proteins, but equilibrium is also set rapidly through binding to the complex Skp1/Cullin/F-box (SCF) that drives *Aux/IAA* for degradation by the 26S proteasome (Gray *et al.*, 2001). *CULLIN1* and *CULLIN4*, components the ubiquitin–proteasome system, were found upregulated in B2 MS6 stems but also in WT MS2, suggesting degradation of the *Aux/IAA* proteins may be also actively taking place (Table S3). In this study, we have found two activator (*ARF5*, *ARF19*) and three repressor (*ARF9*, *ARF16*, *ARF18*) putative *ARF* genes differentially expressed. Except for *ARF5*, all *ARF*s genes have a similar pattern of expression in our dataset. They are all found downregulated in the stems of MS2 grown plants (Figure 5 and Table S3). Interestingly *ARF5*, the only *ARF* in our dataset found upregulated by auxin in WT, is a key regulatory factor in cambial identity determination during

early embryogenesis, known to be an activator of AtHB8, an HD-Zip III transcription factor involved in xylem differentiation (Scarpella *et al.*, 2006; Baima *et al.*, 1995; 2001). The auxin regulatory activity is quite complex due to the large sizes of *Aux/IAA* and *ARF* gene families and their roles in repression and modulation of auxin-mediated response (Kalluri *et al.*, 2007). Other components involved in auxin-response and auxin-gradients found in the dataset include the *SAUR* genes involved in the regulation of auxin signaling and auxin-sensitive *GH3* genes (Figure 5 and Table S3).

Thermospermine crosstalk to cytokinin signaling pathway increasing cytokinin sensitivity

The mechanisms in control of maintenance of cambial/meristematic cell identity and proliferation involve the concerted action of auxin and cytokinin (Cui *et al.*, 2011, Bishopp *et al.*, 2011). We found several cytokinin-related genes expression changed in the transgenic stems (Figure 6 and Table S3). A *Populus CYP735A2* putative homolog that encodes a cytokinin trans-hydroxylase catalyzing the biosynthesis of cytokinin *trans*-zeatin (Takei *et al.*, 2004) was found upregulated in B2 MS2 stems, which suggests that thermospermine may positively affect cytokinin biosynthesis. Moreover, the LONELY GUY (LOG5) cytokinin-activating enzyme that converts inactive cytokinin nucleotides to the biologically active forms, in *rice* and *Arabidopsis* shoot meristems (Kurakawa *et al.*, 2007; Kuroha *et al.*, 2009) was found upregulated in B2 transgenic stems in the absence of hormonal external stimulus (B2 MS6), suggesting a crosstalk between thermospermine and the cytokinin activation mechanism (Figure 6). It is known that the *acl5* mutant is hypersensitive to exogenous cytokinin, producing reduced root growth under cytokinin application (Clay and Nelson, 2005) and Stes *et al.* (2011) also correlated increased polyamine synthesis in the host plant to secretion of cytokinins from a pathogen infection. Thus, it is tempting to

suggest that thermospermine may be somehow implicated in the mechanism that converts inactive to bioactive cytokinin or crosstalk to cytokinin biosynthesis.

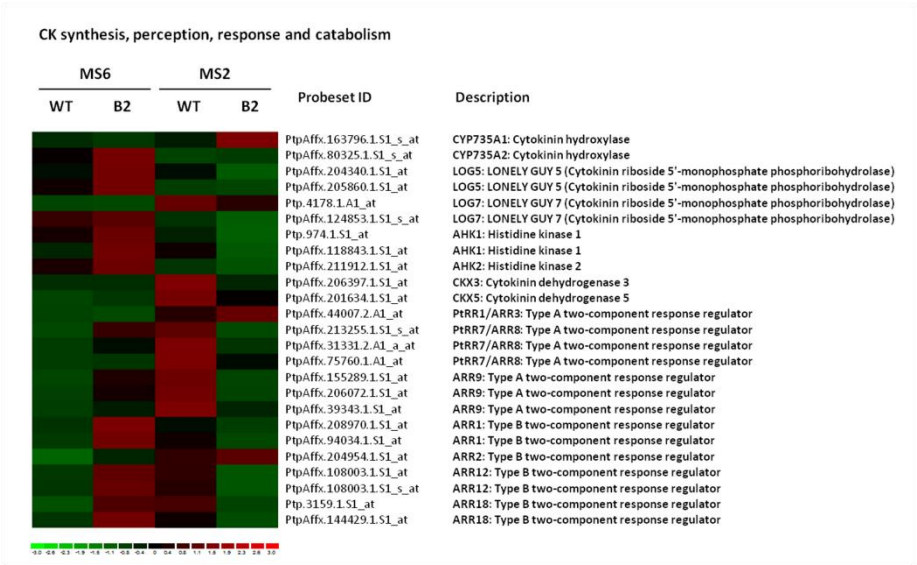


Figure 6. Upregulation of cytokinin-related genes in 35S::*POPACAULIS5*-B2 *Populus* stems. Complete information for each probeset in the genes lists can be found in the Supporting Information, Table S3. WT, wild-type hybrid aspen; B2, 35S::*POPACAULIS5*; MS6, PGRs-depleted growth medium; MS2, PGRs-containing medium.

In *Arabidopsis*, the cytokinin signal is perceived by sensor histidine kinases, namely AHK1, AHK2, AHK3, and CRE1/AHK4 (Inoue *et al.*, 2001; Suzuki *et al.*, 2001; Brenner *et al.*, 2012). We found three *Populus* histidine kinase genes differentially expressed, two homologs to *AHK1* and one to *AHK2*, and all upregulated specifically in B2 MS6 stems. These results suggest cytokinin perception may be activated in the B2 transgenic plants not subjected to the external stimulus of cytokinin signal, meaning thermospermine could have a role in activating the cytokinin signaling pathway. The pathway involves the activation of type B response regulators (type B ARRs) that control the type-A response regulators (type-A ARRs) which on their turn, negatively feedback to the cytokinin signaling pathway

(Hwang and Sheen, 2001). Since type-A ARR_s are rapidly upregulated in response to cytokinin we may infer on the cytokinin availability by their transcript abundance (To *et al.*, 2004). All seven type-A ARR_s *Populus* homologs found in our study were upregulated in WT MS2 stems, which makes sense due to the cytokinin supply from the medium. More importantly, all, except *PtRR1/ARR3*, showed increased expression in B2 MS6 stems suggesting increased cytokinin presence in the 35S::*POPACAULIS5* stems grown under regular PGRs-depleted conditions (Figure 6 and Table S3). Contrary to what happens in *Arabidopsis*, where only type-A ARR_s are induced by cytokinin, in *Populus* it was found that out of the 11 A-type and 11 B-type PtRR_s, seven type As and three type Bs are rapidly induced by exogenous cytokinin (Ramírez-Carvajal *et al.*, 2008). From our results, all type B ARR_s found were induced by cytokinin presence in the medium in WT stems (Table S3). Furthermore, all the 7 *Populus* type-B ARR_s homologs were upregulated in the B2 MS6 stems, which further supports that cytokinin responsiveness is increased in the 35S::*POPACAULIS5* stems. In *Arabidopsis*, the *bushy and dwarf 2 (bud2)* mutant, disrupted in *SAMDC4* (Ge *et al.*, 2006), shows increased cytokinin sensitivity (Cui *et al.*, 2010) and on the other hand, it is known that supplying plants with thermospermine reduces the *BUD2* transcript levels (Takechi *et al.*, 2010). Therefore, our results are in agreement with these reports showing hypersensitivity to cytokinin upon thermospermine levels increase in *Arabidopsis*. Taken together, our results indicate that thermospermine may crosstalk to the cytokinin signaling pathway positively affecting cytokinin sensitivity.

Thermospermine and cytokinin may have cooperative roles in preventing xylem differentiation

In *Populus*, the cambial expression of the catabolic *CYTOKININ OXIDASE2/DEHYDROGENASE (CKX2)* gene reduces apical but mainly radial growth,

showing that cytokinin is an important regulator of cambial development in trees (Nieminen *et al.*, 2008). Reports have shown that *CKX* is induced by auxin (Paponov *et al.*, 2008). In our study, two *CKX*s genes (*CKX3* and *CKX5*) were upregulated in WT stems in the presence of auxin and cytokinin, but not in B2 transgenic stems, suggesting that thermospermine may have a role in sustaining elevated cytokinin levels (Figure 6 and Table S3). Recently, *CKX3* has been shown to be preferentially expressed in xylem tissues and involved in xylem specification (Cui *et al.*, 2011). Adding complexity to the molecular mechanisms governing xylem differentiation it has been shown that SHORT-ROOT (SHR) activates *CKX3* that lowers the cytokinin levels presence, therefore promoting xylem specification, instead of procambial identity (Bishopp *et al.*, 2011; Cui *et al.*, 2011). This suggests that a cooperative action between cytokinin and thermospermine in preventing xylem differentiation and maintaining the cambial identity of the cells may well exist in the cambial region. It is known that *ACL5* is expressed in the procambial region in *Arabidopsis* (Muñiz *et al.*, 2008), however no role has been attributed so far to thermospermine in the cambial cells, prior to xylem specification. Curiously, the other cytokinin oxidase found in our dataset, *CKX5*, that has been found expressed in meristematic tissues in *Arabidopsis* (Bartrina *et al.*, 2011), was also downregulated in B2 transgenic stems. Overall, these results point to a possible role attributed to thermospermine in maintaining cytokinin levels elevated, that we here speculate could maintain the cambial region.

It is not likely that the dwarf phenotype showing a de-organized cambial zone is solely a result of the perturbed cytokinin signalling observed in these stems, but it is compelling to speculate that cytokinin signalling is actually activated in the transgenic line B2 growing under regular conditions. On the other hand, reduced auxin levels are known to decrease meristem activity that is dependent on cytokinin signaling (Zhao *et al.*, 2010). Polar auxin transport mutants and mutants with reduced auxin levels also show

upregulation of type A *ARRs* genes (Zhao *et al.*, 2010) which could also imply that the effect we here observe on cytokinin signaling is a result of thermospermine negative effect on auxin levels. In previous work where we grew these transgenic *in vitro* plants to trees, we have not observed increased meristematic activity, although a prolonged life span of the differentiating developing xylem suggests a delayed death of the xylem elements (Chapter II). Together with the evidence that genes positively regulating cytokinin perception and signalling are upregulated and cytokinin catabolism related genes are downregulated in 35S::*POPACAULIS5* stems we can speculate that *POPACAULIS5* and/or thermospermine may crosstalk to cytokinin in negatively regulating xylem differentiation by increasing cytokinin levels and sensitivity. But it is also plausible that this effect is happening through auxin. It also raises the question to whether well known cambial regulators are affected by thermospermine increase.

Cambial and phloem identity genes are upregulated in POPACAULIS5 overexpressing stems

Due to clues pointing towards cytokinin and thermospermine contribute to the maintenance of the cambial cell identity in 35S::*POPACAULIS5* stems, we examined our data for known cambial regulators and for vascular specification-related genes profile. *HD-Zip III* members are mainly expressed in procambial and xylem cell precursor cells and are thought to promote xylem differentiation whereas *KANADIs* (*KANs*) are mainly expressed in phloem and seem to act antagonistically on vascular specification (Eshed *et al.*, 2001; Kerstetter *et al.*, 2001; Emery *et al.*, 2003; Ilegems *et al.*, 2010; Carlsbecker *et al.*, 2010). Most of the genes involved in xylem specification were strongly downregulated in the B2 MS2 stems (Figure 7 and Table S4). These transcription factors' expression profile in the B2 MS6 stems was found dramatically altered relatively to the WT MS6 (Figure 7). The *Populus*

Cambial regulation and vascular specification related transcription factors

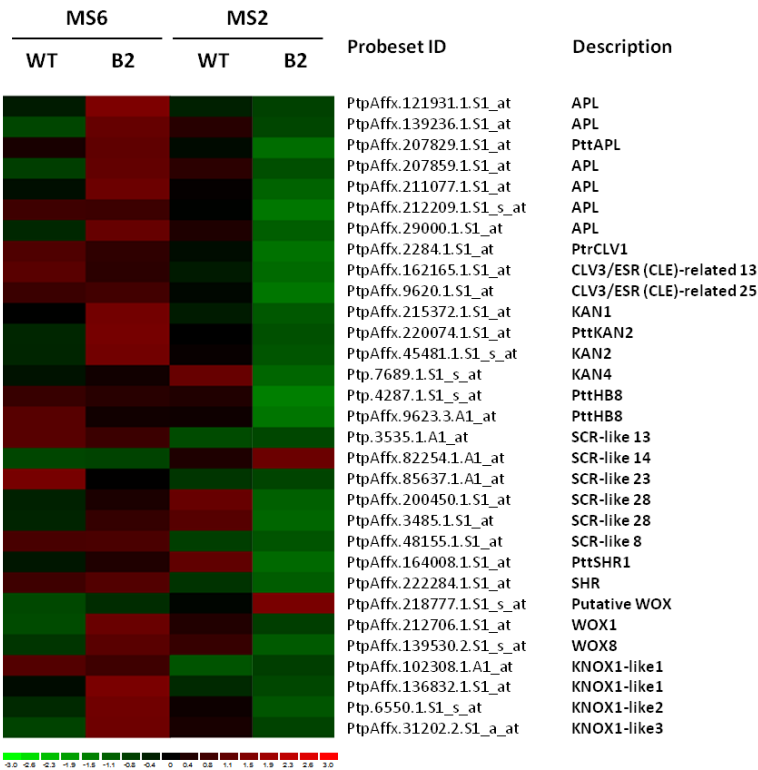


Figure 7. Upregulation of transcription factors involved in cambial regulation and vascular specification in 35S::*POPACAULIS5* stems. Complete information for each probeset in the genes lists can be found in the Supporting Information, Table S4. WT, wild-type hybrid aspen; B2, 35S::*POPACAULIS5*; MS6, PGRs-depleted growth medium; MS2, PGRs-containing medium.

homolog of the procambial HD-Zip III transcription factor *AtHB8*, *PttHB8* was found repressed in the *POPACAULIS5* overexpressors stems; which is in agreement with our previous study showing that *POPACAULIS5* feedback represses its own transcription by repressing *PttHB8*, a transcriptional regulator of *POPACAULIS5* (Chapter II). Interestingly, we found *KAN* genes (*KAN1*, *KAN2*) upregulated in the B2 MS6 stems. Moreover, the phloem specification homolog genes for *ALTERED PHLOEM DEVELOPMENT*

(*APL*) were strongly upregulated in B2 MS6 transgenic stems. Both KAN and APL are required for phloem cell specification (Bonke *et al.*, 2003; Eshed *et al.*, 2001; Kerstetter *et al.*, 2001; Ilegems *et al.*, 2010). This indicates that thermospermine may not be involved exclusively as a xylem cell death delayer, but perhaps a dual role in suppression of xylem cell fate may also be attributed to thermospermine presence. No reports exist on phloem altered morphology upon *ACL5* loss-of-function, but given our observations in severe phenotypes where phloem seems to be the main vascular tissue to be formed (Figure S3) it is quite possible to assume a role in the procambium domain (Muñiz *et al.*, 2008).

It is known that KAN proteins control cambial activity by negatively acting on auxin transport, whereas HD-Zip III trigger the onset of xylem differentiation by having a role in the canalization of auxin flow (Ilegems *et al.*, 2010). Our previous studies showing reduced amounts of endogenous IAA, together with the present study showing altered auxin *PIN* activity suggest a more intricate network is involved in thermospermine action in xylem specification and differentiation. We can speculate whether thermospermine known opposite action on auxin flow/accumulation is connected to KAN proteins function. Furthermore, *KNOX1* homolog proteins, that induce cytokinin synthesis and maintain cytokinin levels increased to maintain cambial cell identity in *Arabidopsis* (Sakamoto *et al.*, 2006; Cui *et al.*, 2011); together with BEL1-like homeodomain protein family that forms complexes with the KNOX homeodomain proteins, such as SHOOTMERISTEMLESS (*STM*) and BREVIPEDICELLUS (*BP*), were found upregulated in B2 MS6 stems (Figure 7; Table S5) further supporting a role in the cambial region. Additionally, *SHR* that controls vascular patterning by controlling cytokinin homeostasis, regulating also this balance between cambial maintenance and specification (Cui *et al.*, 2011), was found represented in two *SHR* genes with altered expression in B2 MS6 stems. Further analyses will be needed to increase the understanding on the crosstalk

here found between cytokinin and thermospermine, but it is very likely this relationship happens within the cambial domain.

Ethylene production is enhanced by auxin and POPACAULIS5 overexpression in Populus stems

Homologs of the ethylene perception members ETHYLENE RECEPTOR 2 (ETR2), ETHYLENE INSENSITIVE 3 (EIN3); ethylene biosynthetic enzymes 1-AMINOCYCLOPROPANE-1-CARBOXYLATE (ACC) SYNTHASE (ACS), ACC OXIDASE (ACO); and ethylene signalling elements, such as ethylene responsive factors (ERFs) and transcription factors related to AP2 (RAP2) have been found differentially expressed in our dataset (Figure 8 and Table S3). Genes representing putative homologs of the enzymes in the biosynthetic pathway of ethylene, such as ACS and ACO, were strongly upregulated in transgenic B2 MS2 dwarf stems. This indicates that high levels of ethylene may be present in the dwarf transgenic stems, which may also explain the large spaces between cells observed, resembling cortical aerenchyma zones typically found in roots of flooded plants (Figure S1 and Figure S2). Since ethylene follows a model of biphasic response, in that growth inhibition and growth stimulation by ethylene is dose-dependent (Pierik *et al.*, 2006; Yoo *et al.*, 2009) it is reasonable to assume that elongation increases with the presence of low concentrations but decreases with increasing concentrations of this volatile gas. Thus, ethylene may have here a preponderant effect in the dwarfism displayed by the transgenic 35S::POPACAULIS5 plants.

Increased expression of the *Populus* putative ethylene receptor ETR2 in WT MS2 and B2 MS2 stems indicates a stimulatory effect of auxin on ethylene perception machinery. After ethylene perception, the signalling pathway relies on the central role EIN3 has in activating ethylene responsive factors (ERFs) (Chao *et al.*, 1997; Solano *et al.*, 1998; Stepanova and Alonso,

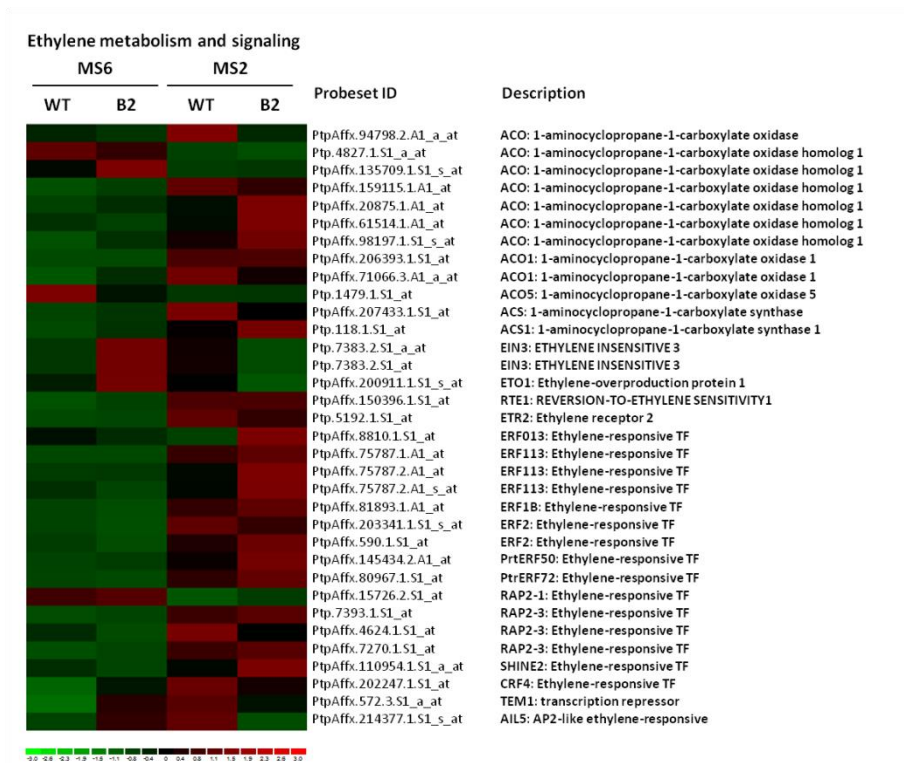


Figure 8. Upregulation of ethylene metabolism and signalling-related genes in the dwarf 35S::*POPACAULIS5* stems. Complete information for each probeset in the genes lists can be found in the Supporting Information, Table S3. WT, wild-type hybrid aspen; B2, 35S::*POPACAULIS5*; MS6, PGRs-depleted growth medium; MS2, PGRs-containing medium.

2009). A *Populus* homolog of *EIN3* was found upregulated in B2 MS6 stems but downregulated in the B2 MS2 dwarf stems. It is known that *EIN3* transcription is not affected in response to ethylene or ethylene precursors but instead *EIN3* accumulates in the nucleus and is degraded constantly through the 26S proteasome (Guo and Ecker, 2003; Yanagisawa *et al.*, 2003). From our results, activation of *EIN3* is not dependent on auxin or cytokinin presence either, but may well be activated by thermospermine, as observed by its increased expression in transgenic B2 stems grown in the absence of any external hormone stimulus. Therefore, increased thermospermine presence in the stems seems to relate to increased ethylene sensitivity. One

more evidence that ethylene may be highly present in the transgenic dwarf stems is the upregulation of a homolog of REVERSION-TO-ETHYLENE SENSITIVITY1 (RTE1), a negative regulator of ethylene signalling, whose expression in *Arabidopsis* is enhanced by ethylene (Resnick *et al.*, 2006). Overall, it is very likely that auxin presence in the medium is enhancing ethylene production in the stems; however, given that increased ethylene responsiveness occurred mainly in B2 stems, we suggest that increased thermospermine results in increased ethylene and thus, ethylene response. This is confirmed by the upregulation of ethylene responsive transcription factors in the B2 MS2 dwarf stems (Figure 8 and Table S3).

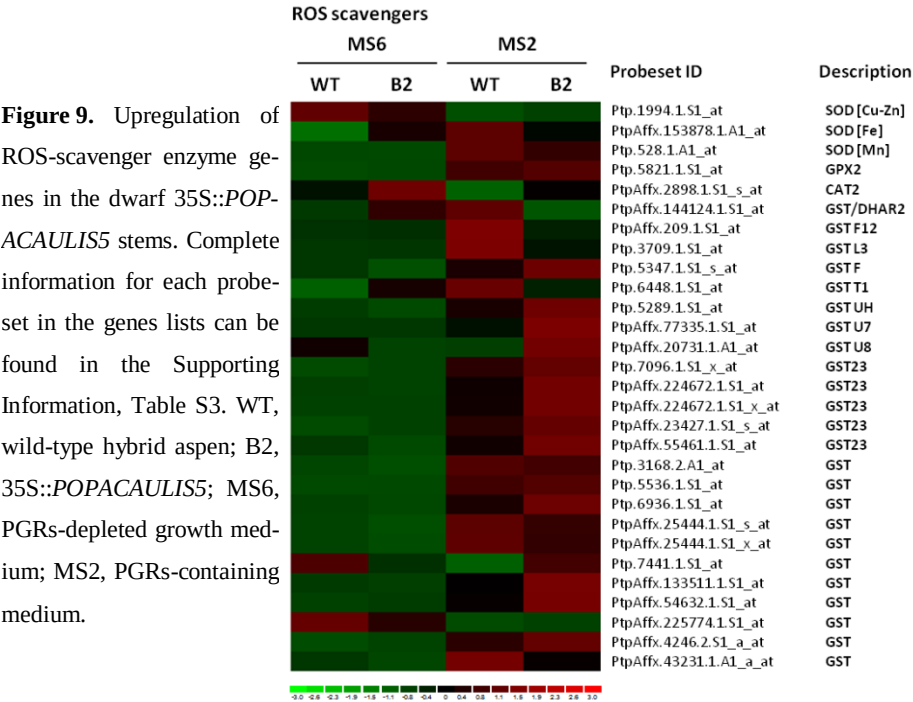
Ethylene and thermospermine actually share common substrates in their biosynthetic pathways, since both require S-adenosylmethionine (SAM). SAM decarboxylase (SAMDC), that decarboxylates SAM to be used for the conversion of spermidine to thermospermine, was found downregulated in the dwarf B2 transgenic stems (Table S1). We may speculate that the SAMDC downregulation may lead to a slight decrease in the channelling of SAM to polyamines biosynthesis. Previous work has shown that in fact there is a slight decrease in spermidine and spermine accumulation in the transgenic B2 stems (Chapter II). Interestingly, the *bud2* mutation shows a disrupted vascular phenotype that also results in dwarf plants. This has been linked to perturbed polyamine homeostasis (Ge *et al.*, 2006) but is yet to link to ethylene response mechanisms.

ROS increase in 35S::POPACAULIS5 dwarf stems

Since ethylene is known to regulate abiotic stress responses, it is possible that the severe defects imposed by thermospermine overproduction together with the hormone stimulus on MS2 medium, are sensed by the plant as a stress condition, and thus the resulting increase in ethylene responses. On the other way around, the stress imposed could also be a cause of the observed

phenotype. Ethylene has a role in triggering reactive oxygen species (ROS) production (de Jong *et al.*, 2002). Increased H₂O₂ levels promote cell death and it is hypothesised that increased ethylene production results in increased ROS accumulation (Wi *et al.*, 2010). In normal growth conditions there is a balance between ROS production and scavenging (Dat *et al.*, 2003; Bailey-Serres and Mittler, 2006). ROS scavengers are antioxidant molecules or enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione S-transferase (GST) and glutathione reductase that maintain this equilibrium. If ethylene is overproduced in the stems of thermospermine overexpressors we would expect these genes transcriptional profile to be altered. In fact, several genes encoding these enzymes were differentially expressed in our study, showing upregulation in stems of plants grown on MS2 medium, but not in stems of WT MS6 or B2 MS6 (Figure 9 and Table S3). Interestingly, there is a stronger upregulation of the ROS scavengers encoding genes in the thermospermine overproducers, suggesting increased detoxification of the cells. Moreover, it has been shown that increased polyamine biosynthesis increases stress tolerance in rice by preventing the ROS accumulation, ethylene accumulation and the resultant cell damage (Jang *et al.*, 2012). This is in line with the findings that thermospermine prevents cell death of the xylem vessel elements in the stems of *Arabidopsis* (Muñiz *et al.*, 2008). It also poses the question to whether increased thermospermine production and/or stability as observed in auxin-rich environment in fact disrupts the balance between the necessary role of ROS as a developmental signalling molecule and the damaging effect of a boost in ROS levels, given that in an auxin-depleted environment we observed that thermospermine levels are prevented from reaching damaging levels (Chapter II).

Recently brassinosteroids have also been shown to stimulate ROS production and that this BR-induced ROS may have a role in plant development (Xia *et al.*, 2009). It would be interesting to unveil if



brassinosteroids known effect on promoting xylem differentiation could be related to ROS production. In our dataset, we have found probesets representing putative homologs to *DET2*, a brassinosteroids biosynthetic gene, strongly upregulated only in the dwarf stems of transgenic B2 MS2 (Table S3). Brassinosteroids, like auxins, are known to promote cell elongation dependent on the expression of xyloglucan endotransglycosylases (XTHs/XETs) induction and stimulate expression of cellulose synthases (CesA) genes (Yang *et al.*, 2011; Xie *et al.*, 2011). The defective transgenic stems showed induction of XTH expression as we below mention. But recently it has been argued that membrane sterols, and not brassinosteroids, are critical for cellulose accumulation (Schrack *et al.*, 2012). How the brassinosteroids relate to polyamines is still unknown, nevertheless they have recently been shown to have cooperative roles in metal stress response in radish (*Raphanus sativus*) plants, which makes it alluring to think they may

have cooperative roles in other developmental processes such as in xylem differentiation (Choudhary *et al.*, 2012), perhaps linking to ROS developmental roles.

Cell death genes are affected by POPACAULIS5 overexpression

Like above mentioned ACL5 is involved in preventing the premature xylem elements death in *Arabidopsis* and inhibition of *Zinnia* TE differentiation (Muñiz *et al.*, 2008; Kakehi *et al.*, 2010). Therefore, we would expect genes that are positive regulators of cell death to be downregulated in the stems of the thermospermine overproducers. The xylem cell death programme is known to involve increased cysteine protease activity (reviewed in Bollh ner *et al.*, 2012). One *Populus* XCP1 homolog and two probesets representing XCP2 – genes encoding xylem cysteine proteases, that are specifically expressed in xylem vessel elements and have been implicated in PCD during xylem vessel differentiation in *Arabidopsis* (Zhao *et al.*, 2000; Avci *et al.*, 2008) – were found downregulated in overexpressing POPACAULIS5 stems; and XCP1 found particularly upregulated by auxin (WT MS2) (Figure 10 and Table S4). These results are in line with the possibility that the transcriptional control pathway of cell death may be preceded by ACL5 negatively regulating xylem cell death (Bollh ner *et al.*, 2012).

Other cysteine proteases, with caspase-like activity and structural homology to animal caspases (the main constituents of apoptosis in animals) have been identified in plants (reviewed in Tsiatsiani *et al.*, 2011). We have found *Populus* homologs genes to metacaspases (AtMC1, AtMC5 and AtMC9), subtilisin-like proteases and vacuolar-processing enzymes (VPEs) represented in our dataset. Both *Populus* MC9 homologs were found downregulated in B2 MS2 stems (Figure 10 and Table S4). AtMC9 and *Populus* homologs have been found specifically expressed in differentiating xylem (Turner *et al.*, 2007; Ohashi-Ito *et al.*, 2010; Courtois-Moreau *et al.*,

2009) prior to cell death or in late maturing xylem elements (Courtois-Moreau *et al.*, 2009; Bollhöner *et al.*, 2012). Our results emphasize that *POPACAULIS5* may negatively affect expression of cell death genes as previously suggested (Vera Sirera *et al.*, 2010; Bollhöner *et al.*, 2012). A putative *Populus* homolog of *AtMC5* was found upregulated exclusively in WT stems and downregulated both by auxin and *POPACAULIS5* increased expression; yet, no function has been so far attributed to *AtMC5*. Furthermore, two putative *Populus* homologs to *AtMC1*, that is a positive regulator of the hypersensitive cell death response (HR; Coll *et al.*, 2010), were found strongly upregulated in B2 MS6 stems, but downregulated on B2 MS2. Actually, several plant defense system genes were upregulated in the dataset (Table I). Programmed cell death plays a critical role during HR and one of components that triggers it is hydrogen peroxide, which is generated through several pathways, one being polyamine oxidation (Yoda *et al.*, 2003, 2006). Therefore, it is quite possible that some ROS accumulation could be triggering defense responses, and hence the antioxidant enzymes upregulation, as we above discussed.

Several subtilisin-like proteases genes were found differentially expressed in our experiment. These serine proteases are constitutively activated to induce cell death upon the death signal, both in biotic and abiotic stresses (Vartapetian *et al.*, 2011). Overall, these results imply that, like other polyamines, thermospermine may also have a role in triggering defense responses to biotic and abiotic stresses. In fact, recently it has been shown that exogenous thermospermine induces a subset of the defense genes and restrict cucumber mosaic virus multiplication, as well as thermospermine catabolism increases *Arabidopsis* resistance to *Pseudomonas viridiflava* (Sagor *et al.*, 2012; Marina *et al.*, 2013). It is not known if, similarly to *XSP1*, the other subtilisin-like proteases found play a role in xylem cell death, but finding them differentially expressed in this dataset raises this hypothesis (Figure 10 and Table S4). Overall, our results also emphasize that the

developmental program of xylem cell death is probably tuned by some of the same signals that trigger programmed cell death in the hypersensitive response and that *POPACAULIS5* increased expression may negatively affect downstream genes thought to be involved in xylem cell death.

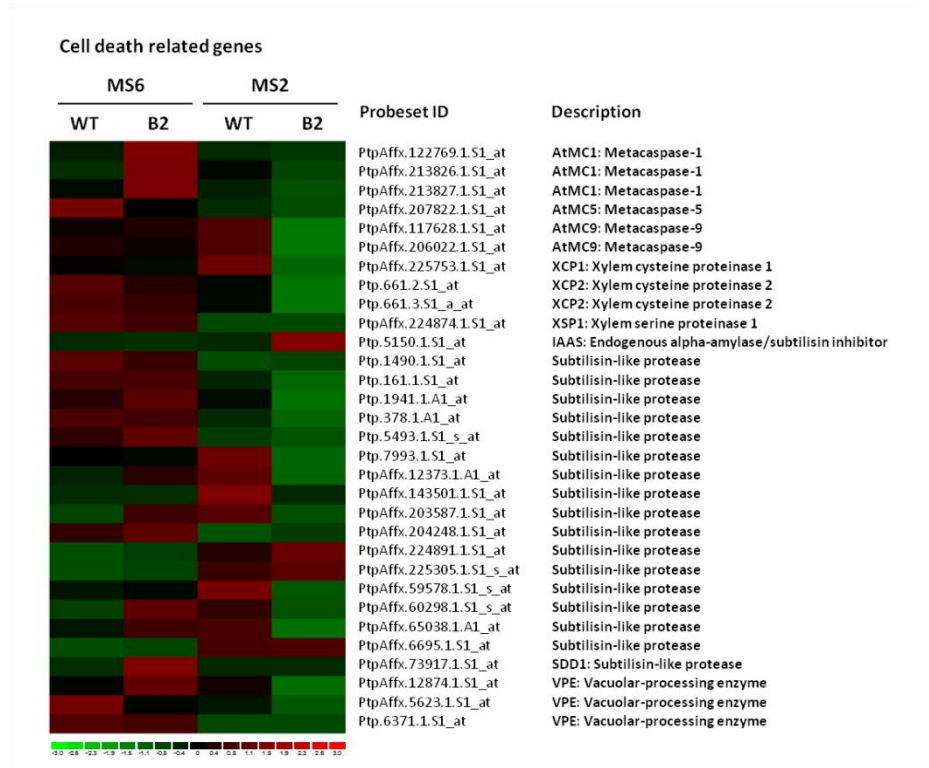


Figure 10. Expression pattern of cell death-related genes in wild-type and 35S::*POPACAULIS5* stems. Complete information for each probeset in the genes lists can be found in the Supporting Information, Table S4. WT, wild-type hybrid aspen; B2, 35S::*POPACAULIS5*; MS6, PGRs-depleted growth medium; MS2, PGRs-containing medium.

Most *Populus* homologs to NAC domain proteins that are involved in secondary cell wall formation and cell death of xylem in *Arabidopsis* have been found downregulated in 35S::*POPACAULIS5* dwarf stems. Probesets representing *NAC SECONDARY WALL THICKENING PROMOTING FACTOR1* (*NST1*), *SECONDARY WALL-ASSOCIATED NAC-DOMAIN1* (*SND1/NST3*) and *SND2* (Zhong *et al.*, 2006; 2007; Mitsuda *et al.*, 2007),

VASCULAR RELATED NAC DOMAIN7 (*VND7*, Kubo *et al.*, 2005), *XYLEM NAC DOMAIN 1* (*XND1*, Zhao *et al.*, 2008) were found downregulated in the B2 transgenic dwarf stems (Figure S5 and Table S4). We also inspected reported xylem marker genes, such as cell wall proteins, cellulose synthase (*CesA*) genes, xyloglucan endotransglycosylase genes, lignin biosynthesis-related genes and xylem-associated transcription factors that were found differentially expressed in our dataset. Most probesets representing cellulose synthase (*CesA*) and *CesA-like* genes involved in cell wall biosynthesis (*PtCesA3/CesA4*, *CesA8/IRX1*, *PtCesA7/CesA9*, *CsIE6* and *CsIH1*) and genes encoding enzymes involved in lignin biosynthesis such as 4-COUMARATE-CoA LIGASE (*4CL1* and *4CLL9*), PHENYLALANINE AMMONIA-LYASE (*PAL2*), HYDROXYCINNAMOYL-CoA SHIKIMATE/QUINATE (*HCT*) exhibited a similar pattern of expression where a reduction of expression could be observed in B2 dwarf stems (Figure S5 and Table S4). However, CINNAMOYL-CoA REDUCTASES (*CCR1* and 2), CAFFEIC ACID 3-O-METHYLTRANSFERASE 1 (*COMT1*), CINNAMYL ALCOHOL DEHYDROGENASES (*CAD1* and 2) were found upregulated in the B2 dwarf stems. Downregulation of *COMT* in transgenic alfalfa and of *CCR1* in poplar has been correlated to reduced lignin content (Guo *et al.*, 2001; Leplé *et al.*, 2007), but the latter's upregulation has been also connected to defense signaling in rice (Kawasaki *et al.*, 2006), which suggests increased lignin content, or may reflect the overall increased response to stress. BETA-1,4-XYLOSYLTRANSFERASES (*IRX*), components of xylan biosynthesis, as well as components involved in cell wall loosening and xyloglucan hydrolysis and breakdown such as XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE PROTEINS (*XTHB*, 6, 9, 23, 31 and 33), ENDO-1,4-BETA-XYLANASE C (*XYNC*), BETA-D-XYLOSIDASES (*BXL1*, 2, 4 and 7) were all found downregulated in B2 dwarf stems with the exception of *XTH31*, found exclusively upregulated in these samples. A steep reduction in expression of genes encoding the cell wall FASCICLIN-LIKE ARABINOGALACTAN

PROTEINS (FLAs) seems to correlate to the increase in thermospermine and could reiterate some increase in lignin content (MacMillan *et al.*, 2010) (Table S4). Overall, the significant changes of expression of the cell wall related genes in the stems of B2 dwarf plants is indicative of the general misregulation of xylem marker genes/specification genes and therefore the loss of a correct xylem differentiation program. Nevertheless, a new set of cell wall-related genes had increased expression in the dwarf transgenic plants, but not in the WT counterpart. Therefore, reprogramming of lignification and secondary cell wall formation could be taking place, but most likely is secondary to upstream events as discussed in the sections above.

Conclusions

A comparative study to identify variations in gene expression and *de novo* transcription as a result of thermospermine increased production in *Populus* stems was performed. We identified several possible hormone crosstalks to thermospermine that suggest globally a positive effect of thermospermine on cytokinin levels, perception, signalling and responsiveness. This could be a consequence of the negative effect thermospermine has on auxin transport, auxin levels and responsiveness. Moreover, we highlighted a positive effect of thermospermine on ethylene levels. We could confirm the earlier identified role of thermospermine in delaying xylem cell death and further observed that a broader role in a provascular stage of development may be attributed to thermospermine, given that many transcripts involved in cambial maintenance were upregulated in transgenic plants overexpressing *POPACAULIS5* (Figure 11). We demonstrate that proper xylem development depends on the correct physiological and metabolic cellular environment and that maintaining thermospermine levels controlled is essential to ensure the

xylem differentiation program. Based upon microscopic structure observations and transcriptome profiling, the results here presented may drive further hypothesis-testing studies on yet unknown roles of thermospermine in xylem differentiation.

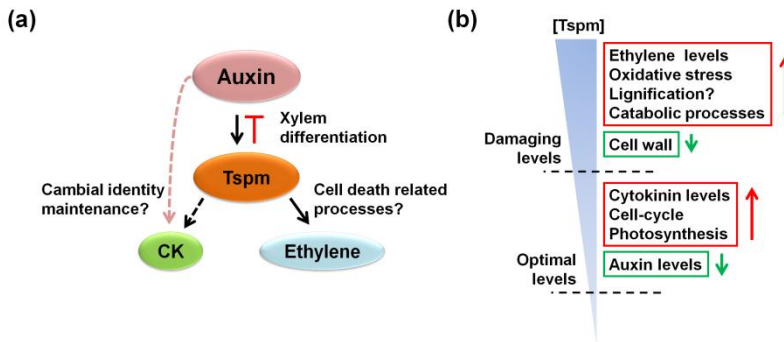


Figure 11. Schematic representation of hormone crosstalk and effect of increased thermospermine on the transcriptome of *Populus* stems. (a) Thermospermine suppresses the auxin-induced xylem differentiation, whereas auxin stimulates thermospermine accumulation. Cytokinin and thermospermine may crosstalk in the cambium domain, or through thermospermine effect on auxin polar transport and auxin levels, whereas ethylene is induced by thermospermine increased levels, as both may have coordinated actions in xylem cell death related processes. (b) Excessive thermospermine accumulation up to damaging levels induces the depicted transcriptomic changes (red arrows indicate upregulation, green arrows indicate downregulation).

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Supporting information

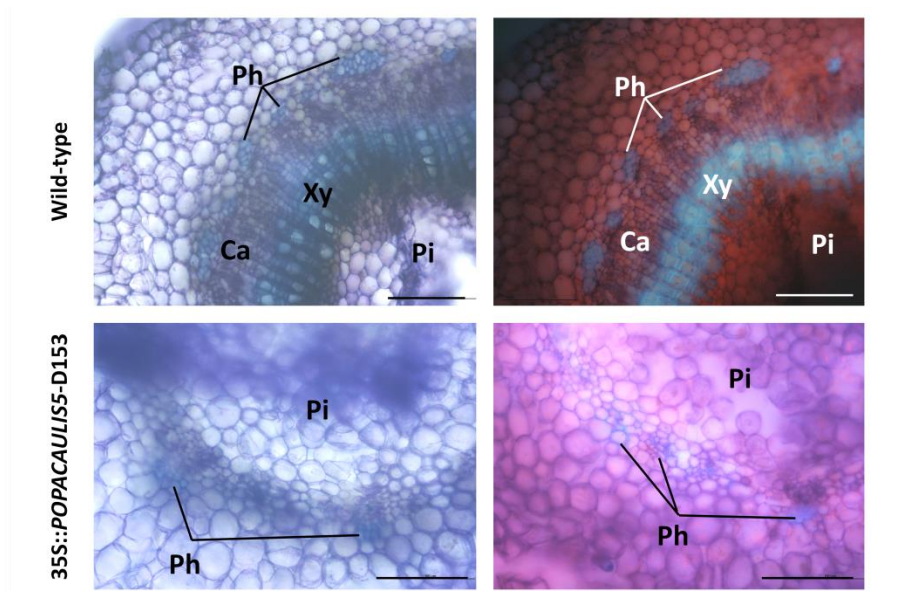


Figure S1. Cross sections of 35S::POPACAULIS5-D153 hybrid aspen stems after Toluidine Blue-O staining. Ph, phloem; Xy, xylem; Ca, cambial zone; Pi, pith. Scale bars: 50 μ m.

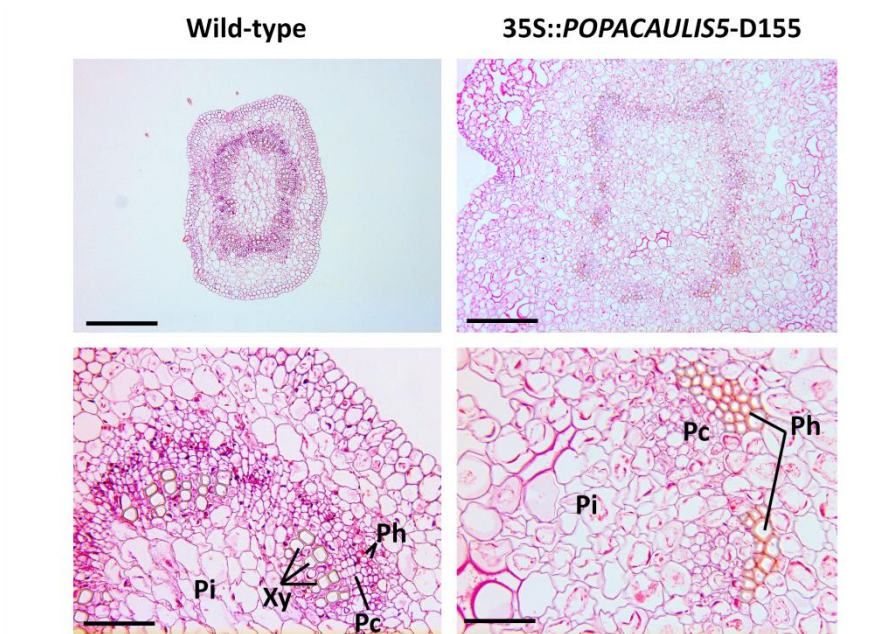


Figure S2. Cross sections of 35S::POPACAULIS5-D155 hybrid aspen stems after Hematoxylin-Eosin staining, with an atypical (pro)cambial zone. Ph, phloem; Xy, xylem; Pc, (pro)cambial zone; Pi, pith. Scale bars: upper panels, 500 μ m; lower panels, 50 μ m.

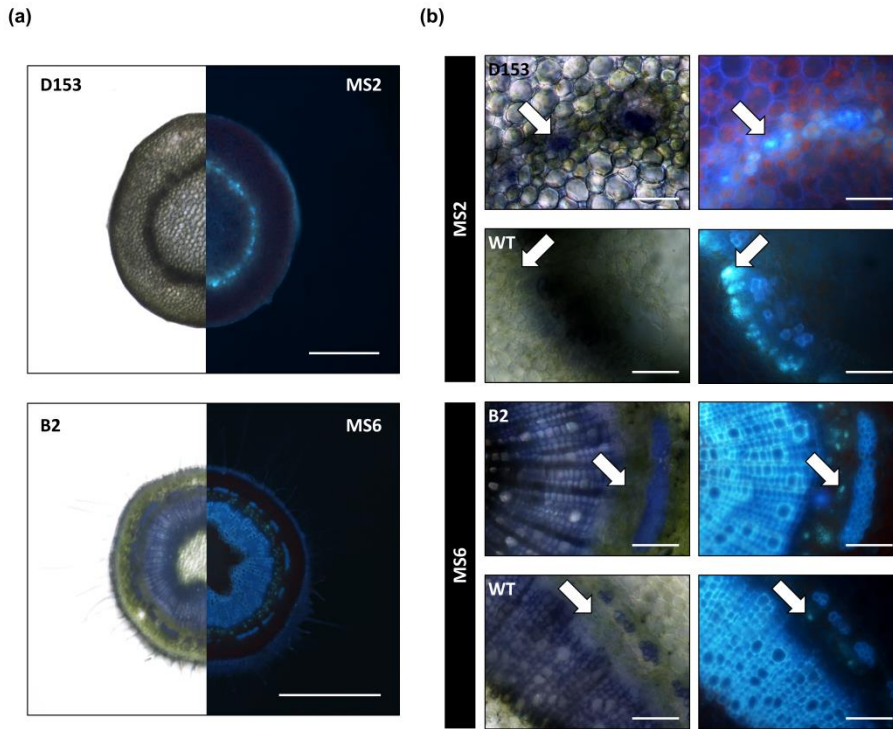


Figure S3. Cross sections of 35S::*POPACAULIS5* hybrid aspen stems stained with aniline blue and observed under light and UV fluorescence microscopy. (a) Stem cross section of transgenic line D153 (dwarf) grown on MS2 medium observed under bright-field and UV fluorescence (*upper panel*), where cells within the cambial zone show strong staining with intense light blue fluorescence identical to the typically observed in phloem cells. D153 line is representative of transgenic lines showing severe dwarfism without recovery once transferred to MS6 medium. (*Lower panel*) Stem cross section of transgenic line B2 after partially recovering the phenotype when transferred to the MS6 medium. (b) Detail of cambial zone in stem cross sections of transgenic lines D153 and B2 and wild-type (WT). Arrows depict phloem identity cells stained with aniline blue in stem sections observed under bright-field and UV fluorescence. Increased density of chloroplasts was observed in D153 and B2 transgenic lines as well as photosynthesis related genes were found upregulated in the stems of the transgenic line B2 (Table S6). Scale bars: (a) 500µm and (b) 25 µm.

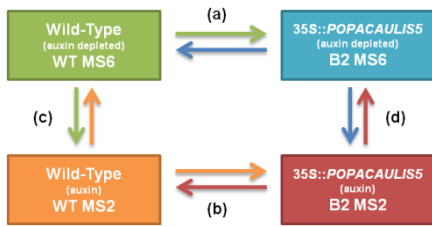


Figure S4. Illustration of the sampling used for global transcripts analysis and the four pair-wise comparisons performed.

Xylem transcriptional network and xylem and cell wall-related marker genes

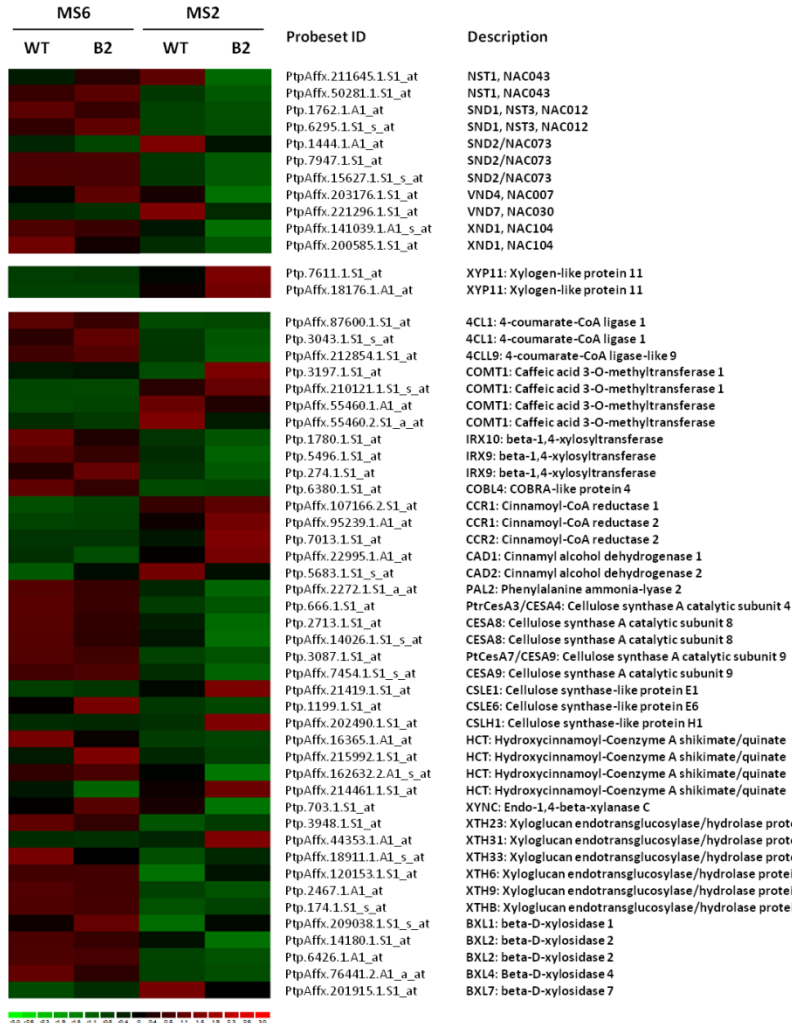


Figure S5. Expression pattern of xylem transcriptional network genes, lignification, xylem-specific and cell wall-related marker genes in wild-type and 35S::POPACAULIS5-B2 *Populus* stems. Complete information for each probeset in the genes lists can be found in the Supporting Information, Table S4. WT, wild-type hybrid aspen; B2, 35S::POPACAULIS5; MS6, PGRs-depleted growth medium; MS2, PGRs-containing medium.

Table S1. Expression data (Mean signal intensities, MSIs) of polyamine metabolism and catabolism related *Populus* homologs ⁽¹⁾.

Probeset ID	Description ⁽³⁾	Samples (MSIs)				Gene Model ⁽²⁾	Pair-wise comparison ⁽⁴⁾							
		WTMS2		B2 MS2			(a)WTMS2/B2 MS2		(b)WTMS2/B2 MS2		(c)WTMS2/WTMS2			
		WTMS6	B2 MS6	WTMS2	B2 MS2		FC	LCB	FC	LCB	FC	LCB		
PipAffx.12147.1.AI_at	Homocysteine S-methyltransferase 2	282.16	345.07	1004.66	882.47	Pp01.005G2131.00	-1.22	-1.04	1.14	0.97	2.56	2.20	3.56	3.06
PipAffx.14459.1.S1_at	Homocysteine S-methyltransferase 3	531.40	560.30	66.29	872.49	AT5G22740.1 Pp01.010C0836.00	-1.05	-0.81	-13.16	-9.03	1.56	1.14	-8.02	-5.50
Pip.3610.1.S1_at	Homocysteine S-methyltransferase 3	759.17	637.99	78.74	558.63	AT5G22740.1 Pp01.008G1559.00	1.19	0.78	-7.09	-4.79	-1.14	-0.98	-9.64	-5.50
PipAffx.13659.1.AI_s_at	Arginine decarboxylase	6002.63	5132.28	7151.70	6134.66	AT4G34710.1 Pp01.004G1633.00	--	--	--	--	--	--	--	--
PipAffx.9791.1.S1_at	Arginine decarboxylase	1012.25	1099.44	1083.69	1009.94	AT5G08170.1 Pp01.015G0553.00	--	--	--	--	--	--	--	--
PipAffx.201583.1.S1_at	Oxalate decarboxylase	30.54	25.31	24.93	39.59	Pp01.002G01580.00	--	--	--	--	--	--	--	--
PipAffx.205650.1.S1_s_at	Oxalate decarboxylase	24.22	18.31	21.84	26.47	Pp01.005G2463.00	--	--	--	--	--	--	--	--
PipAffx.1286.1.S1_s_at	S-adenosylmethionine decarboxylase	7873.34	7573.93	7202.43	7461.26	AT5G02470.3 Pp01.017G1088.00	--	--	--	--	--	--	--	--
PipAffx.208525.1.S1_at	S-adenosylmethionine decarboxylase	7615	50.11	47.36	31.71	AT5G18930.1 Pp01.010C0285.00	--	--	--	--	--	--	--	--
PipAffx.214439.1.S1_at	S-adenosylmethionine decarboxylase	128.88	74.83	66.83	42.78	Pp01.018G10150.00	--	--	--	--	--	--	--	--
PipAffx.39046.1.S1_s_at	SAM-dependent methyltransferase	721.96	537.55	204.09	193.31	Pp01.017G12129.00	-1.34	-1.14	-1.06	-0.85	-3.54	-2.98	-2.78	-2.26
PipAffx.148953.1.AI_at	S-adenosylmethionine decarboxylase	332.34	159.43	158.90	69.74	AT5G18930.1 Pp01.008G1988.00	-2.08	-1.72	-2.28	-1.67	-2.09	-1.66	-2.29	-1.74
Pip.7547.1.S1_at	Spermine synthase	352.98	383.93	541.98	362.59	AT5G53120.5 Pp01.012C0026.00	--	--	--	--	--	--	--	--
PipAffx.17860.4.S1_at	Spermine synthase	65.21	32.97	54.91	76.72	AT5G53120.1 Pp01.015G0188.00	--	--	--	--	--	--	--	--
Pip.5500.1.S1_at	The mosperminase synthase POPACULIS5	1331.37	5861.00	2432.69	7402.14	AT5G19530.1 Pp01.006G2222.00	4.40	3.92	3.04	2.41	1.83	1.46	1.26	1.06
PipAffx.128166.1.AI_at	The mosperminase synthase ACAULIS5	811.50	772.76	1487.60	175.33	AT5G19530.2 Pp01.010C0892.00	-1.05	-0.93	-8.48	-5.61	1.83	1.58	-4.41	-2.94
PipAffx.16965.1.AI_at	The mosperminase synthase ACAULIS5	1286.70	1223.37	1760.06	179.68	AT5G19530.2 Pp01.008G1518.00	-1.05	-0.89	-9.80	-5.47	1.37	1.17	-6.81	-3.78
PipAffx.210560.1.S1_at	Polyamine oxidase	37.95	116.42	116.05	20.68	Pp01.012C0795.00	--	--	--	--	--	--	--	--
PipAffx.46673.1.S1_at	Polyamine oxidase	4137.82	6337.91	7399.40	5472.02	AT5G13700.1 Pp01.015G0746.00	--	--	--	--	--	--	--	--
Pip.6223.1.S1_s_at	Probable polyamine oxidase 2	2467.92	2136.73	1956.37	2286.20	AT2G43020.1 Pp01.005G0730.00	--	--	--	--	--	--	--	--
PipAffx.21946.1.AI_a_at	Probable polyamine oxidase 2	1107.58	817.02	651.82	762.57	Pp01.002G0553.00	--	--	--	--	--	--	--	--
PipAffx.65986.1.S1_at	Probable polyamine oxidase 4	70.33	110.13	346.47	160.09	AT5G5840.1 Pp01.004G0758.00	1.57	1.20	-2.16	-1.96	4.93	3.89	1.45	1.25
PipAffx.35782.2.AI_at	Probable polyamine oxidase 4	2121.77	361.64	1418.66	1032.02	AT5G5840.1 Pp01.0060.00	1.70	1.44	-1.37	-1.03	6.69	5.42	2.85	2.05

(1) bold highlighted probesets represent the polyamine-related differentially expressed genes found in the analysis; in pink highlight, the manipulated transcript POPACULIS5.

(2) genes nomenclature and gene models to the corresponding probesets were search for in PopArray annotation tool (<http://aspendb.uga.edu/poparray>) as well as obtained from the NetAffx annotation as of November 2010.

(3) *Arabidopsis* homolog BLASTX best hit obtained using PlexDB database Microarray Platform Translator tool (<http://www.plexdb.org>), e-value cutoff of $1e^{-20}$.

(4) Fold Change (FC) and 90% Lower Confidence Bound (LCB) of Fold Change is shown for each pair-wise comparison. A positive LCB indicates that the higher signal was from the second member of the pair-wise sample comparison, while a negative LCB indicates that the higher signal was from the first member of the comparison. (--) not found differentially expressed.

Table S2. Expression data (Mean signal intensities, MSIs) of *Populus* cell-cycle related-genes homologs ⁽¹⁾.

Probeset Id	Description ⁽²⁾	Samples (MSIs)		Gene Model ⁽³⁾	Ath ID ⁽³⁾	Pair-wise comparison ⁽⁴⁾									
		WTMS6				B2 MS2		WTMS2		B2 MS6		(G)WTMS6/WTMS2		(D)WTMS2/B2 MS2	
		FC	LCB			FC	LCB	FC	LCB	FC	LCB	FC	LCB	FC	LCB
Cell cycle and MAPK-related genes															
PpAffx.76003.1.A1_at	CDG6	37.51	126.12	364.19	76.22	Porti.001G250200	AT1G07270.1	-4.78	-2.85	9.71	6.91	-1.65	-0.99		
PpAffx.21241.1.S1_at	CDG5L	65.01	144.76	246.44	84.03	Porti.019C018400	AT1G09770.1	-2.93	-2.08	3.79	2.70	-1.72	-1.20		
PpAffx.209155.1.S1_at	CCAR1	51.04	420.21	367.52	54.40	Porti.010C0166100		-6.76	-2.34	7.20	2.50	-7.72	-4.04		
PpAffx.3035.1.S1_a_at	CCAR7	124.76	231.48	288.11	82.47	Porti.012G044400	AT4G37110.1	-3.49	-2.77	2.31	1.89	-2.29	-2.29		
PpAffx.71284.1.S1_at	Cyclin-A1-1	80.57	166.04	231.69	49.88	Porti.005G181400	AT1G44110.1	-4.65	-3.63	2.88	2.20	-3.33	-2.60		
PpAffx.206760.1.S1_at	Cyclin-A2-2	80.36	201.79	255.38	72.55	Porti.006G247200	AT5G13001.1	-3.52	-2.11	3.18	2.13	-2.78	-1.71		
PpAffx.8998.1.S1_a_at	Cyclin-A2-2	22.71	63.50	65.89	14.64	Porti.01R034100	AT5G13001.1	-4.50	-2.60	2.90	1.91	-4.34	-2.63		
PpAffx.132011.1.S1_at	Cyclin-A2-4	10.79	35.81	32.37	13.25	Porti.003G058200		-2.44	-0.84	3.00	1.11	-2.70	-1.19		
PpAffx.200516.1.S1_at	Cyclin-A2-4	55.68	105.12	152.08	47.89	Porti.001G171100	AT1G08370.1	-3.18	-2.39	2.74	1.98	-2.19	-1.70		
PpAffx.5638.1.S1_at	Cyclin-B2-4	159.43	293.86	450.07	107.41	Porti.005G251400	AT1G76310.1	-4.19	-3.22	2.82	2.20	-2.74	-2.11		
PpAffx.721.1.S1_s_at	Cyclin-B2-4	101.37	172.07	225.26	57.48	Porti.009G163800	AT1G76310.1	-3.92	-2.89	2.22	1.74	-2.99	-2.20		
PpAffx.201199.1.S1_at	Cyclin-D4-1	251.79	217.88	425.72	672.46	Porti.001G292300	AT2G24901.1	-1.16	-1.01	1.58	0.94	1.43	1.84		
PpAffx.121547.1.A1_at	DMT F1	40.82	232.08	266.55	39.11	Porti.003G168900		-6.82	-3.53	6.53	3.31	-5.93	-3.79		
PpAffx.6452.1.S1_at	CKR4	262.92	623.99	454.95	97.37	Porti.003G101600	AT4G19110.2	-4.67	-3.34	1.73	1.12	-6.41	-4.47		
PpAffx.202661.1.S1_at	KRP3	92.85	148.63	472.71	456.40	Porti.002G242100	AT5G48820.1	-1.04	-0.87	5.09	4.25	2.47	2.47		
PpAffx.75453.1.A1_at	KRP3	318.61	430.82	1306.77	1456.11	Porti.002G242100	AT5G48820.1	1.11	0.91	4.10	3.76	3.38	2.75		
PpAffx.5937.1.S1_at	Cyclin-P3-1	736.02	1110.75	224.68	278.56	Porti.01G0232000	AT3G21201.1	1.24	0.86	-3.28	-2.45	-3.99	-3.04		
PpAffx.152798.1.S1_at	Cyclin-U1-1	366.25	575.80	169.01	263.60	Porti.007G121500	AT3G21870.1	1.56	1.10	-2.17	-1.72	-2.18	-1.74		
PpAffx.203732.1.S1_at	Purative cyclin-D6-1	67.95	139.82	102.34	30.65	Porti.004G032100	AT4G03270.1	-3.34	-2.19	1.51	1.07	-4.56	-3.41		
PpAffx.209732.1.S1_at	Purative cyclin-D6-1	41.53	71.56	94.05	13.29	Porti.01G040900	AT4G03270.1	-7.08	-4.34	2.26	1.53	-5.38	-3.25		
PpAffx.212506.1.S1_at	Purative cyclin-D6-1	57.65	117.01	145.78	39.82	Porti.019G118600	AT4G03270.1	-3.66	-2.35	2.53	1.92	-2.94	-1.84		
PpAffx.30445.1.A1_at	MPK15	2326.43	3950.47	1024.23	937.26	Porti.008G130000	AT2G01450.4	-1.09	-0.83	-2.27	-1.83	-4.21	-3.57		
PpAffx.30445.2.S1_at	MPK15	454.11	675.76	316.66	177.22	Porti.01G0112200	AT2G01450.4	-1.79	-1.42	-1.43	-0.85	-3.81	-3.19		
PpAffx.92745.1.S1_at	MPK15	256.23	464.92	238.41	85.66	Porti.010C112200	AT3G18040.1	-2.78	-2.14	-1.07	-0.59	-5.43	-4.51		
PpAffx.4765.1.S1_at	MPK19	264.12	824.44	562.73	256.59	Porti.001G381300	AT3G14720.1	-2.19	-1.48	2.13	1.53	-3.21	-2.19		
PpAffx.51779.1.A1_at	MPK19	53.21	156.17	126.42	46.78	Porti.01G102500	AT2G42880.1	-2.70	-2.04	2.38	1.83	-3.34	-2.61		
PpAffx.205653.1.S1_at	MPK20	41.94	150.16	166.17	66.94	Porti.005G201800	AT2G42880.1	-2.48	-1.70	3.96	2.70	-2.24	-1.54		
PpAffx.41158.3.A1_at	MPK3	60.23	96.95	71.89	177.76	Porti.001G271700	AT3G45640.1	1.44	1.44	1.19	0.95	1.83	1.47		
PpAffx.220070.1.S1_at	M2K5	189.27	91.61	36.70	47.89	Porti.001G138800	AT3G21220.1	-2.07	-1.72	1.31	0.85	-1.91	-1.48		
PpAffx.76213.1.A1_at	M3K1	85.01	228.09	71.36	50.07	Porti.002G088900	AT4G08500.1	-1.43	-0.93	-5.16	-3.66	-4.56	-3.23		
PpAffx.208139.1.S1_s_at	M3K3	50.47	96.84	147.04	50.78	Porti.008G149500	AT3G06300.1	1.92	1.50	-2.90	2.33	-1.91	-1.46		
PpAffx.208799.1.S1_at	M3K3	56.07	87.79	147.22	51.45	Porti.010C092000	AT3G06300.1	-2.86	-2.28	2.63	1.93	-1.71	-1.30		
PpAffx.988.1.A1_at	MKKA	222.86	99.18	55.33	53.64	Porti.014G155000	AT4G26890.1	-1.03	-0.73	-4.03	-3.30	-1.85	-1.27		
PpAffx.202496.1.S1_at	MKKA	185.17	109.84	47.60	25.92	Porti.002G228200	AT1G05100.1	-1.69	-1.32	-1.84	-3.02	-4.24	-3.03		
PpAffx.35670.1.A1_at	MKKA	331.53	163.23	66.13	57.15	Porti.002G228200	AT1G05100.1	-2.03	-1.61	-0.87	-5.01	-2.86	-2.01		
PpAffx.28910.1.S1_at	ANP1	53.64	127.51	165.58	52.86	Porti.014G035500		-3.13	-2.24	3.09	2.36	-2.41	-1.84		

⁽¹⁾ probesets represent the cell cycle and MAPK-related differentially expressed genes found in the global analysis, and LCB>1.2 in at least one out of four pair-wise comparisons is indicated.

⁽²⁾ genes nomenclature and gene models to the corresponding probesets were search for in PopArray annotation tool (<http://aspenb.uga.edu/poparray>) as well as obtained from the NetAffx annotation as of November 2010

⁽³⁾ *Arabidopsis* homolog BLASTX best hit obtained using PlexDB database Microarray Platform Translator tool (<http://www.plexdb.org>), e-value cutoff of 1×10^{-10} .

⁽⁴⁾ Fold Change (FC) and 90% Lower Confidence Bound (LCB) of Fold Change is shown for each pair-wise comparison. A positive FC or LCB indicates that the higher signal was from the second member of the pair-wise sample comparison, while a negative FC or LCB indicates that the higher signal was from the first member of the comparison.

Table S3. Expression data (Mean signal intensities, MSIs) of *Populus* hormone-related genes homologs⁽¹⁾.

[illegible]

Table S3. (continued)

Protein ID	Description ⁽¹⁾	Supplies (MSKs)			Gene Model ⁽²⁾	Ref. ⁽³⁾	Ath ID ⁽⁴⁾	GOWT NSC62 MSK			GOWT NSC62 MS2			Bip-allele comparison ⁽⁵⁾		
		WT:MSK	R2:MSK	WT:MS2				L:G	U:G	L:G	U:G	L:G	U:G	L:G	U:G	
G316	PpPpAKR_131103.1.S1_5_at	739.06	437.30	89.42	PpPp001G210400		AT1G54510.1	721	140	15	0.69	75.1	73	3.0	NS2	
G316	PpPpAKR_131140.1.S1_5_at	308.1	597.15	19.67	PpPp011351510.1		AT1G54510.1	191	167	1.32	-0.96	-2.69	-2.20	-4.74	-3.80	
G316	PpPpAKR_131004.1.S1_5_at	693.98	524.46	144.04	PpPp0113512700		AT1G38130.1	116	-19	3.14	2.23	-4.75	-3.59	-2.20	-1.41	
G319	PpPpAKR_135944.1.A1_5_at	558.50	480.04	297.70	PpPp011020360400		AT1G38130.1	11.14	-3.35	-3.25	-2.05	-1.91	-1.64	-5.01	-3.36	
Cullin-1	PpPp_36571.S1_5_at	91.27	304.10	267.20	PpPp0110203800		AT1G02570.3	333	206	-3.52	-2.06	2.93	1.77	-3.33	-2.40	
Cullin-1	PpPpAKR_131455.1.S1_5_at	754.61	124.93	137.55	PpPp001G2115500		AT1G02570.3	435	259	-3.55	-2.46	4.64	3.08	-3.22	-2.95	
Cullin-1	PpPpAKR_125413.2.S1_5_at	196.60	696.50	465.65	PpPp001G217700		AT1G02570.3	335	252	-2.87	-2.23	2.50	1.91	-3.74	-2.95	
Cullin-1	PpPpAKR_68365.1.S1_5_at	215.18	296.45	580.78	PpPp010502079000		AT1G02570.3	138	117	-1.41	-1.00	3.00	2.72	1.96	1.63	
Cullin-4	PpPpAKR_112316.1.S1_5_at	42.03	238.29	79.16	PpPp0110208400		AT1G546210.1	5.67	3.82	-2.52	-1.40	1.88	1.02	-7.59	-5.34	
Cullin-4	PpPpAKR_75563.1.S1_5_at	20.73	71.77	77.81	PpPp010464332900		AT1G546210.1	3.46	2.21	-5.09	-3.66	3.75	2.42	-4.69	-3.34	
Cytokinin signalling																
CK hydrolyase CYP735A1	PpPpAKR_80255.1.S1_5_at	49.53	42.83	57.90	PpPp0119204600		AT1G524910.1	-11.16	-0.85	2.42	1.90	1.27	0.87	3.28	2.49	
CK hydrolyase CYP735A2	PpPpAKR_204240.1.S1_5_at	144.81	31.082	34.38	PpPp011751142000		AT1G538450.1	2.15	1.49	1.78	0.81	-4.22	-2.96	-7.09	-4.69	
LOX5	PpPpAKR_205860.1.S1_5_at	21.15	38.91	21.14	PpPp010464318100		AT1G23805.1	1.84	1.37	-2.00	-1.36	-1.00	-0.63	-3.69	-2.70	
LOX5	PpPpAKR_205860.1.S1_5_at	329.81	55.52	159.01	PpPp010464348900		AT1G35190.1	1.69	1.39	-2.11	-0.70	-2.07	-1.51	-3.86	-2.70	
LOX7	PpPpAKR_12485.1.S1_5_at	56.89	53.89	228.03	PpPp001G210400		AT1G02570.3	1.06	-0.75	-1.36	-0.70	3.97	3.12	3.08	0.82	
LOX7	PpPpAKR_12485.1.S1_5_at	324.86	398.47	169.88	PpPp001G210500		AT1G02570.3	1.16	1.04	-1.78	-1.35	-2.02	-1.69	-4.17	-3.35	
ARK1: His kinase 1	PpPp_9741.S1.S1_5_at	42.24	620.75	295.52	PpPp001G205600		AT1G02570.3	3.15	2.33	-7.01	-4.76	-1.74	-1.24	-4.49	-3.80	
ARK1: His kinase 1	PpPpAKR_118843.1.S1_5_at	81.57	257.13	143.34	PpPp001G205600		AT1G02570.3	1.45	1.10	-1.41	-1.14	-1.79	-1.22	-3.68	-3.03	
ARK2: His kinase 2	PpPpAKR_211912.1.S1_5_at	159.38	231.83	89.03	PpPp011461647000		AT1G02570.3	1.02	-0.47	-1.34	-0.97	-1.39	-1.30	2.34	1.19	
CKX5: CK dehydrogenase 3	PpPpAKR_201624.1.S1_5_at	16.67	16.41	515.76	PpPp010464352500		AT1G02570.3	1.34	0.97	-1.89	-1.38	5.34	2.60	2.00	1.17	
CKX5: CK dehydrogenase 5	PpPpAKR_211912.1.S1_5_at	27.05	36.32	144.50	PpPp0102032800	(d)	AT1G59940.1	-11.10	-0.78	1.34	1.06	2.21	1.60	3.27	2.69	
PpPp1AR32: type A RR	PpPpAKR_44007.2.S1_5_at	131.27	109.79	268.07	PpPp0116203800	(e) (f)	AT1G57040.1	432	323	-6.41	-4.57	3.87	3.11	-5.15	-3.87	
PpPp2AR38: type A RR	PpPpAKR_213255.1.S1_5_at	95.70	470.90	586.66	PpPp0116203800	(e) (f)	AT1G57040.1	1.98	1.39	-3.61	-2.47	4.09	3.11	-1.57	-1.10	
PpPp2AR38: type A RR	PpPpAKR_31321.2.S1_5_at	45.52	91.18	188.18	PpPp0116203800	(e) (f)	AT1G57040.1	1.35	1.15	-3.11	-2.07	12.27	9.82	2.97	1.71	
PpPp2AR38: type A RR	PpPpAKR_75760.1.A1_5_at	64.15	85.34	786.99	PpPp0116203800	(e) (f)	AT1G57040.1	7.05	4.41	-11.53	-6.48	11.85	5.88	4.97	5.40	
AR89: type A RR	PpPpAKR_155280.1.S1_5_at	52.88	373.00	628.46	PpPp001G207200		AT1G57040.1	3.88	2.55	-7.95	-5.22	7.14	4.18	-4.32	-3.34	
AR89: type A RR	PpPpAKR_206027.1.S1_5_at	20.04	108.92	200.18	PpPp001G207200		AT1G57040.1	1.78	1.42	-3.39	-2.71	5.42	4.04	-1.11	-0.99	
AR89: type A RR	PpPpAKR_30343.1.S1_5_at	146.79	261.42	795.02	PpPp001G207200		AT1G57040.1	2.58	1.75	-1.49	-0.91	1.34	0.89	-2.97	-1.78	
AR89: type B RR	PpPpAKR_208970.1.S1_5_at	145.16	374.06	194.46	PpPp0116203800		AT1G57040.1	4.63	2.93	-3.53	-1.88	2.58	1.59	-6.39	-3.48	
AR89: type B RR	PpPpAKR_94034.1.S1_5_at	50.16	232.48	128.51	PpPp0116203800		AT1G57040.1	2.19	1.42	1.21	0.87	3.66	2.47	2.03	1.43	
AR89: type B RR	PpPpAKR_204954.1.S1_5_at	35.77	117.76	197.05	PpPp0116203800		AT1G57040.1	2.94	1.60	-3.71	-2.16	2.31	1.15	-4.73	-3.12	
AR89: type B RR	PpPpAKR_100093.1.S1_5_at	35.97	105.63	82.99	PpPp0116203800		AT1G57040.1	2.67	1.61	-3.03	-1.61	1.99	0.93	-4.08	-3.08	
AR89: type B RR	PpPpAKR_100093.1.S1_5_at	196.88	489.76	371.02	PpPp0116203800		AT1G57040.1	3.37	2.28	-2.73	-1.81	3.27	2.19	-2.82	-1.89	
AR89: type B RR	PpPpAKR_31591.S1_5_at	41.36	139.57	135.06	PpPp0116203800		AT1G57040.1	3.37	2.28	-2.73	-1.81	3.27	2.19	-2.82	-1.89	
AR89: type B RR	PpPpAKR_144429.1.S1_5_at	203.61	530.86	332.38	PpPp001G207200		AT1G57040.1	2.61	2.03	-2.12	-1.66	1.63	1.24	-3.39	-2.70	
Ethylene metabolism and signalling																
ACO	PpPpAKR_94798.2.A1_5_at	940.19	527.15	4769.58	PpPp0111202900		AT1G346640.2	2.19	1.42	-1.78	-1.52	5.07	4.42	1.55	0.48	
ACGH1	PpPp_4827.1.S1_5_at	260.81	21.403	81.25	PpPp001G212200		AT1G346640.2	-1.25	-1.06	-1.22	-0.97	-3.30	-2.76	-3.21	-2.58	
ACGH1	PpPpAKR_135709.1.S1_5_at	67.36	199.90	16.47	PpPp01102073200		AT1G0650.2	1.29	1.37	1.40	-0.98	-3.37	-3.12	-8.68	-6.55	
ACGH2	PpPpAKR_150115.1.S1_5_at	108.69	140.31	419.88	PpPp001G0650.2		AT1G0650.2	1.29	1.37	1.40	-0.98	-3.37	-3.12	-8.68	-6.55	
ACGH3	PpPpAKR_150115.1.S1_5_at	25.16	35.52	51.03	PpPp001G0650.2		AT1G0650.2	1.29	1.37	1.40	-0.98	-3.37	-3.12	-8.68	-6.55	
ACGH4	PpPpAKR_615141.1.A1_5_at	249.25	200.35	359.20	PpPp01132045000		AT1G0650.2	-1.24	-1.08	2.17	1.62	1.43	1.39	3.89	3.03	
ACGH5	PpPpAKR_98197.1.S1_5_at	33.53	47.44	75.39	PpPp01132045000		AT1G0650.2	-1.24	-1.08	2.17	1.62	1.43	1.39	3.89	3.03	
ACOH1	PpPpAKR_75413.2.S1_5_at	13.96	13.39	247.60	PpPp001G214500		AT1G235450.1	1.04	-0.55	1.02	0.75	2.25	1.61	2.39	1.21	
ACOH1	PpPpAKR_206393.1.S1_5_at	812.48	1252.34	2894.36	PpPp001G214500		AT1G235450.1	1.04	-0.55	1.02	0.75	2.25	1.61	2.39	1.21	
ACOH1	PpPpAKR_71066.3.A1_5_at	848.66	191.01	20.24	PpPp001G214500		AT1G235450.1	1.54	1.46	-1.52	-1.24	3.56	3.02	1.52	1.31	
ACOH5	PpPpAKR_206393.1.S1_5_at	22.84	30.51	116.28	PpPp001G214500		AT1G0501.1	-4.44	-3.13	1.46	0.00	-4.53	-29.90	-4.66	-2.63	
ACS	PpPp_118.1.S1_5_at	16.26	20.64	30.53	PpPp001G214500		AT1G0501.1	-4.44	-3.13	1.46	0.00	-4.53	-29.90	-4.66	-2.63	
ACS1	PpPpAKR_207433.1.S1_5_at	126.25	355.59	227.28	PpPp001G214500		AT1G0501.1	-4.44	-3.13	1.46	0.00	-4.53	-29.90	-4.66	-2.63	
EN13	PpPp_2383.2.S1_5_at	136.10	77.02	200.06	PpPp001G214500		AT1G0501.1	-4.44	-3.13	1.46	0.00	-4.53	-29.90	-4.66	-2.63	
EN3	PpPp_2383.2.S1_5_at	77.02	200.06	136.10	PpPp001G214500		AT1G0501.1	-4.44	-3.13	1.46	0.00	-4.53	-29.90	-4.66	-2.63	

Table S3. (continued)

Probe/Std ID	Description ^{a)}	Pair-wise comparison ^{b)}													
		WTMS6					WTMS2								
		WTMS6	B2 MS6	Sample's (MSIs)	Gene Model ^{c)}	Ref ^{d)}	Ath ID ^{e)}	LCB	FC	QW TMS6/WTMS2	QW TMS2/WTMS2	LCB	FC		
PipAffx.20091.1.SI_s_at	ET01	168.95	388.87	210.50	82.29	Port.0010906229300	1.27	1.13	-2.56	-1.96	1.25	0.89	-4.73	-3.64	
PipAffx.150396.1.SI_at	RT01	93.70	119.40	338.23	335.69	Port.0010906229300	1.04	0.88	-1.26	-1.00	3.61	3.13	2.81	2.49	
Pip.5192.1.SI_at	ET02	444.97	463.64	1655.68	1314.14	Port.01010074300	1.27	1.13	-1.01	-0.88	3.72	3.02	2.83	2.32	
PipAffx.8810.1.SI_at	ER013	163.85	118.32	79.81	401.80	Port.00102085600	-1.38	-1.08	5.03	4.07	-2.05	-1.58	3.40	2.80	
PipAffx.75787.1.A1_at	ERF13	36.54	37.47	569.25	737.92	Port.00103612500	1.03	0.78	1.30	1.02	15.58	11.74	19.69	15.59	
PipAffx.75787.2.A1_s_at	ERF13	12.31	13.52	36.06	97.26	Port.00101067600	1.10	0.67	2.70	1.74	2.93	1.96	7.19	4.42	
PipAffx.75787.2.A1_s_at	ERF13	72.34	50.57	108.46	227.14	Port.00101067600	-1.43	-1.20	2.09	1.71	1.50	1.22	4.49	3.74	
PipAffx.81893.1.A1_s_at	ERF1B	20.35	15.16	81.98	105.60	Port.00105223200	-1.34	-0.71	1.29	0.47	4.03	2.02	6.96	2.60	
PipAffx.20341.1.SI_s_at	ERF2	350.00	340.86	997.97	762.70	Port.0010305107000	-1.13	-0.98	-1.19	-0.79	2.59	1.99	2.45	1.49	
PipAffx.590.1.SI_at	PrERF50	182.91	141.76	392.06	570.87	Port.001010379900	-1.29	-0.95	1.46	0.70	2.14	1.68	4.03	1.93	
PipAffx.145434.2.A1_at	PrERF72	111.09	120.39	203.78	331.12	Port.001010379900	1.08	0.79	1.62	1.27	1.83	1.37	2.75	2.13	
PipAffx.157262.SI_at	RAP2-1	19.18	12.43	178.23	245.52	Port.001020180500	-1.54	-0.80	1.43	0.72	9.29	6.45	20.53	9.26	
PipAffx.157262.SI_at	RAP2-1	548.22	602.57	181.23	245.52	Port.001020180500	1.10	1.03	1.35	0.87	-3.02	-2.74	-2.45	-1.80	
PipAffx.4624.1.SI_at	RAP2-3	252.72	330.05	167.482	1994.28	Port.001020210900	1.31	1.09	1.19	0.89	6.63	5.07	6.04	4.77	
PipAffx.7270.1.SI_at	RAP2-3	1567.37	1098.56	3863.20	2136.82	Port.001008210900	-1.43	-1.12	-1.81	-1.67	2.46	2.09	1.95	1.64	
PipAffx.110954.1.SI_s_at	RAP2-3	426.84	589.06	2642.18	3107.99	Port.01014021600	1.38	1.12	1.18	0.86	6.19	4.97	5.28	3.89	
PipAffx.110954.1.SI_s_at	SHINE2	44.05	31.47	63.15	129.02	Port.01018028000	-1.40	-1.10	2.04	1.48	1.43	1.03	4.10	3.30	
PipAffx.20247.1.SI_at	TEM1	14.09	33.03	63.34	43.04	Port.001020167400	2.34	1.57	-1.47	-1.08	4.50	3.03	1.30	0.88	
PipAffx.572.3.SI_at	TEM4	272.64	1339.48	1689.52	941.96	Port.010100219200	4.91	3.60	-1.79	-1.28	6.20	4.49	-1.42	-1.02	
PipAffx.214377.1.SI_s_at	AUL5	141.44	332.31	414.35	122.65	Port.001006167700	2.35	1.49	-3.38	-2.62	2.93	1.90	-2.71	-2.04	
ROS scavengers															
Pip.1994.1.SI_at	SOD [Cu-Zn]	2873.03	2041.06	556.38	715.79	Port.001040216700	-1.31	-0.95	1.29	1.08	-4.80	-3.48	-2.85	-2.41	
PipAffx.153878.1.A1_at	SOD [Fe]	991.16	4509.10	6303.53	3710.12	Port.010150110400	4.55	3.16	-1.70	-1.29	6.36	4.45	-1.22	-0.91	
Pip.528.1.A1_at	SOD [Mn]	3391.97	3340.29	9191.37	7724.09	Port.01019057300	-1.02	-0.94	-1.19	-1.10	2.71	2.53	2.31	2.13	
Pip.592.1.SI_at	GPX2	283.28	298.73	823.57	897.95	Port.001070216600	1.05	0.84	1.09	0.98	2.91	2.52	3.01	2.50	
PipAffx.2898.1.SI_s_at	CAT2	378.24	816.66	100.08	449.67	Port.001005004000	2.16	1.86	4.49	3.46	-3.78	-3.01	-1.82	-1.51	
PipAffx.144124.1.SI_at	CST/DHAR2	20.44	44.72	55.37	13.80	Port.010100211600	2.19	1.41	-4.01	-2.27	2.71	1.69	-3.24	-1.88	
PipAffx.209.1.SI_at	CST F12	332.92	376.73	3164.88	599.64	Port.0101702138000	1.13	0.89	-5.28	-3.05	9.51	6.46	1.59	0.80	
Pip.3709.1.SI_at	CST L3	448.22	456.05	1825.06	725.21	Port.010160083500	1.02	0.76	-2.52	-2.05	4.07	3.10	1.59	1.22	
Pip.5347.1.SI_s_at	CST F	1041.97	489.52	2756.58	4519.47	Port.01035400	-2.13	-1.93	1.64	1.46	2.65	2.44	9.23	8.14	
Pip.6448.1.SI_at	CST T1	309.19	799.18	1145.19	574.81	Port.001010105600	2.98	2.08	-1.99	-1.47	3.70	2.82	-1.39	-1.07	
Pip.5289.1.SI_at	CST UH	742.61	546.36	1891.26	3035.55	Port.010100235500	-1.36	-1.22	1.61	1.35	2.53	2.05	5.56	5.00	
PipAffx.77335.1.SI_at	CST U7	35.57	35.11	55.06	127.06	Port.01181500	-1.01	-0.65	2.31	1.16	1.55	0.94	3.62	1.88	
PipAffx.77335.1.SI_at	CST UB	206.00	61.03	66.35	377.69	Port.010604500	-3.38	-2.11	5.69	1.83	-3.10	-1.50	6.19	2.27	
Pip.2096.1.SI_at	CST23	1427.77	1679.87	4565.07	6103.19	Port.001008217400	1.18	0.90	1.34	1.14	3.20	2.57	3.63	2.98	
PipAffx.24672.1.SI_at	CST23	28.66	25.54	64.43	113.15	Port.0106218500	-1.04	-0.68	1.76	1.21	2.42	1.73	4.43	2.94	
PipAffx.23427.1.SI_s_at	CST23	31.52	31.35	80.38	138.59	Port.01016018500	-1.01	-0.63	1.72	1.10	2.55	1.64	4.42	2.76	
PipAffx.23427.1.SI_s_at	CST23	1603.83	1920.24	5232.59	7224.58	Port.0106218500	-1.20	-0.95	1.38	1.16	3.26	2.62	3.76	3.13	
PipAffx.54661.1.SI_at	CST23	220.56	154.60	493.58	848.12	Port.001008217500	-1.43	-1.01	1.72	1.12	2.24	1.61	5.49	3.52	
Pip.3168.2.A1_at	CST	40.09	34.48	136.09	126.37	Port.01010026900	-1.16	-0.84	-1.08	-0.88	3.39	2.63	3.67	2.86	
Pip.5536.1.SI_at	CST	306.35	309.82	1007.28	1090.98	Port.01010026900	1.01	0.82	1.08	0.93	3.29	2.73	3.52	2.97	
Pip.6936.1.SI_at	CST	150.54	137.33	365.12	536.07	Port.01015042000	-1.10	-0.89	1.52	1.04	2.43	1.98	4.05	2.74	
PipAffx.25444.1.SI_s_at	CST	1179.03	732.77	2171.26	5938.80	Port.01015042000	-1.57	-1.02	-1.26	-1.09	6.25	4.94	7.76	5.48	
PipAffx.25444.1.SI_s_at	CST	506.62	516.94	3866.11	2944.66	Port.01010026900	-1.60	-1.02	-1.31	-1.11	7.63	5.89	9.29	6.54	
Pip.7441.1.SI_at	CST	1125.01	493.72	254.47	1052.19	Port.001010437200	-2.28	-1.87	4.19	3.52	-4.42	-3.60	2.16	1.84	
PipAffx.13351.1.SI_at	CST	275.09	228.43	868.77	2012.99	Port.01010026900	-1.20	-0.86	2.32	1.77	3.16	2.25	8.81	6.86	
PipAffx.54632.1.SI_at	CST	336.71	355.62	615.02	1054.46	Port.01010431200	1.06	0.93	1.71	1.57	1.83	1.60	2.97	2.73	
PipAffx.225774.1.SI_at	CST	287.46	187.10	39.19	52.49	Port.00105037800	-1.43	-1.28	1.34	0.94	-4.82	-3.53	-2.79	-2.30	
PipAffx.4246.2.SI_s_at	CST	90.99	168.21	976.78	1396.08	Port.010190303000	1.85	1.41	1.43	0.98	10.74	8.21	8.30	5.70	
PipAffx.4331.1.A1_at	CST	348.41	188.94	2071.55	958.02	Port.010130317200	-1.84	-1.29	-2.16	-1.48	5.95	5.10	5.07	2.57	

Table S3. (continued)

ProbeSet Id	Description ^(a)	Samples (MSs)			Gene Model ^(b)	Ref ^(c)	Att ID ^(d)	Pair-wise comparison ^(e)							
		Stratigic related Brassicasteroids						FC	LCB	FC	LCB				
		WT MS6	B2 MS6	WT MS2											
Prp1Atx.72480.1.S1_at	2,3-enoyl-CoA reductase	47.61	43.55	85.89	311.22	Pratt0.00245200	AT5G16010.1	-1.09	-0.90	3.62	2.30	1.80	1.41	7.15	4.62
Prp1Atx.154504.1.S1_at	SSA2	266.57	273.84	830.87	1763.92	Pratt0.0080012700	AT5G16010.1	1.03	0.87	2.12	1.93	3.12	2.68	6.44	5.79
Prp1Atx.200436.1.S1_at	ASAT1	64.24	49.72	183.73	150.45	Pratt0.0010106800	AT5G16010.1	-1.29	-0.96	-1.22	-0.83	2.86	1.91	3.03	2.35
Prp1Atx.204864.1.S1_at	ASAT1	433.15	199.61	123.30	129.93	Pratt0.0090551000	AT5G16010.1	-2.17	-1.80	1.05	0.81	-3.51	-2.93	-1.54	-1.20
Prp1Atx.211300.1.S1_at	DWR6, DET2	34.94	25.77	46.81	95.21	Pratt0.0090551000	AT2G38030.1	-1.36	-1.05	2.03	1.55	1.34	1.03	3.69	2.87
Prp1Atx.79902.1.S1_at	Cycochrome P450 90A1	1152.76	1660.26	530.05	531.21	Pratt0.0010018800	AT5G05690.1	1.44	1.18	1.00	0.90	-2.17	-1.73	-3.13	-2.80
Prp1Atx.261561.1.S1_at	Cycochrome P450 90B1	93.55	138.88	33.25	41.29	Pratt0.0050124000	AT5G05690.1	1.48	1.20	1.24	0.84	-2.81	-2.02	-3.36	-2.60
Prp1Atx.70900.1.S1_at	HSOL1	2226.71	1824.62	634.54	648.94	Pratt0.0070409000	AT3G50660.1	-1.22	-1.13	1.02	0.70	-3.51	-3.29	-2.81	-2.13
Prp1Atx.205515.1.S1_at	ORP1C	34.37	92.97	95.41	36.40	Pratt0.0050127900	AT3G50660.1	2.71	2.04	-2.62	-2.09	2.78	2.06	-2.55	-2.07
Prp1Atx.212978.1.S1_at	ORP1C	100.38	197.30	264.31	90.91	Pratt0.00320000	AT1G58440.1	1.97	1.66	-2.91	-2.45	2.63	2.25	-2.17	-1.81
Prp1Atx.212998.1.S1_at	Squalene monoxygenase	25.75	19.77	29.01	103.64	Pratt0.1504209000	AT1G58440.1	-1.30	-0.64	3.57	1.94	1.13	0.73	5.24	2.81
Prp1Atx.212653.1.S1_at	BMI1	33.85	84.32	106.25	28.89	Pratt0.1504080000	AT1G58440.1	2.49	1.73	-3.68	-2.43	3.14	2.18	-2.92	-1.93
Gibberellin signaling															
Prp1Atx.1091.1.S1_at	GA 3-b-deoxygase 1	267.18	209.62	84.41	61.57	Pratt0.0030574000	AT5G59845.1	-1.27	-1.07	-1.37	-0.97	-3.17	-2.57	-3.40	-2.48
Prp1Atx.146201.1.S1_at	Snakin-1	221.46	222.84	101.70	48.84	Pratt0.0402010000	AT5G59845.1	1.01	0.71	-2.08	-0.89	-2.18	-1.17	-4.56	-2.86
Prp1Atx.46665.1.S1_at	Snakin-2	616.05	365.89	953.04	1155.10	Pratt0.0020226000	AT1G75750.1	-1.68	-1.39	1.21	0.62	1.55	0.90	3.16	1.76
Prp1Atx.8875.1.S1_at	Snakin-2	537.20	1252.19	524.28	437.27	Pratt0.15510000	AT1G75750.1	2.33	1.45	8.62	3.63	-2.72	-0.55	3.61	1.75
Prp1Atx.6701.1.S1_at	GA 20 oxidase 2	236.73	316.52	87.18	61.17	Pratt0.0050184400	AT1G75750.1	1.34	0.98	-1.43	-0.88	-2.02	-1.61	-5.17	-4.07
Prp1Atx.138338.1.S1_at	GA 2-b-deoxygase 1	82.14	92.73	189.58	55.16	Pratt0.0010137800	AT1G78440.1	1.13	0.86	-3.44	-2.18	2.31	1.66	-1.68	-1.10
Prp1Atx.3575.1.S1_at	GA-regulated protein 1	763.90	691.13	1084.40	2897.80	Pratt0.0050239100	AT1G78440.1	-1.11	-0.83	2.67	1.69	1.34	0.88	4.19	2.76
Prp1Atx.45488.2.S1_at	Snakin-1	82.25	84.89	114.50	500.33	Pratt0.0010129700	AT2G18420.1	-1.24	-1.16	-1.35	-0.99	-5.13	-4.48	-5.59	-4.34
Prp1Atx.45488.2.S1_at	DELLA protein GAI	3692.06	2983.54	719.59	534.05	Pratt0.0010129700	AT2G18420.1	-1.80	-1.45	2.40	1.81	2.85	2.14	12.30	9.67
Prp1Atx.46984.1.S1_at	GA receptor GID1B	294.88	163.89	839.05	2016.24	Pratt0.0020231000	AT3G63010.1	-1.61	-1.21	-1.20	-0.88	-2.84	-2.07	-2.13	-1.65
Prp1Atx.42169.1.S1_at	GA 3-b-deoxygase 1	93.23	58.06	32.84	27.30	Pratt0.0040288800	AT1G68350.1	-1.24	-1.10	1.06	0.74	-2.95	-2.34	-2.24	-1.74
Prp1Atx.45488.4.S1_at	DELLA protein GAI	340.43	273.96	115.41	123.51	Pratt0.0040288800	AT1G68350.1	1.21	0.85	-1.30	-0.82	2.98	2.09	1.90	0.95
Prp1Atx.218966.1.S1_at	DELLA protein GAI	11.49	13.91	34.26	26.40	Pratt0.1700186000	AT1G68350.1	1.50	1.23	-4.64	-3.52	2.15	1.73	-3.25	-2.49
Prp1Atx.200450.1.S1_at	SCK-like protein 26	76.56	115.21	164.60	35.46	Pratt0.0010109400	AT1G68350.1	1.42	1.04	-4.18	-3.12	4.12	3.24	-1.44	-1.02
Prp1Atx.203250.1.S1_at	GA 3-b-deoxygase	47.24	67.16	194.76	46.60	Pratt0.0030128100	AT1G68350.1	-1.04	-0.73	-1.32	-0.98	-2.76	-2.20	-3.52	-2.13
ABA															
Prp1Atx.209941.1.S1_at	NCED1	158.23	152.86	57.27	43.42	Pratt0.1010124000	AT3G63520.1	-1.04	-0.73	-1.32	-0.98	-2.76	-2.20	-3.52	-2.13
Prp1Atx.130684.1.S1_at	CCD1	40.24	167.85	250.89	61.50	Pratt0.0050605000	AT3G63520.1	4.17	3.14	-4.08	-2.82	6.23	4.04	-5.73	-4.91
Prp1Atx.101067.1.S1_at	CCD4	3255.57	5006.56	1642.78	841.82	Pratt0.1500934000	AT5G67030.2	1.54	1.16	-1.95	-1.59	1.38	0.71	-3.80	-2.95
Prp1Atx.141359.1.S1_at	ABA2	25.02	62.45	25.85	15.95	Pratt0.0050138400	AT5G67030.2	2.50	1.75	-1.82	-1.20	-1.32	-0.89	-3.15	-2.45
Prp1Atx.218036.1.S1_at	ABA2	65.07	136.15	64.99	50.69	Pratt0.0050138400	AT5G67030.2	1.86	1.38	-2.10	-1.59	1.32	0.71	-3.77	-2.95
Prp1Atx.153114.1.S1_at	ABA 8-hydroxylase 1	102.41	137.58	239.57	318.45	Pratt0.0040235400	AT3G67030.2	1.34	1.21	1.45	1.21	2.53	1.74	3.77	2.74
Prp1Atx.22527.1.S1_at	ABA 8-hydroxylase 1	90.46	131.16	226.55	344.21	Pratt0.0040235400	AT3G67030.2	1.45	1.21	1.45	1.21	2.53	1.74	3.77	2.74
Prp1Atx.200165.1.S1_at	ABA 8-hydroxylase 4	194.87	188.97	344.36	36.55	Pratt0.0020230000	AT3G67030.2	1.56	1.20	-3.98	-2.88	2.44	1.76	3.31	2.63
Prp1Atx.218036.1.S1_at	ABA 8-hydroxylase 4	114.41	144.41	344.36	36.55	Pratt0.0020230000	AT3G67030.2	1.77	1.37	-26.98	-14.85	1.04	0.78	-45.68	-35.73
Prp1Atx.20080.1.S1_at	ABA receptor PVL2	257.33	236.70	174.27	174.68	Pratt0.0040230000	AT3G67030.2	-1.09	-0.82	1.37	1.11	4.95	3.70	7.37	6.11
Prp1Atx.206688.1.S1_at	ABA receptor PVL2	15.09	19.35	70.22	134.19	Pratt0.0040230000	AT3G67030.2	1.38	0.70	4.11	3.25	2.00	1.22	6.45	4.62
Prp1Atx.4847.1.S1_at	ABA INSENSITIVE 5-L2	182.35	73.14	206.62	73.14	Pratt0.0040230000	AT1G01360.1	-1.84	-1.84	2.82	2.04	-2.49	-1.84	2.82	2.04
Prp1Atx.2573.1.S1_at	ABA INSENSITIVE 5-L2	606.73	395.70	114.41	89.33	Pratt0.0060258000	AT1G01360.1	-1.53	-1.34	-1.30	-0.82	-5.30	-4.13	-4.48	-3.10
Prp1Atx.206017.1.S1_at	ABA INSENSITIVE 5-L2	343.37	230.77	43.74	15.87	Pratt0.0060258000	AT1G01360.1	-1.56	-1.43	-2.76	-1.62	-7.85	-6.37	-13.91	-8.70
Prp1Atx.204675.1.S1_at	ABA INSENSITIVE 5-L6	84.29	231.44	146.30	36.51	Pratt0.0090101200	AT1G01360.1	2.73	1.91	-4.01	-3.01	3.15	1.15	-6.34	-5.14

Table S3. (continued)

Probe set id	Description ^(a)	Samples (MSIs)				Gene Model ^(a)	Ref ^(a)	Alt ID ^(b)	Pair-wise comparison ^(c)											
		WTMS6		WTMS2					R2 MS6		R2 MS2		QW TMS6/R2 MS6		QW TMS2/R2 MS2		QW TMS6/WTMS2		QW TMS2/WTMS2	
		FC	LCB	FC	LCB	FC			LCB	FC	LCB	FC	LCB	FC	LCB	FC	LCB	FC	LCB	
PupAffx.20234.1.SI_s_at	GEN-like protein 4	25.31	95.17	44.09	14.20	Porti.005G088400			3.76	2.92	-3.10	-2.03	1.74	1.24	-6.70	-4.68				
PupAffx.221548.1.SI_a_at	HVA22-like protein a	46.93	78.17	41.08	16.74	Porti.0017G139000			1.67	1.28	-2.45	-1.72	-1.14	-0.81	-4.67	-3.41				
PupAffx.11051.1.A1_s_at	Mb cofactor sulfatase	28.97	187.62	156.79	31.13	Porti.007G266400			6.48	4.05	-5.04	-3.55	5.41	3.40	-6.03	-4.23				
PupAffx.93201.1.SI_a_at	ABA-responsive protein	57.60	112.88	49.05	30.79		AT5G23350.1		1.96	1.62	-1.59	-1.05	-1.17	-0.90	-3.67	-2.54				

⁽¹⁾ probesets represent the hormone-related differentially expressed genes found in the global analysis, and LCB>1.2 in at least one out of four pair-wise comparisons is indicated.

⁽²⁾ genes nomenclature and gene models to the corresponding probesets were search for in PopArray annotation tool (<http://aspendb.uga.edu/poparray>) as well as obtained from the NetAffx annotation as of November 2010; and in literature when indicated (Ref).

⁽³⁾ *Arabidopsis* homolog BLASTX best hit obtained using PlexDB database Microarray Platform Translator tool (<http://www.plexdb.org>), e-value cutoff of 1.e⁻¹⁰.

⁽⁴⁾ Fold Change (FC) and 90% Lower Confidence Bound (LCB) of Fold Change is shown for each pair-wise comparison. A positive FC or LCB indicates that the higher signal was from the second member of the pair-wise sample comparison, while a negative FC or LCB indicates that the higher signal was from the first member of the comparison.

(a) Carraro et al (2012); (b) Schrader et al (2003); (c) Kalluri et al (2007); (d) Zhang et al (2011); (e) Ramirez-Carvajal et al (2008); (f) Nieminen et al (2008)

Table S4. Expression data (Mean signal intensities, MSIs) of *Populus* vascular development-related genes homologs⁽¹⁾.

Protein ID	Catalytic regulators, system and phenotypic specification related genes	Description ^(a)	Samples (MSK)			Gene Model ^(b)	Ref ^(c)	Pair-wise comparison ^(d)					
			WT MS6					DWTMS6/B2 MS2		DWTMS6/WT MS2		DWTMS6/WT MS2	
			WT MS6	B2 MS6	WT MS2			FC	LCB	FC	LCB	FC	LCB
PipA1Xc.121931.1.S1_at	APL		34.57	93.88	32.61	19.71	Pont.001.C133400	2.72	2.19	-1.65	-1.06	-0.79	-3.26
PipA1Xc.139236.1.S1_at	APL, MYR2		40.22	234.79	164.04	40.63	AT1G304030.1	4.54	4.16	-4.04	-2.57	-1.08	-5.78
PipA1Xc.207929.1.S1_at	PipA1PL		379.01	549.65	296.48	59.65	Pont.000.0087600	7.45	5.84	-1.12	-1.28	-0.98	-9.21
PipA1Xc.207929.1.S1_at	APL		35.38	273.27	191.81	9.54	AT1G793430.2	1.72	1.18	-4.97	-1.35	-0.98	-17.28
PipA1Xc.210177.1.S1_at	APL, MYR1		432.74	903.66	506.55	102.37	Pont.01.C13360200	2.09	1.71	-4.95	-3.61	1.17	0.90
PipA1Xc.212209.1.S1.S1_at	APL		123.78	89.01	24.24	89.01	Pont.01.193032700	2.01	0.85	-3.67	-2.80	-1.40	-5.11
PipA1Xc.212209.1.S1.S1_at	APL		123.78	89.01	24.24	89.01	Pont.01.193032700	2.01	0.85	-3.67	-2.80	-1.40	-5.11
PipA1Xc.2284.1.S1_at	PipC1VL		9478.91	6192.52	5904.31	1877.40	(b)	-1.16	-1.05	-3.14	-2.37	-1.61	-1.41
PipA1Xc.162465.1.S1_at	CLVASE3 (CLE)-13		133.42	108.26	71.27	27.63	Pont.008C215600	-1.23	-1.03	-2.58	-1.61	-1.87	-3.36
PipA1Xc.162465.1.S1_at	CLVASE3 (CLE)-25		374.31	387.87	255.62	52.58	Pont.01.70274600	1.40	0.90	-4.86	-3.45	-1.46	-1.21
PipA1Xc.162465.1.S1_at	KANI		174.37	299.13	145.97	80.17	Pont.01.15031600	1.72	1.32	-1.82	-1.51	-1.49	-0.86
PipA1Xc.215372.1.S1_at	PipK1AN		29.96	86.11	43.97	15.17	Pont.001.C137600	2.87	2.13	-2.90	-1.81	1.47	0.99
PipA1Xc.240074.1.S1_at	PipK2AN		63.51	158.85	92.07	32.81	Pont.003.C096300	2.50	1.92	-2.81	-1.95	1.45	0.68
PipA1Xc.46401.1.S1.S1_at	PipK4		168.07	212.53	321.32	62.19	Pont.01.04037200	1.26	1.11	-5.17	-4.27	1.91	1.68
PipA1Xc.171.S1.S1_at	PipH8B		233.48	216.21	208.11	28.52	Pont.01.006237500	-1.08	-0.90	-7.30	-5.24	-1.12	-0.94
PipA1Xc.3623.3.A1_at	PipH8B		154.12	110.67	1093.25	272.45	Pont.006C237500	-1.40	-1.26	-4.01	-2.95	-1.42	-1.26
PipA1Xc.3623.3.A1_at	PipH8B		154.12	110.67	1093.25	272.45	Pont.006C237500	-1.40	-1.26	-4.01	-2.95	-1.42	-1.26
PipA1Xc.3535.1.A1_at	SCR-like 13		1093.82	939.96	301.03	371.32	Pont.000.0010200	-1.14	0.01	1.05	0.84	-3.32	-2.03
PipA1Xc.3535.1.A1_at	SCR-like 13		1093.82	939.96	301.03	371.32	Pont.000.0010200	-1.14	0.01	1.05	0.84	-3.32	-2.03
PipA1Xc.6657.1.A1_at	SCR-like 23		30.78	17.36	11.23	9.48	AT1G013501.1	-1.77	-1.22	-1.18	-0.75	-2.74	-1.03
PipA1Xc.200450.1.S1_at	SCR-like 28		76.56	115.21	164.60	35.46	Pont.001.C02109400	1.50	1.23	-4.64	-3.52	1.73	-2.49
PipA1Xc.3485.1.S1_at	SCR-like 28		86.27	151.66	177.18	37.41	Pont.01.C02109400	1.76	1.36	-4.74	-3.24	2.05	1.00
PipA1Xc.48155.1.S1_at	SCR-like 8		1130.38	1148.23	424.06	307.22	Pont.01.17042400	1.01	0.88	-1.38	-1.16	-2.67	-2.21
PipA1Xc.164008.1.S1_at	PipSHR1		213.56	318.79	450.81	46.30	AT1G376501.1	1.49	1.06	-9.74	-7.52	2.11	1.51
PipA1Xc.222884.1.S1_at	SHR		135.56	147.84	65.69	40.18	AT1G376501.1	1.09	0.92	-1.63	-1.25	-2.06	-1.79
PipA1Xc.218777.1.S1.S1_at	PipA1Xc.218777.1.S1.S1_at	Positive WOX	13.16	16.99	22.24	39.46	Pont.001.07012900	1.29	0.87	1.77	1.06	1.69	1.04
PipA1Xc.212706.1.S1.S1_at	PipA1Xc.212706.1.S1.S1_at	WOX1	14.27	48.94	35.20	16.78	Pont.01.15031600	3.43	2.63	-2.10	-1.31	2.42	1.44
PipA1Xc.139350.2.S1.S1_at	PipA1Xc.139350.2.S1.S1_at	WOX8	138.82	363.05	307.35	81.46	Pont.000.00542800	2.62	1.80	-3.77	-2.45	2.21	1.52
PipA1Xc.102308.1.A1_at	PipA1Xc.102308.1.A1_at	KNOX-like1	390.94	540.94	188.22	140.85	Pont.000.00542800	1.09	0.99	-1.14	-1.11	-3.14	-2.81
PipA1Xc.255123.1.S1.S1_at	PipA1Xc.255123.1.S1.S1_at	KNOX-like2	51.28	79.28	51.28	51.28	AT1G373801.1	1.09	0.99	-1.14	-1.11	-3.14	-2.81
PipA1Xc.6550.1.S1.S1_at	PipA1Xc.6550.1.S1.S1_at	KNOX-like3	522.82	1358.85	882.15	231.83	Pont.001.0213300	2.94	1.98	-3.81	-2.95	1.69	1.13
PipA1Xc.31202.2.S1.S1_at	PipA1Xc.31202.2.S1.S1_at	KNOX-like2	845.05	939.99	216.53	216.53	Pont.01.18C11400	3.95	2.78	-2.49	-1.58	2.52	1.66
Cell death (metacaspases, proteases)													
PipA1Xc.122769.1.S1_at	AMC1: Metacaspase-1		52.01	134.12	44.47	37.95	Pont.01.70053000	2.58	1.89	-1.17	-0.87	-1.17	-2.57
PipA1Xc.213826.1.S1_at	AMC1: Metacaspase-1		107.03	324.32	156.66	77.62	Pont.01.70052900	3.03	2.23	-2.02	-1.59	1.46	1.10
PipA1Xc.213827.1.S1_at	AMC1: Metacaspase-1		217.00	602.46	156.40	20.00	Pont.01.70053000	2.78	2.00	-7.82	-4.79	-3.20	-18.29
PipA1Xc.207822.1.S1.S1_at	AMC5: Metacaspase-5		82.72	45.73	32.37	21.61	Pont.000.008081100	-1.81	-1.11	-1.50	-1.04	-1.58	-2.12
PipA1Xc.176281.1.S1.S1_at	AMC5: Metacaspase-9		90.25	100.24	126.00	17.88	AT1G793440.1	1.11	0.87	-7.05	-4.54	1.08	-5.61
PipA1Xc.255123.1.S1.S1_at	AMC5: Metacaspase-9		296.05	265.94	372.92	50.54	AT1G504200.1	-1.11	-0.90	-7.38	-4.68	1.26	0.99
PipA1Xc.260222.1.S1.S1_at	Xylen cyclase protease 1		242.16	242.16	180.17	24.60	Pont.000.00624500	1.14	0.88	-1.35	-1.01	1.28	0.82
PipA1Xc.260222.1.S1.S1_at	Xylen cyclase protease 2		242.16	242.16	180.17	24.60	Pont.000.00624500	1.14	0.88	-1.35	-1.01	1.28	0.82
PipA1Xc.661.3.S1.S1_at	XP2: Xylen serine protease 1		4217.81	3768.23	2554.75	390.28	Pont.002.0005700	-1.12	-0.97	-6.55	-4.25	-1.65	-1.46
PipA1Xc.24474.1.S1.S1_at	XP2: Xylen serine protease 2		779.32	635.57	49.60	47.28	Pont.01.140274500	-1.15	-0.91	-1.05	-0.60	-14.70	-11.89
PipA1Xc.51501.1.S1.S1_at	IAAS: subtilisin inhibitor		30.33	9.10	32.79	655.83	Pont.007.C111500	-0.99	-0.99	7.94	1.08	0.62	72.11
PipA1Xc.14901.1.S1.S1_at	Sautilisin-like protease		3911.04	3369.96	1149.41	1335.99	Pont.01.041018900	-1.16	-0.99	1.16	0.34	-3.06	-2.52
PipA1Xc.161.1.S1.S1_at	Sautilisin-like protease		5269.24	5742.43	2750.49	969.82	Pont.002.C204000	0.88	0.88	-2.19	-2.04	-1.68	-5.52
PipA1Xc.1941.1.S1.S1_at	Sautilisin-like protease		1329.63	1557.81	938.55	304.93	Pont.001.C455800	1.26	0.98	-3.08	-2.79	-1.32	-0.86
PipA1Xc.378.1.A1.S1.S1_at	Sautilisin-like protease		1669.45	1611.55	760.10	275.98	Pont.001.C03071000	-1.04	-0.88	-2.75	-2.31	-2.02	-1.86
PipA1Xc.5493.1.S1.S1.S1_at	Sautilisin-like protease		1043.42	1290.22	517.60	410.11	Pont.01.041018900	1.24	0.84	-1.26	-1.05	-2.12	-1.55
PipA1Xc.7993.1.S1.S1.S1_at	Sautilisin-like protease		1006.28	935.50	1934.84	134.56	Pont.01.140274500	-1.08	-0.97	-14.38	-7.87	1.92	1.49
PipA1Xc.122706.1.S1.S1.S1_at	Sautilisin-like protease		138.82	363.05	307.35	81.46	Pont.000.00542800	2.62	1.80	-3.77	-2.45	2.21	1.52
PipA1Xc.122706.1.S1.S1.S1_at	Sautilisin-like protease		138.82	363.05	307.35	81.46	Pont.000.00542800	2.62	1.80	-3.77	-2.45	2.21	1.52
PipA1Xc.122706.1.S1.S1.S1_at	Sautilisin-like protease		138.82	363.05	307.35	81.46	Pont.000.00542800	2.62	1.80	-3.77	-2.45	2.21	1.52

Table S4. (continued)

Probeset Id	Description ^(a)	Samples (MSIs)			Ref ^(b)	Ath ID ^(b)	Pair-wise comparison ^(c)							
		WT MS6	B2 MS6	WT MS2			B2 MS2	(a) WT MS6/B2 MS6	(b) WT MS2/B2 MS2	(c) WT MS6/B2 MS2	(d) B2 MS6/B2 MS2			
PipAfx.203587.1.S1_at	Satellin-like protease	22.01	64.93	73.19	18.48			2.95	2.18	-3.96	3.32	2.49	-3.51	-2.42
PipAfx.204248.1.S1_at	Satellin-like protease	64.57	81.82	12.03	20.91			1.27	1.06	1.74	1.19	-5.37	-3.90	-3.10
PipAfx.224489.1.S1_at	Satellin-like protease	72.10	126.42	456.32	681.10			1.75	1.42	1.49	1.28	6.33	5.14	5.39
PipAfx.225305.1.S1_s_at	Satellin-like protease	855.21	1032.25	3084.14	3610.84			1.21	0.96	1.17	1.05	3.61	2.92	3.50
PipAfx.259781.1.S1_s_at	Satellin-like protease	281.73	321.15	750.19	54.54		AT1(GD1900.1)	1.14	0.86	-13.76	-9.44	2.66	1.95	-5.89
PipAfx.602981.1.S1_s_at	Satellin-like protease	73.60	221.74	181.32	57.42		AT1(GD2001.1)	3.01	1.98	-3.16	-2.39	2.46	1.54	-3.86
PipAfx.650381.1.A1_at	Satellin-like protease	115.08	190.66	200.23	31.51		AT1(GD2050.1)	1.66	1.39	-4.36	-4.34	1.74	1.30	-6.05
PipAfx.66951.1.S1_at	Satellin-like protease	463.91	724.91	1800.31	1600.31		AT1(GD7090.1)	1.23	1.13	1.04	0.85	4.57	3.89	3.68
PipAfx.67051.1.S1_at	Satellin-like protease	377.16	463.91	124.51	254.26		AT1(GD4200.1)	1.23	1.13	1.04	0.85	4.57	3.89	3.68
PipAfx.739171.1.S1_at	SD1: Satellin-like protease	244.25	891.51	170.44	24.24		AT1(GD4200.1)	3.65	2.95	-1.06	-0.79	1.11	0.86	-3.51
PipAfx.128741.1.S1_at	VPE: Variable-processing enzyme	58.42	170.92	114.16	24.24		AT1(GD2900.1)	1.74	1.20	-4.71	-3.54	1.16	0.80	-7.05
PipAfx.5623.1.S1_at	VPE: Variable-processing enzyme	179.06	103.10	92.64	49.93		AT1(GD2900.1)	-1.74	-1.41	-1.86	-1.42	-1.93	-1.59	-2.06
PipAfx.6371.1.S1_at	VPE: Variable-processing enzyme	670.61	622.59	49.04	53.16		AT1(GD2700.1)	-1.08	-0.96	1.08	0.64	-13.67	-11.69	-11.71
Satellin transcriptional network														
PipAfx.211645.1.S1_at	NST1, NAC043	36.92	55.25	68.83	16.18			1.50	1.10	-4.31	-2.77	1.89	1.33	-3.41
PipAfx.214565.1.S1_at	ND1, NAC043	423.74	516.00	116.47	20.60			1.22	0.91	-5.65	-3.66	-3.64	-2.45	-25.05
PipAfx.20281.1.S1_at	ND1, NAC043	413.83	333.57	67.24	32.43		AT1(G32770.1)	-1.24	-1.00	-2.07	-0.97	-6.15	-4.67	-10.29
PipAfx.1762.1.A1_at	ND1, NST3, NAC012	178.67	219.47	79.56	67.23		AT1(G32770.1)	1.23	0.97	-1.18	-0.96	-2.25	-1.65	-3.26
PipAfx.62951.1.S1_s_at	ND1, NST3, NAC012	193.05	124.17	534.43	232.20		AT1(G32770.1)	-1.55	-1.14	-2.30	-1.61	2.77	2.10	1.87
PipAfx.1444.1.A1_at	ND2/NAC073	167.73	168.90	52.87	20.61		Porti.0042049300	1.01	0.77	-2.57	-1.85	-3.17	-2.63	-8.20
PipAfx.7947.1.S1_at	ND2/NAC073	863.74	841.70	243.45	59.10		Porti.01702016700	-1.03	-0.78	-4.12	-2.91	-3.55	-3.15	-14.24
PipAfx.15627.1.S1_s_at	VND4, NAC007	35.51	56.65	41.65	12.22		Porti.0003213000	1.69	1.22	-3.41	-1.99	1.17	0.85	-4.63
PipAfx.203176.1.S1_at	VND7, NAC007	21.70	17.68	97.19	20.80		Porti.01321131000	-1.23	-0.95	-3.21	-1.78	-1.74	-1.28	-5.10
PipAfx.221286.1.S1_at	VND7, NAC007	124.44	113.80	11.68	22.31		Porti.00302208000	-1.09	-0.95	-3.21	-1.78	-1.74	-1.28	-5.10
PipAfx.141039.1.A1_s_at	ND1, NAC004	134.33	89.72	60.44	40.67		Porti.00020069000	-1.50	-1.12	-1.48	-0.97	-2.22	-1.80	-2.20
PipAfx.200365.1.S1_at	ND1, NAC004	134.33	89.72	60.44	40.67		Porti.00020069000	-1.50	-1.12	-1.48	-0.97	-2.22	-1.80	-2.20
Xtcm markers, liliatation related genes														
PipAfx.476001.1.S1_at	4CL1	127.94	108.96	39.39	41.70		Porti.0060201697000	-1.16	-0.88	1.06	0.74	-3.25	-2.40	-2.64
PipAfx.211284.1.S1_at	4CL1	2994.60	4002.03	1176.34	592.03		Porti.002030389000	1.34	0.87	-1.99	-1.48	-2.55	-1.40	-6.76
PipAfx.212854.1.S1_at	COMT1	57.88	63.02	27.55	17.28		Porti.012030389000	1.09	0.83	-1.59	-1.12	-2.10	-1.52	-3.65
PipAfx.210121.1.S1_s_at	COMT1	261.52	274.73	137.44	627.94		Porti.012030389000	1.05	0.93	4.57	2.85	-1.90	-1.40	2.29
PipAfx.55460.1.A1_s_at	COMT1	22.38	21.44	94.41	134.18		Porti.012030389000	-1.04	-0.63	1.42	0.88	4.22	2.88	6.26
PipAfx.55460.2.S1_s_at	COMT1	15.34	16.82	77.49	51.86		Porti.012030389000	1.10	0.70	-1.49	-0.80	5.05	3.22	3.08
PipAfx.1780.1.S1_at	IRX10; b-1,4-xylosyltransferase	50.47	44.06	138.42	60.06		Porti.0002001005000	-1.15	-0.83	-2.30	-1.15	2.74	1.75	1.36
PipAfx.5496.1.S1_at	IRX3; b-1,4-xylosyltransferase	3195.85	2138.23	993.67	522.70		Porti.00102081000	-1.49	-1.13	-1.90	-1.48	-3.22	-2.84	-4.09
PipAfx.274.1.S1_at	IRX3; b-1,4-xylosyltransferase	409.59	342.72	152.75	46.37		Porti.000620310000	-1.20	-0.90	-3.29	-2.36	-2.68	-2.26	-7.39
PipAfx.6380.1.S1_at	COBL4; COBL4-like 4	645.39	959.13	253.78	69.52		Porti.016208640000	1.49	1.04	-3.65	-2.33	-2.54	-1.87	-13.80
PipAfx.107166.2.S1_at	CCR1; Cinnamoyl-CoA reductase 1	725.61	546.00	27.10	42.22		Porti.0004041729000	-1.33	-0.71	1.56	0.64	-26.77	-13.12	-12.93
PipAfx.55239.1.A1_at	CCR1; Cinnamoyl-CoA reductase 2	107.38	135.76	385.39	466.38		Porti.000102564000	1.26	1.01	1.21	1.05	3.59	2.89	3.44
PipAfx.22065.1.A1_at	CCR1; Cinnamoyl-CoA reductase 2	119.25	126.16	272.54	482.12		Porti.000102564000	1.06	0.83	1.70	1.50	2.29	1.88	3.06
PipAfx.12905.1.A1_at	CAD2; Cinnamoyl-CoA reductase 2	22.41	15.50	34.79	29.92		Porti.0010101480000	1.15	0.88	1.77	1.20	1.55	1.05	3.97
PipAfx.12905.1.A1_at	CAD2; Cinnamoyl-CoA reductase 2	22.41	15.50	34.79	29.92		Porti.0010101480000	1.15	0.88	1.77	1.20	1.55	1.05	3.97
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000	-1.17	-0.79	-3.41	-2.99	-3.37	-1.74	-6.91
PipAfx.2272.1.S1_s_at	PAL2	305.75	600.66	1090.17	596.55		Porti.0010224240000							

Table S4. (continued)

Probeset ID	Description ^(a)	Pair-wise comparison ^(b)									
		WT MS6		Samples (MSIs)		B2 MS2		Gene Model ^(d)		Ref ^(e)	
Probeset ID	Description ^(a)	WT MS6		Samples (MSIs)		B2 MS2		Gene Model ^(d)		Ref ^(e)	Alt ID ^(a)
		WT MS6	WT MS6	WT MS6	WT MS6	B2 MS2	B2 MS2	Gene Model ^(d)	Gene Model ^(d)		
Pip.703.1.S1_at	XYNC: Ind-1,4-bisylase C	2090.36	3181.03	2383.20	514.14	514.14	514.14	Pori.002.G113100	Pori.002.G113100	ATG25810.1	
Pip.3548.1.S1_at	XTX23	379.14	306.93	96.20	134.25	134.25	134.25	Pori.002.G113100	Pori.002.G113100	ATG25810.1	
Pip.Afx.4435.1.S1_at	XTX31	239.87	191.68	301.28	179.35	179.35	179.35	Pori.009.G066000	Pori.009.G066000	ATG10550.1	
Pip.Afx.1891.1.S1_at	XTX31	314.12	162.53	59.18	110.00	110.00	110.00	Pori.004.G113100	Pori.004.G113100	ATG10550.1	
Pip.Afx.12055.1.S1_at	XTH6	614.04	192.04	48.08	192.22	192.22	192.22	Pori.009.G066000	Pori.009.G066000	ATG10550.1	
Pip.703.1.S1_at	XTH6	614.04	192.04	48.08	192.22	192.22	192.22	Pori.009.G066000	Pori.009.G066000	ATG10550.1	
Pip.703.1.S1_at	XTH6	614.04	192.04	48.08	192.22	192.22	192.22	Pori.009.G066000	Pori.009.G066000	ATG10550.1	
Pip.Afx.20908.1.S1_at	BXL1: b-Dxylosidase 1	644.82	615.59	1677.88	2167.24	2167.24	2167.24	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.Afx.14180.1.S1_at	BXL2: b-Dxylosidase 2	371.43	609.57	397.35	3090.36	3090.36	3090.36	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.6426.1.S1_at	BXL2: b-Dxylosidase 2	495.77	444.35	262.47	1692	1692	1692	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.Afx.7644.2.S1_at	BXL4: b-Dxylosidase 4	863.61	831.23	117.88	36.81	36.81	36.81	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.Afx.20195.1.S1_at	BXL7: b-Dxylosidase 7	184.09	131.72	25.41	13.17	13.17	13.17	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.7611.1.S1_at	XYP1: Xyloglucan-like protein 11	25.29	115.71	599.12	232.55	232.55	232.55	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.Afx.18176.1.S1_at	XYP1: Xyloglucan-like protein 12							Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.Afx.18176.1.S1_at	XYP1: Xyloglucan-like protein 12							Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Expansin and Fusicin-like genes											
Pip.Afx.12086.1.S1_at	Expansin-A1	56.01	51.31	66.28	152.29	152.29	152.29	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.Afx.27616.1.S1_at	Expansin-A11	53.47	139.37	520.21	560.27	560.27	560.27	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.Afx.89446.1.S1_at	Expansin-A15	3536.81	3578.28	675.72	126.37	126.37	126.37	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.Afx.17914.3.S1_at	Expansin-A4	239.73	21.01	710.29	135.69	135.69	135.69	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.1101.1.S1_at	Expansin-A6	2889.91	5479.79	1783.56	1807.62	1807.62	1807.62	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.Afx.12086.1.S1_at	Expansin-B1	56.01	51.31	66.28	152.29	152.29	152.29	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.Afx.22008.1.S1_at	Expansin-B1	22.46	21.46	591.80	41.73	41.73	41.73	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.792.1.S1_at	Fusicin-like arabinogalactan 11	1054.27	457.94	211.63	119.96	119.96	119.96	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.Afx.25130.1.S1_at	Fusicin-like arabinogalactan 11	2361.71	1413.03	1118.14	670.70	670.70	670.70	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.Afx.25130.1.S1_at	Fusicin-like arabinogalactan 11	2361.71	1413.03	1118.14	670.70	670.70	670.70	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.30581.1.S1_at	Fusicin-like arabinogalactan 12	2480.76	862.89	6.86	30.51	30.51	30.51	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.3083.1.S1_at	Fusicin-like arabinogalactan 12	1497.47	488.68	69.51	104.05	104.05	104.05	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.3500.1.S1_at	Fusicin-like arabinogalactan 12	7041.83	3957.82	70.16	279.42	279.42	279.42	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.3701.1.S1_at	Fusicin-like arabinogalactan 12	7040.61	3997.27	143.04	292.85	292.85	292.85	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.5517.1.S1_at	Fusicin-like arabinogalactan 12	3285.76	1844.83	783.25	160.01	160.01	160.01	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.Afx.141260.1.S1_at	Fusicin-like arabinogalactan 12	5551.84	2818.98	348.21	593.21	593.21	593.21	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.Afx.142864.1.S1_at	Fusicin-like arabinogalactan 12	5738.17	2496.21	98.25	312.70	312.70	312.70	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.Afx.162947.1.S1_at	Fusicin-like arabinogalactan 12	7479.74	4560.56	74.75	324.47	324.47	324.47	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.Afx.211342.1.S1_at	Fusicin-like arabinogalactan 12	2744.70	1280.62	32.46	89.22	89.22	89.22	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.Afx.249461.1.S1_at	Fusicin-like arabinogalactan 12	535.07	40.06	386.18	93.23	93.23	93.23	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.963.1.S1_at	Fusicin-like arabinogalactan 13	29.23	29.23	971.44	36.81	36.81	36.81	Pori.002.G113100	Pori.002.G113100	ATG13870.1	
Pip.Afx.210588.1.S1_at	Fusicin-like arabinogalactan 17	991.13	263.31	172.18	31.90	31.90	31.90	Pori.002.G113100	Pori.002.G113100	ATG13870.1	

(1) probesets represent the vascular development-related differentially expressed genes found in the global analysis, and LCB>1.2 in at least one out of four pair-wise comparisons is indicated.

(2) genes nomenclature and gene models to the corresponding probesets were search for in PopArray annotation tool (<http://aspenb.uga.edu/poparray>) as well as obtained from the NetAffx annotation as of November 2010; and in literature when indicated (Ref).

(3) *Arabidopsis* homolog BLASTX best hit obtained using PlexDB database Microarray Platform Translator tool (<http://www.plexdb.org>), e-value cutoff of $1.e^{-10}$.

(4) Fold Change (FC) and 90% Lower Confidence Bound (LCB) of Fold Change is shown for each pair-wise comparison. A positive FC or LCB indicates that the higher signal was from the second member of the pair-wise sample comparison, while a negative FC or LCB indicates that the higher signal was from the first member of the comparison.

(a) Bonke et al (2003); (b) Schrader et al (2004); (c) Zhao et al (2005); (d) Helariutta et al (2000); (e) Courtois-Moreau et al (2009); (f) Hu et al (2010).

Table S5. Expression data (Mean signal intensities, MSIs) of *Populus* transcription factors homologs ⁽¹⁾.

Protein ID	Description ⁽²⁾	Samples (MSI)		Gene Model ⁽³⁾	Auh ID ⁽⁴⁾	Pair-wise comparison ⁽⁵⁾			
		WTMS6	B2 MS6			(O)WTMS6/B2 MS6	(O)WTMS7/B2 MS7	(O)WTMS6/WTMS2	(O)B2 MS6/B2 MS2
						FC	LCB	FC	LCB
PpAffx.27300.1.S1_at	Homeobox protein ATH1	39.28	102.88	182.71	35.37	2.62	1.82	2.62	1.82
PpAffx.77300.1.S1_at	Homeobox protein HATH3.1	66.13	224.48	222.95	72.26	3.55	2.95	3.55	2.95
PpAffx.18687.1.A1_at	Homeobox protein HD1	1333.40	1317.12	508.12	362.40	-1.18	-0.95	-1.18	-0.95
PpAffx.34805.1.S1_at	Homeobox protein HD1	200.53	247.05	106.41	49.93	1.23	0.81	1.23	0.81
PpAffx.206918.1.S1_at	Homeobox-LZ protein ATHB52	99.07	71.43	193.40	37.06	-1.39	-1.10	1.85	1.10
PpAffx.209946.1.S1_s_at	Homeobox-LZ protein ATHB52	111.02	125.57	25.55	84.01	1.13	0.86	3.29	1.39
PpAffx.76129.1.S1_at	Homeobox-LZ protein ATHB52	230.15	251.83	46.41	100.05	1.09	0.90	2.16	1.29
PpAffx.4806.3.S1_a_at	Homeobox-LZ protein ATHB6	374.04	1417.42	1167.50	474.83	3.79	2.91	-2.46	-2.02
PpAffx.21034.1.S1_at	Homeobox-LZ protein ATHB7	21.57	35.10	8.97	12.45	1.63	1.26	1.39	0.87
PpAffx.200626.1.S1_s_at	Homeobox-LZ protein GLABRA2	198.67	410.94	299.55	94.72	2.07	1.63	-3.16	-2.61
PpAffx.133756.2.A1_s_at	Homeobox-LZ protein HAT1.4	1507.96	1161.23	597.43	327.65	-1.30	-1.09	-1.79	-1.44
PpAffx.133756.2.A1_at	Homeobox-LZ protein HAT1.4	607.41	525.13	220.64	159.68	-1.16	-0.94	-1.38	-1.08
PpAffx.58744.1.A1_at	Homeobox-LZ protein HAT1.4	1057.16	925.09	254.96	220.13	-1.06	-1.06	-1.16	-1.06
PpAffx.3022.3.A1_at	Homeobox-LZ protein HAT2	22.98	48.92	77.65	28.96	2.13	1.49	-2.68	-1.79
PpAffx.7795.1.S1_at	Homeobox-LZ protein HAT2	494.42	333.40	144.29	91.48	-1.48	-1.38	-1.58	-1.49
PpAffx.20215.1.S1_at	Homeobox-LZ protein HAT2	279.23	223.60	97.84	65.62	-1.25	-1.09	-1.11	-0.95
PpAffx.20781.1.S1_at	Homeobox-LZ protein HAT2	91.59	261.19	300.13	95.30	2.86	2.09	-3.15	-2.36
PpAffx.208056.1.S1_s_at	GLABRA 3	87.14	63.48	386.27	1240.53	-1.37	-0.63	1.44	1.04
PpAffx.20781.1.S1_at	LOB domain-containing protein 12	3400.63	2517.02	956.84	1240.53	-1.35	-1.24	1.30	0.80
PpAffx.30182.1.S1_at	LOB domain-containing protein 21	491.34	592.46	289.87	133.81	-1.83	-1.16	2.21	1.82
PpAffx.204722.1.S1_at	LOB domain-containing protein 38	181.30	99.29	994.54	2200.24	-1.22	-1.48	2.75	2.05
PpAffx.203919.1.S1_at	LOB domain-containing protein 41	165.94	74.73	418.03	1148.17	-1.31	-0.66	3.22	2.88
PpAffx.215454.1.S1_at	LOB domain-containing protein 41	75.97	57.99	598.17	1926.37	-1.20	-0.63	1.17	0.93
PpAffx.200951.1.S1_at	LOB domain-containing protein 42	17.82	14.80	30.67	21.46	-1.24	-0.63	1.17	0.93
PpAffx.3680.1.S1_at	MADS-box protein SOCL	47.41	92.24	163.15	141.88	1.24	0.85	-1.03	-0.79
PpAffx.40156.2.S1_at	MADS-box 1b	293.45	525.85	169.15	141.88	1.29	0.84	7.78	5.92
PpAffx.40156.2.S1_at	MADS-box 1b	49.49	64.27	67.85	527.96	1.31	0.97	-2.29	-1.25
PpAffx.1307.1.S1_at	Putative acid regulator or YABBY 2	183.07	239.41	62.34	27.17	1.31	0.97	-2.29	-1.25
PpAffx.150043.1.A1_at	Putative acid regulator or YABBY 2	595.54	1707.95	595.83	543.08	2.87	2.38	-1.10	-0.84
PpAffx.204158.1.S1_at	Myb-related protein 305	69.94	84.70	192.44	187.08	1.39	1.08	-1.03	-0.79
PpAffx.213932.1.S1_at	Myb-related protein 306	38.82	59.12	113.83	267.84	1.52	1.17	2.35	1.75
PpAffx.162211.2.S1_at	Myb-related protein 308	36.05	71.71	107.17	177.58	1.99	1.48	1.66	1.09
PpAffx.89303.1.A1_at	Myb-related protein B	327.29	391.97	151.03	108.78	1.20	0.97	-1.39	-1.02
PpAffx.104683.1.S1_at	Myb-related protein Myb4	45.86	1256.83	695.08	293.74	1.37	1.17	-2.37	-1.68
PpAffx.224262.1.S1_at	Myb-related protein Myb4	15.58	16.71	101.83	27.39	2.61	2.15	-4.61	-3.32
PpAffx.35610.1.A1_at	Myb-related protein Myb4	28.84	31.75	280.15	315.13	1.07	0.64	-3.72	-2.76
PpAffx.202578.1.S1_s_at	Transcription factor MYB113	7.58	4.97	62.75	3.72	-1.53	-0.66	-16.87	-6.17
PpAffx.207742.1.S1_s_at	Transcription factor MYB181	1993.61	1318.68	470.82	185.75	-1.51	-1.32	-2.53	-2.33
PpAffx.47974.1.S1_at	Transcription factor MYB21	2158.82	2054.97	1526.16	347.72	-1.05	-1.00	-4.39	-3.55
PpAffx.12991.1.S1_at	Transcription factor MYB21	31.98	33.93	309.03	170.46	1.06	0.75	-1.81	-1.05
PpAffx.4185.1.S1_at	Transcription factor MYB44	513.88	679.24	1407.64	562.22	1.32	1.17	-2.50	-1.99
PpAffx.1161.1.S1_at	Transcription factor MYB44	93.20	143.50	54.43	40.97	1.54	0.98	-1.33	-1.01
PpAffx.159678.1.A1_at	Transcription factor MYB44	171.47	122.56	920.75	550.39	-1.07	-1.07	-1.23	-1.01
PpAffx.20579.1.S1_at	Transcription factor MYB44	69.87	73.72	15.52	26.94	1.06	0.69	1.74	1.16
PpAffx.59575.1.A1_at	Transcription factor MYB44	201.50	138.34	32.88	37.80	-1.46	-1.06	1.15	0.74
PpAffx.7131.3.S1_at	Transcription factor MYB48	371.38	856.02	223.85	40.76	2.30	1.57	-5.49	-3.58
PpAffx.7131.3.S1_s_at	Transcription factor MYB48	840.14	1474.28	450.83	142.75	1.76	1.38	-3.16	-2.29
PpAffx.206149.1.S1_s_at	Transcription factor MYB82	73.58	83.13	330.50	63.12	1.13	0.85	-5.24	-3.20
PpAffx.206149.1.S1_s_at	Transcription factor MYB82	170.24	198.96	37.63	17.92	1.17	0.83	-2.10	-1.36
PpAffx.102380.2.S1_s_at	Transcription factor MYB86	348.89	360.82	57.68	22.62	1.03	0.64	-2.55	-1.53
PpAffx.131940.1.S1_at	Transcription factor MYB86	1666.95	1553.99	417.02	492.90	-1.07	-1.01	1.18	0.82
PpAffx.195311.S1_at	Transcription factor MYB86	156.38	175.25	63.22	54.58	1.12	0.91	-1.16	-0.86

Table S5. (continued)

Protein Id	Description ^(a)	Samples (MSIs)		Gene Model ^(b)	Ah ID ^(c)	Pair-wise comparison ^(d)				Pair-wise comparison ^(e)			
		WTMS6	B2 MS6	WTMS2	B2 MS2	WTMS6/B2 MS6	LCB	FCB	LCB	WTMS6/B2 MS2	LCB	FCB	LCB
PipAffx.22325.LAI_at	Transcription factor MYB86	870.16	426.35	197.08	217.68	-1.34	-1.14	1.10	0.77	-1.34	-1.14	1.10	-2.98
PipAffx.22453.L1.SI_s_at	Transcription factor MYB86	220.46	426.35	123.23	116.55	1.93	1.27	-1.06	-0.84	1.93	1.27	-1.06	-3.66
PipAffx.22453.L1.SI_s_at	Transcription factor MYB86	48.57	141.83	26.51	11.82	2.92	1.54	-2.24	-1.39	2.92	1.54	-2.24	-12.00
PipAffx.11710.LAI_at	Transcription repressor MYB4	193.14	125.30	102.85	406.53	-1.54	-1.23	-2.89	-1.94	-1.54	-1.23	-2.89	-6.63
PipAffx.11208.LAI_at	Probable WRKY transcription factor 11	146.22	135.91	502.40	406.53	1.05	0.69	-1.23	-0.91	1.05	0.69	-1.23	-2.26
PipAffx.4594.1.SI_s_at	Probable WRKY transcription factor 12	573.14	849.63	453.34	135.26	-1.56	1.36	-3.57	-2.38	-1.56	1.36	-3.57	-6.38
PipAffx.4594.1.SI_s_at	Probable WRKY transcription factor 12	573.14	849.63	453.34	135.26	-1.56	1.36	-3.57	-2.38	-1.56	1.36	-3.57	-6.38
PipAffx.12708.LAI_at	Probable WRKY transcription factor 23	80.51	69.14	533.42	903.51	-1.46	-0.48	1.69	1.13	-1.46	-0.48	1.69	9.16
PipAffx.1575.LAI_at	Probable WRKY transcription factor 23	248.95	138.17	477.64	1936.76	-1.80	-1.30	3.05	2.96	-1.80	-1.30	3.05	4.74
PipAffx.114251.LAI_at	Probable WRKY transcription factor 28	153.75	78.32	44.20	166.31	-1.96	-1.58	3.76	1.88	-1.96	-1.58	3.76	12.12
PipAffx.5261.LAI_at	Probable WRKY transcription factor 28	76.21	48.18	34.87	137.41	-1.58	-1.09	3.94	2.65	-1.58	-1.09	3.94	2.12
PipAffx.20652.L1.SI_s_at	Probable WRKY transcription factor 32	59.11	143.06	124.65	37.66	2.42	1.81	-3.31	-2.34	2.42	1.81	-3.31	-2.79
PipAffx.107379.LSI_s_at	Probable WRKY transcription factor 33	23.43	125.80	157.76	31.99	4.52	2.70	-4.93	-2.96	4.52	2.70	-4.93	-3.31
PipAffx.107379.LSI_s_at	Probable WRKY transcription factor 33	81.32	228.88	184.26	87.37	2.81	1.95	-2.11	-1.64	2.27	1.59	-2.62	-1.99
PipAffx.213965.LSI_s_at	Probable WRKY transcription factor 4	45.72	130.65	84.09	36.42	2.86	1.99	-2.21	-1.53	1.84	1.24	-1.73	-2.44
PipAffx.220415.LSI_s_at	Probable WRKY transcription factor 4	21.86	65.18	50.04	21.93	2.98	2.27	-2.28	-1.66	2.29	1.73	-2.57	-2.18
Pip2164.L1.SI_s_at	Probable WRKY transcription factor 40	66.85	178.94	554.82	388.84	-1.81	-1.39	3.76	1.88	-1.81	-1.39	3.76	1.75
PipAffx.10586.L1.SI_s_at	Probable WRKY transcription factor 40	186.60	422.79	828.88	398.04	2.68	2.28	-2.28	-1.43	-0.90	8.30	5.32	2.17
PipAffx.1554.L1.SI_s_at	Probable WRKY transcription factor 41	29.10	112.43	80.71	32.87	2.27	1.90	-2.08	-1.34	4.44	3.20	-1.06	-0.76
PipAffx.15729.LAI_at	Probable WRKY transcription factor 47	109.69	113.82	70.69	360.27	3.86	2.51	-2.46	-1.63	2.77	1.78	-3.42	-2.31
PipAffx.4303.LAI_at	Probable WRKY transcription factor 57	161.49	160.34	331.05	503.88	1.04	0.87	5.10	3.50	-1.55	-1.21	3.17	2.26
Pip726.L1.SI_at	Probable WRKY transcription factor 7	269.42	121.71	94.38	118.70	-2.21	-1.66	1.26	0.86	-2.85	-2.14	1.03	-0.77
PipAffx.116166.LSI_s_at	Probable WRKY transcription factor 7	132.24	258.47	179.18	64.73	1.95	1.23	-2.77	-2.24	1.35	0.86	-3.09	-3.19
PipAffx.156569.LSI_s_at	Probable WRKY transcription factor 7	36.31	69.19	155.18	144.81	1.91	1.44	-1.07	-0.77	4.27	3.21	2.09	1.36
PipAffx.63488.LSI_s_at	Probable WRKY transcription factor 71	106.26	122.03	312.79	425.60	-2.46	-1.70	2.22	1.82	-1.05	-0.80	5.22	3.81
PipAffx.140839.LAI_at	Probable WRKY transcription factor 75	194.11	79.00	185.74	412.48	1.46	1.28	2.10	1.25	2.23	1.73	3.21	1.95
Pip6836.LSI_at	Probable WRKY transcription factor 75	164.40	286.16	525.78	660.05	1.74	1.40	1.26	0.54	3.20	2.26	2.31	1.00
Pip3786.L1.SI_s_at	WRKY transcription factor 22	20.39	68.57	50.10	16.09	3.36	2.25	-3.11	-2.16	2.46	1.65	-4.26	-2.94
PipAffx.21910.LSI_s_at	WRKY transcription factor 22	44.93	57.34	94.08	231.83	1.28	0.97	2.46	1.34	2.09	1.61	4.04	2.19
PipAffx.139444.LAI_at	WRKY transcription factor 6	46.41	107.48	820.24	855.17	2.32	1.45	1.04	0.79	17.67	11.26	7.96	6.07
PipAffx.209605.LSI_s_at	WRKY transcription factor 6	45.02	32.44	117.53	90.72	-1.39	-1.05	-1.30	-0.92	2.61	2.01	2.80	1.84
PipAffx.23042.LSI_s_at	RING finger and CHY ZFI	2060.76	321.441	833.50	1074.28	1.56	1.27	1.29	0.71	-2.47	-2.02	-2.99	-2.01
PipAffx.35062.LSI_s_at	RING-H2 finger protein ATL12	25.22	66.44	19.64	21.75	1.56	1.27	1.29	0.71	-2.47	-2.02	-2.99	-2.01
PipAffx.65561.LSI_at	RING-H2 finger protein ATL22	474.37	366.25	148.04	157.16	-1.55	-1.37	1.06	0.87	-3.19	-2.74	-1.95	-1.66
PipAffx.4429.LSI_at	RING-H2 finger protein ATL44	142.59	99.27	136.30	331.53	1.51	1.00	2.30	1.99	4.24	2.88	6.45	5.34
Pip7225.LSI_at	RING-H2 finger protein ATL51	108.07	93.57	139.07	111.5	-1.15	-0.81	1.09	0.81	-2.62	-2.02	-1.96	-1.56
Pip689.LAI_at	RING-H2 finger protein ATL52	118.52	108.86	49.06	20.86	1.01	0.81	1.09	0.81	-2.62	-2.02	-1.96	-1.56
PipAffx.11910.LSI_s_at	RING-H2 finger protein ATL72	1531.50	1694.12	493.56	380.18	1.19	1.04	-1.30	-1.01	-3.20	-2.83	-4.47	-3.50
PipAffx.44551.LSI_s_at	RING-H2 finger protein ATL73	133.83	175.03	47.03	28.67	1.14	1.01	-1.64	-1.21	-3.57	-3.27	-6.11	-4.84
PipAffx.19866.LSI_s_at	RING-H2 finger protein ATL78	609.62	289.22	174.06	122.26	-2.11	-1.95	-1.42	-1.09	-3.50	-3.10	-2.37	-1.85
PipAffx.201133.LSI_at	RING-H2 finger protein ATL78	153.90	69.05	22.15	24.79	-1.68	-1.12	0.47	-0.63	-5.18	-4.79	-2.79	-1.65
PipAffx.17953.LSI_at	RING-H2 finger protein ATL78	448.05	144.70	165.70	33.13	-2.57	-2.02	-5.00	-3.45	-2.70	-2.32	-2.37	-2.95
PipAffx.67538.LAI_at	RING-H2 zinc finger protein BHA4a	340.88	416.59	262.25	853.19	1.22	0.87	3.25	2.08	-1.30	-0.74	2.05	1.41
Pip1601.LAI_s_at	Transcription factor BHLH10	409.37	375.84	420.43	66.72	-1.09	-0.94	-6.30	-5.35	1.03	0.88	-5.63	-4.80
Pip4186.LAI_s_at	Transcription factor BHLH10	198.87	182.59	123.28	33.91	-1.09	-0.93	-3.64	-2.75	-1.61	-1.33	-5.38	-4.20
PipAffx.129036.LSI_s_at	Transcription factor BHLH11	124.47	118.33	87.56	327.49	1.03	0.76	-6.10	-5.33	-3.76	-3.76	2.77	1.74
PipAffx.20830.LSI_s_at	Transcription factor BHLH11	403.37	415.12	75.75	12.43	1.03	0.76	-6.10	-5.33	-3.76	-3.76	2.77	1.74
Pip7962.LSI_s_at	Transcription factor BHLH12	183.65	428.67	195.62	191.5	2.33	1.66	-10.21	-6.66	1.07	0.69	-22.38	-16.09
PipAffx.152720.LSI_at	Transcription factor BHLH12	55.27	122.52	70.77	30.88	2.22	1.86	-2.29	-1.61	1.28	0.96	-3.97	-3.02
PipAffx.59055.LAI_at	Transcription factor BHLH12	689.30	749.15	357.56	125.41	1.09	1.02	-2.85	-2.18	-1.67	-1.57	-5.97	-4.77
PipAffx.21077.LSI_at	Transcription factor BHLH20	14.16	14.06	97.79	22.18	-1.01	-0.59	-4.41	-2.95	6.91	4.57	1.38	0.95
Pip1010.LAI_at	Transcription factor BHLH22	969.36	1217.22	484.97	323.65	1.26	1.15	-1.50	-1.21	-2.00	-1.70	-3.76	-3.25

Table S5. (continued)

Probeset Id	Description ⁽ⁱ⁾	WT MS6		Samples (MSD)		B2 MS2	Gene Model ⁽ⁱⁱ⁾	Aib ID ⁽ⁱ⁾	Pair-wise comparison ⁽ⁱⁱ⁾				Pair-wise comparison ⁽ⁱⁱ⁾			
		WT MS6	B2 MS6	B2 MS6	WT MS2				(G) WT MS6/B2 MS6	(D) WT MS2/B2 MS2	(G) WT MS6/B2 MS6	(D) WT MS2/B2 MS2	(G) WT MS6/B2 MS6	(D) WT MS2/B2 MS2	(G) WT MS6/B2 MS6	(D) WT MS2/B2 MS2
PipAffx.200175.1.SI_at	Transcription factor BHLH22	486.65	131.81	510.64	75.43	223.66	Porti.001.GU17000	AT2G42280.1	2.15	1.73	1.63	1.05	1.73	1.63	1.05	-5.43
PipAffx.130267.1.SI_at	Transcription factor BHLH23	44.10	94.86	484.27	75.43	141.13	Porti.001.GU10700	AT2G42280.1	2.50	1.63	1.73	1.11	1.63	1.73	1.11	-5.06
PipAffx.127862.1.SI_at	Transcription factor BHLH30	216.83	618.58	484.27	220.96	164.00	Porti.01.GC50500	AT2G42280.1	2.85	2.20	-2.95	2.23	2.20	-2.95	2.23	-3.77
PipAffx.203501.1.SI_at	Transcription factor BHLH30	145.43	549.87	484.27	220.96	57.64	Porti.003.GC20700	AT2G42280.1	3.78	2.58	-3.83	-2.60	1.52	1.04	-9.54	-6.47
Pip.2081.1.SI_s_at	Transcription factor BHLH30	2966.38	2941.95	1449.87	1449.87	844.86	Porti.01.GU14700	AT3G17100.1	-1.01	-0.95	-1.72	-1.47	-2.05	-1.88	-3.48	-3.02
PipAffx.17885.1.SI_at	Transcription factor BHLH47	1642.26	1065.34	373.81	373.81	550.16	Porti.008.GC43000	AT3G17100.1	-1.54	-1.32	1.47	1.24	-4.39	-3.53	-1.94	-1.80
PipAffx.141577.2.SI_at	Transcription factor BHLH55	1072.36	1765.61	2098.60	2098.60	523.71	Porti.005.GC22200	AT1G06150.1	1.65	1.48	-4.01	-3.41	1.96	1.68	-3.37	-2.99
PipAffx.141577.2.SI_at	Transcription factor BHLH55	25.11	67.31	54.99	54.99	14.53	Porti.002.GC40400	AT1G06150.1	2.68	1.93	-3.78	-2.15	2.19	1.55	-4.63	-2.66
Pip.1757.1.SI_at	Transcription factor BHLH30	213.21	248.24	265.56	265.56	33.30	Porti.01.GC130000	AT1G06150.1	1.16	0.99	-7.98	-5.97	1.25	1.11	-7.46	-5.46
PipAffx.207993.1.SI_at	Transcription factor BHLH30	23.30	47.22	52.81	107.34	17.55	Porti.008.GC16000	AT1G06150.1	1.86	1.38	-6.12	-3.31	2.18	1.66	-5.20	-2.79
PipAffx.208977.1.SI_at	Transcription factor BHLH30	694.17	630.75	172.01	172.01	213.07	Porti.01.GC130000	AT1G06150.1	2.03	1.50	-4.05	-3.14	2.27	1.71	-3.62	-2.75
PipAffx.160035.1.SI_at	Transcription factor BHLH35	413.06	879.75	658.89	658.89	91.02	Porti.01.GC141700	AT1G06150.1	-1.10	-0.89	1.26	0.98	-4.04	-2.96	-2.91	-2.64
PipAffx.326463.1.SI_at	Transcription factor BHLH35	20.09	23.71	72.27	72.27	13.78	Porti.01.GC141700	AT1G06150.1	1.18	0.84	-5.24	-4.11	3.60	2.79	-1.72	-1.23
PipAffx.65797.1.SI_at	Transcription factor BHLH35	83.41	34.83	391.64	391.64	5.91	Porti.001.GC063000	AT1G06150.1	-2.39	-1.80	-5.16	-2.56	-2.73	-1.95	-5.89	-3.06
PipAffx.41858.1.SI_at	Transcription factor BHLH36	808.66	1194.99	391.64	391.64	279.17	Porti.001.GC063000	AT1G06150.1	1.48	1.29	-1.40	-1.20	-2.06	-1.80	-4.28	-3.70
PipAffx.65220.1.SI_at	Transcription factor BHLH48	117.20	302.58	111.66	111.66	49.28	Porti.006.GC05700	AT1G06150.1	2.58	1.59	-2.27	-1.59	-1.05	-0.41	-6.14	-4.34
PipAffx.157333.1.SI_at	Transcription factor BHLH60	24.52	49.10	18.35	9.93	75.64	Porti.006.GC05700	AT1G06150.1	1.04	0.86	-1.03	-0.87	-2.96	-2.43	-3.17	-2.63
PipAffx.36157.1.SI_at	Transcription factor BHLH60	231.35	239.88	78.05	78.05	372.28	Porti.004.GC156000	AT1G06150.1	1.16	1.00	-1.36	-1.02	-2.44	-2.13	-3.84	-2.87
PipAffx.3113.1.SI_at	Transcription factor BHLH63	205.47	499.46	87.63	87.63	183.63	Porti.009.GC17300	AT1G06150.1	1.98	1.36	1.21	0.79	-1.60	-1.34	-2.61	-1.82
PipAffx.207364.1.SI_s_at	Transcription factor BHLH64	54.62	194.05	38.33	38.33	74.77	Porti.007.GC23600	AT1G06150.1	2.43	1.99	-1.17	-0.63	-2.34	-1.64	-6.68	-4.30
PipAffx.65822.1.SI_at	Transcription factor BHLH66	324.08	194.03	1629.50	1629.50	309.16	Porti.006.GC186000	AT1G06150.1	1.32	1.12	-3.27	-4.53	3.11	2.33	-2.57	-2.18
PipAffx.1312.1.SI_at	Transcription factor BHLH66	190.07	782.15	782.15	782.15	248.32	Porti.001.GC083700	AT1G06150.1	-1.31	-1.11	-1.11	-1.11	-1.11	-1.11	-4.35	-4.11
PipAffx.1312.1.SI_at	Transcription factor BHLH68	125.36	178.00	923.29	923.29	348.32	Porti.001.GC083700	AT1G06150.1	-1.31	-1.11	-1.11	-1.11	-1.11	-1.11	-4.35	-4.11
PipAffx.1021.1.SI_at	Transcription factor BHLH68	854.54	684.03	923.29	923.29	498.82	Porti.001.GC083700	AT1G06150.1	-1.25	-1.15	-1.73	-1.24	-2.46	-2.17	-3.44	-2.69
PipAffx.613.1.SI_at	Transcription factor BHLH70	200.04	242.34	272.51	272.51	620.05	Porti.01.GC106300	AT1G06150.1	1.21	0.97	2.28	1.94	1.36	1.10	2.56	2.16
PipAffx.215458.1.SI_at	Transcription factor BHLH75	83.31	209.82	78.41	78.41	44.60	Porti.01.GC115300	AT1G06150.1	2.52	1.79	-1.76	-1.29	-1.06	-0.82	-4.70	-3.28
PipAffx.160510.1.SI_at	Transcription factor BHLH77	202.28	182.71	60.29	60.29	81.92	Porti.005.GC35400	AT1G06150.1	-1.11	-0.95	1.36	1.14	-3.36	-2.89	-2.23	-1.88
PipAffx.202624.1.SI_s_at	Transcription factor BHLH77	419.24	499.33	169.05	169.05	175.36	Porti.005.GC35400	AT1G06150.1	1.19	1.09	1.04	0.91	-2.48	-2.27	-2.85	-2.52
PipAffx.150004.1.SI_s_at	Transcription factor BHLH80	219.69	490.44	284.53	284.53	104.51	Porti.01.GC079900	AT1G06150.1	2.23	1.70	-2.72	-2.10	1.30	0.97	-4.69	-3.66
PipAffx.8849.1.SI_at	Transcription factor BHLH93	462.79	594.30	282.24	282.24	309.11	Porti.003.GC108400	AT1G06150.1	1.28	1.09	1.10	0.96	6.10	5.10	5.20	4.63
PipAffx.11967.1.SI_at	Transcription factor BHLH96	177.13	242.48	282.40	282.40	810.96	Porti.003.GC108400	AT1G06150.1	1.37	1.16	2.87	1.92	1.59	1.35	3.34	2.24
PipAffx.207090.1.SI_at	Transcription factor PIF3	73.15	56.12	318.12	318.12	303.09	Porti.007.GC097000	AT1G22490.1	-1.30	-0.73	1.26	0.80	3.29	2.25	5.40	3.28
PipAffx.55882.1.SI_at	Transcription factor PIF3	424.93	636.46	790.23	790.23	172.18	Porti.002.GC52800	AT1G22490.1	1.50	1.23	-1.85	-1.38	-1.34	-1.18	-3.70	-2.65
PipAffx.33059.1.SI_at	Transcription factor PIF5	470.62	953.39	790.23	790.23	257.50	Porti.01.GC114000	AT1G22490.1	2.03	1.72	-3.07	-2.47	1.68	1.35	-3.70	-3.15
PipAffx.1627.1.SI_at	Transcription factor PIF5	990.57	182.38	633.08	633.08	663.72	Porti.002.GC55400	AT1G22490.1	1.84	1.57	-1.10	-0.94	-1.35	-1.14	-2.75	-2.34
Pip.7895.1.SI_s_at	Transcription factor PIF1	233.77	326.29	73.08	73.08	31.48	Porti.01.GC114000	AT1G09530.1	1.40	1.21	-2.00	-1.14	-3.71	-2.67	-10.36	-7.14
PipAffx.140558.1.SI_at	Transcription factor RAX2	20.04	26.68	81.20	81.20	228.14	Porti.004.GC21500	AT1G09530.1	1.33	0.67	2.81	1.86	4.05	2.52	8.55	5.16
Pip.3869.1.SI_at	Transcription factor RAX3	314.30	357.95	81.61	81.61	120.60	Porti.007.GC07000	AT1G09530.1	1.14	0.94	1.48	1.02	-3.85	-3.18	-2.97	-2.19
Pip.5001.2.SI_at	Transcription factor TCP2	46.27	145.37	64.70	64.70	19.55	Porti.004.GC065800	AT1G09530.1	3.14	2.14	-3.31	-2.19	1.40	0.87	-7.44	-5.46
PipAffx.203798.1.SI_at	Transcription factor TCP2	93.56	233.10	107.43	107.43	55.70	Porti.004.GC065800	AT1G09530.1	2.49	2.22	-1.93	-1.12	1.15	0.72	-4.19	-3.06
Pip.7471.1.SI_at	Transcription factor TCP20	71.49	121.36	66.72	66.72	31.94	Porti.001.GC327000	AT1G53230.1	1.70	1.20	-2.09	-1.50	-1.07	-0.72	-3.80	-2.67
Pip.6257.1.SI_at	Transcription factor TCP3	647.23	834.89	276.01	276.01	221.94	Porti.001.GC327000	AT1G53230.1	1.29	0.95	-1.24	-0.76	-2.34	-1.41	-3.76	-2.93
PipAffx.144790.1.SI_at	Transcription factor TCP3	67.43	129.36	50.74	50.74	33.53	Porti.001.GC327000	AT1G53230.1	1.92	1.65	-1.51	-0.98	-1.33	-0.97	-3.86	-3.06
PipAffx.201326.1.SI_s_at	Transcription factor TCP3	810.56	127.05	468.30	468.30	350.48	Porti.001.GC327000	AT1G53230.1	1.57	1.26	-1.54	-1.02	-1.73	-1.14	-3.64	-3.21
PipAffx.103645.1.SI_at	Transcription factor TCP4	78.69	173.29	142.32	142.32	35.58	Porti.01.GC194000	AT1G53230.1	2.20	1.82	-4.00	-2.48	1.80	1.29	-4.87	-3.30
PipAffx.12315.1.SI_at	Transcription factor VIP1	110.30	185.68	71.52	71.52	591.13	Porti.01.GC124400	AT1G53230.1	1.68	1.36	-1.21	-0.99	-1.54	-1.29	-3.14	-2.48
PipAffx.201317.1.SI_at	Transcription factor VIP1	347.23	898.02	276.77	276.77	76.13	Porti.01.GC240000	AT1G53230.1	2.59	1.76	-2.52	-1.25	-0.75	-1.80	-8.76	-6.76
PipAffx.210564.1.SI_at	Transcription factor WER	78.77	369.71	392.14	392.14	145.90	Porti.01.GC240000	AT1G53230.1	4.69	3.54	-2.69	-1.64	4.98	3.72	-2.53	-1.56
PipAffx.204119.1.SI_at	Zr-HD homeobox protein	85.01	205.58	152.16	152.16	64.15	Porti.004.GC13000	AT1G53230.1	2.40	1.92	-2.40	-1.69	1.77	1.32	-3.20	-2.44
PipAffx.23284.1.SI_at	Zr-HD homeobox protein	46.46	81.87	37.12	37.12	15.79	Porti.004.GC29600	AT1G53230.1	1.76	1.34	-2.35	-1.38	-1.25	-0.85	-5.19	-3.50

Table S5. (continued)

Probeset Id	Description ⁽²⁾	WTMS6		R2 MS2		Gene Model ⁽³⁾	Ab ID ⁽⁴⁾	GW TMS6/R2 MS2		Bis-seq comparison ⁽⁵⁾		GW TMS6/R2 MS2		Bis-seq comparison ⁽⁵⁾		GW TMS6/R2 MS2		Bis-seq comparison ⁽⁵⁾	
		WTMS6	R2 MS2	WTMS6	R2 MS2			FC	LCB	FC	LCB	FC	LCB	FC	LCB	FC	LCB	FC	LCB
PipAffx.15732.1.SI_at	ZE-HD homeobox protein	48.77	264.36	325.93	47.09	Parl.005G122500	AT1G75440.1	5.42	3.61	-6.92	-4.59	6.68	4.34	-5.61	-3.82	-2.70	-2.07	-5.61	-3.82
PipAffx.120656.1.AI_at	ZE-HD homeobox protein	82.52	229.29	79.18	84.98	Parl.002G335300	AT3G28210.1	2.78	2.11	-3.24	-2.68	3.33	2.72	-2.40	-1.98	2.40	1.98	-2.40	-1.98
PipAffx.1658.1.AI_at	Zinc finger_1 AI SAP12	20.47	23.73	79.14	57.04	Parl.011G138500	AT3G28210.1	1.16	0.75	-1.37	-0.90	3.82	2.42	-1.78	-1.20	1.78	1.20	-1.78	-1.20
PipAffx.202609.1.SI_s_at	Zinc finger_1 CCHC14	175.90	72.63	38.92	40.85	Parl.004G295100		-2.42	-1.83	1.05	0.72	-4.52	-3.61	-3.87	-2.73	-3.87	-2.73	-3.87	-2.73
PipAffx.203942.1.SI_s_at	Zinc finger_1 CCHC14	73.88	70.57	38.16	18.21	Parl.017G119900		-1.05	-0.71	-2.10	-1.50	-1.94	-1.33	-1.44	-1.09	-1.33	-1.09	-1.44	-1.09
PipAffx.770741.1.AI_at	Zinc finger_1 CCHC14	87.49	61.08	32.06	27.57	Parl.004G295100	AT1G68200.1	-1.46	-1.08	-1.16	-0.90	-2.73	-2.22	-2.18	-1.68	-2.18	-1.68	-2.18	-1.68
PipAffx.6581.10.SI_s_at	Zinc finger_1 CCHC17	301.73	307.49	155.40	59.07	Parl.017G119900	AT1G68200.1	1.05	0.67	-2.63	-1.99	-1.94	-1.07	-5.38	-3.85	-5.38	-3.85	-5.38	-3.85
PipAffx.6581.10.SI_s_at	Zinc finger_1 CCHC17	217.22	707.94	62.32	290.48	Parl.008G144200	AT2G02160.1	3.26	2.39	-2.14	-1.67	2.86	2.10	-3.45	-2.23	-3.45	-2.23	-3.45	-2.23
PipAffx.120668.1.AI_at	Zinc finger_1 CCHC18	88.84	331.63	270.51	108.65	Parl.008G144200	AT2G02160.1	3.73	2.62	-2.49	-1.82	3.04	2.14	-3.05	-2.23	-3.05	-2.23	-3.05	-2.23
PipAffx.16090.2.SI_s_at	Zinc finger_1 CCHC18	1221.69	2228.78	528.97	581.13	Parl.014G165500		1.82	1.55	1.10	0.85	-2.31	-1.90	-3.83	-3.14	-2.85	-2.51	-3.83	-3.14
PipAffx.202456.1.SI_s_at	Zinc finger_1 CCHC18	947.70	1687.33	377.09	593.23	Parl.002G221100		1.78	1.32	1.57	1.19	-2.51	-1.70	-2.85	-2.07	-2.85	-2.07	-2.85	-2.07
PipAffx.204889.1.SI_s_at	Zinc finger_1 CCHC22	160.10	307.30	100.36	111.26	Parl.009G250700		1.92	1.37	1.11	0.77	-1.60	-1.00	-2.76	-2.07	-2.76	-2.07	-2.76	-2.07
PipAffx.6610.2.SI_s_at	Zinc finger_1 CCHC22	126.20	298.91	350.12	125.63	Parl.003G204300		2.37	1.77	-2.79	-2.09	2.77	2.08	-2.38	-1.78	-2.38	-1.78	-2.38	-1.78
PipAffx.215522.1.SI_s_at	Zinc finger_1 CCHC22	34.38	105.88	48.41	24.96	Parl.003G204300		3.08	2.30	-1.94	-1.40	1.41	0.96	-4.24	-3.41	-4.24	-3.41	-4.24	-3.41
PipAffx.6384.1.SI_s_at	Zinc finger_1 CCHC29	60.36	88.10	85.65	43.86	Parl.011G156600		-1.04	-0.82	-3.71	-2.63	1.82	1.49	-1.96	-1.39	-1.96	-1.39	-1.96	-1.39
PipAffx.149742.1.AI_at	Zinc finger_1 CCHC38	347.04	272.99	547.67	910.49	Parl.008G069400	AT2G40140.2	1.78	1.14	-2.24	-1.56	-1.10	-0.66	-4.38	-3.42	-4.38	-3.42	-4.38	-3.42
PipAffx.206671.1.SI_s_at	Zinc finger_1 CCHC38	61.62	193.78	216.26	75.86	Parl.002G223700	AT2G29330.1	-1.27	-1.05	1.66	1.36	1.58	1.28	3.34	2.82	3.34	2.82	3.34	2.82
PipAffx.147253.1.AI_at	Zinc finger_1 CCHC39	18.65	63.46	56.62	25.81	Parl.006G223700		3.14	2.06	-2.85	-2.13	3.51	2.29	-2.55	-1.91	-2.55	-1.91	-2.55	-1.91
PipAffx.202905.1.SI_s_at	Zinc finger_1 CCHC39	60.62	80.09	211.59	132.49	Parl.003G061500	AT3G19360.1	2.07	1.76	-1.71	-1.36	4.55	3.69	1.28	1.02	1.28	1.02	1.28	1.02
PipAffx.223276.1.SI_s_at	Zinc finger_1 CCHC39	142.18	294.46	646.99	377.28	Parl.004G223700		1.32	1.05	-1.60	-1.23	3.49	2.62	1.65	1.35	1.65	1.35	1.65	1.35
PipAffx.207141.1.SI_s_at	Zinc finger_1 CCHC55	43.82	108.14	143.96	47.60	Parl.004G223700		3.40	2.35	-3.02	-2.07	3.28	2.45	-2.27	-1.55	-2.27	-1.55	-2.27	-1.55
PipAffx.204890.1.SI_s_at	Zinc finger_1 CCHC55	71.77	354.64	383.47	95.98	Parl.004G223700		2.47	1.84	-3.02	-2.07	3.28	2.45	-2.27	-1.55	-2.27	-1.55	-2.27	-1.55
PipAffx.201842.1.SI_s_at	Zinc finger_1 CCHC7	114.59	412.53	311.19	125.46	Parl.002G077700		3.60	2.80	-2.48	-1.87	2.72	2.10	-3.29	-2.50	-3.29	-2.50	-3.29	-2.50
PipAffx.441.1.AI_at	Zinc finger_1 CCHC7	725.35	386.19	163.56	274.38	Parl.002G077700		-2.37	-2.11	1.68	1.01	-4.43	-4.05	-1.12	-0.78	-1.12	-0.78	-1.12	-0.78
PipAffx.209359.1.SI_s_at	Zinc finger_1 CCHC7	4020.96	3489.46	126.53	126.53	Parl.004G064100	AT5G25160.1	-1.15	-1.06	-1.02	-0.91	-3.14	-2.86	-2.76	-2.50	-2.76	-2.50	-2.76	-2.50
PipAffx.209359.1.SI_s_at	Zinc finger_1 CCHC7	383.30	128.23	131.84	39.76	Parl.013G122400	AT1G66140.1	-2.79	-2.34	-2.21	-1.83	-2.72	-2.22	-2.15	-1.86	-2.15	-1.86	-2.15	-1.86
PipAffx.201172.1.SI_s_at	Zinc finger_1 CCHC7	57.46	128.23	32.97	58.13	Parl.013G122400	AT1G66140.1	-1.52	-1.13	-1.13	-0.83	-1.13	-0.83	-1.13	-0.83	-1.13	-0.83	-1.13	-0.83
PipAffx.201172.1.SI_s_at	Zinc finger_1 CCHC7	57.46	128.23	32.97	58.13	Parl.013G122400	AT1G66140.1	-1.52	-1.13	-1.13	-0.83	-1.13	-0.83	-1.13	-0.83	-1.13	-0.83	-1.13	-0.83
PipAffx.201172.1.SI_s_at	Zinc finger_1 CCHC7	371.82	430.48	64.37	38.02	Parl.009G298700		-1.13	-0.93	-1.48	-1.13	-5.78	-4.25	-7.63	-5.86	-7.63	-5.86	-7.63	-5.86
PipAffx.66810.1.SI_s_at	Zinc finger_1 CCHC7	1979.37	1264.22	169.77	128.87	Parl.009G298700		-1.13	-0.93	-1.48	-1.13	-5.78	-4.25	-7.63	-5.86	-7.63	-5.86	-7.63	-5.86
PipAffx.101182.1.SI_s_at	Zinc finger_1 CCHC7	14.95	154.52	46.92	34.57	Parl.018G206300	AT4G31420.1	-1.57	-1.40	-1.32	-1.13	-1.66	-1.44	-9.81	-8.38	-9.81	-8.38	-9.81	-8.38
PipAffx.200249.1.SI_s_at	Zinc finger_1 CCHC7	22.88	37.91	152.25	68.19	Parl.018G206300		1.66	1.14	-2.23	-1.83	6.65	4.89	1.71	1.31	1.71	1.31	1.71	1.31
PipAffx.108.1.AI_s_at	Zinc finger_1 CCHC7	382.09	936.53	117.03	67.56	Parl.018G206300		2.45	1.98	-1.73	-1.36	6.65	4.89	1.71	1.31	1.71	1.31	1.71	1.31
PipAffx.1032.1.SI_s_at	Zinc finger_1 CCHC7	3652.33	347.27	896.17	52.32	Parl.017G07500	AT1G73870.1	1.29	1.14	-1.12	-0.81	-4.08	-3.33	-4.71	-3.74	-4.71	-3.74	-4.71	-3.74
PipAffx.289.1.SI_s_at	Zinc finger_1 CCHC7	3652.33	347.27	896.17	52.32	Parl.017G07500	AT1G73870.1	1.29	1.14	-1.12	-0.81	-4.08	-3.33	-4.71	-3.74	-4.71	-3.74	-4.71	-3.74
PipAffx.289.1.SI_s_at	Zinc finger_1 CCHC7	3652.33	347.27	896.17	52.32	Parl.017G07500	AT1G73870.1	1.29	1.14	-1.12	-0.81	-4.08	-3.33	-4.71	-3.74	-4.71	-3.74	-4.71	-3.74
PipAffx.289.1.SI_s_at	Zinc finger_1 CCHC7	3652.33	347.27	896.17	52.32	Parl.017G07500	AT1G73870.1	1.29	1.14	-1.12	-0.81	-4.08	-3.33	-4.71	-3.74	-4.71	-3.74	-4.71	-3.74
PipAffx.289.1.SI_s_at	Zinc finger_1 CCHC7	3652.33	347.27	896.17	52.32	Parl.017G07500	AT1G73870.1	1.29	1.14	-1.12	-0.81	-4.08	-3.33	-4.71	-3.74	-4.71	-3.74	-4.71	-3.74
PipAffx.289.1.SI_s_at	Zinc finger_1 CCHC7	3652.33	347.27	896.17	52.32	Parl.017G07500	AT1G73870.1	1.29	1.14	-1.12	-0.81	-4.08	-3.33	-4.71	-3.74	-4.71	-3.74	-4.71	-3.74
PipAffx.289.1.SI_s_at	Zinc finger_1 CCHC7	3652.33	347.27	896.17	52.32	Parl.017G07500	AT1G73870.1	1.29	1.14	-1.12	-0.81	-4.08	-3.33	-4.71	-3.74	-4.71	-3.74	-4.71	-3.74
PipAffx.289.1.SI_s_at	Zinc finger_1 CCHC7	3652.33	347.27	896.17	52.32	Parl.017G07500	AT1G73870.1	1.29	1.14	-1.12	-0.81	-4.08	-3.33	-4.71	-3.74	-4.71	-3.74	-4.71	-3.74
PipAffx.289.1.SI_s_at	Zinc finger_1 CCHC7	3652.33	347.27	896.17	52.32	Parl.017G07500	AT1G73870.1	1.29	1.14	-1.12	-0.81	-4.08	-3.33	-4.71	-3.74	-4.71	-3.74	-4.71	-3.74
PipAffx.289.1.SI_s_at	Zinc finger_1 CCHC7	3652.33	347.27	896.17	52.32	Parl.017G07500	AT1G73870.1	1.29	1.14	-1.12	-0.81	-4.08	-3.33	-4.71	-3.74	-4.71	-3.74	-4.71	-3.74
PipAffx.289.1.SI_s_at	Zinc finger_1 CCHC7	3652.33	347.27	896.17	52.32	Parl.017G07500	AT1G73870.1	1.29	1.14	-1.12	-0.81	-4.08	-3.33	-4.71	-3.74	-4.71	-3.74	-4.71	-3.74
PipAffx.289.1.SI_s_at	Zinc finger_1 CCHC7	3652.33	347.27	896.17	52.32	Parl.017G07500	AT1G73870.1	1.29	1.14	-1.12	-0.81	-4.08	-3.33	-4.71	-3.74	-4.71	-3.74	-4.71	-3.74
PipAffx.289.1.SI_s_at	Zinc finger_1 CCHC7	3652.33	347.27	896.17	52.32	Parl.017G07500	AT1G73870.1	1.29	1.14	-1.12	-0.81	-4.08	-3.33	-4.71	-3.74	-4.71	-3.74	-4.71	-3.74
PipAffx.289.1.SI_s_at	Zinc finger_1 CCHC7	3652.33	347.27	896.17	52.32	Parl.017G07500	AT1G73870.1	1.29	1.14	-1.12	-0.81	-4.08	-3.33	-4.71	-3.74	-4.71	-3.74	-4.71	-3.74
PipAffx.289.1.SI_s_at	Zinc finger_1 CCHC7	3652.33	347.27	896.17	52.32	Parl.017G07500	AT1G73870.1	1.29	1.14	-1.12	-0.81	-4.08	-3.33	-4.71	-3.74	-4.71	-3.74	-4.71	-3.74
PipAffx.289.1.SI_s_at	Zinc finger_1 CCHC7	3652.33	347.27	896.17	52.32	Parl.017G07500	AT1G73870.1	1.29	1.14	-1.12	-0.81	-4.08	-3.33	-4.71	-3.74	-4.71	-3.74	-4.71	-3.74
PipAffx.289.1.SI_s_at	Zinc finger_1 CCHC7	3652.33	347.27	896.17	52.32	Parl.017G07500	AT1G73870.1	1.29	1.14	-1.12	-0.81	-4.08	-3.33	-4.71	-3.74	-4.71	-3.74	-4.71	-3.74
PipAffx.289.1.SI_s_at	Zinc finger_1 CCHC7	3652.33	347.27	896.17	52.32	Parl.017G07500	AT1G73870.1	1.29	1.14	-1.12	-0.81	-4.08	-3.33	-4.71	-3.74	-4.71	-3.74	-4.71	-3.74
PipAffx.289.1.SI_s_at	Zinc finger_1 CCHC7	3652.33	347.27	896.17	52.32	Parl.017G07500	AT1G73870.1	1.29	1.14	-1.12	-0.81	-4.08	-3.33	-4.71	-3.74	-4.71	-3.74	-4.71	-3.74
PipAffx.289.1.SI_s_at	Zinc finger_1 CCHC7	3652.33	347.27	896.17	52.32	Parl.017G07500	AT1G73870.1	1.29	1.14	-1.12	-0.81	-4.08	-3.33	-4.71	-3.74	-4.71	-3.74	-4.71	-3.74
PipAffx.289.1.SI_s_at	Zinc finger_1 CCHC7	3652.33	347.27	896.17	52.32	Parl.017G07500	AT1G73870.1	1.29	1.14	-1.12	-0.81	-4.08	-3.33	-4.71	-3.74	-4.71	-3.74	-	

Table S5. (continued)

WTMS6		Samples (MS6)		WTMS2		B2 MS2		Gene Model ^(b)		Ath ID ^(c)		(a)WTMS6/B2 MS6		(b)WTMS2/B2 MS2		Pair-wise comparison ^(d)		(b)WTMS6/R2 MS2		(b)WTMS6/R2 MS2	
WTMS6	B2 MS6	WTMS6	B2 MS6	WTMS2	B2 MS2	WTMS2	B2 MS2	WTMS2	B2 MS2	WTMS2	B2 MS2	FC	LCB	FC	LCB	FC	LCB	FC	LCB	FC	LCB
385.86	382.34	140.47	162.19	162.19	Parti.002.GI19300	Parti.002.GI19300	Parti.002.GI19300	Parti.002.GI19300	Parti.002.GI19300	Parti.002.GI19300	Parti.002.GI19300	-1.01	-0.76	-1.01	-0.76	1.15	1.15	-2.75	-2.08	-2.36	-1.54
597.59	243.90	86.09	399.06	399.06	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	-2.45	-1.89	-2.45	-1.89	4.64	3.02	-6.94	-5.54	1.64	1.04
726.55	648.40	172.75	155.21	155.21	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	-1.12	-1.05	-1.12	-1.05	-1.11	-0.85	-4.21	-3.64	-4.18	-3.34
86.14	269.47	563.28	123.26	123.26	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	-4.34	-3.30	-4.34	-3.30	-6.87	-5.35	-2.39	-2.21	-3.78	-2.44
922.30	212.50	385.73	56.18	56.18	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	-1.37	-0.98	-1.37	-0.98	1.26	0.77	-5.28	-4.61	-3.06	-2.19
1367.01	1001.34	258.86	327.43	327.43	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	-1.29	-1.26	-1.29	-1.26	-5.89	-4.51	-1.23	-0.93	-5.59	-4.25
413.61	320.43	337.34	57.31	57.31	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	Parti.01.0G209400	-1.10	-1.00	-1.10	-1.00	-1.39	-1.04	-3.32	-2.98	-4.21	-3.18
789.58	720.63	237.99	171.28	171.28	Parti.004.GI21800	Parti.004.GI21800	Parti.004.GI21800	Parti.004.GI21800	Parti.004.GI21800	Parti.004.GI21800	Parti.004.GI21800	1.23	1.08	1.23	1.08	-1.37	-1.14	-2.38	-1.98	-4.00	-3.53
1003.14	123.69	422.16	308.50	308.50	Parti.004.GI21800	Parti.004.GI21800	Parti.004.GI21800	Parti.004.GI21800	Parti.004.GI21800	Parti.004.GI21800	Parti.004.GI21800	-1.16	-1.00	-1.16	-1.00	2.80	2.31	-1.24	-1.06	2.62	2.21
608.79	523.03	489.97	137.29	137.29	Parti.008.GI055100	Parti.008.GI055100	Parti.008.GI055100	Parti.008.GI055100	Parti.008.GI055100	Parti.008.GI055100	Parti.008.GI055100	1.28	1.08	1.28	1.08	-4.80	-4.00	-1.19	-0.97	-7.29	-6.14
327.69	418.88	275.44	57.44	57.44	Parti.013.GI018700	Parti.013.GI018700	Parti.013.GI018700	Parti.013.GI018700	Parti.013.GI018700	Parti.013.GI018700	Parti.013.GI018700	2.19	1.44	2.19	1.44	-4.70	-2.97	1.78	1.17	-5.76	-3.65
62.01	137.01	81.74	23.77	23.77	Parti.013.GI018700	Parti.013.GI018700	Parti.013.GI018700	Parti.013.GI018700	Parti.013.GI018700	Parti.013.GI018700	Parti.013.GI018700	1.08	1.01	1.08	1.01	-2.68	-1.89	-1.64	-1.47	-4.78	-3.40
1468.41	1395.16	119.99	332.68	332.68	Parti.003.GI044500	Parti.003.GI044500	Parti.003.GI044500	Parti.003.GI044500	Parti.003.GI044500	Parti.003.GI044500	Parti.003.GI044500	-1.26	-1.14	-1.26	-1.14	-3.37	-2.57	-1.58	-1.43	-4.23	-3.22
572.00	454.51	365.21	107.52	107.52	Parti.009.GI029500	Parti.009.GI029500	Parti.009.GI029500	Parti.009.GI029500	Parti.009.GI029500	Parti.009.GI029500	Parti.009.GI029500	1.25	1.11	1.25	1.11	-0.86	-0.86	-2.85	-2.42	-3.78	-3.17
1936.44	244.53	882.38	645.86	645.86	Parti.015.GI064800	Parti.015.GI064800	Parti.015.GI064800	Parti.015.GI064800	Parti.015.GI064800	Parti.015.GI064800	Parti.015.GI064800	1.32	1.14	1.32	1.14	-1.70	-1.48	-1.66	-1.40	-3.72	-3.28
1620.67	2114.90	977.27	576.24	576.24	Parti.015.GI064800	Parti.015.GI064800	Parti.015.GI064800	Parti.015.GI064800	Parti.015.GI064800	Parti.015.GI064800	Parti.015.GI064800	1.25	1.14	1.25	1.14	-1.70	-1.48	-1.66	-1.40	-3.72	-3.28
92.81	105.89	122.15	282.36	282.36	Parti.003.GI020700	Parti.003.GI020700	Parti.003.GI020700	Parti.003.GI020700	Parti.003.GI020700	Parti.003.GI020700	Parti.003.GI020700	-1.37	-1.32	-1.37	-1.32	1.27	1.02	-4.52	-4.32	-2.61	-2.18
3830.29	2803.79	846.68	1076.20	1076.20	Parti.012.GI020700	Parti.012.GI020700	Parti.012.GI020700	Parti.012.GI020700	Parti.012.GI020700	Parti.012.GI020700	Parti.012.GI020700	-1.37	-1.32	-1.37	-1.32	1.27	1.02	-4.52	-4.32	-2.61	-2.18
253.24	124.20	84.60	464.62	464.62	Parti.012.GI020700	Parti.012.GI020700	Parti.012.GI020700	Parti.012.GI020700	Parti.012.GI020700	Parti.012.GI020700	Parti.012.GI020700	-2.04	-1.74	-2.04	-1.74	5.49	3.59	-2.99	-2.41	3.74	2.51
102.52	85.37	30.24	21.04	21.04	Parti.005.GI24500	Parti.005.GI24500	Parti.005.GI24500	Parti.005.GI24500	Parti.005.GI24500	Parti.005.GI24500	Parti.005.GI24500	-1.20	-1.06	-1.20	-1.06	-1.44	-0.93	-3.39	-2.54	-4.06	-3.17
180.97	505.27	215.04	53.96	53.96	Parti.006.GI031200	Parti.006.GI031200	Parti.006.GI031200	Parti.006.GI031200	Parti.006.GI031200	Parti.006.GI031200	Parti.006.GI031200	2.79	1.87	2.79	1.87	-3.99	-2.76	1.19	0.78	-9.36	-6.64
280.33	534.19	1080.58	505.99	505.99	Parti.01.GI37200	Parti.01.GI37200	Parti.01.GI37200	Parti.01.GI37200	Parti.01.GI37200	Parti.01.GI37200	Parti.01.GI37200	1.91	1.37	1.91	1.37	-2.14	-1.81	3.85	2.79	-1.06	-0.88
325.25	717.90	413.83	136.33	136.33	Parti.01.GI37200	Parti.01.GI37200	Parti.01.GI37200	Parti.01.GI37200	Parti.01.GI37200	Parti.01.GI37200	Parti.01.GI37200	-1.48	-1.30	-1.48	-1.30	-3.04	-2.29	1.27	0.85	-5.27	-3.96
496.44	583.26	205.91	139.20	139.20	Parti.01.GI066700	Parti.01.GI066700	Parti.01.GI066700	Parti.01.GI066700	Parti.01.GI066700	Parti.01.GI066700	Parti.01.GI066700	1.17	1.05	1.17	1.05	-1.48	-1.08	-2.41	-2.08	-4.19	-3.12
232.49	213.98	226.15	531.32	531.32	Parti.003.GI21900	Parti.003.GI21900	Parti.003.GI21900	Parti.003.GI21900	Parti.003.GI21900	Parti.003.GI21900	Parti.003.GI21900	-1.09	-0.93	-1.09	-0.93	2.35	1.85	-1.03	-0.87	-2.48	-1.98
1107.80	831.60	193.76	465.67	465.67	Parti.013.GI036200	Parti.013.GI036200	Parti.013.GI036200	Parti.013.GI036200	Parti.013.GI036200	Parti.013.GI036200	Parti.013.GI036200	-1.33	-1.25	-1.33	-1.25	2.40	1.58	-5.72	-4.76	-1.79	-1.35
30.29	24.94	140.53	54.44	54.44	Parti.013.GI036200	Parti.013.GI036200	Parti.013.GI036200	Parti.013.GI036200	Parti.013.GI036200	Parti.013.GI036200	Parti.013.GI036200	1.61	1.31	1.61	1.31	-1.28	-0.96	-2.32	-1.72	-4.78	-3.80
340.26	340.26	553.10	1297.30	1297.30	Parti.006.GI096900	Parti.006.GI096900	Parti.006.GI096900	Parti.006.GI096900	Parti.006.GI096900	Parti.006.GI096900	Parti.006.GI096900	-1.21	-0.95	-1.21	-0.95	-2.58	-2.58	-4.47	-4.64	0.84	0.84
3230.07	3040.26	553.10	1297.30	1297.30	Parti.006.GI096900	Parti.006.GI096900	Parti.006.GI096900	Parti.006.GI096900	Parti.006.GI096900	Parti.006.GI096900	Parti.006.GI096900	-1.06	-0.85	-1.06	-0.85	2.35	1.47	-5.84	-4.63	-2.34	-1.70
1027.80	747.70	140.68	331.09	331.09	Parti.013.GI036200	Parti.013.GI036200	Parti.013.GI036200	Parti.013.GI036200	Parti.013.GI036200	Parti.013.GI036200	Parti.013.GI036200	-1.37	-1.33	-1.37	-1.33	3.05	1.64	-7.31	-6.14	-2.26	-1.77
69.59	130.97	261.49	146.29	146.29	Parti.013.GI036200	Parti.013.GI036200	Parti.013.GI036200	Parti.013.GI036200	Parti.013.GI036200	Parti.013.GI036200	Parti.013.GI036200	1.88	1.46	1.88	1.46	-1.79	-1.62	3.76	3.01	-6.14	-4.96
2032.17	2620.46	690.12	1266.10	1266.10	Parti.001.GI078300	Parti.001.GI078300	Parti.001.GI078300	Parti.001.GI078300	Parti.001.GI078300	Parti.001.GI078300	Parti.001.GI078300	-1.75	-1.39	-1.75	-1.39	6.79	3.79	-2.66	-2.06	-4.45	-2.51
515.52	295.41	193.53	1314.19	1314.19	Parti.001.GI078300	Parti.001.GI078300	Parti.001.GI078300	Parti.001.GI078300	Parti.001.GI078300	Parti.001.GI078300	Parti.001.GI078300	-1.43	-0.95	-1.43	-0.95	2.10	1.64	-2.94	-2.44	-2.07	-1.59
31.52	21.97	56.24	118.36	118.36	Parti.007.GI050100	Parti.007.GI050100	Parti.007.GI050100	Parti.007.GI050100	Parti.007.GI050100	Parti.007.GI050100	Parti.007.GI050100	-1.95	-1.62	-1.95	-1.62	1.47	1.23	-3.05	-2.59	-1.06	-0.88
863.45	443.01	283.50	416.01	416.01	Parti.002.GI187000	Parti.002.GI187000	Parti.002.GI187000	Parti.002.GI187000	Parti.002.GI187000	Parti.002.GI187000	Parti.002.GI187000	2.01	1.70	2.01	1.70	-1.50	-1.09	-1.40	-1.11	-4.23	-3.28
167.94	338.01	119.80	79.93	79.93	Parti.013.GI077800	Parti.013.GI077800	Parti.013.GI077800	Parti.013.GI077800	Parti.013.GI077800	Parti.013.GI077800	Parti.013.GI077800	1.39	1.18	1.39	1.18	-1.13	-0.76	-1.91	-1.57	-2.99	-2.03
96.32	133.60	50.32	44.64	44.64	Parti.005.GI221300	Parti.005.GI221300	Parti.005.GI221300	Parti.005.GI221300	Parti.005.GI221300	Parti.005.GI221300	Parti.005.GI221300	1.39	1.18	1.39	1.18	-1.13	-0.76	-1.91	-1.57	-2.99	-2.03
92.90	226.30	108.71	77.13	77.13	Parti.006.GI0661700	Parti.006.GI0661700	Parti.006.GI0661700	Parti.006.GI0661700	Parti.006.GI0661700	Parti.006.GI0661700	Parti.006.GI0661700	2.44	1.84	2.44	1.84	-1.41	-0.90	-1.17	-0.82	-2.93	-1.99
46.52	161.92	68.54	23.67	23.67	Parti.006.GI0661700	Parti.006.GI0661700	Parti.006.GI0661700	Parti.006.GI0661700	Parti.006.GI0661700	Parti.006.GI0661700	Parti.006.GI0661700	3.48	2.33	3.48	2.33	-2.90	-1.68	1.47	0.81	-6.84	-4.93
144.34	24.02	71.29	24.69	24.69	Parti.01.GI010200	Parti.01															

Table S5. (continued)

Probeset id	Description ⁽¹⁾	Samples (MSs)				Gene Model ⁽²⁾	Ath ID ⁽³⁾	Pair-wise comparison ⁽⁴⁾					
		WTMS6	B2 MS6	WTMS2	B2 MS2			Δ0W TMS6/R2 MS6		Δ0W TMS2/R2 MS2		Δ0R2 MS6/R2 MS2	
								FC	LCB	FC	LCB	FC	LCB
PipAffx.205791.1.SI_at	BEL1-like homeodomain protein 11	16.03	30.96	40.77	10.78	Potri.005G232000	AT1G75430.1	1.93	1.07	-3.78	-2.13	2.54	1.47
PipAffx.209317.1.SI_s_at	BEL1-like homeodomain protein 9	116.79	206.03	211.90	55.95	Potri.008G061000		1.76	1.22	-3.79	-2.74	1.81	1.25
PipAffx.222526.1.SI_at	BEL1-like homeodomain protein 8	291.06	502.90	249.24	148.39	Potri.004G213300	AT2G27990.1	1.73	1.20	-1.68	-1.36	-1.17	-0.67
PipAffx.45681.1.SI_at	BEL1-like homeodomain protein 3	411.97	284.19	116.13	101.18	Potri.002G031000		-1.45	-1.08	-1.15	-0.90	-3.55	-2.77
PipAffx.52988.1.SI_at	BEL1-like homeodomain protein 7	235.81	800.73	382.43	138.54	Potri.009G120800		3.40	2.49	-2.76	-1.71	1.62	0.98
												-2.87	-1.55
												-3.68	-2.67
												-3.39	-2.83
												-2.81	-2.05
												-5.78	-4.41

- (1) probesets represent some of the transcription factors encoding genes differentially expressed in the analysis.
- (2) genes nomenclature and gene models to the corresponding probesets were search for in PopArray annotation tool (<http://aspendb.uga.edu/poparray>) as well as obtained from the NetAffx annotation as of November 2010.
- (3) *Arabidopsis* homolog BLASTX best hit obtained using PlexDB database Microarray Platform Translator tool (<http://www.plexdb.org>), e-value cutoff of $1 \cdot 10^{-20}$.
- (4) Fold Change (FC) and 90% Lower Confidence Bound (LCB) of Fold Change is shown for each pair-wise comparison. A positive LCB indicates that the higher signal was from the second member of the pair-wise sample comparison, while a negative LCB indicates that the higher signal was from the first member of the comparison. n.s. - non-significant (not differentially expressed in this comparison).

Table S6. Expression data (Mean signal intensities, MSIs) of *Populus* photosynthesis-related homologs⁽¹⁾.

Description ^{a)}	WT:MS6			Samples (MS1s)			Gene Model ^{b)}	Ath ID ^{c)}			QDWT:MS6/2 MS6			QDWT:MS2/2 MS2			P-value comparison ^{d)}		
	WT:MS6			Samples (MS1s)				Ath ID ^{c)}			QDWT:MS6/2 MS6			QDWT:MS2/2 MS2			P-value comparison ^{d)}		
	WT:MS6	WT:MS6	WT:MS6	WT:MS6	WT:MS6	WT:MS6		Ath ID ^{c)}			QDWT:MS6/2 MS6			QDWT:MS2/2 MS2			P-value comparison ^{d)}		
Lhc3a-1; light-harvesting complex II protein	11861.45	14818.71	9688.95	1024.42	1064.42	1064.42	P001.004170.100	AT1G542470.1			12.5	13.9	1.08	0.96	1.20	-1.07	-1.39	1.34	LCB
Lhc2-1; light-harvesting complex II protein	3892.15	5546.60	3388.17	4522.12	4522.12	4522.12	P001.01461500.100	AT2G05070.1			1.53	1.34	1.33	1.11	-1.15	-0.93	-1.32	-1.18	LCB
Lhc3-1; light-harvesting complex II protein	12872.98	14635.74	10424.61	11274.72	11274.72	11274.72	P001.01079500.100	AT2G34430.1			1.15	1.09	1.08	0.99	-1.22	-1.10	-1.30	-1.23	LCB
Lhc4-1; light-harvesting complex I protein	12228.32	10779.14	15056.41	10389.26	10389.26	10389.26	P001.015029200.100	AT3G47270.1			1.51	1.15	-1.04	-0.93	-1.13	-1.03	-1.45	-1.33	LCB
Lhc5-2; light-harvesting complex I protein	624.86	1158.60	443.16	625.48	625.48	625.48	P001.014026700.100	AT1G45474.1			1.85	1.51	1.41	1.13	-1.41	-1.03	-1.85	-1.67	LCB
Lhc7-1; light-harvesting complex II protein	193.30	228.86	87.16	124.78	124.78	124.78	P001.00538600.100	AT1G16570.1			1.19	1.05	1.43	1.11	-2.22	-1.75	-1.85	-1.61	LCB
Lhc2-1; light-harvesting complex II protein	1546.00	3609.07	975.02	974.78	974.78	974.78	P001.008034100.100	AT3G54890.1			2.38	2.09	-1.00	-0.59	-1.59	-1.12	-3.79	-3.14	LCB
Lhc3-1; light-harvesting complex I protein	12605.94	14003.30	8673.04	10338.05	10338.05	10338.05	P001.00105700.100	AT3G61470.1			1.20	1.18	1.19	1.04	-1.45	-1.30	-1.46	-1.36	LCB
Lhc6-1; light-harvesting complex I protein	12382.02	14003.30	9414.17	11358.81	11358.81	11358.81	P001.014472400.100	AT1G61520.1			1.11	1.06	1.23	1.11	-1.34	-1.22	-1.42	-1.15	LCB
Lhc5-1; light-harvesting complex I protein	7078.35	5794.42	4976.24	7376.51	7376.51	7376.51	P001.0138200.100	AT1G13820.1			-1.22	-1.12	-1.48	-1.22	-1.42	-1.27	-1.27	-1.06	LCB
Lhc2-1; light-harvesting complex II protein	12605.94	14003.30	8673.04	10338.05	10338.05	10338.05	P001.00105700.100	AT3G61470.1			1.20	1.18	1.19	1.04	-1.45	-1.30	-1.46	-1.36	LCB
Lhc6-1; light-harvesting complex I protein	12382.02	14003.30	9414.17	11358.81	11358.81	11358.81	P001.014472400.100	AT1G61520.1			1.11	1.06	1.23	1.11	-1.34	-1.22	-1.42	-1.15	LCB
Lhc5-1; light-harvesting complex I protein	7078.35	5794.42	4976.24	7376.51	7376.51	7376.51	P001.0138200.100	AT1G13820.1			-1.22	-1.12	-1.48	-1.22	-1.42	-1.27	-1.27	-1.06	LCB
Lhc2-1; light-harvesting complex II protein	12605.94	14003.30	8673.04	10338.05	10338.05	10338.05	P001.00105700.100	AT3G61470.1			1.20	1.18	1.19	1.04	-1.45	-1.30	-1.46	-1.36	LCB
Lhc6-1; light-harvesting complex I protein	12382.02	14003.30	9414.17	11358.81	11358.81	11358.81	P001.014472400.100	AT1G61520.1			1.11	1.06	1.23	1.11	-1.34	-1.22	-1.42	-1.15	LCB
Lhc5-1; light-harvesting complex I protein	7078.35	5794.42	4976.24	7376.51	7376.51	7376.51	P001.0138200.100	AT1G13820.1			-1.22	-1.12	-1.48	-1.22	-1.42	-1.27	-1.27	-1.06	LCB
Lhc2-1; light-harvesting complex II protein	12605.94	14003.30	8673.04	10338.05	10338.05	10338.05	P001.00105700.100	AT3G61470.1			1.20	1.18	1.19	1.04	-1.45	-1.30	-1.46	-1.36	LCB
Lhc6-1; light-harvesting complex I protein	12382.02	14003.30	9414.17	11358.81	11358.81	11358.81	P001.014472400.100	AT1G61520.1			1.11	1.06	1.23	1.11	-1.34	-1.22	-1.42	-1.15	LCB
Lhc5-1; light-harvesting complex I protein	7078.35	5794.42	4976.24	7376.51	7376.51	7376.51	P001.0138200.100	AT1G13820.1			-1.22	-1.12	-1.48	-1.22	-1.42	-1.27	-1.27	-1.06	LCB
Lhc2-1; light-harvesting complex II protein	12605.94	14003.30	8673.04	10338.05	10338.05	10338.05	P001.00105700.100	AT3G61470.1			1.20	1.18	1.19	1.04	-1.45	-1.30	-1.46	-1.36	LCB
Lhc6-1; light-harvesting complex I protein	12382.02	14003.30	9414.17	11358.81	11358.81	11358.81	P001.014472400.100	AT1G61520.1			1.11	1.06	1.23	1.11	-1.34	-1.22	-1.42	-1.15	LCB
Lhc5-1; light-harvesting complex I protein	7078.35	5794.42	4976.24	7376.51	7376.51	7376.51	P001.0138200.100	AT1G13820.1			-1.22	-1.12	-1.48	-1.22	-1.42	-1.27	-1.27	-1.06	LCB
Lhc2-1; light-harvesting complex II protein	12605.94	14003.30	8673.04	10338.05	10338.05	10338.05	P001.00105700.100	AT3G61470.1			1.20	1.18	1.19	1.04	-1.45	-1.30	-1.46	-1.36	LCB
Lhc6-1; light-harvesting complex I protein	12382.02	14003.30	9414.17	11358.81	11358.81	11358.81	P001.014472400.100	AT1G61520.1			1.11	1.06	1.23	1.11	-1.34	-1.22	-1.42	-1.15	LCB
Lhc5-1; light-harvesting complex I protein	7078.35	5794.42	4976.24	7376.51	7376.51	7376.51	P001.0138200.100	AT1G13820.1			-1.22	-1.12	-1.48	-1.22	-1.42	-1.27	-1.27	-1.06	LCB
Lhc2-1; light-harvesting complex II protein	12605.94	14003.30	8673.04	10338.05	10338.05	10338.05	P001.00105700.100	AT3G61470.1			1.20	1.18	1.19	1.04	-1.45	-1.30	-1.46	-1.36	LCB
Lhc6-1; light-harvesting complex I protein	12382.02	14003.30	9414.17	11358.81	11358.81	11358.81	P001.014472400.100	AT1G61520.1			1.11	1.06	1.23	1.11	-1.34	-1.22	-1.42	-1.15	LCB
Lhc5-1; light-harvesting complex I protein	7078.35	5794.42	4976.24	7376.51	7376.51	7376.51	P001.0138200.100	AT1G13820.1			-1.22	-1.12	-1.48	-1.22	-1.42	-1.27	-1.27	-1.06	LCB
Lhc2-1; light-harvesting complex II protein	12605.94	14003.30	8673.04	10338.05	10338.05	10338.05	P001.00105700.100	AT3G61470.1			1.20	1.18	1.19	1.04	-1.45	-1.30	-1.46	-1.36	LCB
Lhc6-1; light-harvesting complex I protein	12382.02	14003.30	9414.17	11358.81	11358.81	11358.81	P001.014472400.100	AT1G61520.1			1.11	1.06	1.23	1.11	-1.34	-1.22	-1.42	-1.15	LCB
Lhc5-1; light-harvesting complex I protein	7078.35	5794.42	4976.24	7376.51	7376.51	7376.51	P001.0138200.100	AT1G13820.1			-1.22	-1.12	-1.48	-1.22	-1.42	-1.27	-1.27	-1.06	LCB
Lhc2-1; light-harvesting complex II protein	12605.94	14003.30	8673.04	10338.05	10338.05	10338.05	P001.00105700.100	AT3G61470.1			1.20	1.18	1.19	1.04	-1.45	-1.30	-1.46	-1.36	LCB
Lhc6-1; light-harvesting complex I protein	12382.02	14003.30	9414.17	11358.81	11358.81	11358.81	P001.014472400.100	AT1G61520.1			1.11	1.06	1.23	1.11	-1.34	-1.22	-1.42	-1.15	LCB
Lhc5-1; light-harvesting complex I protein	7078.35	5794.42	4976.24	7376.51	7376.51	7376.51	P001.0138200.100	AT1G13820.1			-1.22	-1.12	-1.48	-1.22	-1.42	-1.27	-1.27	-1.06	LCB
Lhc2-1; light-harvesting complex II protein	12605.94	14003.30	8673.04	10338.05	10338.05	10338.05	P001.00105700.100	AT3G61470.1			1.20	1.18	1.19	1.04	-1.45	-1.30	-1.46	-1.36	LCB
Lhc6-1; light-harvesting complex I protein	12382.02	14003.30	9414.17	11358.81	11358.81	11358.81	P001.014472400.100	AT1G61520.1			1.11	1.06	1.23	1.11	-1.34	-1.22	-1.42	-1.15	LCB
Lhc5-1; light-harvesting complex I protein	7078.35	5794.42	4976.24	7376.51	7376.51	7376.51	P001.0138200.100	AT1G13820.1			-1.22	-1.12	-1.48	-1.22	-1.42	-1.27	-1.27	-1.06	LCB
Lhc2-1; light-harvesting complex II protein	12605.94	14003.30	8673.04	10338.05	10338.05	10338.05	P001.00105700.100	AT3G61470.1			1.20	1.18	1.19	1.04	-1.45	-1.30	-1.46	-1.36	LCB
Lhc6-1; light-harvesting complex I protein	12382.02	14003.30	9414.17	11358.81	11358.81	11358.81	P001.014472400.100	AT1G61520.1			1.11	1.06	1.23	1.11	-1.34	-1.22	-1.42	-1.15	LCB
Lhc5-1; light-harvesting complex I protein	7078.35	5794.42	4976.24	7376.51	7376.51	7376.51	P001.0138200.100	AT1G13820.1			-1.22	-1.12	-1.48	-1.22	-1.42	-1.27	-1.27	-1.06	LCB
Lhc2-1; light-harvesting complex II protein	12605.94	14003.30	8673.04	10338.05	10338.05	10338.05	P001.00105700.100	AT3G61470.1			1.20	1.18	1.19	1.04	-1.45	-1.30	-1.46	-1.36	LCB
Lhc6-1; light-harvesting complex I protein	12382.02	14003.30	9414.17	11358.81	11358.81	11358.81	P001.014472400.100	AT1G61520.1			1.11	1.06	1.23	1.11	-1.34	-1.22	-1.42	-1.15	LCB
Lhc5-1; light-harvesting complex I protein	7078.35	5794.42	4976.24	7376.51	7376.51	7376.51	P001.0138200.100	AT1G13820.1			-1.22	-1.12	-1.48	-1.22	-1.42	-1.27	-1.27	-1.06	LCB
Lhc2-1; light-harvesting complex II protein	12605.94	14003.30	8673.04	10338.05	10338.05	10338.05	P001.00105700.100	AT3G61470.1			1.20	1.18	1.19	1.04	-1.45	-1.30	-1.46	-1.36	LCB
Lhc6-1; light-harvesting complex I protein	12382.02	14003.30	9414.17	11358.81	11358.81	11358.81	P001.014472400.100	AT1G61520.1			1.11	1.06	1.23	1.11	-1.34	-1.22	-1.42	-1.15	LCB
Lhc5-1; light-harvesting complex I protein	7078.35	5794.42	4976.24	7376.51	7376.51	7376.51	P001.0138200.100	AT1G13820.1			-1.22	-1.12	-1.48	-1.22	-1.42	-1.27	-1.27	-1.06	LCB
Lhc2-1; light-harvesting complex II protein	12605.94	14003.30	8673.04	10338.05	10338.05	10338.05	P001.00105700.100	AT3G61470.1			1.20	1.18	1.19	1.04	-1.45	-1.30	-1.46	-1.36	LCB
Lhc6-1; light-harvesting complex I protein	12382.02	14003.30	9414.17	11358.81	11358.81	11358.81	P001.014472400.100	AT1G61520.1			1.11	1.06	1.23	1.11	-1.34	-1.22	-1.42	-1.15	LCB
Lhc5-1; light-harvesting complex I protein	7078.35	5794.42	4976.24	7376.51	7376.51	7376.51	P001.0138200.100	AT1G13820.1			-1.22	-1.12	-1.48	-1.22	-1.42	-1.27	-1.27	-1.06	LCB
Lhc2-1; light-harvesting complex II protein	12605.94	14003.30	8673.04	10338.05	10338.05	10338.05	P001.00105700.100	AT3G61470.1			1.20	1.18	1.19	1.04	-1.45	-1.30	-1.46	-1.36	LCB
Lhc6-1; light-harvesting complex I protein	12382.02	14003.30	9414.17	11358.81	11358.81	11358.81	P001.014472400.100	AT1G61520.1			1.11	1.06	1.23	1.11	-1.34	-1.22	-1.42	-1.15	LCB
Lhc5-1; light-harvesting complex I protein	7078.35	5794.42	4976.24	7376.51	7376.51	7376.51	P001.0138200.100	AT1G13820.1			-1.22	-1.12	-1.48	-1.22	-1.42	-1.27	-1.27	-1.06	LCB
Lhc2-1; light-harvesting complex II protein	12605.94	14003.30	8673.04	10338.05	10338.05	10338.05	P001.00105700.100	AT3G61470.1			1.20	1.18	1.19	1.04	-1.45	-1.30	-1.46	-1.36	LCB
Lhc6-1; light-harvesting complex I protein	12382.02	14003.30	9414.17	11358.81	11358.81	11358.81	P001.014472400.100	AT1G61520.1			1.11	1.06	1.23	1.11	-1.34	-1.22	-1.42	-1.15	LCB
Lhc5-1; light-harvesting complex I protein	7078.35	5794.42	4976.24	7376.51	7376.51	7376.51	P001.0138200.100	AT1G13820.1			-1.22	-1.12	-1						

Table S6. (continued)

Probeset Id	Description ⁽¹⁾	Samples (MSIs)		Gene Model ⁽²⁾	Atr ID ⁽³⁾	Pair-wise comparison ⁽⁴⁾					
		WTMS6	B2 MS6	WT MS2	B2 MS2	GWTMS6B2 MS6	(GWTMS6B2 MS6)	(GWTMS6B2 MS6)	GWTMS6WT MS2	(GWTMS6WT MS2)	LCB
PpAtrfx157021.S1_s_at	Photosystem II reaction center protein M	351.35	2203.14	1164.38	345.54	P001013G142100					
PpAtrfx25311.S1_s_at	Photosystem II reaction center protein M	436.85	1671.49	876.57	630.60	P001013G142100	6.27	4.29	-3.37	-2.06	3.31
PpAtrfx23105.1A1_s_at	Photosystem II reaction center protein M	86.91	525.18	339.50	143.66	P001013G142100	3.83	2.73	-1.39	-1.09	2.01
PpAtrfx43881.S1_s_at	Photosystem II reaction center P680 protein	3393.29	2820.01	2321.56	3241.86	P001002G256400	6.04	4.40	-2.36	-1.69	3.91
PpAtrfx45645.S1_s_at	Photosystem II reaction center P680 protein	416.46	1549.77	816.18	256.86	P001002G256400	-1.20	-1.15	1.40	1.26	-1.46
PpAtrfx45645.S1_s_at	Photosystem II reaction center P680 protein	671.46	1549.77	816.18	256.86	P001002G256400	-1.13	-1.08	1.41	1.26	-1.46
PpAtrfx44861.S1_s_at	Photosystem II subunit X	7948.12	8849.80	439.19	713.42	P001002G244300	1.11	1.03	1.62	1.39	-1.32
PpAtrfx44861.S1_s_at	Photosystem II subunit X	5921.48	7607.31	4514.61	7725.85	P001002G244300	1.29	1.15	1.61	1.41	-1.31
PpAtrfx20736.1.S1_s_at	Photosystem Qb protein	16401.00	13327.55	8646.83	13325.88	P001013G138300	-1.07	-0.94	1.54	1.15	-1.90
PpAtrfx2247551.S1_s_at	Protein pab3	1131.62	2953.41	1461.57	666.19	P001019G28300	2.61	1.51	-2.19	-1.09	1.29

(1) probesets represent the photosynthesis-related differentially expressed genes found in the analysis

(2) genes nomenclature and gene models to the corresponding probesets were search for in PopArray annotation tool (<http://aspenb.uga.edu/poparray>) as well as obtained from the NetAffx annotation as of November 2010.

(3) *Arabidopsis* homolog BLASTX best hit obtained using PlexDB database Microarray Platform Translator tool (<http://www.plexdb.org>), e-value cutoff of $1.e^{-20}$.

(4) Fold Change (FC) and 90% Lower Confidence Bound (LCB) of Fold Change is shown for each pair-wise comparison. A positive LCB indicates that the higher signal was from the second member of the pair-wise sample comparison, while a negative LCB indicates that the higher signal was from the first member of the comparison. n.s. - non-significant (not differentially expressed in this comparison).

CHAPTER IV

HD-ZIP III REGULATORY FUNCTIONS IN *POPULUS*[†]

[†] Milhinhos A., Matos A., Vera-Sirera F., Rambla J.L., Carbonell J., Blázquez M.A., Goulão L. and Miguel C.M. HD-Zip III regulatory functions in *Populus* (*in preparation*)

Elucidating the regulatory function of PttHB8 on *POPACAULIS5* expression

Summary

Class III Homeodomain-Leucine Zipper (HD-Zip III) transcription factors have been implicated in several fundamental roles in plant vascular development. The regulatory network upstream of *HD-Zip III* genes has become quite well studied over the years, yet the downstream targets of these transcription factors are missing from the literature. In *Populus*, we have previously suggested that PttHB8, homolog to the HD-Zip III AtHB8, is part of a feedback regulatory mechanism that controls *POPACAULIS5* (*ACL5* *Populus* ortholog) expression, thus controlling the delaying role thermospermine has on xylem differentiation in the stem. Here we further investigate the role of PttHB8 in the transcriptional control of *POPACAULIS5* in *Populus*. By making use of a heterologous system miRNA-resistant PttHB8 gain-of-function *Arabidopsis* mutants were obtained. Gene expression changes support that *PttHB8* ectopic overexpression upregulates *ACL5* expression while *POPACAULIS5* overexpression is accompanied by *AtHB8* reduced expression. Thus, suggesting that the control of thermospermine accumulation is quite conserved among vascular plants. Furthermore, we here illustrate a new function for PttHB8 in plant organ polarity. Whole plant morphology analysis suggests this neofunctionalization of HD-Zip III AtHB8 lineage has occurred in *Populus*, but latter evolutionary duplication events in the *Populus* genome have maintained those new functions conserved.

Keywords

Class III Homeodomain leucine zipper (HD-Zip III), vascular development, xylem, organ polarity, thermospermine, *POPACAULIS5*

Introduction

The HD-Zip transcription factors family that controls a large set of developmental genes combines a homeodomain (HD) tightly linked to a downstream leucine-zipper motif. The homeodomain is a conserved 60-aminoacid motif which folds into a characteristic three-helix structure that is able to specifically bind DNA. The downstream b-ZIP acts as a dimerization motif, essential for the binding ability of HD-Zip proteins to DNA (Sessa *et al.*, 1998). The two motifs are present in transcription factors from other eukaryotic kingdoms, but their association is unique to plants (Schna and Davis, 1992; Ariel *et al.*; 2007). The class III homeodomain-leucine zipper (HD-Zip III) proteins are further characterized by having a START (STeroidogenic Acute Regulatory protein related lipid Transfer) domain that is a lipid binding domain followed by a SAD domain (START Associated conserved Domain; De Caestecker *et al.*, 2000). Also, a MEKHLA domain is found in the C-terminal end of plant HD-Zip III proteins (Ponting and Aravind, 1999; Schrick *et al.*, 2004; Mukherjee and Bürglin, 2006). In *Arabidopsis*, HD-Zip III genes are implicated in apical-basal polarity in early embryogenesis (Grigg *et al.*, 2009; Smith and Long, 2010), meristem formation (McConnell and Barton, 1998; Otsuga *et al.*, 2001), lateral organ polarity in the shoot (Bowman *et al.*, 2002; Grigg *et al.*, 2009), vascular patterning (McConnell *et al.*, 2001; Emery *et al.*, 2003; Green *et al.*, 2005; Ohashi-Ito and Fukuda, 2005; Ochando *et al.*, 2008) and vascular specification and differentiation (Zhong and Ye, 1999; Baima *et al.*, 2001; Kim *et al.*, 2005; Ohashi-Ito and Fukuda, 2005; Prigge *et al.*, 2005). Five HD-Zip III genes can be found in the *Arabidopsis* genome: *REVOLUTA/INTERFASCICULAR FIBERLESS1 (REV/IFL1)*, *PHABULOSA/AtHB14 (PHB)*, *PHAVOLUTA/AtHB9 (PHV)* which form one clade, and *CORONA/AtHB15 (CNA)* and *AtHB8* that form a separate lineage (Baima *et al.*, 1995; McConnell *et al.*, 2001; Emery *et al.*, 2003; Green *et al.*, 2005).

This family has a complex pattern of expression, with distinct and antagonistic but also overlapping functions (Prigge *et al.*, 2005). It was early documented that all *Arabidopsis* HD-Zip III gain-of-function mutants were disrupted in the microRNA165/166 ability to target the START domain and cleave the *HD-Zip III* mRNA (Kim *et al.*, 2005; McConnell *et al.*, 2001; Emery *et al.*, 2003; Ochando *et al.*, 2006; Zhong and Ye, 2007; Zhou *et al.*, 2007). While miRNA166 is thought to target *PHB*, *PHV* and *REV*, overexpression of miRNA165 has been shown to downregulate all *HD-Zip III* genes (Zhou *et al.*, 2007). Another important aspect is that the expression of *HD-Zip III* family members, such as *PHB*, *PHV* and *REV* promotes the adaxial fate (McConnell and Barton, 1998; McConnell *et al.*, 2001; Emery *et al.*, 2003), therefore suggesting that adaxialization of tissues at some stages of development is attributed to a balance between *HD-Zip III* and *KANADI* genes that promote abaxialization of tissues (Eshed *et al.*, 2001; Kerstetter *et al.*, 2001; Eshed *et al.*, 2004).

The *HD-Zip III* expression patterns have been intensively studied in *Arabidopsis*, *Zinnia* and, more recently, in *Populus* with the characterization of *REV*, *AtHB15* and *AtHB8* *Populus* homologs *POPREVOLUTA*, *POPCORONA* and *PtrHB7*, respectively (Robischon *et al.*, 2011; Du *et al.*, 2011; Zhu *et al.*, 2013). The family members *AtHB8* and *AtHB15* are of particular interest for their roles in xylem differentiation. In *Arabidopsis* and *Zinnia*, *AtHB15/ZeHB13* is expressed in procambial cells and proposed to be a regulator of procambium formation and cambium cell identity maintenance thus preventing xylem differentiation (Ohashi-Ito and Fukuda 2003; Kim *et al.*, 2005) but in *Populus* *POPCORONA* is expressed in secondary xylem (Du *et al.*, 2011). Therefore, xylem formation in trees could implicate the HD-Zip III family with roles yet to uncover. *AtHB8* is found expressed in tracheary elements precursors and thought to promote xylem differentiation (Baima *et al.*, 2001; Ohashi-Ito *et al.*, 2005). The *Populus* *PtrHB7* has been proposed to promote cambial activity and secondary xylem differentiation (Zhu *et al.*,

2013). Due to the duplication of the *Populus* genome, a set of paralog genes has been identified for each *Arabidopsis* homolog (Ko *et al.*, 2006a). As a result, in the *Populus* genome the *AtHB15* homolog *POPCORONA* (also named *PtrHB5*) has a paralog gene - *PtrHB6*; and *AtHB8* homolog *PtrHB7* has also one paralog gene - *PtrHB8*. It is not currently understood whether the paralog pairs have conserved or redundant functions in xylem development.

Despite the unequivocal role HD-Zip III transcription factors have in xylem differentiation the knowledge on the downstream target genes is absent from the literature. We have previously presented data suggesting that the *AtHB8* *Populus* homolog, *PttHB8*, targets the thermospermine synthase encoding gene *POPACAULIS5*, by unveiling the presence of a feedback regulatory mechanism where increased levels of *POPACAULIS5* suppress *PttHB8* expression and increased *PttHB8* expression is accompanied by upregulation of *POPACAULIS5* (Chapter II). In the current work, we wanted to further elucidate the role of *HD-Zip III* genes in the transcriptional control of *POPACAULIS5* and whether this control is conserved in vascular plants. For that, we engaged on generating a set of tools to perform gene functional analysis making use of *Populus* and *Arabidopsis* model plants. Putative *HD-Zip III* *Populus* orthologs of *AtHB8* (*PtrHB8/PttHB8* and *PtrHB7/PttHB7*) and *AtHB15* (*PtrHB5/PttHB5* and *PtrHB6/PttHB6*) were cloned from hybrid aspen (*Populus tremula* × *Populus tremuloides*) and black cottonwood (*Populus trichocarpa*) and their expression levels were altered in *Populus* trees. The heterologous expression of either *PttHB8* or *PtrHB7* in *Arabidopsis* resulted in gain-of-function mutants with aberrant phenotypes exhibiting polarity defects, showing not only that both paralogs have conserved functions, but that new functions may be attributed to *HD-Zip III* genes in *Populus*. Heterologous expression of *POPACAULIS5*, *PttHB8* and *PtrHB7* in *Arabidopsis* plants followed by quantification of endogenous transcript levels of *ACL5* and *AtHB8* showed that *POPACAULIS5* feedback

represses *AtHB8* and that *PttHB8* induces *ACL5* expression, which supports the regulatory mechanism we previously found (Chapter II). Additionally, whole mount *in situ* hybridization located *POPACAULIS5* mRNA in the vasculature of *Populus* stems. Taken together, the results support that HD-Zip III PtrHB8/PtrHB7 control thermospermine levels by transcriptional regulation of *POPACAULIS5* expression in *Populus* and that this mechanism of maintenance of thermospermine homeostasis is conserved in *Arabidopsis* and *Populus* plants.

Experimental procedures

Plant material, growth conditions and sampling

Hybrid aspen (*Populus tremula* L $\times$ *P. tremuloides* MICHX.; clone T89) was subcultured every five-weeks on MS basal salt medium at half-strength (Murashige and Skoog, 1962). *In vitro* hybrid aspen plants were grown in growth chambers at 21°C and 16 h light/8 h dark photoperiod. Transgenic and wild-type hybrid aspen plants were transferred to soil and trees grown for 2 months in the greenhouse at 21°C and 16 h light/8 h dark photoperiod. *Populus trichocarpa* Nisqually-1 clone was maintained in the greenhouse. *Arabidopsis thaliana* (ecotype Columbia (Col-0)) were grown in growth chambers at 22°C, 70% humidity and 16/8h light/dark cycle. Seeds were surface-sterilized in a 35% commercial bleach solution and rinsed four times in sterile distilled water, sown and stratified for 72h at 4°C in the dark and germinated.

Sampled tissues were immediately flash-frozen in liquid nitrogen and stored at -80°C. Hybrid aspen leaves, stem between the third and the seventh internode from the top (IN3-IN7), stem between the eighth and the basal internode (IN8-basal) and root apices from five week-old *in vitro* grown

plants were pooled from six to ten individual plants, ground to powder and used in gene expression analysis. For RNAi::*POPACAULIS5*-L149 and RNAi::*PttHB8*-L146 and L147 lines three pools of six to ten plants were ground to powder and portioned for gene expression and polyamine quantification analyses. *Arabidopsis* rosette leaves, cauline leaves, inflorescence stems and flowers were sampled in pools of six plants (or as specified), ground to powder and used for gene expression analysis.

Sequence analysis

To identify *P. trichocarpa* (*Ptr*) and *P. tremula* × *P. tremuloides* (*Ptt*) putative *HD-Zip III* coding regions we carried BLAST/browse searches in different databases [JGI *Populus trichocarpa* v.1.1 (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html; Tuskan *et al.*, 2006), Phytozome *Populus* v.2 (Goodstein *et al.*, 2012), and *Populus* DB (<http://www.populus.db.umu.se/>; Sterky *et al.*, 2004)] using the *Arabidopsis* (*AtHB15/CNA*, AT1G52150; *AtHB8*, AT4G32880; *REV/IFL*, AT5G60690; *PHV/AtHB9*, AT1G30490; *PHB/AtHB14*, AT2G34710) sequences as query. Alignment of the genomic regions of *Populus* and the coding sequences of *Arabidopsis*, allowed inferring on exon and intronic regions of *Populus* sequences. Predicted aminoacid sequence alignments were performed using ClustalX (Larkin *et al.*, 2007) or MUSCLE (Edgar, 2004; <http://www.ebi.ac.uk/>).

For the phylogenetic analysis, putative *AtHB15* homologs *PtrHB5/POPCORONA* (also named Pt-ATHB.12; Joint Genome Institute (JGI) *Populus* v.1.1 gene model fgenes4_pm.C_LG_I000560; Phytozome *Populus* v2.0 gene model POPTR_0001s18930; Potri.001G188800) and *PtrHB6* (also named Pt-ATHB.11; JGI *Populus* v1.1 gene model estExt_fgenes4_pg.C_LG_III0436; Phytozome *Populus* v2.0 gene model POPTR_0003s04860; Potri.003G050100); putative *AtHB8* homologs *PtrHB8*

(Pt-HB1.6; JGI Populus v1.1 gene model estExt_fgenes4_pm.C_LG_VI0713; Phytozome Populus v2.0 gene model POPTR_0006s25390; Potri.006G237500) and *PtrHB7* (Pt-HB1.5; JGI Populus v1.1 gene model fgenes4_pg.C_LG_XVIII000250; Phytozome Populus v2.0 gene model POPTR_0018s08110; Potri.018G045100); putative *REV/IFL* homologs *PtrHB1* (also named Pt-HB1.7; JGI Populus v1.1 estExt_Genewise1_v1.C_660759; Phytozome Populus v2.0 gene model POPTR_0004s22090; Potri.004G211300) and *PtrHB2* (also named Pt-HB1.8; JGI Populus v1.1 gene model gw1.IX.4748.1; Phytozome Populus v2.0 gene model POPTR_0009s01990; Potri.009G014500); putative *PHV/AtHB9* homolog *PtrHB3* (also named Pt-PHB.1; JGI Populus v1.1 gene model estExt_fgenes4_pg.C_2360002; Phytozome Populus v2.0 gene model POPTR_0011s10070; Potri.011G098300); and putative *PHB/AtHB14* homolog *PtrHB4* (also named Pt-PHB.2; JGI Populus v1.1 gene model estExt_fgenes4_pg.C_LG_I2905; Phytozome Populus v2.0 gene model POPTR_0001s38120; Potri.001G372300) predicted amino acid sequences were used together with predicted sequences from genomes of *Arabidopsis thaliana*, *Zinnia elegans* and *Pinus taeda*. Evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). Phylogenetic analyses were conducted in MUSCLE and MEGA4 (Edgar, 2004; Tamura *et al.*, 2007).

Identification of TF-binding sites in POPACAULIS5 gene promoter

PlantPAN database was used to identify *cis*-elements related to HD-Zip III transcription factors in *POPACAULIS5* putative promoter region, ranging from -1 to -3487 bp upstream of the translation starting site (<http://plantpan.mbc.nctu.edu.tw/>; Chang *et al.*, 2008). The footprintDB was scanned with *PtrHB8* (POPTR_0006s25390) and *PtrHB7* (POPTR_0018s08110) protein sequences to identify DNA-binding proteins

that bind to similar DNA motifs and to recognize the amino acids residues that interact with DNA (<http://floresta.eead.csic.es/>, Contreras-Moreira, 2010). The identified *cis*-elements in the putative promoter regions of *POPACAULIS5* (POPTR_0006s23880), *ACL5* (AT5G19530) and *SPDS1* (AT1G23820) were compared for comprehensive analysis of thermospermine synthase encoding gene characteristic DNA binding motifs.

Isolation of Populus HB5, HB6, HB7 and HB8 coding regions

One µg of total RNA, extracted with RNeasy Plant Mini Kit (Qiagen) from shoot apices of hybrid aspen and black cottonwood were used for cDNA synthesis using 1st Strand cDNA synthesis kit for RT-PCR (Roche Applied Science) and oligo-dT, following the manufacturer's instructions. Amplification of the full length *PttHB5*, *PtrHB5*, *PttHB6*, *PtrHB6*, *PttHB7*, *PtrHB7*, *PttHB8* and *PtrHB8* sequences from cDNA was performed using the primers described in Table I. All sequences were cloned through TA ligation into the pCR2.1 vector (Invitrogen). For PCR amplifications we used HF Phusion High Fidelity DNA polymerase (Finnzymes) and orientation and sequence identity was confirmed by sequencing.

Site-directed mutagenesis of HD-Zip III miRNA 165/166 binding site

Prior to preparing *HD-Zip III* genes overexpression constructs, a site-directed mutagenesis approach was followed as described in Chapter II *Experimental Procedures* section. Briefly, because the microRNA binding site is located in the START domain of the protein, we designed primers that have two T and G nucleotides substituted by A nucleotides, maintaining the encoded aminoacids but blocking the miRNA from cleaving the transcript. The mutated primers for *PtrHB5/PttHB5* and *PtrHB6/PttHB6* fragments were 5'-CTGGAATGAAGCCTGGACCAGATTCC-3' and 5'-GCATTTGGAC-

-CCACTCTACAGCAGTTCCAGT-3'. For *PtrHB8/PttHB8* the primers used are described in Chapter II. For *PtrHB7/PttHB7* the primers used were 5'-CTGGGATGAAGCCTGGACCAGATTCCATTGG-3' and 5'-GCATT-TGGACCCACTCGACGGCAGTTCCTGT-3'. The mutated PCR product was then re-circularized by ligation with T4 Quick DNA ligase (Biolabs, UK). The point synonymous mutations were confirmed by sequencing.

Table I. Oligonucleotide sequences used for isolation of *HD Zip III* putative homologs from *P. trichocarpa* and *P. tremula* × *P. tremuloides*. The upstream and downstream regions to the start and stop translation sites included in the isolated sequences are indicated, as well as the full-length sizes.

Gene	Primer sequence (5' - 3')	5'-UTR (bp)	3'-UTR (bp)	Amplicon size (bp)
<i>HB5</i>	<i>F</i> AAGTTTGGATCGGCAATACG	669	139	3364
	<i>R</i> TCTGTATICTAAACTTCATTGT			
<i>HB6</i>	<i>F</i> TCTATGATTAAGGGAGGTTACG	234	247 ^(a) /486 ^(b)	2995 ^(a) /3234 ^(b)
	<i>R</i> TAAGTCCTCACCTGGGTCTATGTTG			
<i>HB8</i>	<i>F</i> ATCTCTAATCCGATCTACGCCAGG	225 ^(a) /92 ^(b)	330	3042 ^(a) /2909 ^(b)
	<i>R</i> GCTCCCAAAGGTTTTTAGGC			
<i>HB7</i>	<i>F</i> GAAGTTTCGCCAAACGGTAA	205	56	2733
	<i>R</i> CAGTTTCAGTTTGTTCTAATCTG			

^(a) *P. trichocarpa* (*Ptr*); ^(b) *P. tremula* × *P. tremuloides* (*Ptt*); *F*, forward; *R*, reverse.

Construction of expression plasmids

To construct the overexpression clones, the mutated *HD-Zip III* full-length cDNA in pCR2.1 vector were used as template for PCR amplification with primers bearing Gateway adapters and the amplified fragments were recombined into pDONR221 vector and then recombined with Gateway vector pK7GW2.0. For the silencing constructs, recognition sites of

recombination were introduced by PCR, cloned into the pDONR221 vector with Gateway BP recombinase and then LR recombined with Gateway vector pK7GWIWG2(I) for hairpin silencing expression (Karimi *et al.*, 2002; Gateway Technology, Invitrogen). Primers used are described in Table II. BP and LR recombination reactions followed the manufacturer's instructions. Fragments were sequenced after the first cloning step into pDONR221 vector.

Table II. Oligonucleotide sequences used in the Gateway cloning procedures. The size of the cloned fragments is indicated. The underlined nucleotides correspond to the adapter sequences and the remaining is specific to the target DNA.

Gene		Primer sequence (5'- 3')	Size (bp)
Overexpression			
HB5	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC <u>TGTTGCGACTTTGGTGGATTT</u>	2983
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCACAATGAAGTTTAGAATACAGA	
HB6	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC <u>TCTATGATTAAGGGAGGTTACG</u>	3056
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCAACATAGACCCAGGTGAGGACTTA	
HB8	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATCTCTAATCCGATCTACGCCAGG	2836
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCGAAAGACAGTGAAGGAG	
HB7	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAAGTTTCGCCAAACGGTAA	2671
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCGTTTCAGTTTGTCTAATCTG	
ACL5	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGTACTGAGGCAGTTGAG	1100
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCAATTTTGTAGCCACCCCATG	
Silencing			
HB5	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC <u>TGTTGCGACTTTGGTGGATTT</u>	265
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAATAGGATCTTACCATCCTT	
HB6	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC <u>TCTATGATTAAGGGAGGTTACG</u>	280
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCATTGTCATGATAGGCTG	
HB8	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATCTCTAATCCGATCTACGCCAGG	315 ^(a)
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCATAGAGCTTGGCTT	182 ^(b)
HB7	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAAGTTTCGCCAAACGGTAA	299
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCGACGCGTAGAAGTTGGTTT	
ACL5	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGTACTGAGGCAGTTGAG	369
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTCAGAGCAGAGCAGGTTGAAT	

^(a) *P. trichocarpa* (Ptr); ^(b) *P. tremula* × *P. tremuloides* (Ptt); F, forward; R, reverse.

Hybrid aspen transformation

Expression clones were introduced into *Agrobacterium tumefaciens* strain GV3101pMP90 (Koncz and Schell, 1986). Hybrid aspen was transformed as described in Chapter II (following Nilsson *et al.*, 1992). For confirming insertions, PCR was performed using the primers described in Table II and also: CaMV 35S promoter forward, 5'-CTCATCAAGACGATCTACC-CGAG-3' and reverse, 5'-TGGGCAATGGAATCCGAGGAGGT-3'; *NPTII* forward 5'-GAATCGGGAGCGGCGATACCGTAAA-3', and reverse, 5'-CAAGATGGATTACACGCAGGTTCTC-3'; and *virBG* forward 5'-GCGGTGAGACAATAGGCG-3', and reverse 5'-GAACTGCTTGCTG-TCGGC-3' for false positives screening. After shoot elongation, plants were transferred to half-strength MS medium for rooting.

Arabidopsis plant transformation

Expression clones bearing *POPACAULIS5* and the miRNA-misregulated forms *PtrHB7* and *PttHB8* were introduced into *Agrobacterium tumefaciens* strain LBA4404 (Koncz and Schell, 1986). At the flowering stage, *Arabidopsis* plants were transformed for heterologous expression of the *Populus* genes by the floral-dip method (Clough and Bent, 1998). T0 seeds were surface-sterilized in a 35% commercial bleach solution and rinsed four times in sterile distilled water, stratified for 72h at 4°C in the dark and screened on MS plates containing 1% sucrose, 50 mg.l⁻¹ kanamycin monosulphate (Duchefa) and 150 mg.l⁻¹ cefotaxime (Duchefa). Three resistant 35S::*PtrHB7*-miRNA_Δ and three resistant 35S::*PttHB8*-miRNA_Δ seedlings were transplanted to soil and grown in growth chambers. Genomic DNA was isolated to confirm insertions by PCR analysis as described in the above section.

Forty day-old *Arabidopsis* plants resulting from the transformation

with 35S::*POPACAULIS5* were left to dry and seeds were collected to generate the T1 progeny. T0 seeds were grown in selection medium and the resistant plants were planted in soil for further T1 seed collection and selection. The resultant progenies were screened for homozygosity of the transgenes by antibiotic resistance, until the T3 generation. 35S::*POPACAULIS5 Arabidopsis* plants were analysed at T3 for gene expression.

Quantitative real-time RT-qPCR

Total RNA was extracted from 100 mg of frozen powdered tissues from *Populus in vitro* grown material and from *Arabidopsis* two-month old plant tissues with RNeasy Plant Mini kit (Qiagen) as described in the sampling section. cDNA synthesis was performed on 1 µg of DNase-treated total RNA using Transcriptor HF cDNA synthesis kit with oligo-dT primers (Roche Applied Science). RT-qPCR was performed in LightCycler 480 PCR system with LightCycler480 SYBR Green I Master (Roche Applied Science), to monitor double stranded DNA products. Specific primer pairs were designed to generate amplicons of *AtHB8* (AT4G32880), *AtACL5* (AT5G19530), *PttHB8* (POPTR_0006s25390), *PttHB6* (POPTR_0003s04860) and *POPACAULIS5* (POPTR_0006s23880) used in detection. *Pt1* (POPTR_0002s12910) or *CYP2* (POPTR_0009s13270) were used as reference genes for *Populus* samples (Czechowski *et al.*, 2005; Gutierrez *et al.*, 2008) and *EIF4α* (AT3G13920) for *Arabidopsis* samples. The amount of target transcripts was normalized by the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) using wild-type as the calibrator sample in each tissue and experimental condition and relative to *Pt1*, *CYP2* or *EIF4α* reference genes. For all experiments, the mean of triplicate qPCR reactions was determined and at least three biological replicates or pooled biological samples were used as specified. The quantifications were repeated twice. Primers used are

described in Table III.

Table III. Oligonucleotide sequences used in RT-qPCR analysis.

Gene		Primer sequence (5'- 3')
<i>POPACAULIS5</i>	F	AAGATGCAGAGTGCCGAAGT
	R	GACTTGTGCTTGAGGGCTTC
<i>PttHB8</i>	F	ATCTCTAATCCGATCTACGCCAGG
	R	CGCATAGAGCTTGGCTTAGG
<i>AtACL5</i>	F	ACCGTTAACCAGCGATGCTTT
	R	CCGTAACTCTCTCTTTGATTCTTCGATCC
<i>AtHB8</i>	F	TAGAGAATGAAGAGTTTATGGAAGT
	R	ACATCTATAAGAGTGAGAGATCCAG
<i>PttHB6</i>	F	TCTATGATTAAGGGAGGTTACG
	R	CATCCTTGCAGGACATTTCCAT
<i>Pt1</i>	F	GCGGAAAGAAAACTGCAAG
	R	TGACAGCACAGCCCAATAAG
<i>CYP2</i>	F	TAAGACCGAATGGCTTGACG
	R	AGAACGCACCCCAAACTACTA
<i>EIF4</i>	F	TCATAGATCTGGTCCTTAAACC
	R	GGCAGTCTCTTCGTGCTGAC

F, forward; R, reverse.

mRNA in situ hybridization

Localization of the *POPACAULIS5* mRNA was determined by *in situ* hybridization, using 10 µm sections of hybrid aspen stems embedded in paraplast as described by Harding *et al.* (2003). Briefly, antisense and sense riboprobes were generated from the complete cDNA of *POPACAULIS5* subcloned into pCRII vector (Invitrogen), using SP6 and T7 RNA polymerases, respectively. Sequence orientation was confirmed by sequencing. Stems of five week-old hybrid aspen plants were collected and

fixed in 4% para-formaldehyde, with overnight vacuum-infiltration at 4°C. The tissues were washed twice with 20% ethanol at 4°C for 5 min, and subjected to a graded ethanol/tert-butanol/85%NaCl solution series for 2 h each. The gradient consisted of 40/0/60, 45/15/40, 50/28/22, 45/55/0, 25/75/0, 0/100/0 (twice). The first three infiltration steps were performed at 4°C, whereas the final steps were conducted at room temperature. Dehydrated tissues were infiltrated with paraplast. Sectioning was performed with a RM2155 microtome (Leica Microsystems), and sections placed in polysine-treated surface glass slides.

Sections were re-hydrated and pre-treated in $10\mu\text{g.mL}^{-1}$ Protease K (Sigma-Aldrich) in PBS for 10 min, fixed with 4% paraformaldehyde (in phosphate-buffered saline, PBS pH 7.0) for 10 min and acetylated for non-specific blocking with 0.5% acetic anhydride in 0.1M triethanolamine for 5 min. Sections were hybridized with equal concentrations ($0,75\text{ ng.}\mu\text{L}^{-1}$) of either sense or antisense RNA probes in hybridization solution [20x SSPE, 10% blocking reagent (Roche), 20% SDS, 1 mg.mL^{-1} tRNA (Roche Applied Science), RNA inhibitor (Roche)] in 50% deionized formamide, covered with an RNase-free coverslip and incubated at 52°C overnight in a humid chamber. The sections were subjected to a series of washes for post-hybridization washing and blocking (two changes of 50% formamide/2× SSPE for 30 min at 52°C; 2× maleic acid buffer (Sigma-Aldrich) 15 min at room temperature; 0.5x maleic acid buffer for 1 h at 65°C; 1× maleic acid, 1% blocking reagent, 0.3% Tween-20 for 1 h at room temperature; 1× maleic acid, 0,1% blocking reagent for 10min at room temperature). For immunodetection of the DIG probes the sections were incubated with 1:750 dilution of alkaline phosphatase conjugated anti-digoxigenin antibody (Roche Applied Science) in 1× maleic acid buffer containing 0,1% blocking reagent for 2h at room temperature. Sections were washed in 1× maleic acid buffer containing 1% blocking reagent for 15 min and then re-washed for 1 h. Afterwards, sections were washed twice with 1× maleic acid buffer

containing 0,1% blocking reagent for 1h and with AP buffer (100 mM Tris pH 9.5, 150 mM NaCl, 50 mM MgCl₂) for 15 min. Colour-development buffer (AP buffer containing PVA, NBT and BCIP) was placed covering the sections and colour development monitored over the course of several hours. After color development, the reaction was stopped by rinsing in 10mM EDTA pH8.0. Sections were observed with an Eclipse TE300 light microscope (Nikon Instruments).

Polyamine quantification

Polyamines were extracted from about 100 mg of frozen tissues collected as described in the sampling section and purified (Rambla *et al.*, 2010), derivatized (Fernandes and Ferreira, 2000), identified and quantitated as described by Rambla *et al.* (2010).

Growth parameters

Plant height, distance between third and seventh internodes (IN3-IN7), internode length, total internode number and number of main roots were measured from five week-old *in vitro* grown and greenhouse plants. Greenhouse grown trees were also additionally measured for expanded leaf dimensions.

Statistical analysis

Non parametric Mann-Whitney *U* test was employed to assess significant differences in gene expression, polyamine content and growth parameters. A significance level of $\alpha=0.05$ was considered. Statistics were performed using software Statistica (Statsoft) after Zar (1998).

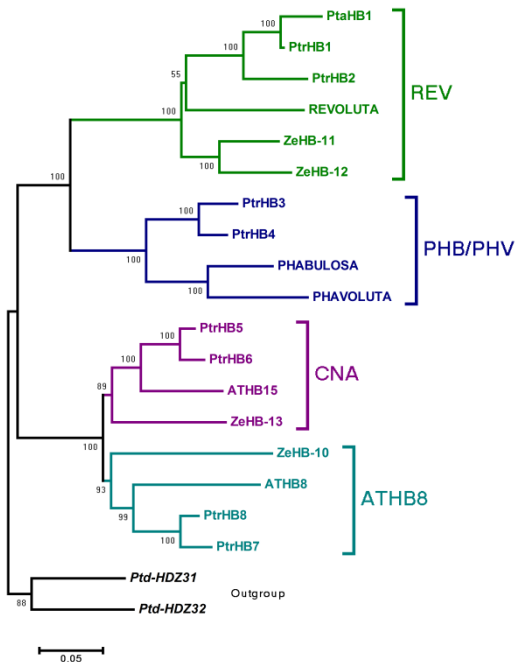
Results

Protein and phylogenetic analysis of Populus HD-Zip III transcription factors

In *Populus trichocarpa* genome eight putative orthologs of HD-Zip III proteins have been identified (Ko *et al.*, 2006a; Côté *et al.*, 2010; Hu *et al.*, 2012). *Arabidopsis* and *Populus* diverged about 100-120 million years ago and the duplication events of the *Populus* genome explain the presence of duplicated sequences (Tuskan *et al.*, 2006). Recent work on HD-Zip III family members POPREVOLUTA (ortholog of REV), POPCORONA (ortholog of ATHB15/CNA) and PtrHB7 (ortholog of ATHB8) in *Populus* has been reported (Hertzberg and Olsson, 1998; Ko *et al.*, 2006a; Robischon *et al.*, 2011; Du *et al.*, 2011; Zhu *et al.*, 2013). To evaluate the evolutionary relationships within the HD-Zip III gene family, we performed a combined phylogenetic analysis of *Arabidopsis*, *Populus*, *Zinnia* and *Pinus* protein sequences (Figure 1). Three clusters of orthologous groups were identified and represented in the phylogenetic tree as putative paralogs pairs PtrHB1/PtrHB2 (orthologs of REV), PtrHB3/PtrHB4 (putative orthologs of PHV/PHB), PtrHB5/PtrHB6 (orthologs of ATHB15/CNA) and PtrHB7/PtrHB8 (putative orthologs of ATHB8). Gymnosperm *Pinus taeda* PtdHDZ31 and PtdHDZ32 do not form a cluster of orthologous groups of proteins and were therefore chosen to represent the outgroup. In agreement with previous reports (Prigge and Clark, 2006; Du *et al.*, 2011) we have found AtHB15/CNA protein to have higher similarity to *Populus* proteins PtrHB5 and PtrHB6; whereas AtHB8 shows higher identity to PtrHB7 and PtrHB8, which are also more closely related with each other than the remaining HD-Zip III proteins. Presently, it is not clear if the paralogs pairs have evolved different functions. Multiple sequence alignment of *Populus* putative HD-Zip III orthologs was performed to identify conserved domains.

High conservation of functional domains and motifs was found amongst these proteins (Figure 2), and several motifs between the START and MEKHLA domains are also greatly conserved suggesting the existence of additional novel functional domains (Prigge & Clark, 2006).

Figure 1. Evolutionary relationships of Class III HD-Zip protein sequences. Phylogenetic analysis of putative *Populus trichocarpa* HD-Zip III family, based on the protein sequences identified from *Populus trichocarpa* PtrHB1 (AY919616), PtrHB2 (AY919617), PtrHB3 (AY919618), PtrHB4 (AY919619), PtrHB5 (AY919620), PtrHB6 (AY919621), PtrHB7 (AY919622), PtrHB8 (AY919623); *Arabidopsis thaliana* AtHB15 (AJ439449), AtHB8 (NP_195014), REVOLUTA (AAF42938), PHAVOLUTA (NP_174337), PHABULOSA (AAC 16263); *Populus alba* x *P. tremula*



PtaHB1 (AY497772); *Zinnia elegans* ZeHB-10 (AB084380), ZeHB-11 (AB084381), ZeHB-12 (AB084382), ZeHB-13 (AB109562) and *Pinus taeda* PtdHDZ31 (DQ657210), PtdHDZ32 (DQ6557211) as outgroup to root the tree. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the proteins analyzed (Felsenstein, 1985). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 767 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

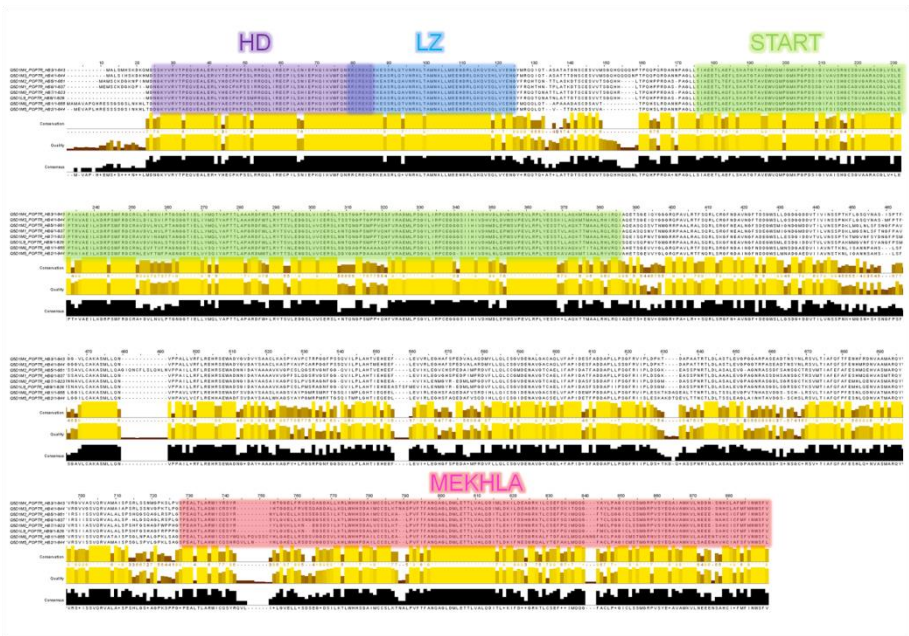


Figure 2. Schematic representation of HD-Zip III family and multiple sequence alignment of putative HD-Zip III protein sequences from *P. trichocarpa*, using MUSCLE software (Edgar, 2004). The most conserved blocks of sequences amongst the proteins of this family are: homeodomain (HD), leucine-zipper motif (LZ), START-domain and MEKHLA domain.

Identification of putative cis-elements in POPACAULIS5 promoter

To determine whether HD-Zip III PtrHB8 is a good candidate in binding to *POPACAULIS5* promoter as suggested in Chapter II, we used the PlantPAN database (Chang *et al.*, 2008) in search of known motifs already reported to be bound by TFs and present in the Jaspar, Transfac and Place databases (Matys *et al.*, 2003; Sandelin *et al.*, 2004; Higo *et al.*, 1999). To identify *cis*-elements related to DNA binding homeobox domain (HD) we extracted the first 3487 nucleotides upstream of the translation initiation codon. Several HD *cis*-elements were identified, amongst them, the binding site for the *Arabidopsis* Homeobox1 (ATHB1, HAT5), Homeobox5 (ATHB5) and Homeobox9 (ATHB9, PHV) TFs (Figure 3). Within the target promoter region five ATHB-9 sequences with the consensus sequence

NNNGTAATGATTRCNYBS were found. ATHB9 *cis*-element is described as present in the promoter sequences of 54 *Arabidopsis* genes (AthaMap, www.athamap.de/, Steffens *et al.*, 2004; Bülow *et al.*, 2010). We then interrogated FootprintDB database to identify HD-Zip III AtHB8 and

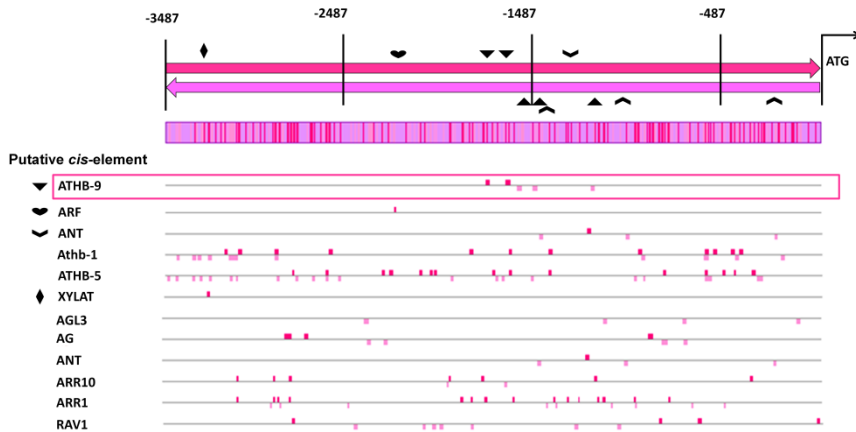


Figure 3. Putative *cis*-elements present in the *POPACAULIS5* gene promoter. The promoter region -3487 bp to -1 bp upstream of the translation starting site (ATG) was scanned at the PlantPAN database (Chang *et al.*, 2008). Highlighted in pink is the putative HD-Zip III *cis*-element.

PtrHB8/PtrHB7 protein binding interface signatures to DNA (Figure 4a). The search detected high homology to AtHB9 protein signature at the level of the homeobox domain, suggesting that AtHB8 and PtrHB8/PtrHB7 recognize similar DNA motifs (Figure 4b; Contreras-Moreira, 2010).

In addition, we found ARF *cis*-element that are known to be enriched in the 5' flanking region of genes upregulated by IAA and brassinosteroids (auxin response factor; consensus sequence, TGTCTC; Ulmasov *et al.*, 1999; Goda *et al.*, 2004); ARR1 and ARR10 (cytokinin activating *Arabidopsis* response regulators; consensus sequence, NGATT; Sakai *et al.*, 2000; Taniguchi *et al.*, 2007; Yokoyama *et al.*, 2007); RAV1 (AP2 domain interacting factor; consensus sequence NNGCAACAKAWN, Kagaya *et al.*, 1999); ANT, a positive regulator of meristematic activity and highly present



Figure 4. Analysis of the PtrHB8/PtrHB7 homeobox domain. (a) Alignment of PtrHB8 and PtrHB7 with the AtHB9 protein sequence, obtained by querying the footprintDB database, to detect the amino acid residues involved in DNA binding interface. The arrows depict conserved amino acid residues that are identical in the protein sequences, and the numbers represent the position of the residues in the protein sequence. (b) AtHB9 consensus DNA binding motif.

in vascular cambium (AINTEGUMENTA, AP2-domain transcription factor; consensus sequence CACANWTCCRAKG; Mizukami and Fischer, 2000; Zhao *et al.*, 2005) and XYLAT, a *cis*-element identified among the promoters of the core xylem gene set (consensus sequence ACAAAGAA; Ko *et al.*, 2006b); all were present in the target promoter region of *POPACAULIS5* (Figure 3). Furthermore, comparing the putative promoter regions of *ACL5*, *POPACAULIS5* and *SPDS1* (*Arabidopsis* spermidine synthase, AT1G23820) we found ARF, ANT and XYLAT motifs were shared between the thermospermine synthase gene promoter regions of *Arabidopsis* and *Populus* but were not found in the spermidine synthase counterpart. This suggests that thermospermine transcriptional regulatory mechanisms involve additional factors from those of other polyamines.

PtrHB7/PttHB7 and PtrHB8/PttHB8 share conserved functions

To study the functions of PtrHB7/PttHB7 and PtrHB8/PttHB8 we

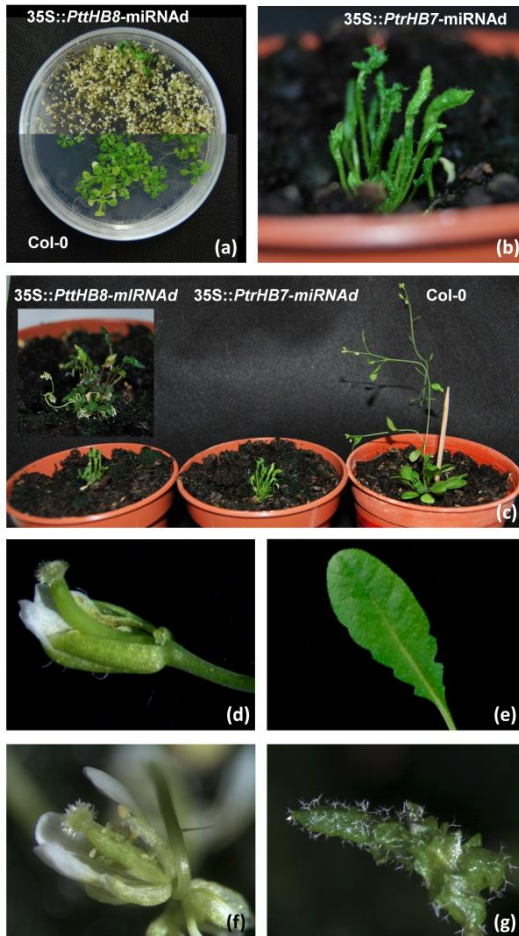


Figure 5. Heterologous over-expression of *PttHB8* and *PtrHB7* effects on *Arabidopsis* development. (a) Early stage of *35S::PttHB8-miRNA* seedlings development showing curled cotyledonary leaves. (b) Strong defective phenotype of three week-old *35S::PtrHB7-miRNA* *Arabidopsis* plants with curled leaves. (c) Three week-old *35S::PtrHB7-miRNA* and *35S::PttHB8-miRNA* *Arabidopsis* plants. Inset: two month old *35S::PttHB8-miRNA* presented severe defects on lateral organ polarity, bent inflorescence stems, and flower defects. (d) wild-type (*Col-0*) flower and (e) leaf. (f) *35S::PttHB8-miRNA* flower with ectopic ovules and (g) curled leaves.

transformed *Arabidopsis* *Col-0* for the heterologous ectopic expression of the *Populus* genes. Due to the posttranscriptional regulation of Class III HD-Zip transcription factors by microRNAs (miRNA166/165) their site of recognition in the START domain was synonymously mutated, to block the hypothetical microRNA downregulation and obtain the desired overexpression *in planta*. We obtained three miRNA resistant *35S::PtrHB7-miRNA* transgenic *Arabidopsis* T0 seedlings and three miRNA resistant *35S::PttHB8-miRNA*. Early in the selection plates we observed cotyledonary leaves were severely curled and the frequency of viable gain-of-function mutants was reduced (Figure 5a). Three weeks after transplanting

the T1 plants, we observed gross morphological aberrations in all lateral organs and delayed bolting. We observed the abnormal development of leaves, that were narrow and dark green, the loss of leaf polarity, but also the absence of a dominant inflorescence stem as typical in wild-type Col-0 plants (Figure 5b-e). Two month-old plants displayed abnormal fruit development, with ectopic ovules as well as normal aspect ovules inside the carpel. These results are suggestive of polarity defects in the carpel, since adaxial (inside) tissues such as septum tissue and placentae-bearing ovules are duplicated in abaxial (outside) position (Figure 6a). The defects of petiole structure at the base of the flowers resulted in bent inflorescence stems, where several floral structures emerged from the inflorescence stem with poor supporting structure (Figure 5d,f). Leaves displayed complete loss of polarity, with curled adaxial side over the atypical abaxial side that displays increased density of trichomes and loss of abaxial identity (Figure 6b). No T2 seeds were viable when progeny was sown. Both transformations rendered plants with this abnormal phenotype that indicate a conserved function for *PtrHB7* and *PtrHB8* paralogs in organ polarity.

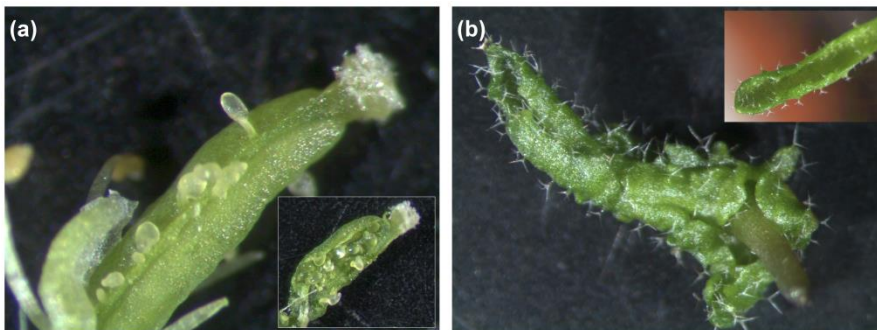


Figure 6. Heterologous overexpression of *PttHB8* and *PtrHB7* polarity defects of reproductive and vegetative organs of *Arabidopsis*. (a) Carpel with ectopic ovules. Inset: presence of ovules inside the carpel. (b) Curled-down leaf showing loss of polarity. Inset: adaxialization of abaxial tissue, with presence of trichomes both on the adaxial and abaxial sides.

PttHB8* heterologous overexpression increases *ACL5* expression in *Arabidopsis

To analyse *PttHB8* gain-of-function mutants we investigated the gene expression profile in the heterologous system. The expression of *PttHB8* in the T1 *Arabidopsis* 35S::*PttHB8*-miRNA_{Ad} plants was analysed by RT-qPCR, using the tissues of one representative line showing the severe defects above described. Overexpression of *PttHB8* in leaf and stem tissues was detected (Figure 7a) and confirmed our primers were specific and were not amplifying the endogenous *AtHB8* gene. Previous work suggested *PttHB8* has regulatory functions in *POPACALIS5* expression (Chapter II). We wanted to further elucidate if *PttHB8* increased expression in the 35S::*PttHB8*-miRNA_{Ad} plants

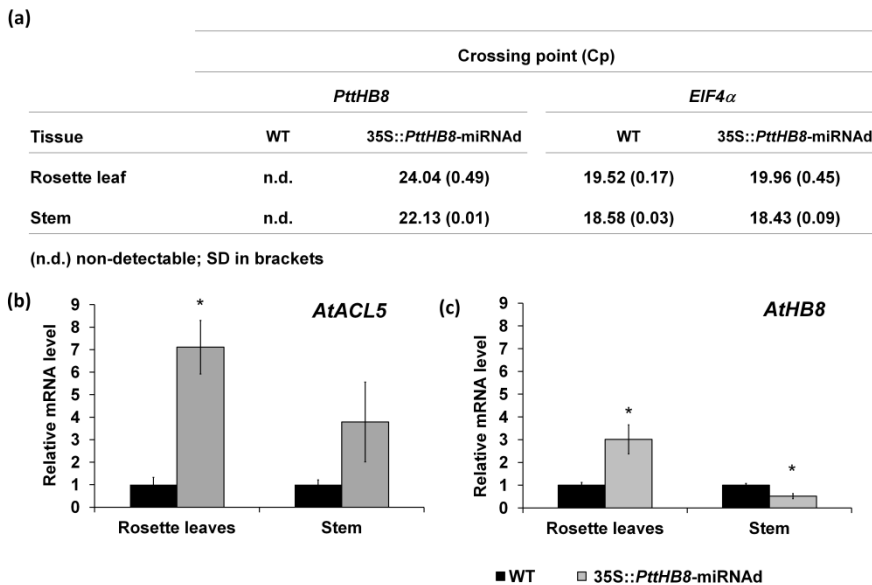


Figure 7. Heterologous expression of *PttHB8* in *Arabidopsis*. (a) *PttHB8* raw expression and relative expression of (b) *ACL5* and (c) *AtHB8* in 35S::*PttHB8*-miRNA_{Ad} and wild-type *Arabidopsis* plants. Values are means $\pm$ SD of three technical replicates from a representative line with strong defective phenotype. Crossing points (Cp) for *PttHB8* and reference *EIF4 α* transcripts are indicated. Asterisks indicate significant differences from the wild-type ($p < 0.05$, Mann-Whitney *U* test). The transcript quantification was performed in duplicate.

led to an increase in *Arabidopsis* *ACL5* transcript accumulation. Indeed, we could detect the increased *Arabidopsis* endogenous *ACL5* expression in response to increased *PttHB8* expression in 35S::*PttHB8*-miRNA plants (Figure 7b) confirming the positive transcriptional regulation that HB8 has on *ACL5* expression. Furthermore, we observed that the endogenous *AtHB8* transcript levels are reduced in stems of these transgenic plants but not in leaf tissues (Figure 7c). These results further sustain our previous studies suggesting *AtHB8* to have a regulatory role in *ACL5* expression and *ACL5* feedback regulation of *AtHB8*.

On the other hand, heterologous overexpression of *POPACAULIS5* in *Arabidopsis* produced slightly shorter plants than Col-0 wild-type (Figure 8a). Expression of *POPACAULIS5* was detected in all tissues tested in the transgenic plants (Figure 8b) and confirmed our primers were specific and

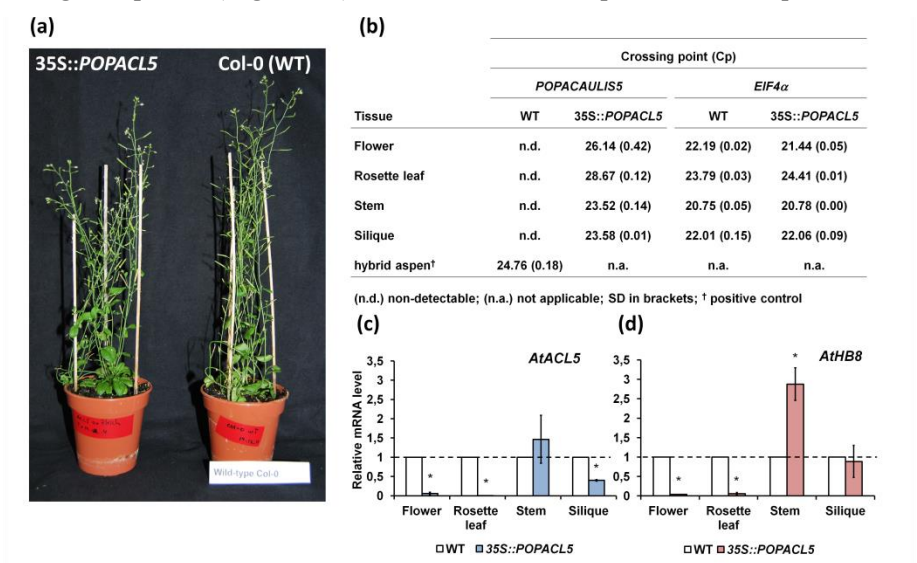


Figure 8. Heterologous expression of *POPACAULIS5* in *Arabidopsis*. (a) 35S::*POPACAULIS5* and wild-type *Arabidopsis* plants. *POPACAULIS5* raw expression and relative expression of (b) *ACL5* and (c) *AtHB8* in 35S::*POPACAULIS5* and wild-type *Arabidopsis* tissues. Values are means $\pm$ SD of three biological replicates (sampled as three pools of three to six individual plants) and three technical replicates. Crossing points (Cp) for *POPACAULIS5* and reference *EIF4 α* transcripts are indicated. Asterisks indicate significant differences from the wild-type ($p < 0.05$, Mann-Whitney *U* test). The transcript quantification was performed twice.

not amplifying the endogenous *ACL5* gene. Ectopic expression of *POPACAULIS5* in *Arabidopsis* was accompanied of suppressed *AtHB8* expression, therefore we hypothesize it consequently led to downregulation of the endogenous *ACL5* transcript levels in most tissues tested, with the exception of stem tissues (Figure 8c,d). A positive correlation between *AtHB8* and *ACL5* was observed in all tissues tested. These results suggest that expressing *POPACAULIS5* in *Arabidopsis* may somehow have a repressive feedback effect on the endogenous *AtHB8* gene resulting in the downregulation of the endogenous *ACL5* expression.

To further understand *PttHB8* involvement in *POPACAULIS5* regulation we silenced *PttHB8* in *Populus* and obtained two RNAi::*PttHB8* transgenic lines of hybrid aspen, L146 and L147 (Figure 9a-d), showing a decrease of *PttHB8* expression levels (Figure 9a). Decreased *POPACAULIS5* expression in stems of L146 and a slight increase in L147 was observed when compared to the wild-type *in vitro* grown plants, suggesting the lack of *POPACAULIS5* upregulation in RNAi::*PttHB8*-L146 (Figure 9b). It would be expected that in the RNAi::*PttHB8* lines less *POPACAULIS5* transcripts would be present, but this is not strikingly evident for both lines. When we quantified polyamines in the tissues RNAi::*PttHB8*, contrary to expected we observed less thermospermine was produced in L147 tissues (Figure 9c). Given that *PttHB8* and *PttHB7* have conserved functions, we can speculate that *PttHB7*, the paralogous gene to *PttHB8* might function in these plants counteracting *PttHB8* down-regulation.

***POPACAULIS5* expression appears restricted to the vascular tissues**

The expression pattern of *POPACAULIS5* was visualized by RNA *in situ* hybridization. *POPACAULIS5* expression was found in the vascular bundles in transverse sections of the hybrid aspen stem (Figure 10a). The antisense probe revealed expression broadly associated with the cambial zone. As

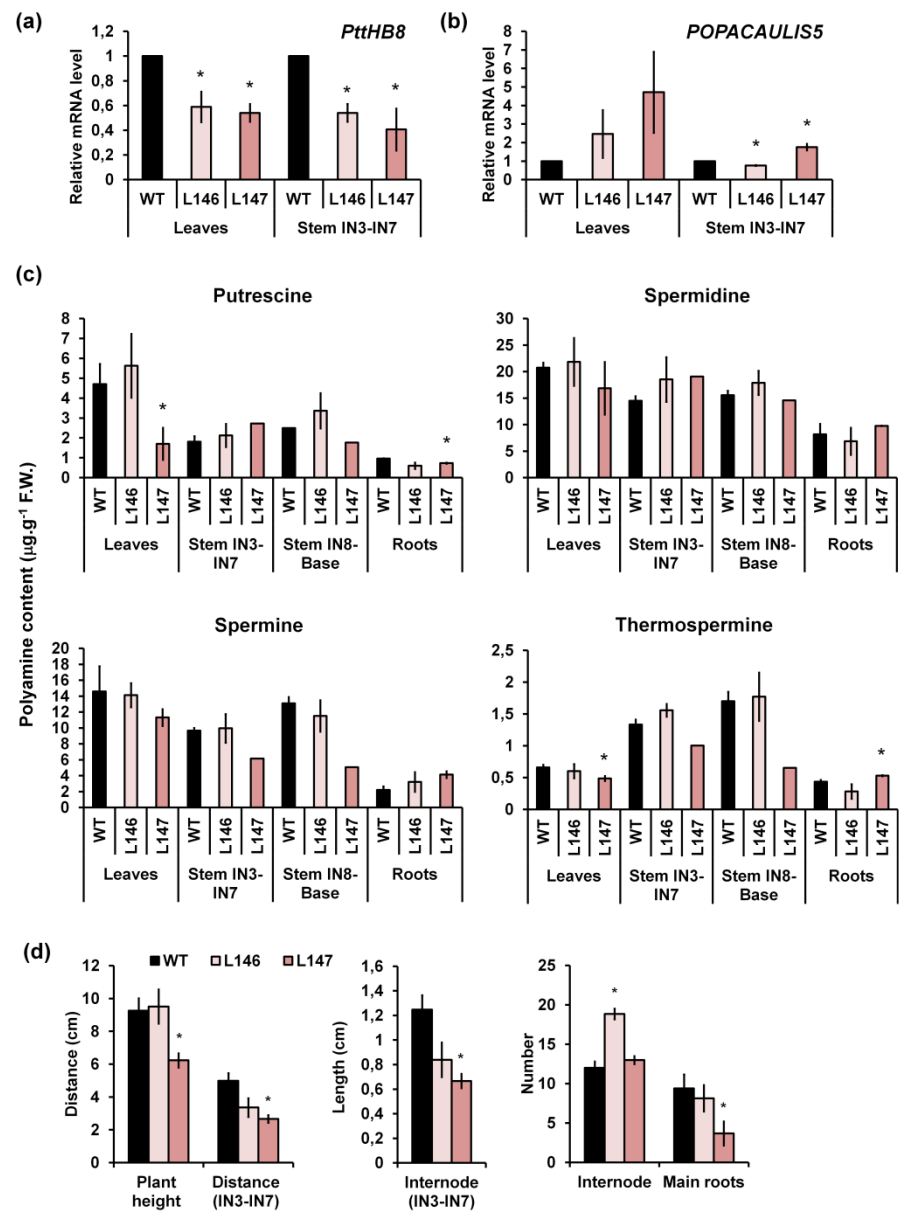


Figure 9. Expression and thermospermine content in tissues of five week-old *in vitro* grown RNAi::PttHB8 plants. (a) Reduced *PttHB8* transcript levels in transgenic lines L146 and L147. (b) *POPACAULIS5* transcript levels are reduced in L146 stems but increased in L147 transgenic line when compared to wild-type. (c) Polyamine levels in organs of five week-old *in vitro* grown plants. Thermospermine levels are similar to wild-type in L146 and slightly reduced in L147 shoot tissues. Other polyamines accumulation is similar to the wild-type, ►

◀ with the exception of a decrease in putrescine levels in leaves and its increase in roots of the L147 line. (d) Growth parameters of L146 and L147 transgenic lines measured in five week-old *in vitro* grown plants ($n = 8$). L147 shorter plants had reduced distance from third (IN3) to seventh (IN7) elongating internodes, reduced internode length and reduced number of main roots. L146, showed similar or even slightly taller plants than WT, mainly due to an increased internode number. Values are means $\pm$ SD of three biological replicates (sampled as three pools of six to ten individual plants and three technical replicates for polyamine and expression data). Asterisks indicate significant differences from the wild-type ($p < 0.05$, Mann-Whitney *U* test). The experiments for gene expression analysis were performed twice. Polyamine measurements to L147 stem internode samples (IN3-IN7 and IN8-basal) were taken from one pooled sample of six plants.

secondary growth proceeds, *POPACAULIS5* expression is weaker and not uniform, but seems restricted to cambium and phloem fibers (Figure 10b). We confirmed broad hybridization of the antisense probe using 35S::*POPACAULIS5* stem sections as positive control (Figure 10c). However, the sections hybridized occasionally with the sense probe showing purple coloration in the vascular tissues (Figure 10d). Muñiz *et al.* (2008)

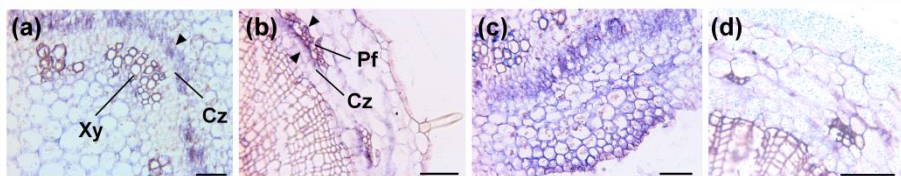


Figure 10. Expression of *POPACAULIS5* during *Populus* stem development revealed by whole mount *in situ* hybridization. (a) Antisense probe hybridized with stem sections of wild-type hybrid aspen first elongating internode. *POPACAULIS5* is expressed broadly during primary growth, with strongest expression associated with the procambial and cambial cells. (b) Antisense probe hybridized with stem sections at the base internode from five week-old *Populus*. *POPACAULIS5* is expressed in cambial region and in the phloem fibers. (c) Antisense probe hybridized with stem sections of the first internode of 35S::*POPACAULIS5*, used as positive control. (d) Sense probe showing non-specific hybridization with stem sections of five week-old hybrid-aspen. CZ-cambial zone, Xy-xylem, Pf-phloem fibers; arrows indicate hybridization. Bars: 25 μ m.

have also encountered some degree of sense probe hybridization in *Arabidopsis* stem sections. In *Arabidopsis*, *ACL5* expression is associated with provascular/procambial cells in early stages of embryogenesis (Clay and Nelson, 2005), that is abundant and specific to procambial cells of primary roots (Birnbaum *et al.*, 2003) and to vessel elements in the inflorescence stem (Muñiz *et al.*, 2008). Therefore, it is not surprising that a weak signal is encountered in the *Populus* stems with secondary xylem growth, suggesting not only that *POPACAULIS5* expression is associated with early events in xylem specification but also feedback repression at the transcriptional level.

Suppression of POPACAULIS5 results in increased tree height

To further explore *POPACAULIS5* role in plant development we post-transcriptionally silenced the *POPACAULIS5* gene in *Populus* plants. We obtained two RNAi::*POPACAULIS5* transgenic lines, L149 and L150, which showed reduced overall growth *in vitro*. Plant height, internode length and number of main roots were found reduced and internode number increased in RNAi::*POPACAULIS5* plants when compared to the wild-type (Figure 11a). The reduced *POPACAULIS5* expression in stem tissues correlated well to the reduced amount of thermospermine measured in stem tissues (Figure 11b,c), but no significant differences to wild-type were observed in *POPACAULIS5* expression and thermospermine content from leaves of L149 plants (Figure 11b,c). Interestingly, a significant increase in plant height was observed in two-month old L149 trees grown in the greenhouse (Figure 11d). Since suppression of *POPACAULIS5* expression was only detected in the stem tissues of one transgenic line, several new transgenic lines showing different levels of *POPACAULIS5* suppression have recently been generated (Figure 12) and are currently under study to understand the growth increase observed in RNAi::*POPACAULIS5*-L149 plants (Figure 11d) and to further elucidate on the roles of *POPACAULIS5* in tree vascular development.

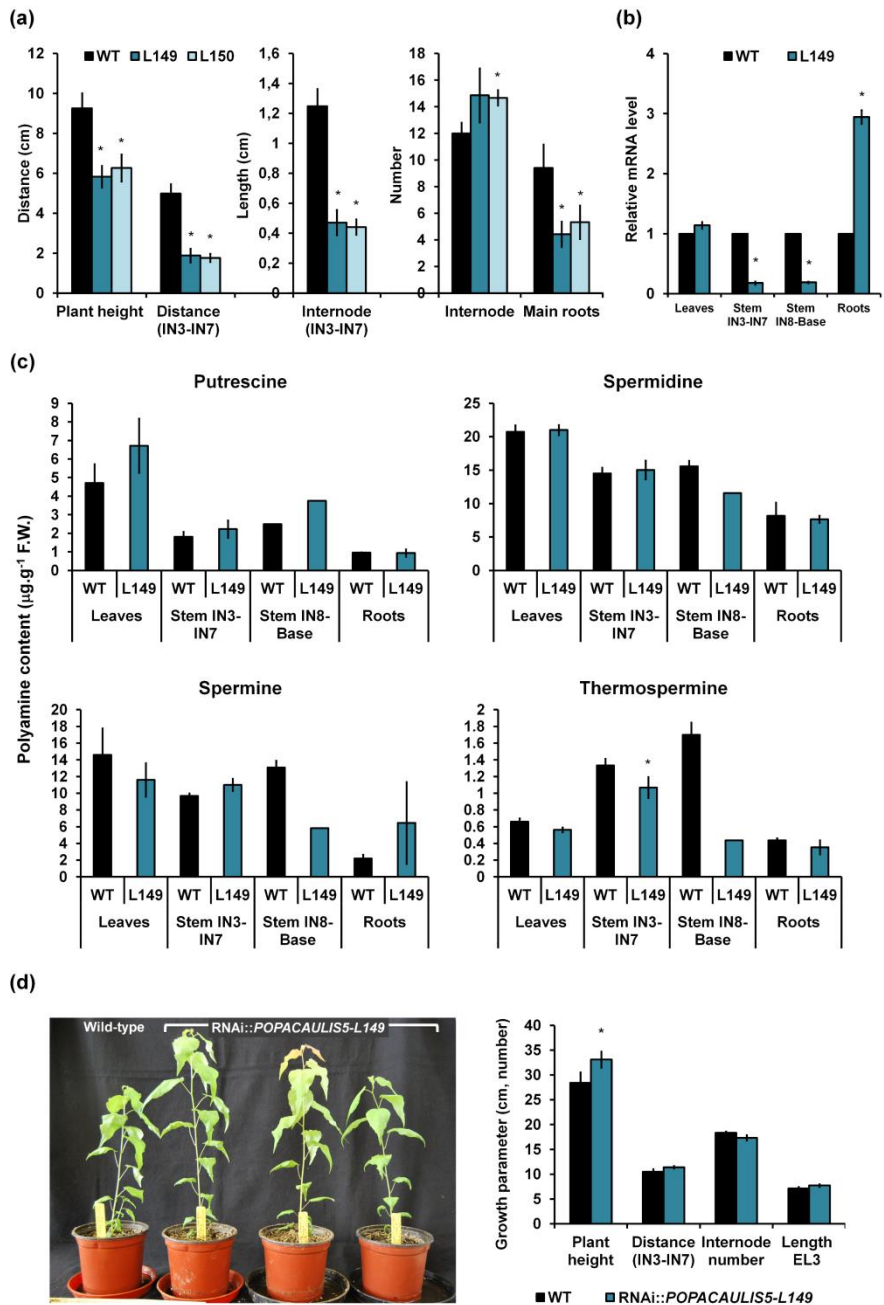


Figure 11. Characterization of RNAi::POPACAULIS5 plants. (a) Growth parameters of L149 and L150 transgenic lines measured in five week-old *in vitro* grown plants ($n = 8$). RNAi::POPACAULIS5 shorter plants had reduced distance from third (IN3) to seventh

◀ (IN7) elongating internodes, reduced internode length and reduced number of main roots, but increased number of internodes compared to the wild-type. (b) Suppressed *POPACAULIS5* expression was only evident in stem tissues of five week-old *in vitro* grown L149 plants. (c) Polyamines content in leaves, stem internodes (IN3-IN7 and IN8-basal internodes from the top) and root tissues from L149 plants. Decreased thermospermine content was only detected in stem tissues of L149 plants and no effect on putrescine, spermidine or spermine levels was observed. (d) Greenhouse-grown 2-month old trees (left) and growth parameters (right) showed increase tree height, increased internode length and increased dimensions of the third fully expanded leaf (EL3). Values for growth parameters represent mean $\pm$ SD of biological replicates (for *in vitro* experiments $n = 8$ and for trees $n = 3$). Relative expression and polyamine content values represent mean $\pm$ SD of three biological replicates (sampled as three pools of six to ten individual plants and three technical replicates). The transcript quantification was performed twice. Polyamine measurements to L149 stem internode sample (IN8-basal) was taken from one pooled sample of six plants. Asterisks indicate significant differences from the wild-type ($p < 0.05$, Mann-Whitney U test).

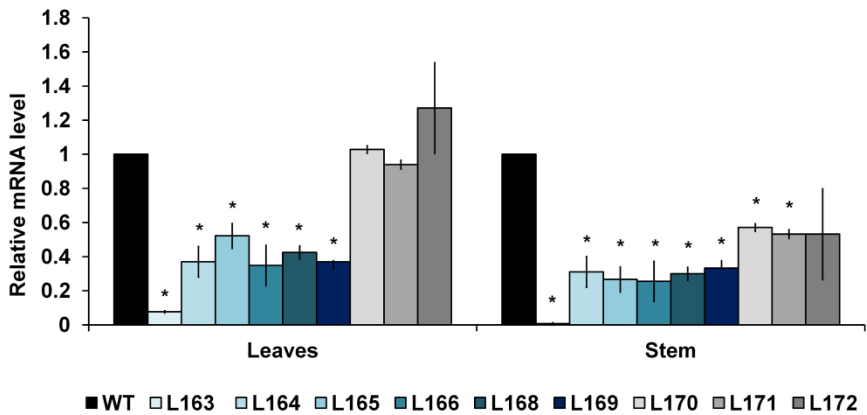


Figure 12. Relative *POPACAULIS5* expression in transgenic lines showing suppressed *POPACAULIS5* expression. Values represent mean $\pm$ SD of three technical replicates from a pool of eight *in vitro* grown plant tissues. Asterisks indicate significant differences from the wild-type ($p < 0.05$, Mann-Whitney U test).

Phenotypic analysis of Populus plants misregulated for suppression or induction of HD-Zip III PttHB5/PttHB6

Due to the clustering of PttHB8/PttHB7 protein sequences with PttHB5/PttHB6, we expect increased similarities between these orthologs pairs of HD-Zip III TFs. To study the effect of altering the HD-Zip III transcript levels *in planta*, we generated tools for future work on HD-Zip III TFs functional analysis and their relation to *POPACAULIS5* transcriptional regulation. We transformed hybrid aspen for silencing and overexpression of the miRNA-resistant forms of the HD-Zip III family members PttHB5/PttHB5 and PttHB6/PttHB6. From 460 *Agrobacterium*-co-cultivated *Populus* petiole explants, we recovered nine 35S::*PttHB5*-miRNA^{ad}, thirty eight RNAi::*PttHB5* and sixteen 35S::*PttHB6*-miRNA^{ad} kanamycin-resistant shoots grown on selection medium. However, contamination frequency was high during the selection period and rooting was only successful for a much reduced number of transgenic lines. Therefore, after confirming transgene insertions, we were able to proceed with four RNAi::*PttHB5* lines (L142, L143, L144, L145) and five lines for *PttHB6* overexpression (L127, L131, L133, L136, L141). Growth parameter analysis of the transgenic lines grown *in vitro* revealed that one transgenic line for silencing of *PttHB5* (L142) showed reduced overall growth, whereas the remaining lines were similar to wild-type (Figure 13a,b) and three lines overexpressing *PttHB6* (L131, L136, L141) were shorter and showed increased number of internodes relatively to the wild-type plants (Figure 13c,d). Analysis of the transgenic lines for altered *PttHB5* and *PttHB6* transcript levels is currently underway. We aim at transferring these plants to soil for greenhouse growth experiments to further elucidate on the effects of altering these genes transcription profile and understand whether PttHB5 and PttHB6 have conserved functions in *Populus*. Since AtHB8 and AtHB15 have rather antagonist functions in *Arabidopsis* stem, and seeing that we have discovered new functions for

PttHB8 in *Populus*, we expect exciting results from PtrHB5 and PtrHB6 functional analysis. So far, we observed 35S::*PttHB6*-miRNA plants have narrower stems, which is suggestive of defects in cambial growth that only further assays including histological analysis will elucidate on.

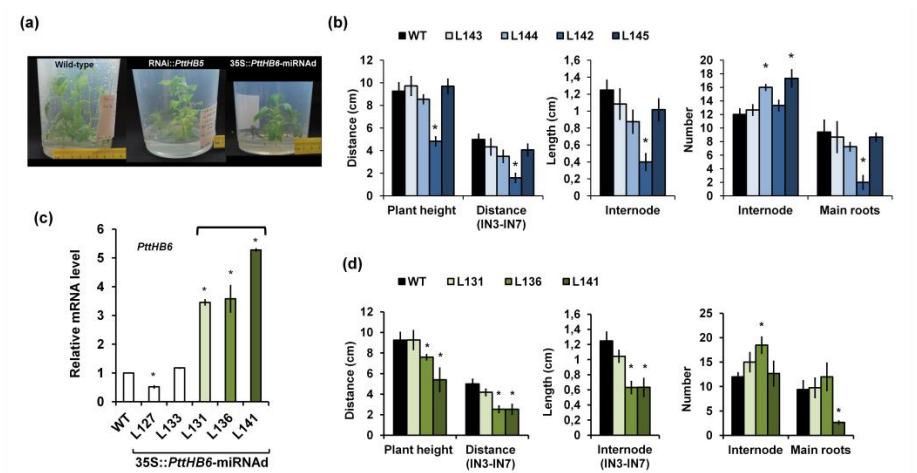


Figure 13. Characterization of transgenic lines for *PttHB5* transcript levels suppression and *PttHB6* overexpression. (a) Representative individuals of wild-type, RNAi::*PttHB5* and 35S::*PttHB6*-miRNA lines. (b) No significant differences to wild-type were observed in the growth patterns of RNAi::*PttHB5* *in vitro* grown lines, with the exception of line L142 that showed reduced overall growth. (c) Relative expression of *PttHB6* confirming induced expression in 35S::*PttHB6*-miRNA lines L131, L136 and L141. (d) Overall growth was reduced in two of the transgenic lines (L136 and L141) that showed increased expression of *PttHB6*. Values for growth parameters represent mean $\pm$ SD of biological replicates (n=8). Relative expression values represent mean $\pm$ SD of three technical replicates from a pool of eight plant tissues. The transcript quantification was performed twice. Asterisks indicate significant differences from the wild-type ($p < 0.05$, Mann-Whitney *U* test).

Discussion

PtrHB8 HD-Zip III is predicted to bind to POPACAULIS5 promoter

Members of the class III HD-Zip family in *Populus* share a homeodomain (HD), a basic leucine-zipper domain (bZIP), as well as START and

MEKHLA domains, which we have found to be highly conserved in agreement with previous reports (Prigge and Clark, 2006; Ariel *et al.*, 2007). The search for HD-Zip III regulatory motifs in *POPACAULIS5* putative promoter region predicted that the transcription factor ATHB9/PHV targets this gene. ATHB9/PHV has high affinity *in vitro* for the pseudopalindromic sequence GTAAT(G/C)ATTAC (Sessa *et al.*, 1998). Due to similarity amongst HD-Zip III proteins, we hypothesized that PtrHB8 targets *POPACAULIS5* promoter and found that the amino acid residues in the DNA binding interface in PtrHB8 follow a similar signature to the one identified for AtHB9/PHV. Interestingly, a *cis*-element related to xylem differentiation ACAAAGAA (XYLAT), overrepresented in the promoter regions of thirteen genes that are co-regulated during secondary xylem differentiation (Ko *et al.*, 2006b) was identified in the putative promoter regions of *POPACAULIS5* and *ACL5* but not in the promoter of the closely related polyamine spermidine synthase gene (*SPDS1*). The functional role of XYLAT element in xylem differentiation is currently not known. Also, finding ARF (auxin response factors) amongst the *cis*-elements further supports our previous data (Chapter II) showing a high dependence of *POPACAULIS5* expression on auxin.

PtrHB7 and PtrHB8 have conserved novel functions on organ polarity

In *Arabidopsis*, PHB and PHV gain-of-function alleles result in adaxialized radial leaves with no lamina expansion (McConnell and Barton, 1998; McConnell *et al.*, 2001) and loss-of-function alleles result in abaxialized radial cotyledons (Emery *et al.*, 2003). The adaxialization of abaxial positions in leaves and carpels from *Arabidopsis* plants transformed to express *PttHB8* and *PtrHB7* suggests that, like other class III HD-Zip genes in *Arabidopsis*, *PtrHB8/PtrHB7* genes may be implicated in the initial establishment of adaxial-abaxial polarity in *Populus* (Figure 5 and Figure 6).

Previous studies in *Arabidopsis* have not reported such polarity defects when *AtHB8* was ectopically expressed (Baima *et al.*, 2001). Neither have vascular defects been observed when *AtHB8* function is inactive. Albeit, posttranscriptional regulation of *AtHB8* may have suppressed the *AtHB8* ectopic expression in the overexpression *Arabidopsis*, and any effect it could have had in abaxial-adaxial domains in *Arabidopsis* plants (Baima *et al.*, 2001). Our results suggest that, in *Populus*, neofunctionalization of the PtrHB8/PtrHB7/*AtHB8* lineage might have occurred, which is consistent with recent work from Zhu *et al.* (2013) that propose PtrHB7 function may have evolved a more specific role in regulating vascular differentiation during secondary growth in woody plants than in *Arabidopsis*.

Similar polarity defects have been observed when KANs activity is compromised. While *KANs* genes are expressed in the abaxial regions of lateral organs (Eshed *et al.*, 2001; Kerstetter *et al.*, 2001; Eshed *et al.*, 2004), class III HD-Zip are already known to be expressed in adaxial regions (McConnell and Barton, 1998; McConnell *et al.*, 2001; Emery *et al.*, 2003) implying that the two gene families act antagonistically during pattern formation. *kan1 kan2 kan3* triple mutant showed loss of abaxial identity of the leaf tissues (Eshed *et al.*, 2004). Interestingly, *kan1 kan2 kan3* plants are phenotypically similar to *phb-1d* mutants in that the lateral organs are adaxialized (Eshed *et al.*, 2001; Eshed *et al.*, 2004). Although the triple loss-of-function *phb-6 phv-5 rev-9* plants exhibit radialized abaxialized cotyledons (Emery *et al.*, 2003), *AtHB8* expression has been shown limited to the procambial domain and as above mentioned no reports exist on the polarity defects upon *athb8* single loss of activity (Baima *et al.*, 2001). Furthermore, *athb8 rev* loss of function is similar to *rev* single mutant (Prigge *et al.*, 2005), which suggests that *AtHB8* in *Arabidopsis* may have higher redundancy to the other HD-Zip III members than PtrHB8/PtrHB7 to other HD-Zip III in *Populus*. Curiously, *rev phv* plants produce trumpet-shaped leaves, with adaxial tissue inside the trumpet leaf cone, abaxial tissue

surrounding proximal portion of the leaf, and normal adaxial/abaxial polarity distally (Prigge *et al.*, 2005). Our transgenic plants displayed some leaves with a similar trumpet-shaped form and the complete adaxialization of the leaf tissues (Figure 6), suggesting that PttHB8/PtrHB7 promotes adaxialization of organ polarity.

To examine the function of PttHB8 on *POPACAULIS5* expression, we created transgenic *Populus* plants silenced for *PttHB8*. Results showed that suppression of only one of the paralogs pairs PttHB8/PttHB7 has little effect on thermospermine levels in *Populus* tissues (Figure 9). Again, we hypothesize that this is related to redundancy between PttHB8 and PttHB7; therefore, decreased expression of one gene would be masked by the presence of the counterpart endogenous paralog. Overall, our results point towards functional redundancy in the *Populus* HD-Zip III paralogs genes and neofunctionalization of the family members may have occurred leading to new roles such as the regulation of organ polarity in the case of PttHB8/PttHB7.

PttHB8 transcriptional control of POPACAULIS5

Similarly to our previous data in 35S::*PttHB8*-miRNA transgenic *Populus* plants, our investigation showed that *PttHB8* expression in *Arabidopsis* has a positive effect on *ACL5* transcription and on the other hand, *POPACAULIS5* seems to have a negative effect on *AtHB8* expression. We were expecting *AtHB8* transcript levels to be downregulated by the increase of thermospermine levels, which was held true and confirmed by the decreased transcript levels observed in stem tissues but not in leaves. We sustain this is related to a more efficient feedback regulation in the stem due to the increased presence of xylem tissues when compared to the leaf organ. Thus, we here provide additional support to the regulatory feedback loop mechanism proposed in Chapter II. More importantly, this work suggests that

the mechanism of thermospermine control is quite conserved in vascular plants.

Functional redundancy within multigene families complicates the attempts to find a single gene member function, particularly when attempting posttranscriptional silencing of these genes. Such redundancy in functions can make the interpretation of results for silencing of *PttHB8* difficult, given the fluctuations in *POPACAULIS5* expression and thermospermine levels in tissues from the silenced lines. It has been previously shown that the endogenous network of stress-related genes, for instance, responds to heterologous expression of other species genes (Dubouzet *et al.*, 2003; Jing *et al.*, 2009). Therefore, the heterologous expression in *Arabidopsis* and the overexpression of such genes could be a good approach to overcome such difficulties and to study the related network of genes responses.

Localization of *POPACAULIS5* transcript was observed in the vascular tissues in the *Populus* stems and it would be interesting to also localize *PttHB8* expression to understand if expression patterns overlap. Since the sense probe occasionally showed the same hybridization pattern as the antisense probe, further confirmatory assays will be needed. Several researchers favour instead the use of promoter::GUS fusion constructs to observe the localization of a given gene expression. In fact, we have isolated the herein discussed putative promoter region of *POPACAULIS5* gene and in future work transgenic *Populus* bearing the promoter::GUS fusion will be generated to confirm our preliminary data from the *in situ* hybridization showing specific expression in the vasculature domain. However, it should also be noted that expression of the promoter-GUS reporter gene may not represent the endogenous gene expression pattern. In fact, the 5' upstream sequence of any given gene does not always contain all the regulatory elements essential for its expression (Zhong and Ye, 2007) and, therefore, additional techniques (such as antibody staining) should be also tested.

Silencing of *POPACAULIS5* in *Populus* stems correlated well with

the reduced levels of *POPACAULIS5* transcripts and thermospermine production in the stem tissues. Curiously, plant growth was slightly stimulated in transgenic trees silenced for *POPACAULIS5*, opposite to the parallel situation in the *Arabidopsis acl5* mutants, which are dwarf. Caution should be taken given that only one transgenic line was available for these studies. It will be important to determine the nature of increased plant growth observed in this transgenic line and if it correlates with decreased *POPACAULIS5* expression. Nevertheless, this data supports that the defects previously observed in stems of 35S::*POPACAULIS5* *Populus* trees are due to increased thermospermine levels at earlier stages of growth (see for instance transgenic trees from lines B13 and B4, Figure 2c, Chapter II) that are subsequently controlled by the feedback repression mechanism we have unveiled.

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CHAPTER V

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In the present work it was for the first time demonstrated that thermospermine homeostasis in secondary xylem tissues relies on the negative feedback control of its own production. Maintaining thermospermine levels within a safe range ensures that xylem differentiation occurs in a timely manner. In this proposed mechanism, IAA mediates *POPACAULIS5* expression through *PttHB8*, while thermospermine levels feedback control *PttHB8* and consequently *POPACAULIS5* transcript levels through repression of IAA in a loop. The presence of this negative feedback loop mechanism was uncovered by the surprising failure to induce upregulation of *POPACAULIS5* in xylem tissues following its ectopic expression under the control of 35S constitutive promoter (Chapter II). Reduced levels of auxin found in the *Populus* stems indicate that auxin biosynthesis may be suppressed by thermospermine overproduction. It is not evidently clear how thermospermine feeds back to auxin. From the transcriptome analysis of 35S::*POPACAULIS5* stems we could conclude that auxin distribution is disrupted (Chapter III). Since thermospermine is thought to bypass the auxin-promoting effect on xylem differentiation, one hypothesis is that thermospermine also affects polar auxin transport, and in this way perturbs the auxin maxima necessary for auxin channeling and xylem differentiation. Another hypothesis is that inactivation of IAA occurs either by conjugation to amino acids, or in the free form by binding to TIR/AFB F-box proteins which promotes their interaction with Aux/IAA proteins that are subsequently targeted for degradation. Either way, the molecular mechanism of the thermospermine action on auxin signaling or biosynthesis is currently unknown. It would be interesting to cross available *Arabidopsis* reporter lines for auxin-response, auxin-signaling and transport genes with 35S::*POPACAULIS5* *Arabidopsis* line we generated to understand the nature of this interaction.

It should be noted that fluctuations in thermospermine levels in the stem tissues are expected to occur as a result of the strong overexpression of

POPACAULIS5 from the 35S promoter in the stem that needs to be counteracted by the negative feedback loop. It is clear from our data that the feedback loop cannot cope with too big disturbances, as addition of auxin into the growth medium of the transgenic 35S::*POPACAULIS5* trees resulted in higher expression levels of *POPACAULIS5*. It is intriguing that overproduction of thermospermine produces the same phenotype as the one observed in *acl5* loss of function mutant in *Arabidopsis*. However, this is in line with the mode of action of most hormones in that a dose-dependent effect is observed until a threshold is reached, after which we hypothesize thermospermine becomes detrimental to general plant growth. In the future, it will be interesting to determine the dose-response profile for thermospermine. This would allow understanding if increased levels of thermospermine are responsible for the slight defects observed in xylem of the transgenic 35S::*POPACAULIS5* trees or if these were instead the result of slightly decreased thermospermine levels. We intend to make use of the *POPACAULIS5* RNAi lines to further elucidate these open questions (Chapter IV). Furthermore, since the feedback mechanism is unable to cope with excessive auxin we hypothesize that post-transcriptional control of *POPACAULIS5* by auxin may as well take place. The fact that the phenotype is dependent on increased auxin levels suggests that stabilisation of the *POPACAULIS5* transcript by auxin might occur. It is plausible that the *POPACAULIS5* transcript may be unstable and rapidly degraded similarly to what happens to genes that must be rapidly or strictly controlled, like many transcripts involved in regulating cell growth and differentiation. This may justify that, in the absence of auxin stimulus which would provide stabilization of *POPACAULIS5* mRNA, the transcript is rapidly degraded in the transgenic plant stems. In fact, auxin is known to exert both transcriptional and post-transcriptional control on other genes as well, such as the *Aux/IAA* genes. Furthermore, we have found an auxin-responsive element in *POPACAULIS5* putative promoter region which indicates that auxin also

exerts a transcriptional regulation role.

We found that the dwarfism imposed by high levels of thermospermine is accompanied by the general shut-down of the xylem differentiation program (Chapter III). A role for thermospermine at a procambial stage of development seems likely as suggested by the defects observed at the cambial zone. The fact that only phloem identity cells could be found within the abnormal cambial region raises several questions. One relevant question is whether thermospermine has some role in cell fate decision at the cambial domain. This is not easy to answer; on the one hand, we observed upregulation of several cambial regulators and of phloem identity genes (e.g. *KAN*, *APL*), which could suggest this is the case. On the other hand, even though we observe only phloem identity within dwarf stems cambial region, we were unable to clarify whether increased functional phloem was produced as a result of thermospermine accumulation in the dwarf transgenic plants. Furthermore, we have not observed in the transgenic trees any indication that the increased levels of thermospermine at earlier stages of plant growth subsequently affected secondary phloem production. Also, no indication exists of phloem defects in the *acl5 Arabidopsis* loss-of-function mutant. Therefore, at this point it would be speculative to link thermospermine to the balance between phloem and xylem differentiation. Interestingly, *KAN* proteins, as earlier mentioned, are also known to limit polar auxin transport and inhibit procambium formation at early stages of embryogenesis. It would be interesting to understand if the negative effect thermospermine has on auxin transport happens through elements such as *KAN*. Consequently, a more relevant question is whether thermospermine has in fact a dual role, not only preventing the premature cell death of xylem, but also preventing xylem specification at an earlier stage. We believe this might be the case. First, evidence shows the lack of xylem identity cells in the most extreme dwarf *POPACAULIS5* overexpressor plants that was correlated to the *POPACAULIS5* transcript levels. Second, the reduced auxin levels

imposed by the feedback effect of *POPACAULIS5* on auxin indicate that thermospermine presence bypasses the auxin-induced xylem formation. Additionally, crosstalk to cytokinin was also highlighted, as increased levels of cytokinin and activation of the signaling pathway occurs in the transgenic plants (Chapter III). Since cytokinin is known for its role in suppressing xylem differentiation and maintaining cambial cell identity it would be interesting to further pursue thermospermine and cytokinin crosstalk. It is not currently clear whether the increased cytokinin signaling is indirect (perhaps a consequence of reduced auxin levels) or whether a direct interaction between thermospermine and cytokinin molecular machinery takes place.

Another aspect also evidenced by the transcriptomic analysis is the increase in ethylene production (Chapter III). We believe this increase could be accountable for the dwarf phenotype observed. Large air filled spaces between cells were observed in the stems of dwarf transgenic plants which may be an indication of increased ethylene presence. This also evidences that the phenotype observed is very likely the cause or the result of a stress response. Nevertheless, ethylene signaling was activated in stems of 35S::*POPACAULIS5* plants that were not dwarf. This makes it alluring to link ethylene to the modulation of signaling molecules, such as hydrogen peroxide, that may be implicated in thermospermine mode of action in xylem cell death, having a putative role in modulating hydrogen peroxide signal. In fact, hydrogen peroxide is also a by-product of polyamine catabolism and it is unclear yet if the degradation of polyamines could itself be a triggering mechanism to xylem cell death.

The present work also demonstrates how the intricate nature of the molecular networks that govern xylem development can be difficult to disentangle since results may seem at first glimpse difficult to interpret or even contradictory. For instance, the roles for HD-Zip III transcription factors in vascular development are known to be antagonistic, overlapping and distinct as observed from the manipulation of their transcription in

Arabidopsis. We have here shown that HD-Zip III members *PttHB8* and *PttHB7* may have evolved novel roles in organ polarity in *Populus* after separation of *Arabidopsis* and *Populus* lineages (Chapter IV). Our study further strengthens that the presence of HD-Zip III is crucial across vascular plants. We will need to deepen our investigation to the nature of the aberrant phenotype we recovered by further exploring the defects on the vascular patterning that are likely underlying the adaxialization of abaxial tissues. Clarifying the roles of these elements is not straightforward. When considering a broad network of elements that evolved and function in a coordinate manner rapidly adapting to developmental cues and environmental changes, a careful examination of the several stages of growth in trees is needed, given that these transcription factors roles may be different during embryogenesis, primary and secondary growth. Moreover, the dosage effect should be taken into account as different outcomes in xylem differentiation result from high or low dosages of these transcription factors.

Another important aspect is that most functional studies use transgenic approaches that increase or repress hormone signaling components or downstream transcriptional regulators in the whole plant such as we present in this study. It would be important to use cambial specific promoters to determine the effect of manipulating hormone signaling components in a tissue-specific manner; in a way that maintains apical meristem unaffected. Although it is most likely that the functioning of the feedback loop could addle these efforts, the use of an inducible-overexpression system in short time-course experiments could be of some aid to overcome such difficulties. However, there are many constraints in using an inducible system in trees and the analysis would probably have to be restricted to *in vitro* growth. The use of *Arabidopsis* would allow further genetic studies that are not possible in *Populus*. In the past years a profusion of work has increased significantly our knowledge of the molecular mechanisms involved in wood development, mainly due to the use of this simple model plant. The early processes of

xylem specification in trees most probably follow the same developmental main leads or cues as in *Arabidopsis*. At least, several parallels have been found between *Arabidopsis* and *Populus* and many reports have shown that a number of mechanisms that control xylem specification and differentiation are conserved among vascular plants. Due to the amenability of *Arabidopsis* to diverse experimental approaches this model plant has been widely used to solve fundamental questions in plant biology including xylem formation. A fundamental question arises though. How are the molecular mechanisms we know to exist in common among vascular plants integrated with other molecular information that defines tree secondary growth? What makes a tree a tree? The ontogeny of some of the cells in the xylem is different in trees. For instance, in *Populus* stem we can account for the presence of xylem living ray parenchymatic cells that are completely absent in the xylem tissues of *Arabidopsis*. This probably reflects differences in terms of nutrient or signal supply that may be required for the sustainability of such large stems as the ones found in trees. These are some of the challenges in tree biology research. Nonetheless, we believe that the current knowledge of the mode of action of molecules such as thermospermine in the secondary xylem of trees can greatly benefit from the use of both model plants.

As a final note and as stated in the first sentence of this thesis “Wood is one of the most remarkable natural renewable resources”. One of the remarkable properties of wood is its time duration. One of the most impressive examples of this is the majestic wood warship Vasa (Stockholm, Sweden) which was submerged in water for 333 years and still preserved its wood structure. Another notable example are the downtown Lisbon (Portugal) “Pombaline” buildings, from the 18th century, built after the 1755 earthquake, and made with a composite wood-masonry structure, that still maintains its anti-seismic properties after all these years (Ramos and Lourenço, 2005). It is this astonishing durability and adaptable properties to the surrounding environment that make wood such an elite raw material.

How plants were able to evolve such an amazing structure has more to do with their need to compete to capture light energy while guaranteeing the needed structural support as well as water and nutrients transport, than to the multiple applications mankind has ingeniously set to wood. But it is due to its applicability that so many studies aim at understanding the processes behind wood formation. It is evident that the increase in cambial division results in increased xylem biomass production which is valuable information from a biotechnological point of view given the high demands for improved biomass yields. This study has provided a small piece of the puzzle on xylem development but also shows that much research is still needed to fully disclose the mechanisms underlying wood formation.

References

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