Wood anatomy and cytokinin-related responses in poplar (*Populus* sp.) under environmental stress

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CONTENTS

Abstract

Woody plants like poplar are of great importance as a second generation bioenergy crop. However, wood formation is dynamic and strongly affected by exogenous factors such as drought or seasonality, and endogenous factors. Drought negatively affects wood growth and results in significant changes in wood anatomy in poplar. But the intraspecific variations in drought-induced wood anatomical changes and the underlying molecular responses are not clear. In addition to the exogenous factors, endogenous factors such as the phytohormone, cytokinins affect wood formation. The analysis of cytokinin activity is of particular interest, as growth maintenance under unfavourable environmental conditions is the basis to increase poplar productivity. Our current knowledge about cytokinins comes mainly from studies of the herbaceous annual model plant, *Arabidopsis thaliana*, whereas a few studies have been conducted with woody model plant, poplar. For instance, the cytokinin levels differ between the season of active growth and dormancy and under drought. But the localization pattern of active cytokinins in different organs and cells in poplar is unknown.

To address these research gaps, the goals of this study were:

(1) Investigation of the presence and cellular localization pattern of active cytokinins in apical buds, leaves, along the stem and fine roots of *Populus* × *canescens* in the active growth phase and during dormancy.

(2) Investigation of drought-induced changes of active cytokinins at tissue and cellular levels in different organs of *P.* × *canescens* and to compare the patterns with growth responses, physiological and morphological drought acclimation.

(3) Analysis of the intraspecific variation in the drought-induced changes in wood anatomy in *P. nigra* and the molecular responses underlying them.

(4) Analysis of the time dependent progress in drought-induced wood anatomical changes in *P. nigra* and to examine whether these changes are accompanied by changes in the transcript abundance of cytokinin signalling, biosynthesis and degradation genes in the transcriptome of developing xylem.

To achieve the first goal, poplars transformed with *ARR5::GUS* reporter construct were tested for GUS staining. Lines with similar patterns of GUS activity were chosen and tested for cytokinin inducibility. Selected lines were grown outdoors for 1.5 years and used to monitor

growth and GUS activity. The transgenic poplar lines "showed no influence of *ARR5::GUS* reporter construct on the growth performance compared with the wildtype, but one line lost the reporter activity during the time course of the study. *ARR5::GUS* activity indicated changes in the tissue- and cell type-specific pattern of cytokinin activity during dormancy compared with the growth phase. *ARR5::GUS* activity, which was present in the root tips in the growing season, disappeared in winter. In the stem apex ground tissue, *ARR5::GUS* activity was higher in winter than in summer. Leaf primordia in summer showed *ARR5::GUS* activity, but not the expanded leaves of outdoor plants or leaf primordia in winter. In stem cross sections, the most prominent *ARR5::GUS* activity was detected in the cortex region and in the rays of bark in summer and in winter. In the cambial zone, the *ARR5::GUS* activity was more pronounced in the dormant than in growth phase. The pith and the parts of ray cells associated with the vessels also displayed *ARR5::GUS* activity. *In silico* analyses of the tissue-specific expression patterns of the whole PtRR type-A family of poplar showed that *PtRR10*, the closest ortholog to the Arabidopsis *ARR5* gene, was usually the most highly expressed gene in all tissues. In this study, gene expression and tissue-localization indicated high activity of cytokinins not only in summer, but also in winter. The presence of the signal in meristematic tissues supports their role in meristem maintenance."*

To meet the second goal, a mild drought treatment, which did not abolish growth completely, was applied to poplars transformed with *ARR5::GUS* reporter construct. "Young leaves showed strong cytokinin activity in the veins and low staining under drought stress, accompanied by diminished leaf expansion. Leaf scars, at positions where drought-shedding occurred, showed strong reduction of cytokinin activity. The pith in the differentiation zone of stem showed high cytokinin activity with distinct, very active parenchymatic cells and enhanced activity close to primary xylem. This pattern was maintained under drought but the cytokinin activity was reduced. Mature phloem parenchymatic cells showed high cytokinin activity and mature wood showed no detectable cytokinin activity. Cytokinin activity in the cambium was apparent as a clear ring, which faded under drought. Xylem-localized cytokinin activities were also mirrored by the relative expression of *PtaRR3*, whereas *PtaRR10* showed developmental but no drought-induced changes. Primary meristems exhibited high cytokinin activity regardless of drought stress, supporting a function of this phytohormone in meristem maintenance, whereas declining cytokinin activities in apical pith tissues and cambium of drought-stressed poplars linked cytokinin in these cell types with the control of primary and

secondary growth processes. Changes in cytokinin activity further imply a role in drought avoidance mechanisms of poplars, especially in the reduction of leaf area. ”†

In order to reach the third goal, three *Populus nigra* genotypes originating from a dry, a mesic or a wet habitat were grown under control or drought-stressed conditions and wood anatomy was analyzed. “Drought resulted in reduced cambial activity, decreased vessel and fiber lumina, and increased the saccharification potential. The saccharification potential was unrelated to lignin content as well as to most wood anatomical traits. RNA sequencing of the developing xylem revealed that 1.5% of the analyzed genes were differentially expressed in response to drought, while 67% differed among the genotypes. Weighted gene correlation network analysis identified modules of co-expressed genes correlated with saccharification potential. These modules were enriched in gene ontology terms related to cell wall polysaccharide biosynthesis and modification and vesicle transport, but not to lignin biosynthesis. Among the most strongly saccharification-correlated genes, those with regulatory functions, especially kinases, were prominent. We further identified transcription factors whose transcript abundances differed among genotypes, and which were co-regulated with genes for biosynthesis and modifications of hemicelluloses and pectin.”#

To meet the fourth goal, a five week moderate drought treatment was applied to *P.nigra* plants. The plants were harvested weekly and wood anatomy was analyzed. During the five weeks of drought treatment, radial growth, relative width of developing xylem, number of cambial cell layers and lumen area per fibre were significantly reduced when compared to the control plants. The other anatomical traits analyzed did not show a significant effect of drought. Regression analyses revealed significant positive correlations between radial growth and number of cambial cell layers, radial growth and relative width of the developing xylem, radial growth and lumen area per fibre, number of cambial cell layers and relative width of the developing xylem. Analysis on the transcript abundance of cytokinin response genes in the transcriptome of developing xylem revealed that the response regulators, *RR7* and *RR9*, showed a significant decline under drought as well as with the duration of the experiment which suggested reduced cytokinin signalling under drought. Among the cytokinin biosynthetic genes, transcript abundance of *IPT2*, *5a* and *5b* showed variation with time and only marginal induction under drought. The transcript abundance of cytokinin degradation gene, *CKX6* was significantly increased under drought. The analysis of transcript abundance of cytokinin related genes showed reduced cytokinin signalling and increased degradation under drought. As cytokinins are the central regulators of cambial development

in poplar, this reduction in cytokinin signalling and levels may have resulted in the significantly reduced number of cambial layers and thereby bringing about significant changes in wood anatomy which will eventually help the plants in survival under drought.

Transgenic poplars transformed with *ARR5::GUS* construct have been introduced for the first time in this study and used to shed light on tissue and cellular level cytokinin activity during active growth and dormancy, as well as in response to drought. Fine root tips, cambial cells and xylem rays were the main tissues that showed differences in cytokinin activity under varying environmental conditions studied here. The *ARR5::GUS* poplar "reporter lines can be used to investigate the involvement of cytokinins in mediating growth constraints and growth-promoting treatments for vascular development and cell type identities in the future. Thereby, these poplars may become an important tool to enhance our understanding of woody biomass production."*

* Paul S. et al. **2016**. *Frontiers in Plant Science* **7**: 652.

† Paul S. et al. **2018** *AoB PLANTS* **10**: plx067.

Wildhagen et al. **2017**. *Tree Physiology* **24**:1-20.

Zusammenfassung

Die Holzgewächse, wie die Pappel, sind als Energiepflanzen zweiter Generation von großer Bedeutung. Die Holzbildung ist jedoch dynamisch und durch exogene Faktoren wie Trockenheit und Saisonalität stark beeinträchtigt als auch von endogenen Faktoren. Trockenheit beeinträchtigt das Holzwachstum negativ und führt zu signifikanten Veränderungen in der Holzanatomie der Pappel. Die intraspezifischen Variationen in der Holzanatomie, welche durch Trockenheit induziert werden und zu holzanatomischen Veränderungen führen, sowie die molekularen Antworten sind jedoch nicht klar. Neben den exogenen Faktoren wird die Holzbildung auch durch endogene Faktoren wie den Phytohormonen beeinträchtigt. Die Analyse der Cytokininaktivität ist von besonderem Interesse, weil die Erhaltung des Wachstums unter ungünstigen Umweltbedingungen die Basis für die Verbesserung der Pappelproduktion darstellt. Unser aktuelles Wissen über Cytokinine wird hauptsächlich von Studien über die krautige, einjährige Modellpflanze *Arabidopsis thaliana,* bezogen und nur wenige Studien wurden an der holzigen Modellpflanze, der Pappel, durchgeführt. Beispielszweise verändert sich das Level an Cytokinin zwischen der Phase des aktiven Wachstums und der Winterruhe sowie unter Trockenheit. Die Lokalisation von aktivem Cytokininen in den verschiedenen Organen und Zellen ist jedoch in der Pappel nicht bekannt.

Um diese Lücke in der Forschung zu berücksichtigen, waren die Ziele dieser Studie folgende:

(1) Die Untersuchung des Auftretens und der zellulären Lokalisation von aktiven Cytokininen in der Apikalknospe, den Blättern, den Feinwurzeln und entlang des Stammes in der aktiven Wachstumsphase und während der Ruhephase anhand von *Populus* × *canescens*.

(2) Die Untersuchung der durch Trockenheit induzierten Veränderungen der aktiven Cytokinine im Gewebe und auf zellulärer Ebene verschiedener Organe von *P.* × *canescens* und der Vergleich von Mustern bezüglich Wachstumsreaktion sowie physiologische und morphologische Anspassungen unter Trockenheit.

(3) Die Analyse in *P. nigra* von intraspezifischen Variationen in der durch Trockenheit induzierten Holzanatomie und der molekularen Reaktion, die diesem unterliegt.

(4) Das Analysieren von Trockenheit induzierten Veränderungen der Holzanatomie anhand von *P. nigra* im zeitabhängigen Verlauf um aufzuzeigen, ob diese Veränderungen von der

Transkripthäufigkeit von Genen des Cytokininsignals, der Biosynthese und der Zersetzung im Transkriptom des sich entwickelnden Xylems begleitet werden.

Um das erste Ziel zu erreichen wurden Pappeln mit einem *ARR5::GUS* Reporter Konstrukt transformiert und mittels GUS Färbung getestet. Linien, welche ähnliche Muster der GUS Aktivität zeigten wurden ausgewählt und auf Cytokininduzierbarkeit getestet. Die ausgewählten Linien wurden für 1,5 Jahre draußen wachsen gelassen und das Wachstum sowie die GUS Aktivität wurden beobachtet. Im Vergleich von transgenen Pappellinien mit dem Wildtyp wurde bezüglich ihres Wachstums kein Einfluss des *ARR5::GUS* Reporter Konstrukts beobachtet, jedoch verlor während der Zeitreihe der Studie eine Line die Reporter Aktivität. Während der Ruhephase im Vergleich zur Wachstumsphase traten durch die *ARR5::GUS* Aktivität Veränderungen im Gewebe- und Zelltyp spezifische Muster für Cytokinin Aktivität auf. *ARR5::GUS* Aktivität, welche in den Wurzelspitzen während der Wachstumsphase vertreten war, verschwand im Winter. *ARR5::GUS* Aktivität war im basalen Gewebe des Stammapexs im Winter höher als im Sommer. Blattanlagen zeigten im Sommer *ARR5::GUS* Aktivität, jedoch nicht die ausgebildeten Blätter der Pflanzen, die draußen standen oder Blattanlagen im Winter. Die markanteste *ARR5::GUS* Aktivität wurde in den Stammquerschnitten in der Kortexregion und, im Sommer sowie im Winter, in den Strahlenzellen der Rinde detektiert. Die *ARR5::GUS* Aktivität im Kambium war während der Ruhephase mehr ausgeprägt als in der Wachstumsphase. Das Mark sowie die mit dem Xylem verbundenen Strahlenzellen zeigten ebenfalls *ARR5::GUS* Aktivität. *In silico* Analysen des gewebespezifischen Expressionsmusters der ganzen PtRR type-A Familie der Pappel zeigte, dass *PtRR10,* das naheste ortholog zum Arabidopsis *ARR5* Gen, für gewöhnlich am höchsten exprimierte Gen war. Durch diese Studie wurde gezeigt, dass Genexpression und Gewebelokalisation hohe Cytokininaktivität nicht nur im Sommer, sondern auch im Winter zeigt. Die Rolle von Cytokinin zur Erhaltung des Mersitems wird durch die Signalpräsenz im meristematischen Gewebe unterstützt.

Um das nächste Ziel zu erreichen, einen milden Trockenstress, welches das Wachstum nicht komplett zum Stillstand bringt, wurden transformierten Pappeln mit dem *ARR5::GUS* Reporter Konstrukt genutzt. Junge Blätter zeigten starke Cytokininaktivität im Geäder und schwache Färbung unter Trockenstress begleitet von vermindertem Blattwachstum. Blattnarben an denen trockeninduzierter Blattverlust auftrat, zeigten eine starke Reduktion von Cytokinaktivität. Das Mark in der Differenzierungszone des Stammes zeigte hoche Cytokininaktivität mit eindeutingen, sehr aktiven Parenchymzellen und verbessertet Aktivität

nahe am primären Xylem. Dieses Muster wurde under Trockenheit erhalten, aber die Cytokininaktivität war reduziert. Reifes Phloemparenchym zeigte hohe Cytokininaktivität während im reifen Holz keine Cytokininaktivität detektierbar war. Das Kambium war durch einen klaren Ring offensichtlich, welcher unter Trockenheit verblasste. Die Cytokininaktivitätk, welche im Xylem lokalisiert wurde, wurde auch durch relative Expression von *PtaRR3* bestätigt, wohingegen *PtaRR10* sich entwickelnde aber nicht signifikante Trockenheit induzierte Veränderungen zeigte. Primäre Meristeme wiesen trotz Trockenstress hohe Cytokininaktivität auf, was eine Meristem erhaltende Funktion dieses Phytohormons unterstützt. Eine sinkende Cytokininaktivität im apikalen Markgewebe und Kambium bei Pappeln unter Trockenstress verbinden Cytokinin, hingegen, in diesen Zelltypen mit der Kontrolle von primären und sekundären Wachstumsprozessen. Veränderungen in der Cytokiniaktivität implizieren eine Rolle von Trockenheit vermeidenen Mechanismen der Pappel insbesondere in der Reduktion von Blattfläche.

Um das dritte Ziel zu erreichen, wurden drei *Populus nigra* Genotypen, die aus einem trockenen, einem mäßigen und einem feuchten Habitat stammen, unter Kontroll- und Trockenstress-Bedingungen angezogen und die Holzanatomie analysiert. Trockenheit führte zu reduzierter kambischer Aktivität, zu verminderter Gefäß- und Faserweite und zur gesteigerten Saccharifizierung. Zwischen dem Potential der Saccharifizierung und dem Ligningehalt sowie den meisten holzanatomischen Eigenschaften konnte kein Bezug festgestellt werden. Durch die RNA-Sequenzierung des entwickelnden Xylems zeigte sich, dass 1,5 % der analysierten Gene unterschiedlich unter Trockenheit exprimiert waren (DDEGs), während 67 % sich zwischen den Genotypen unterschieden (GDEGs). Durch die WGCNA (weighted gene correlation network analysis) wurden Module identifiziert, welche koexprimierte Gene enthielten, die mit dem Saccharifizierungsptential korrelierten. Diese Module bestanden hauptsächlich aus GO (gene ontology) Begriffen, welche im Zusammenhang mit der Biosynthese von Zellwandpolysacchariden, Modifizierung und Vesikeltransport stehen, jedoch aber nicht mit Ligninbiosynthese. Besonders Gene mit Regulationsfunktionen befanden sich unter den am stärksten sacchafizeriungskorrelierten Genen. Dabei wareninsbesondere Kinasen sehr auffällig. Des Weiteren wurden Transkriptionsfaktoren identifiziert, die eine unterschiedliche Transkripthäufigkeit zwischen den Genotypen zeigten und zudem, mit Genen der Biosynthese und Modifikation von Hemizellulosen und Pektinenkoreguliert waren.

Für das vierte Ziel wurde moderater Trockenstress für fünf Wochen an *P. nigra* Pflanzen angewandt. Die Pflanzen wurden wöchentlich geerntet und die Holzanatomie analysiert. Während der fünf Wochen Trockenheitsbehandlung wurden Radialwachstum, relative Weite des entwickelnden Xylems, Anzahl an kambialen Zelllagen und Lumenareal pro Gefäß, verglichen mit den Kontrollpflanzen, signifikant reduziert. Die anderen analysierten anatomischen Merkmale zeigten keinen signifikanten Trockenheitseffekt. Regressionsanalysen deckten signifikante positive Korrelationen zwischen Radialwachstum und Anzahl an kambialen Zelllagen, Radialwachstum und relativer Weite des entwickelnden Xylems, Radialwachstum und Lumenareal pro Gefäß und zwischen Anzahl an kambialen Zelllagen und relativer Weite des entwickelnden Xylems auf. Eine Analyse der Transkript-Abundanzen von Cytokinin-Antwort-Genen im Transkriptom des entwickelnden Xylems zeigte auf, dass die Antwort-Regulatoren RR7 und RR9 eine signifikante Reduzierung sowohl unter Trockenstress zeigten, als auch mit der Dauer des Experiments, was eine reduzierte Cytokinin-Signalübertragung unter Trockenheit vermuten lässt. Unter den Cytokinin-Biosynthese-Genen zeigten die Transkript-Abundanzen von IPT2, 5a und 5b Variationen mit der Zeit und nur marginale Induktion bei Trockenheit. Die Transkript-Abundanz des Cytokinin-Degradation-Gens CKX6 war bei Trockenheit signifikant erhöht. Die Analyse der Transkriptabundanzen von mit Cytokinin im Zusammenhang stehenden Genen zeigte eine reduzierte Cytokinin-Signalübertragung und erhöhte Degradierung bei Trockenheit. Da Cytokinine die zentralen Regulatoren der kambialen Entwicklung in Pappeln sind, könnte diese Reduktion der Cytokinin-Signalübertragung und -Level zur signifikant reduzierten Anzahl an kambialen Lagen geführt und damit signifikante Änderungen in der Holzanatomie bedingt haben, die den Pflanzen letztendlich helfen bei Trockenheit zu überleben.

Transgene Pappeln, transformiert mit dem *ARR5::GUS* Reporter Konstrukt wurden in dieser Studie zum ersten Mal bekannt gemacht und eingesetzt, um die Cytokinin-Aktivität auf Gewebe- und Zellebene während des aktiven Wachstums, während der Dormanz, und auch als Antwort auf Trockenheit zu beleuchten. Die hauptsächlichen Gewebe, welche Unterschiede in ihrer Cytokininaktivität unter den hier untersuchten verschiedenen Umweltbedingungen aufwiesen, waren Feinwurzeln, Kambium und Strahlenzellen. Die *ARR5::GUS* Pappel Reporter Linien können genutzt werden, um zukünftig die Beteiligung von Cytokininen an der Vermittlung von Wachstumszwängen und wachstumsfördernden Behandlungen für die vaskuläre Entwicklung und die Zelltypen-Identität zu erforschen.

Dabei könnten diese Pappeln ein wichtiges Mittel werden, um unser Verständnis der Produktion an Holzbiomasse zu steigern.

Chapter 1

Introduction

1.1 Wood formation

Wood is one of the most important renewable resources. It can be used for construction, pulping, paper making, burning for energy and lignocellulosic biofuel production. Together with the growing population, climate change and the need for having alternatives for fossil fuels, the demand for wood is also increasing (Buongiorno et al., 2011). The abundance of lignocellulose in wood makes it attractive for production of biofuels. Wood can be employed for the production of secondary biofuels by enzymatic saccharification and subsequent fermentation. Woody plants like poplar are of particular interest in this regard as they are of great importance as a second generation bioenergy crop (Allwright and Taylor, 2016). Poplars grown under short rotation coppice (SRC) have been extensively studied with regard to bioenergy production (Karp and Shield, 2008; Dickmann, 2006; Laureysens et al., 2004).

'Wood' is the secondary xylem which is produced as a result of the secondary growth in woody plants. Wood formation is dynamic and strongly affected by various exogenous and endogenous factors (Antonova and Stasova, 1997; Puech et al., 2000; Escalante-Perez et al., 2009; Wind et al., 2004; Luo et al., 2005; Deslauriers and Morin, 2005; Arend and Fromm, 2007; Nieminen et al., 2012). Exogenous factors that affect the wood formation are the natural environmental variations and abiotic stresses. Among the abiotic stresses faced by the plants, drought stress is of serious concern because periods of drought stress are likely to

increase along with the rising global temperature in many areas of the world (IPCC, 2007). Drought negatively affects wood growth and results in significant changes in wood anatomy in poplars such as, reduced cambial layers, vessel and fibre lumen, increased number of vessels and cell wall area (Bogeat-Triboulot et al., 2007; Arend and Fromm, 2007; Beniwal et al., 2010).

Wood, i.e., xylem comprises of the majority of secondary tissues in plants. It is produced by the activity of vascular cambium (Fig. 1). Secondary xylem is composed of three main cell types: (1) the interconnected xylem tracheary elements (vessel elements and tracheids), which enable water and solute transport (2) xylem fibres, having thick secondary cell walls and give structural support for the plants and (3) xylem parenchyma cells that does not possess a clear secondary cell wall and are involved in the storage of reserve food (Fig. 1) (Schuetz et al., 2013). A stem cross section of poplar highlighting the xylem parts is shown in Fig. 1.

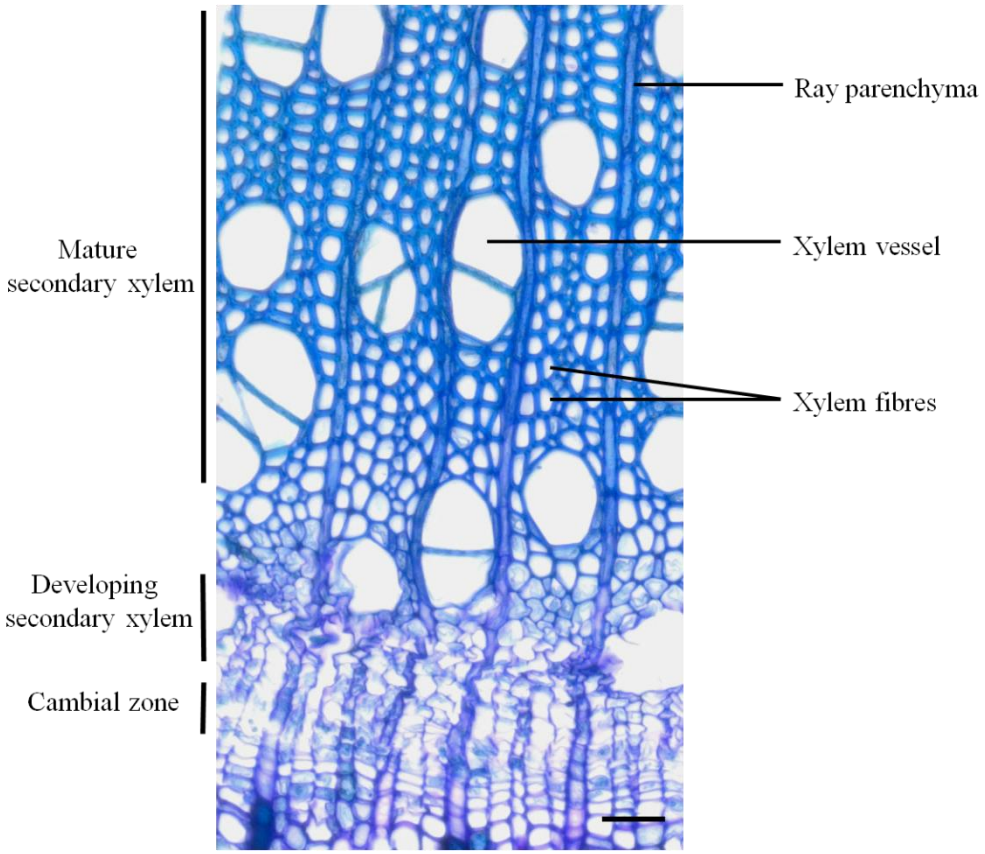

Figure 1. Secondary xylem in the stem cross section of *Populus nigra*. The cross section is stained using toluidine blue O solution. Scale = 50µm (Source S. Paul).

1.1.1. Intraspecific variation as a factor that affects wood properties

Ecotypes of a given plant species that are adapted to different environmental conditions show significant genetic differentiation as well as phenotypic variation in growth rates, plant architecture and leaf size (DeWoody et al., 2015; Viger et al., 2016). Morphological differences in these progenies are mainly due to the adaptation to local climate and significant genetic differentiation underlying the adaptive traits within the populations (DeWoody et al., 2015). The intraspecific differences and phenotypic variations of a plant species can also be expected in their wood anatomical adaptations to unfavourable environmental conditions for wood growth such as drought. A few studies reported intraspecific variation in wood anatomical traits (Guet et al., 2015) and also within hybrids of poplar (Harvey and Driessche, 1997; Fichot et al., 2009; Fichot et al., 2010; Schreiber et al., 2011). However, the molecular responses that lead to differences in wood anatomy among different genotypes are unknown.

1.1.2 Hormonal regulation of wood formation

Phytohormones and various factors acting downstream of hormones are the most important endogenous factors affecting wood growth (Nieminen et al., 2012). Phytohormones such as auxins, cytokinins, brassinosteroids, gibberellins, ethylene and strigolactones are reported to have a role in wood formation (Aloni 2001; Nieminen et al., 2008; Matsumoto-Kitano et al., 2008; Dayan et al., 2012; Sehr et al., 2010, Agusti et al., 2011; Sorce et al., 2013).

Auxins, in high concentration, stimulate cell division and maintain cambial cell identity while, in low concentration, they stimulate the expansive growth of xylem initials as well as the process of xylem cell maturation (Sorce et al., 2013; Sieburth and Deyholos, 2006; Moyle et al., 2002; Tuominen et al., 1997, Uggla et al., 1996). Auxins also help in the vessel density enhancement (Sorce et al., 2013; Lovisolo et al., 2002; Aloni, 2001). Cytokinins, along with auxins, stimulate cambial cell division and determine the vascular cell types other than protoxylem (Argyros et al., 2008; Nieminen et al., 2008; Matsumoto-Kitano et al., 2008; Hutchison et al., 2006; Yokoyama et al., 2007; Mähönen et al., 2000). Brassinosteroids promote division of procambial cells, programmed cell death and secondary cell wall deposition during tracheary element differentiation (Sorce et al., 2013; Turner et al., 2007; Cano-Delgado et al., 2004; Yamamoto et al., 2001; Fukuda, 1997; Demura and Fukuda, 1994; Iwasaki and Shibaoka, 1991). Gibberellins, along with auxins, stimulate cambial cell proliferation and elongation of xylem fibres (Sorce et al., 2013; Dayan et al., 2012; Ragni et al., 2011; Dayan et al., 2010; Björklund et al., 2007; Israelsson et al., 2005; Eriksson et al.,

2000; Wang et al., 1997). Abscisic acid, through a hypothesized negative interaction, inhibits the cambial growth (Little and Wareing, 1981). Ethylene promotes cambial cell division and expansion (Sorce et al., 2013; Love et al., 2009; Du and Yamamoto, 2003). Strigolactones also stimulate cambial cell divisions (Agusti et al., 2011). A simplified model of hormonal crosstalk which is operational in the division of cambial cells and xylem differentiation after Sorce et al. (2013) is presented in Fig. 2.

Cytokinins are of great importance among the phytohormones that play a role in wood formation since they are responsible for the increased sensitivity shown by cambium towards auxin, and thus, function as major regulators of wood quality and quantity (Aloni 1991, 2001). Therefore, the analysis of cytokinin activities is of particular interest because growth maintenance under unfavourable environmental conditions is the basis to enhance poplar productivity.

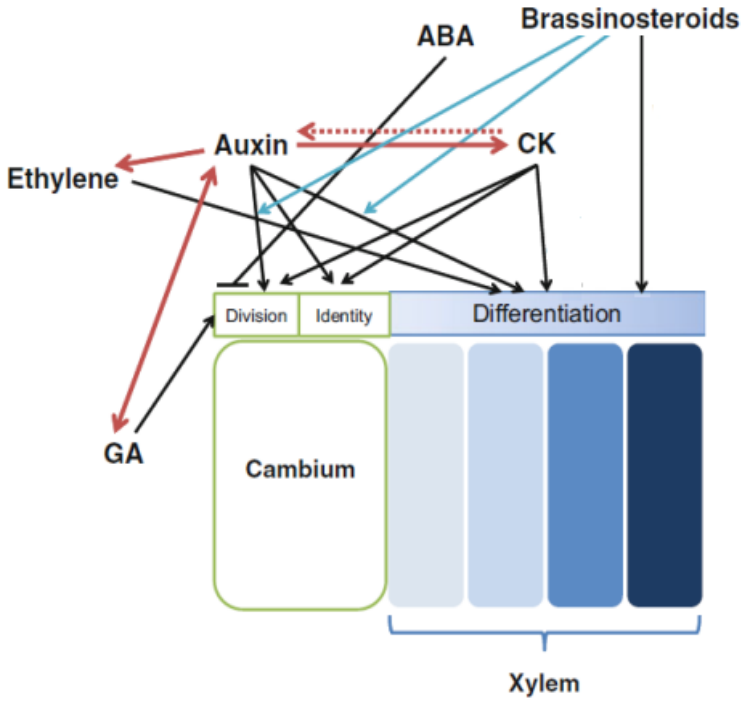

Figure 2. A simplified model of crosstalk between hormones operational in the identity and cell division of cambium and xylem differentiation. Black arrows show positive regulation, cyan arrows show positive interactions with hormone signaling pathways, red arrows show upregulation of hormonal metabolism, dotted red arrows show hypothesized regulation of hormonal metabolism. Here ABA is for abscisic acid, CK for cytokinins and GA for gibberellins (modified after Sorce et al., (2013)).

1.1.3 Functions of cytokinins in plants

Cytokinins are adenine derivatives and are synthesized primarily in root tips (Dieleman et al., 1997; Aloni et al., 2005; Miyawaki et al., 2004). Studies also indicate that cytokinins are locally synthesized in shoot tissues (Kamada-Nobusada and Sakakibara, 2009; Hirose et al., 2008; Sakakibara, 2006; Tanaka et al., 2006). Root-derived cytokinins are mainly tZ-type (trans-zeatin) and are transported via xylem sap acropetaly under the transpiration pull (Aloni et al., 2005) whereas shoot-derived cytokinins are mainly iP-type (isopentenyladenine) and are transported through phloem (Bishopp et al., 2011). ATP/ADP isopentenyltransferases (IPTs) catalyse an important step in the biosynthesis of cytokinin and they are also responsible for the biosynthesis of majority of the iP and tZ type cytokinins (Immanen et al., 2013).

Cytokinins with free bases are the active forms of cytokinins found in plant cells while they are also present in inactive forms as ribosides, ribotides and sometimes conjugated to glucose (Romanov et al., 2006; Mok and Mok, 2001). The proteins encoded by the *LONELY GUY (LOG)* gene family and the β-glucosidase enzymes convert the cytokinin ribotides and glycosyl conjugates respectively into active cytokinins (Kurakawa et al., 2007; Brzobohaty, 1993). Cytokinin oxidases/dehydrogenases (CKX) irreversibly inactivate active cytokinins (Galuszka et al., 2001). In *Arabidopsis, ABCG14* (Zhang et al., 2014; Ko et al., 2014), some of the *PUP* (purine permease) genes and *ENT* (equilibrative nucleoside transporter) genes have been reported to play an important role in cytokinin transport inside the plant body (Bürkle et al, 2003; Li et al., 2003).

Extensive studies have been carried out on the cytokinin perception and signaling in *Arabidopsis* and the involvement of a His-Asp phosphorelay, similar to that characterized in the bacterial two component phosphorelay pathway, has been understood (Mizuno, 2005; Schaller et al., 2008). Cytokinin receptors which are hybrid histidine kinases (AHKs) get autophosphorylated when cytokinins are perceived (Fig. 3). The histidine phosphotransfer proteins (AHP) receive the phosphoryl group from CK receptors and transfer them to nuclear type-B ARRs (*Arabidopsis* response regulators), the positive response regulators of cytokinin signaling pathway in *Arabidopsis*. This will turn on the type-A ARRs which are the primary response genes for cytokinin (Fig. 3). The targets of Type-A ARR genes include the genes involved in cell expansion, auxin (eg. SHY2/IAA3, AXR3/IAA17) and gibberellins (eg. GNL/CGA1/GATA22, GNC/GATA21) pathway, pathogen-responsive, light regulated (eg.

phy B) and cell cycle regulating (eg. cyclinD3) genes, thereby bringing about the secondary responses by cytokinin (Argueso et al., 2010). Type-A ARRs, together with type-B ARRs form cytokinin signaling negative feedback loops (Fig. 3). This negative feedback loop plays an important role to stabilize variations in signalling or to shut down the pathway activity suddenly in response to an unexpected stimuli (Müller, 2011).

Ten type-A RRs have been specified in *Arabidopsis* (Schaller et al., 2008; D'Agostino et al., 2000) while eleven have been reported in *Populus trichocarpa* (Immanen et al., 2013; Ramírez-Carvajal et al., 2008). They are transcriptionally upregulated by exogenous cytokinin treatment (Paul et al. 2016; D'Agostino et al., 2000). The induction by type-A RRs does not require *de novo* protein synthesis (D'Agostino et al., 2000). Their promoters have multiple type-B RR binding sites which turn out to be a reason for the strong upregulation due to cytokinin (Ramireddy et al., 2013; Taniguchi et al., 2006). Their induction is very

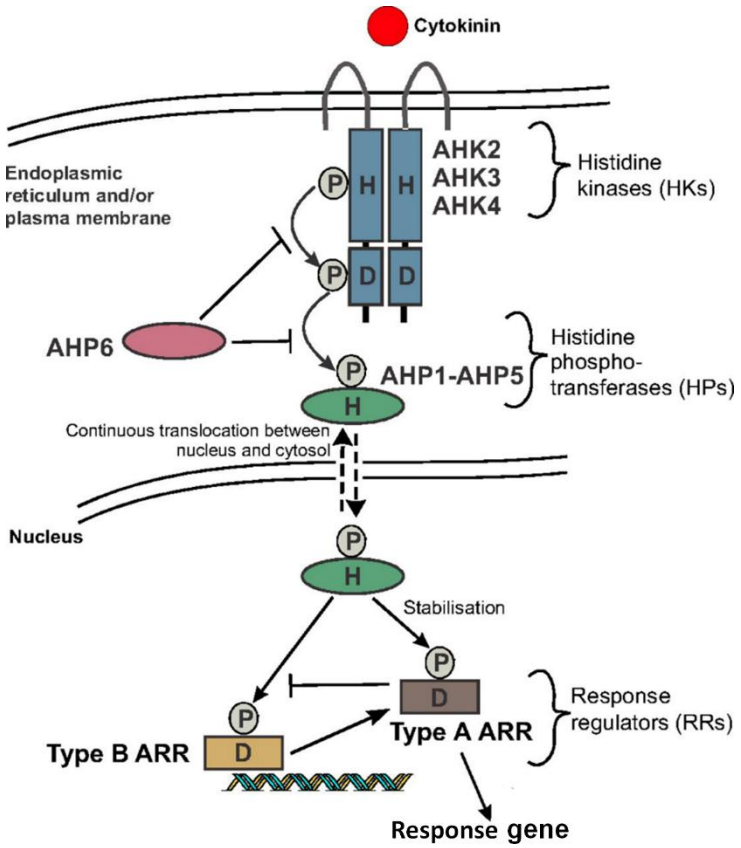

Figure 3. The two-component cytokinin signaling system in *Arabidopsis*. AHK stands for *Arabidopsis* histidine kinases which are the cytokinin receptors, AHP stands for *Arabidopsis* histidine phosphotransferase proteins which accept and transfer the phosphoryl group to the response regulators. ARR stands for *Arabidopsis* Response Regulators (modified after El-Showk et al., (2013)).

specific to cytokinin, i.e., they are not induced by any other phytohormones (Taniguchi et al., 1998). Because of these reasons type-A RR promoters, especially *ARR5* gene promoter, has been used in the reporter system development that visualize the cytokinin localization pattern in plants. Cytokinin localization has been reported in transgenic *Arabidopsis* seedling transformed with *ARR5::GUS* reporter construct (D'Agostino et al., 2000). This reporter gene construct was produced by comprising the GUS reporter gene of *Escherichia coli* encoding β–glucuronidase under the control of the *Arabidopsis ARR5* gene promoter (D'Agostino et al., 2000). Studies done with *ARR5::GUS* transformed transgenic *Arabidopsis* show that this construct helped to shed some light on the recent advances that has been made in cytokinin research (D'Agostino et al., 2000; Werner et al., 2003; Aloni et al., 2004; Lohar et al., 2004; Aloni et al., 2005; Aloni et al., 2006; Kudryakova et al 2008; Stolz et al., 2011; Kudryakova et al., 2013; Ko et al., 2014; Zhang et al., 2014). But a study on the tissue and cellular level active cytokinin localization in different parts of a woody plant like poplar was lacking when this thesis started.

"Cytokinins have roles in almost all aspects of plant growth and development including cell division, shoot initiation and growth, nutrient uptake, breaking of bud dormancy, delay of leaf senescence and regulation of vascular development" (Paul et al., 2016; Kieber and Schaller, 2014; Hwang et al., 2012). A major role of cytokinin in the vascular development is on promoting the protoxylem differentiation and the cambium development (Dettmer et al., 2009). Mähönen et al. (2000) showed a role for the phytohormone, cytokinin, in vascular development in the model plant, *Arabidopsis*, on the basis of the analysis of the *woodenleg* (wol) mutant that was characterized with a root having mainly protoxylem, but both phloem and metaxylem were absent. Other studies which also used mutations that reduced cytokinin signaling showed a similar root vasculature comprising mainly of protoxylem (Argyros et al., 2008; Yokoyama et al., 2007; Hutchison et al., 2006). Thus, cytokinins determine the vascular cell types other than protoxylem. Cytokinins play a main role in the specification of vascular pattern by controlling the amount of PIN auxin efflux proteins (Bishopp et al., 2011). High cytokinin signaling promotes the expression of PIN7 which will in turn regulates the radial distribution of PIN3 and PIN1 in the root meristem which creates an auxin signaling maximum in the xylem axis and eventually specifies protoxylem identity (Bishopp et al., 2011).

The positive role of cytokinins in the cambium development is backed by studies where a complete absence of cambium in the stem and root was noted due to the disruption of

multiple *IPT* genes, involved in the biosynthesis of cytokinins in *Arabidopsis* (Matsumoto-Kitano et al., 2008). A high expression peak for the genes involved in cytokinin signaling was observed in the actively dividing cells of cambium of *Populus* and *Betula* and a reduction in endogenous cytokinin levels by ectopic expression of cytokinin degrading enzyme, cytokinin oxidase 2 (*CKX2*) in the cambial cells resulted in impaired cambial growth in poplar (Nieminen et al., 2008). Also cytokinins determine wood quantity and quality by increasing the cambial sensitivity to auxin (Aloni 1991, 2001). Our knowledge about cytokinins, an important plant hormone involved in stimulating cambial activity and wood production, comes mainly from studies done on the herbaceous annual plant, *Arabidopsis*. More studies to this end have to be conducted on model woody perennial plants like poplar.

1.1.4 Environmental fluctuations in cytokinin levels: Tree physiology and wood production undergoes seasonal changes**.** Therefore, in "trees, changes in endogenous cytokinin levels in relation to seasonality have been studied for a long time. Most of these studies focused on the endogenous cytokinin levels in xylem or phloem sap of the trees (Hewett and Wareing, 1973; Alvim et al., 1976; Weiler and Ziegler, 1981; Tromp and Ovaa, 1990; Cook et al., 2001) or reported the endogenous cytokinin concentrations in different organs (Hewett and Wareing, 1973; Van Staden and Dimalla, 1981; Cook et al., 2001). Furthermore active and inactive forms of cytokinins were distinguished (Hewett and Wareing, 1973; Van Staden and Dimalla, 1981; Tromp and Ovaa, 1990) and their changes were related to seasonal fluctuations (Tromp and Ovaa, 1990). For example, in the xylem sap of apple trees, the active trans-zeatin type (tZ) levels were high during the growing season, dropped during dormancy and showed an increase during bud burst, whereas they continued to increase during the growing season (Tromp and Ovaa, 1990)" (Paul et al., 2016).

Even though knowledge about cytokinin levels in xylem and phloem sap under different seasons gives us information on cytokinin levels on a whole plant basis, it is unclear in which tissue and cell type active cytokinins are localized during active growth phase and dormancy. Active cytokinin localization throws light on the specific tissues and cells and thus may be related to the growth pattern.

Cytokinins also play important roles in response to drought. "Plants respond to environmental constraints by physiological and morphological adjustments such as decreases in stomatal conductance and reduced plant growth. Since cytokinins are negative regulators of root meristem activity and positive regulators of shoot meristem activity (Werner et al. 2003), it is

conceivable that growth reduction under drought involves altered phytohormone levels. For example, a decreased content of cytokinins was found in alfalfa under drought (Goicoechea et al. 1996) which was accompanied by accelerated leaf senescence. The xylem sap of sunflower contained decreased cytokinin concentrations under drought (Bano et al. 1994; Shashidhar et al. 1996; Hansen and Dörffling 2003). Because drought-stressed plants often show increased root production and decreased shoot growth, it has been suggested that enhanced drought tolerance can be achieved by decreasing cytokinin levels through overexpression of systemic or root-specific cytokinin-degrading enzymes (Werner et al. 2010; Nishiyama et al. 2011; Mackova et al. 2013). In contrast to this proposal, rice (Peleg et al. 2011), tobacco (Rivero et al. 2007), peanut (Qin et al. 2011) and cotton plants (Kuppu et al. 2013) transformed with the Agrobacterium *IPT* gene, i.e. a cytokinin biosynthetic gene, under a stress inducible (SARK) promoter resulted in enhanced drought tolerance. These findings show that cytokinins play an important role in drought susceptibility although the underlying mechanisms are not yet fully understood (Peleg and Blumwald, 2011; Zwack and Rashotte, 2015)" (Paul et al. 2018). Also, it is not yet known whether the morphological alterations caused by drought are accompanied by changes in cytokinin activity.

1.2 Scope of this study

The identified research gaps include the need (i) to localize tissue and cellular level active cytokinins in different parts of a woody perennial plant like poplar in seasons of active growth and dormancy (ii) to study the drought response of active cytokinins at the tissue and cellular level in different parts of poplar and (iii) to study the intraspecific variations in drought induced wood anatomical traits and molecular responses underlying them. To address these research gaps, this study has been designed to address the following main questions:

(1) Is there any change in the tissue and cell-specific cytokinin activity in different organs of *Populus* × *canescens* during the seasons, summer and winter?

To address this question *ARR5::GUS* poplar reporter lines were grown in field conditions for one year. Tissue level active cytokinin localization area was monitored in different organs of poplar during active growth phase in summer and also in dormancy. A special focus was the cellular level localization of active cytokinins along the different stem positions. Here in this study, the *ARR5::GUS* construct was used for the first time in a woody plant, poplar. The

results from this study were expected to give an insight into the sites of cytokinin activity particularly during dormancy. This study comprises the chapter 2 of this thesis and it was published as Paul et al., (2016).

2. Is there any drought induced variations in the active cytokinin localization at tissue and cellular level in different organs of *P.* × *canescens*?

Here active cytokinin localization was monitored in different organs of poplars grown under well-watered and drought-stressed conditions. Active cytokinin was localized in the different cell types along the stem. The drought-induced changes in the cytokinin localization patterns were compared with physiological, morphological and growth responses to drought. This study comprises the chapter 3 of this thesis and it was published as Paul et al. (2018).

3. Is there intraspecific variation in the drought induced changes in wood anatomical traits in *P. nigra* and what are the molecular responses underlying them?

To address this question, a drought treatment was applied to *Populus nigra* genotypes originating from a dry, mesic or wet habitat. Drought-induced wood anatomical changes were compared between three genotypes. Genes and co-expressed gene clusters related to genotypes and drought-induced variation in wood traits were analyzed. The study comprises the chapter 4 of this thesis and it was published in Wildhagen et al. (2017).

4. How do the drought-induced wood changes in wood anatomy progress in a time dependent manner? Are these changes accompanied by variations in transcript abundance of genes related to cytokinin signaling, biosynthesis and degradation?

To address these questions, a five-week moderate drought treatment was applied to *P. nigra* plants. The plants were harvested weekly and wood anatomy was analysed. A possible relation between these changes and the transcript abundance of genes belonging to cytokinin primary response, biosynthesis and degradation in the transcriptome of developing xylem was also examined. The study has been described in detail in the chapter 5 of this thesis.

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Chapter 2

Tissue- and cell-specific cytokinin activity in *Populus* × *canescens* monitored by *ARR5::GUS* reporter lines in summer and winter

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2.1 Declaration

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Chapter 3

Drought effects on the tissue- and cell-specific cytokinin activity in poplar

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Chapter 4

Genes and gene clusters related to genotype and drought-induced variation in saccharification potential, lignin content, and wood anatomical traits in *Populus nigra*

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4.1 Declaration

I contributed to harvest of plants, anatomical analyses, description of these data and their interpretation and writing the manuscript (introduction, materials and methods, results and discussion sections related to wood anatomical analyses).

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Chapter 5

Drought-induced changes in wood anatomical traits - a time course study

5.1 Introduction

Drought brings about morphological, physiological, biochemical and anatomical changes in a plant. These changes will eventually help the plant for its survival under drought. Among these changes, drought-induced wood anatomical changes are important as xylem is involved in water transport. Drought-induced anatomical changes have been studied in many plants including poplar. In poplar, drought stress has massive consequences for the wood anatomy and cell wall metabolism (Le Gall et al., 2015; Guet et al., 2015; Cao et al., 2014; Schreiber et al., 2011; Beniwal et al., 2010; Fichot et al., 2010, 2009; Arend and Fromm, 2007; Harvey and Van Den Driessche, 1997). For example, drought affects the cambial cells and their derivatives (Arend and Fromm, 2007; Le Gall et al., 2015). Drought results in significantly greater number of vessels with smaller lumen area, reduced fibre lumen area and increased cell wall area in poplars (Beniwal et al., 2010; Arend and Fromm, 2007). A study on the time-dependent progression of drought-induced variations in wood anatomical traits of *Populus euphratica*, a species growing in semiarid areas, has been conducted by Bogeat-Triboulot et al. (2007). The study reports significant drought-induced reduction in vessel and fibre lumen and an increase in the fibre cell wall thickness were noted on a weekly basis under varying drought levels (Bogeat-Triboulot et al., 2007). The significant drought-induced changes were evident in the plants harvested after the first week of drought treatment (Bogeat-Triboulot et al., 2007).

However, studies on the relation between time-dependent drought-induced wood anatomical changes and cytokinin-related gene expression in the transcriptome of developing xylem are lacking. Since the sensitivity of the cambial cells to auxin is increased by cytokinins, and thereby wood quantity and quality are determined (Paul et al., 2016; Aloni 1991, 2001), we expect that the analysis of genes involved in cytokinin metabolism and signaling can give insights into the cytokinin involved molecular mechanisms underlying drought-induced wood anatomical changes. The main goal of this study was to investigate the drought-induced variations in wood anatomical traits of *Populus nigra* on a time-dependent manner (weekly). Here a gradual drought treatment was applied by withholding water supply and thereafter constant drought levels were maintained. In addition to wood anatomy, the transcript abundance of cytokinin primary response genes (type-A Response Regulators (*RRs*)), biosynthesis genes (isopentenyltransferases; *IPTs*) and degradation genes (cytokinin oxidases/dehydrogenases; *CKXs*) in the transcriptome of developing secondary xylem were examined.

5.2 Materials and methods

5.2.1 Plant cultivation and drought treatment

The plant material, used for this experiment are the *Populus nigra* L.(origin- La Zelata; IT1) plants. Planting in the greenhouses, cultivation and drought treatment were conducted as described in Wildhagen et al. (2017). Air temperature and daily water consumption per plant was recorded regularly during the treatment period (Unpublished data were provided by Bogeat-Triboulot M.B. [INRA, France]).Water consumption per pot was determined as the amount of water added to each pot with plants. Water loss determined for control pots without plants were subtracted from this.

5.2.2 Harvests

During the five-week treatment, harvests were conducted at the end of each week. The collection of samples for molecular analyses (developing xylem) and wood anatomical analyses (2-3 cm stem bottom) from four plants per treatment, were collected as described in Wildhagen et al. (2017). The same plants were used for wood anatomical analyses and extracting RNA.

5.2.3 Plant radial growth

The stem diameter was measured weekly twice on four biological replicates by taking photos of the stem bottom with an attached scale bar at a fixed position. The software ImageJ was then used to determine stem diameter (Schneider et al., 2012). Based on these measurements, the weekly radial growth increment was calculated.

5.2.4 Wood anatomical analyses

For wood anatomical analyses, stem cross sections of 10 µm thickness stained with 0.05% (w/v, pH = 7.0) toluidine blue O solution (O'Brien et al. 1964) were photographed with the help of a digital camera (Axio Cam MRC, Carl Zeiss Microimaging GmbH, Göttingen, Germany) which was attached to a microscope (Axioplan Observer.Z1, Carl Zeiss GmbH, Oberkochen, Germany).

Image J was used to analyse the images (Schneider et al. 2012). Areas of 90, 000 μm^2 (in the form of two rectangles of 100 µm width × 450 µm length) or of 100 µm width× 200 µm length in the mature secondary xylem adjacent to the developing xylem region were used for trait measurements of vessel frequency, average vessel lumen area, predicted conductivity, vessel wall thickness, fibre frequency, average fibre lumen area and fibre double wall thickness, percentage of cell wall area of vessels and fibres and number of cambial cell layers. The predicted hydraulic conductivity of vessels in the analysed xylem area ($0.09 mm^2$) was calculated as $\Sigma r^4\ mm^{-2}$ according to Hagen–Poiseuille law (Zimmermann, 1983). For this, radius of each vessel was derived from the vessel lumen area (A). By approximating the individual vessel lumens (only full vessels) as circles, the radius of each vessel (r) was calculated using the formula:

$$r = \sqrt{\frac{A}{\pi}}$$

Four biological replicates per treatment and week were analyzed. The wood anatomical analyses are described in detail in Wildhagen et al. (2017).

5.2.5 Transcriptome profiling

Here a processed count table with RNA seq data was obtained from the WATBIO project. Briefly, total RNA was extracted from homogenized samples of developing xylem of four biological replicates per treatment and time point using the CTAB protocol (Chang et al.

1993). RNA was used for library preparation and sequenced in 50 bp single-end mode at 6-fold multiplex on the Illumina HiSeq2000 (Illumina, San Diego, CA, USA). After sequencing, filtering, normalization and annotation, a normalized count table was generated, from which type-A *RR* genes, cytokinin biosynthesis and degradation genes were extracted and further analysed. Library preparation, sequencing, filtering, normalization and annotation were performed as described in Wildhagen et al. (2017).

The count table was used for the extraction of data for cytokinin type-A *RRs*, *IPTs* and *CKX* genes. A detailed gene list that was used for transcript abundance analyses has been provided in Table 1.

5.2.6 Statistical analyses

Statistical analyses were conducted using the free statistical software R v3.1.1 (R Core Team 2015). Two-way ANOVA was conducted for daily water consumption and cumulative water consumption per plant with drought and time as fixed factors and plant number as random factor to account for repeated measurements.

For wood anatomical analyses, Two-factorial mixed linear models with 'drought' and 'time' included as main and interaction effect and also a random effect for greenhouse chamber were fitted to the data using the function 'lme', package 'nlme' (Pinheiro et al., 2015). Normality and homogeneity of variance were checked visually by plotting residuals. Logarithmic (log2) transformation (vessel frequency, vessel wall thickness, fibre lumen area per fibre, fibre double wall thickness and relative width of developing xylem) or square root transformation (predicted conductivity) was done to meet these criteria. Homogeneous subsets were calculated using Post-hoc Tukey HSD test. Regression analyses were done using the R function 'cor.test'.

A Two-way ANOVA was conducted for transcript abundance data for each gene with drought and time as factors. The main effects (drought, time) and their interaction effect were tested. Homogeneous subsets were identified using a post-hoc Tukey HSD test computed for drought-time interaction.

Table 1. Poplar genes that belong to the two component type-A response regulator gene family (Paul et al., 2016), cytokinin biosynthesis and degradation (Immanen et al., 2013), that were used for analysis of transcript abundance in the transcriptome of developing xylem. The homolog of each gene in *Arabidopsis* was procured by blasting the protein sequence obtained from Phytozome for each poplar gene, into TAIR (www.arabidopsis.org).

***Populus trichocarpa* gene name**	***Populus trichocarpa* gene ID**	***Arabidopsis* gene name**	**AGI**	**References**
Type-A Response Regulators (Cytokinin signalling)				
PtRR1	Potri.010G037800	*ARR3*	AT1G59940	Paul et al. 2016
PtRR2	Potri.008G193000	*ARR3*	AT1G59940	
PtRR3	Potri.002G082200	*ARR9*	AT3G57040	
PtRR4	Potri.003G197500	*ARR9/ ARR8*	AT3G57040/ AT2G41310	
PtRR5	Potri.001G027000	*ARR9/ ARR8*	AT3G57040/ AT2G41310	
PtRR6	Potri.006G041100	*ARR9*	AT3G57040	
PtRR7	Potri.016G038000	*ARR8*	AT2G41310	
PtRR8	Potri.019G058900	*ARR17*	AT3G56380	
PtRR9	Potri.013G157700	*UCP030365*	AT5G05240	
PtRR10	Potri.015G070000	*ARR5*	AT3G48100	
PtRR11	Potri.019G133600	*ARR17*	AT3G56380	
ATP/ ADP Isopentenyl transferases (Cytokinin biosynthesis)				
PtIPT2	Potri.009G147600	*AtIPT2*	AT2G27760	Immanen et al., 2013
PtIPT3	Potri.014G139300	*AtIPT3*	AT3G63110	
PtIPT5a	Potri.008G202200	*AtIPT5*	AT5G19040	
PtIPT5b	Potri.010G030500	*AtIPT5*	AT5G19040	
PtIPT6a	Potri.008G121500	*AtIPT1*	AT1G68460	
PtIPT6b	Potri.010G123900	*AtIPT1*	AT1G68460	
PtIPT7a	Potri.004G150900	*AtIPT5*	AT5G19040	
PtIPT7b	Potri.008G033300	*AtIPT5*	AT5G19040	
PtIPT9	Potri.001G200000	*Protein kinase*	AT3G13690	
Cytokinin oxidase/ dehydrogenases (Cytokinin degradation)				
PtCKX1a	Potri.006G047900	*AtCKX1*	AT2G41510	Immanen et al., 2013
PtCKX1b	Potri.016G044100	*AtCKX1*	AT2G41510	
PtCKX3a	Potri.006G152500	*AtCKX3*	AT5G56970	
PtCKX3b	Potri.007G066100	*AtCKX3*	AT5G56970	
PtCKX5a	Potri.002G030500	*AtCKX5/ AtCKX6*	AT1G75450	
PtCKX5b	Potri.005G232300	*AtCKX5/ AtCKX6*	AT1G75450	
PtCKX6	Potri.003G203600	*AtCKX6/ AtCKX7*	AT3G63440	
PtCKX7	Potri.006G221000	*AtCKX5/ AtCKX7*	AT5G21482	

5.3 Results

5.3.1 Drought resulted in reduced daily water loss as well as water consumption per plant

Drought resulted in greater daily water consumption per pot than that of the controls (Fig. 1A). The cumulative water consumption per plant for drought plants was lower than that of the controls (Fig. 1B). The drought-induced reduction in daily water consumption and cumulative water consumption per plant was evident after the first week of drought treatment.

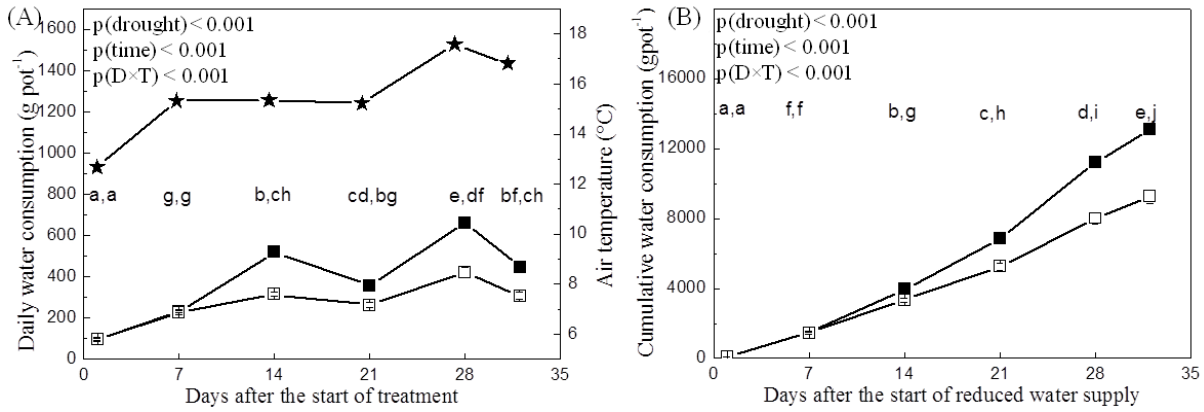

Figure 1. Daily water consumption and cumulative water consumption per plant during the treatment period. Data shown are mean ± SE. Representative data of n = 10 plants per treatment and time point are shown. The closed squares represent the controls while the open squares represent the drought-treated. The star symbol represents the air temperature. Letters indicate homogenous subsets of drought-time interaction identified by post-hoc Tukey HSD test. In each pair of homogeneous subsets at every time point, the first letter(s) belongs to the control group and the second letter(s) belongs to the drought group.

5.3.2 Time-dependent progression of drought-induced variation in wood anatomical traits

Drought resulted in a significantly reduced radial growth of plants (Table 2). The drought-treated plants showed a reduction in radial growth by 6.7% when compared to that of controls. Regarding the wood anatomical traits, drought significantly reduced the relative radial width of developing xylem by 6.8% than that of controls (Table 2). The cambial cell layers were also reduced significantly under drought (Table 2). A reduction of 19.1% in the

number of cambial cell layers was observed under drought than the controls. Lumen area per fibre was significantly reduced under drought by 6.3% when compared to that of controls.

Significant variation with time was noted in case of some traits in control plants (Table 2). The radial growth of controls showed a decrease of 11.2% over the period of the experiment (Table 2). Relative width of developing xylem also varied with time. During the experiment, controls showed a decline of 38.2% in the relative width of developing xylem (Table 2). Over the experiment period, the cambial cell layers of control plants were reduced by 4.1% (Table 2). Among the vessel traits, vessel frequency and vessel wall thickness showed significant variation with time. Vessel frequency of the controls showed an increase of 67.1% over the treatment period (Table 2). A decrease of 8.5% was observed in the vessel wall thickness of the controls (Table 2). Lumen area per fibre of the controls first showed a reduction for four weeks of the treatment period and then showed an increase (Table 2). The fibre double wall thickness of the controls showed a 35.7% increase during the five weeks (Table 2).

Anatomical traits like vessel lumen area per vessel, vessel diameter, predicted conductivity, fibre frequency and cell wall area of vessels and fibres, showed no significant effect for any of the main factors or interaction factor (Table 2).

There was no significant drought-time interaction effect in any of the analyzed wood anatomical traits except for the trait, number of cambial cell layers. The variation with time observed for some traits in controls, the absence of drought-time interaction and no significant drought effect for many of the traits except radial width of developing xylem, number of cambial cell layers and lumen area per fibre, suggest that the control plants also experienced water deficit to some extent.

Overview of the stem cross sections of *P.nigra* under well-watered and drought conditions during the five-week treatment period have been shown in Fig. 2 and 3.

Regression analyses revealed significant correlations between radial growth and number of cambial cell layers, radial growth and relative width of developing xylem, radial growth and lumen area per fibre, number of cambial cell layers and relative width of developing xylem, and number of cambial cell layers and lumen area per fibre (Fig. 4). There was no significant correlation between lumen area per fibre and relative width of developing xylem (Fig. 4)

Table 2. Wood anatomical traits of *Populus nigra,* exposed to a control or drought treatment for five weeks. Mean ± SE are given for n = 4 biological replicates per treatment and time. P-values as computed for 2-factorial linear models including 'drought' and 'time' main effects and the drought-week interaction effect (D×T). Letters indicate homogenous subsets of 'drought-time' interaction identified by post-hoc tests. Here W1 to W5 represent the five weeks of the drought treatment respectively (W1 denotes the harvest point after one week of start of drought treatment).

Anatomical trait	**Time**	**Mean ± SE**		**P-value**		
		Control	**Drought**	**Drought**	**Week**	**D×T**
Radial growth ($mmweek^{-1}$)	W1	1.43 (± 0.10)b	1.06 (± 0.07)bc	**1.0E-04**	**1.0E-04**	0.846
	W2	1.14 (± 0.10)bc	0.76 (± 0.05)ac			
	W3	1.18 (± 0.18)bc	0.98 (± 0.11)abc			
	W4	0.72 (± 0.03)ac	0.48 (± 0.08)a			
	W5	1.27 (± 0.14)bc	0.87 (± 0.21)abc			
Relative radial width of developing xylem (%)	W1	7.07 (± 0.65)c	5.20 (± 0.44)bc	**<0.0001**	**<0.0001**	0.946
	W2	4.65 (± 0.43)abc	3.44 (± 0.51)ab			
	W3	4.65 (± 0.36)abc	3.39 (± 0.37)ab			
	W4	3.88 (± 0.55)ab	2.87 (±0.30)a			
	W5	4.37 (± 0.33)abc	2.86 (±0.31)a			
Number of cambial cell layers	W1	7.3 (± 0.3)bc	6.3 (± 0.3)abc	**<0.0001**	**0.003**	**0.031**
	W2	7.8 (± 0.3)c	6.5 (± 0.3)bc			
	W3	7.0 (± 0.6)bc	6.8 (± 0.3)bc			
	W4	6.5 (± 0.3)bc	6.0 (± 0.0)ab			
	W5	7.0 (± 0.4)bc	4.8 (± 0.3)a			
Vessel frequency (vessel number mm^{-2})	W1	152 (± 08)ab	161 (± 17)ab	0.127	**0.001**	0.213
	W2	182 (± 14)abc	146 (± 15)b			
	W3	166 (± 16)abc	209 (± 14)abc			
	W4	198 (± 17)abc	286 (± 41)ac			
	W5	254 (± 46)abc	315 (± 64)c			
Lumen area per vessel (μm^2)	W1	1135.8 (± 171.5)a	1099.7 (± 80.5)a	0.124	0.299	0.404
	W2	1032.8 (± 225.0)a	1135.2 (± 32.4)a			
	W3	1265.9 (± 167.5)a	971.2 (± 22.9)a			
	W4	1249.6 (± 128.1)a	829.9 (± 80.6)a			
	W5	879.2 (± 146.9)a	800.3 (± 228.4)a			
Vessel diameter (µm)	W1	35.93 (± 3.06)a	35.27 (± 1.57)a	0.209	0.342	0.467
	W2	33.19 (± 3.85)a	35.98 (± 0.93)a			
	W3	37.38 (± 3.22)a	32.86 (± 0.59)a			
	W4	37.10 (± 1.83)a	30.22 (± 1.62)a			
	W5	31.25 (± 2.79)a	29.43 (± 4.75)a			

Predicted conductivity (mm^2)	W1	2.0E-06 (± 5.2E-07)a	1.9E-06 (± 2.1E-07)a	0.076	0.141	0.326
	W2	1.9E-06 (± 6.9E-07)a	1.9E-06 (± 1.0E-07)a			
	W3	2.5E-06 (± 4.3E-07)a	1.5E-06 (± 1.3E-07)a			
	W4	2.5E-06 (± 4.6E-07)a	1.1E-06 (± 1.9E-07)a			
	W5	1.2E-06 (± 3.5E-07)a	1.1E-06 (± 5.0E-07)a			
Vessel wall thickness (µm)	W1	1.30 (± 0.09)a	0.99 (± 0.05)a	0.097	**0.025**	0.086
	W2	0.98 (± 0.04)a	1.00 (± 0.08)a			
	W3	1.16 (± 0.03)a	1.08 (± 0.05)a			
	W4	1.13 (± 0.13)a	1.06 (± 0.04)a			
	W5	1.19 (± 0.08)a	1.25 (± 0.05)a			
Fibre frequency (fibre number mm^{-2})	W1	5019 (± 440)a	4677 (± 775)a	0.945	0.887	0.558
	W2	4566 (± 572)a	4287 (± 281)a			
	W3	4313 (± 431)a	5428 (± 551)a			
	W4	4557 (± 791)a	4497 (± 329)a			
	W5	5056 (± 552)a	4504 (± 359)a			
Lumen area per fibre ($µm^2$)	W1	86.2 (± 2.2)b	77.6 (± 3.3)ab	**0.032**	**0.024**	0.264
	W2	80.9 (± 3.2)ab	83.8 (± 7.6)ab			
	W3	73.0 (± 3.3)ab	62.7 (± 4.4)a			
	W4	77.6 (± 7.4)ab	77.4 (± 5.4)ab			
	W5	92.4 (± 7.3)b	72.7 (± 2.7)ab			
Fibre double wall thickness (µm)	W1	2.69 (± 0.02)a	2.92 (± 0.27)ab	0.331	**2.0E-04**	0.692
	W2	2.72 (± 0.21)ab	2.60 (± 0.05)a			
	W3	2.93 (± 0.25)ab	2.95 (± 0.13)ab			
	W4	3.02 (± 0.06)ab	3.47 (± 0.06)ab			
	W5	3.65 (± 0.21)b	3.70(± 0.03)b			
Cell wall area of vessels and fibres (%)	W1	29.2 (± 2.4)a	32.4 (± 1.9)a	0.464	0.6	0.312
	W2	36.1 (± 1.4)a	31.4 (± 3.1)a			
	W3	32.5 (± 1.6)a	35.4 (± 1.1)a			
	W4	31.9 (± 4.0)a	31.4 (± 1.8)a			
	W5	31.2 (± 3.3)a	36.1 (± 1.8)a			

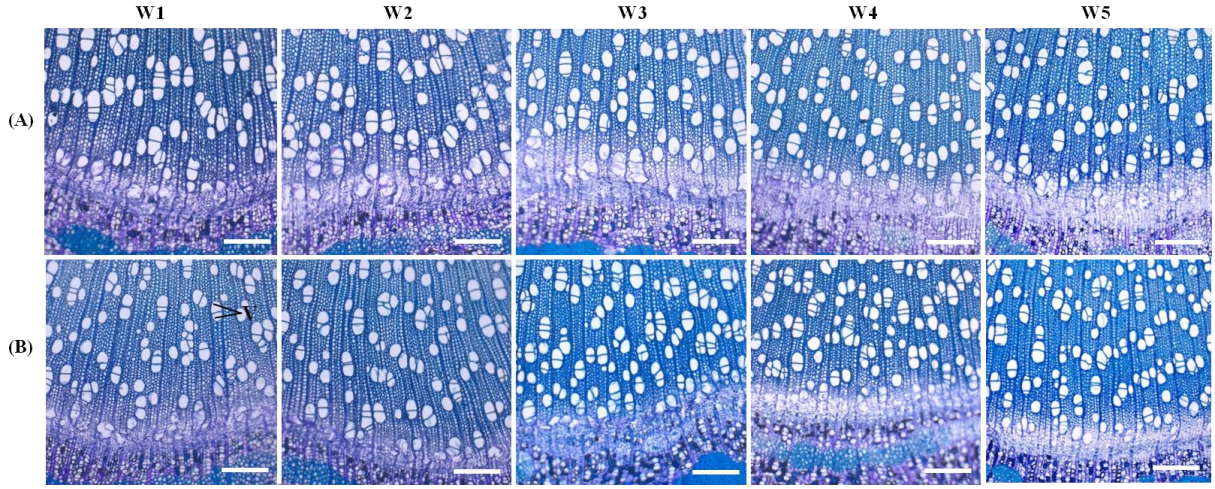

Figure 2. Overview of the stem cross sections of *P.nigra* under well-watered and drought conditions during the five-week treatment period. Representative pictures of n = 4 biological replicates are shown. Row (A) represents cross sections from control plants while row (B) represents those from drought-treated plants. Here 'v' represents vessels. Pictures taken at 100× magnification are shown. Scale bar= 200µm.

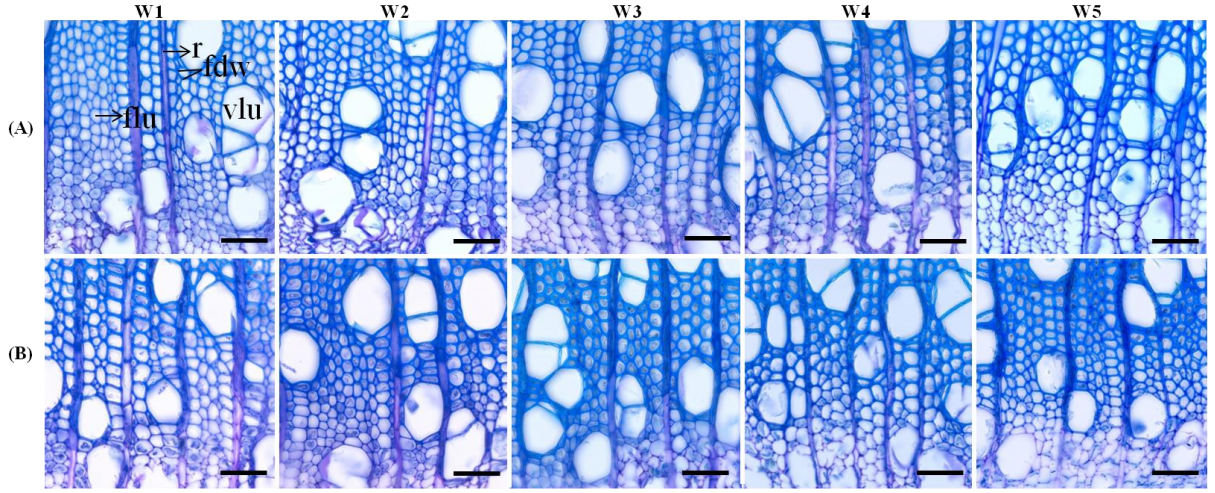

Figure 3. Overview of the stem cross sections of *P.nigra* under well-watered and drought conditions during the five-week treatment period. Representative pictures of n = 4 biological replicates are shown. Row (A) represents cross sections from well-watered plants while row (B) represents those from drought-treated plants. Here 'vlu' represents vessel lumen area, 'flu' represents fibre lumen area, 'r' represents ray parenchyma and 'fdw' represents fibre double wall thickness. Cambium is towards the bottom in each figure. Pictures taken at 400× magnification are shown. Scale bar = 50µm.

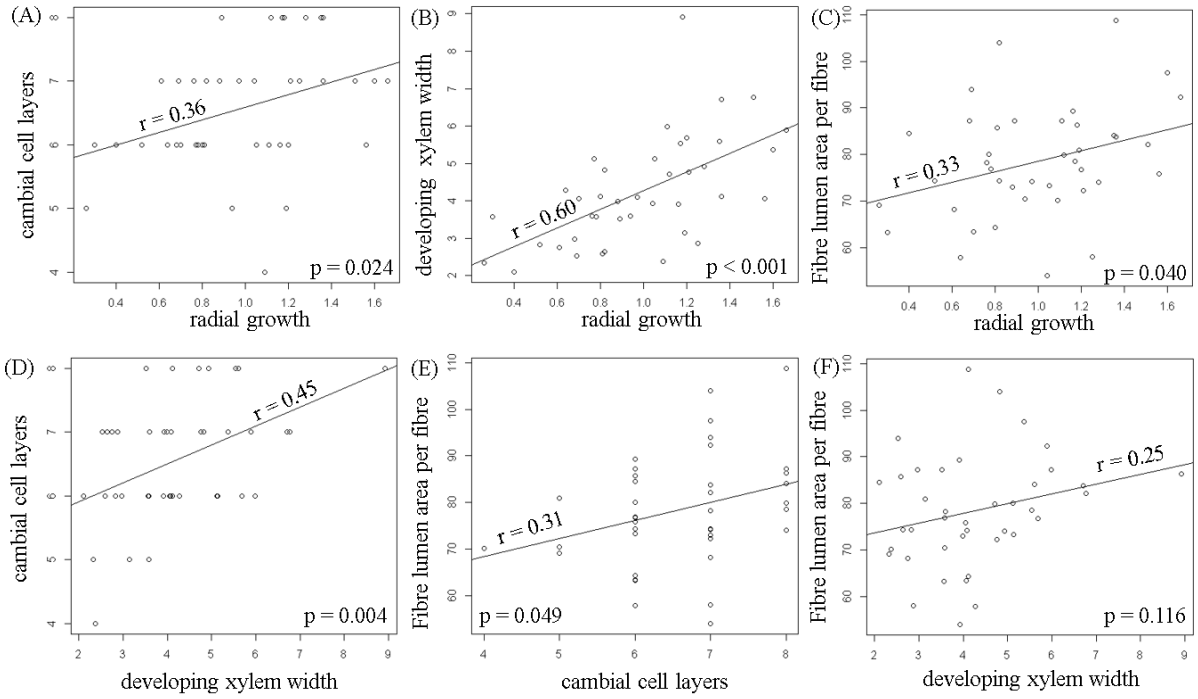

Figure 4. Correlation between drought responsive traits of *Populus nigra*. The letter 'r' denotes Pearson's correlation coefficient. P-value for the correlation test is also shown.

5.3.3 Analysis of transcript abundance of cytokinin-related genes in the transcriptome of the developing xylem under progressive drought

5.3.3.1 Transcript abundance of poplar type-A RR genes

Among the eleven type-A *RRs* in poplar, the transcript abundance of two of the type-A *RR* genes, *RR7* and *RR9* (Fig. 5A, B) showed a significant decline under drought as well as with the duration of the experiment. Among these genes, transcript abundance of *RR9* showed an additional significant drought-time interaction effect. The transcript abundance of *RR10* showed only a drought-time interaction effect during the second and third week of drought treatment (Fig. 5C).

The expression of *RR 1, 2, 3, 4, 6* and *8* showed a significant variation only with time (Fig. 6). Here most of these genes showed lower transcript levels in W5 than in other weeks. The genes *RR5* and *RR11* showed no significant variations.

5.3.3.2 Transcript abundance of cytokinin biosynthetic genes (IPTs)

Among the nine IPTs (*IPT 2,3, 5a, 5b, 6a, 6b, 7a, 7b* and *9*) found in poplar, the transcript levels of none of the IPTs showed a significant drought effect. The transcript abundance of

genes *IPT 2*, *5a* and *5b* showed variation with time and only a marginal stimulation under drought (Fig. 7). No significant drought-week interactions were seen in the case of expression of the *IPTs*.

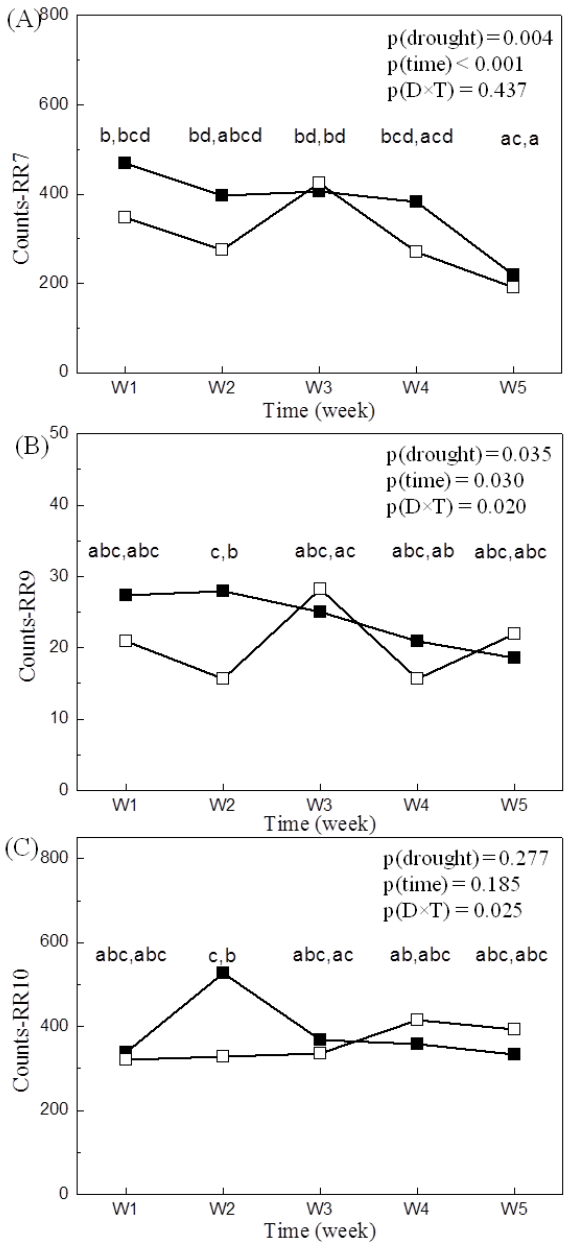

Figure 5. Transcript abundance of poplar type–A *RR7*, *9* and *10* in the transcriptome of the developing xylem under progressive drought. Average value for normalized counts are shown (n = 4 per treatment and time point). The p-values as detected by a two-way ANOVA are provided. Closed symbols represent controls and open symbols represent drought-treated. Letters indicate homogenous subsets of drought-time interaction identified by post-hoc Tukey HSD test. In each pair of homogeneous subsets at every time point, the first letter(s) belongs to the control group and the second letter(s) belongs to the drought group.

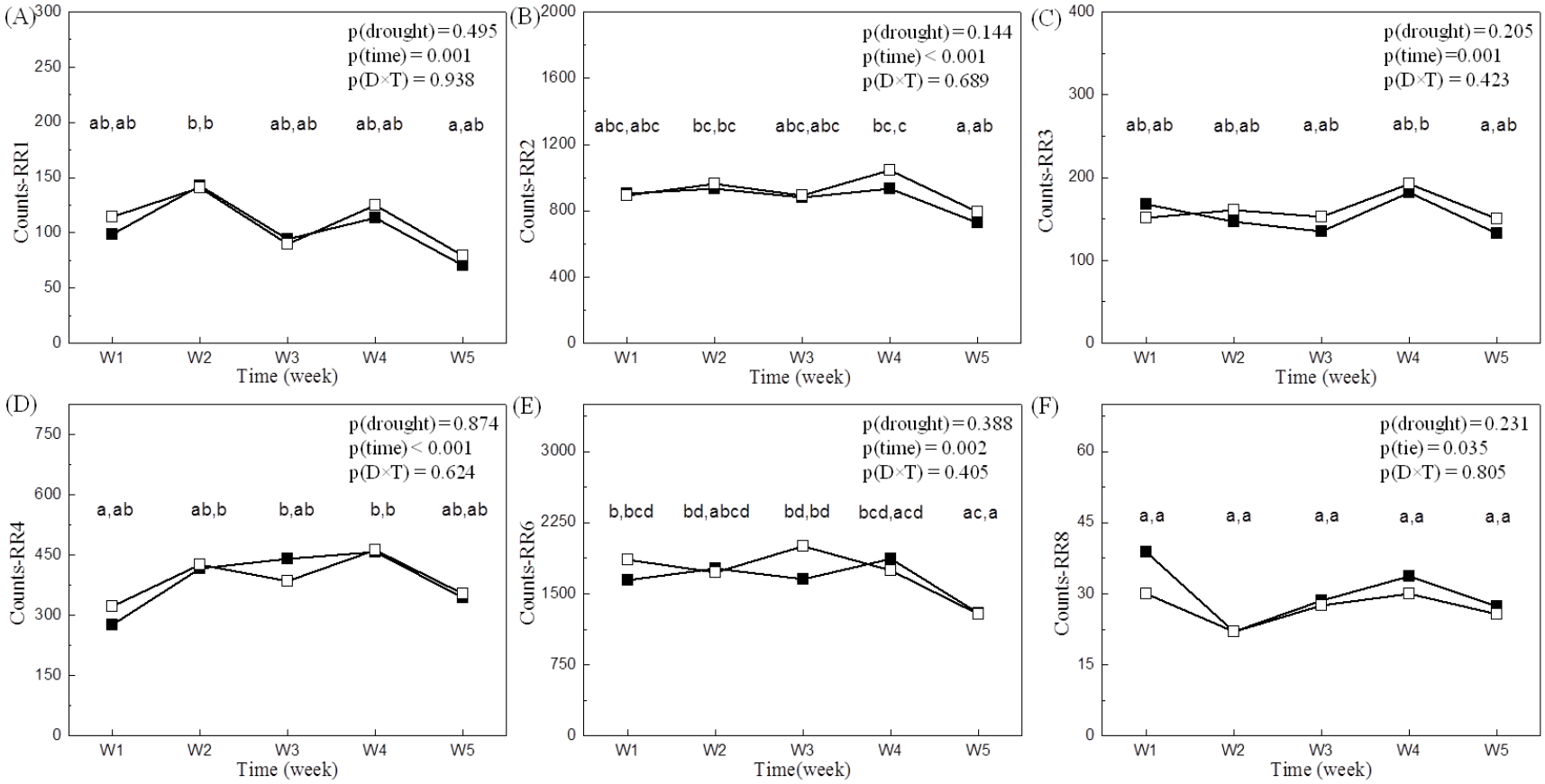

Figure 6. Transcript abundance of poplar type–A *RR1*, *2*, *3*, *4*, *6*, and *8* in the transcriptome of the developing xylem under progressive drought. Average value for normalized counts are shown (n = 4 per treatment and time point). The p-values as detected by a two-way ANOVA are provided. Closed symbols represent controls and open symbols represent drought-treated. Letters indicate homogenous subsets of drought-time interaction identified by post-hoc Tukey HSD test. In each pair of homogeneous subsets at every time point, the first letter(s) belongs to the control group and the second letter(s) belongs to the drought group.

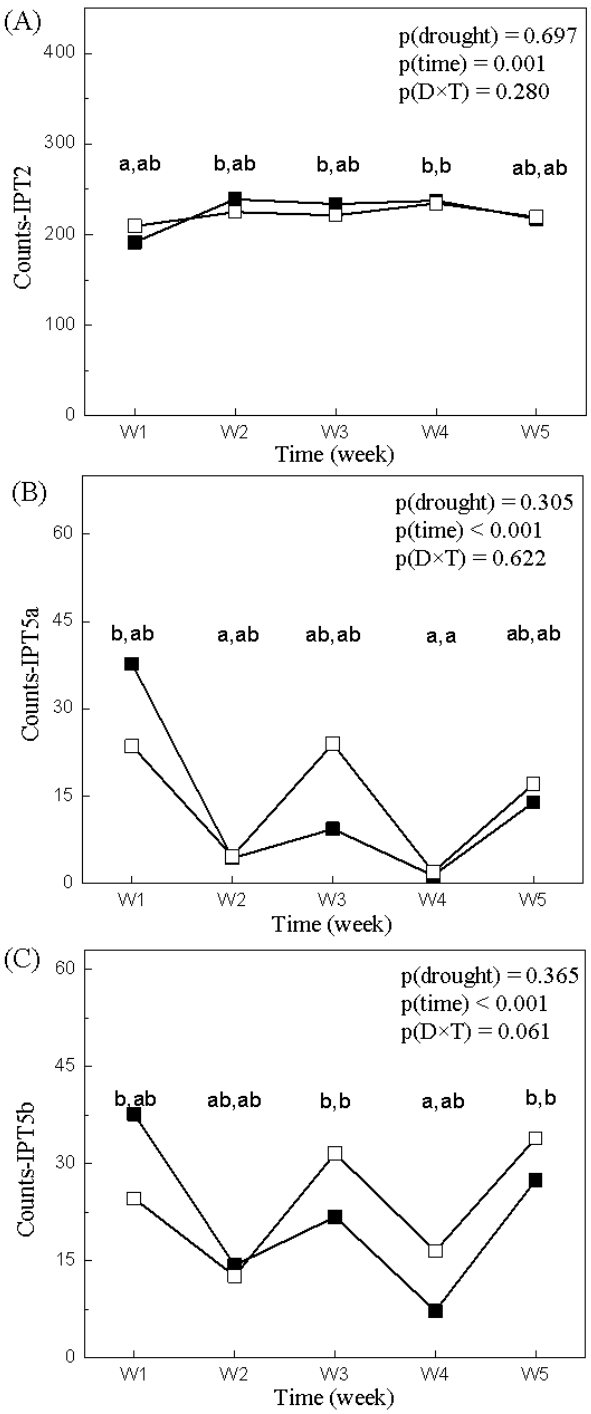

Figure 7. Transcript abundance of poplar *IPT 2*, *5a* and *b* in the transcriptome of the developing xylem under progressive drought. Average value for normalized counts are shown (n = 4 per treatment and time point). The p-values as detected by a two-way ANOVA are provided. Closed symbols represent controls and open symbols represent drought-treated. Letters indicate homogenous subsets of drought-time interaction identified by post-hoc Tukey HSD test. In each pair of homogeneous subsets at every time point, the first letter(s) belongs to the control group and the second letter(s) belongs to the drought group.

5.3.3.3 Transcript abundance of cytokinin degradation genes (CKXs)

Among the eight *CKXs* (*CKX1a, 1b, 3a, 3b, 5a, 5b, 6* and *7*) in poplar, the transcript levels of *CKX6* showed an increase under drought. The transcript levels of *CKX6* also showed a variation during the treatment period (Fig. 8A). The transcript abundance of genes *CKX1b* and 5a showed a variation during the experiment (Fig. 8B, C). The other *CKX* genes did not show any significant effects for main factors or interaction factor.

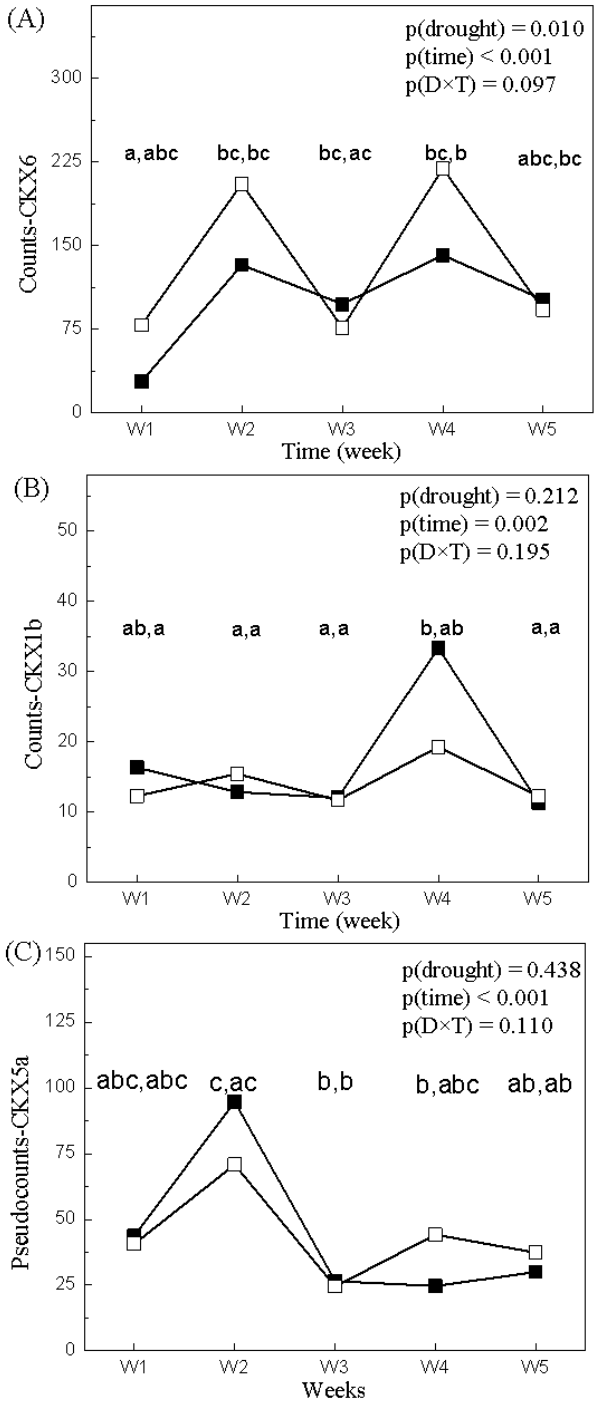

Figure 8. Transcript abundance of poplar *CKX6, 1b* and *5a* in the transcriptome of the developing xylem under progressive drought. Average value for normalized counts are shown (n = 4 per treatment and time point). The p-values as detected by a two-way ANOVA are provided. Closed symbols represent controls and open symbols represent drought-treated. Letters indicate homogenous subsets of drought-time interaction identified by post-hoc Tukey HSD test. In each pair of homogeneous subsets at every time point, the first letter(s) belongs to the control group and the second letter(s) belongs to the drought group.

5.4 Discussion

Drought resulted in the reduction of cambial cell layers. The reduction in the number of cambial cell layers under drought has been reported previously in poplar (Arend and Fromm, 2007). As a consequence of cambial cell layers, radial width of developing xylem declined. This finding is also supported by the positive correlation ($p = 0.004$) between these two traits. This suggests reduced meristematic activity of cambial cell layers under drought.

In addition to the reduction in cambial cell layers and relative width of developing xylem, drought also resulted in the reduction of lumen area per fibre (Table 2). But drought-induced changes were not observed in any other wood anatomical traits. This suggests that after the reduction in cambial cell layers and relative radial width, the reduction in the fibre lumen may be the first change that is notable with regard to wood anatomical traits. In the time course study of progress of drought-induced wood anatomical changes under varying drought levels, Bogeat-Triboulot et al. (2007) also noted that reduction in the vessel and fibre lumen area was notable after the first week of drought treatment. Reduced fibre lumen area under drought has been reported in poplar previously (Beniwal et al., 2010; Arend and Fromm, 2007).

In all the analysed parameters, no interaction between drought and time (except in the case of number of cambial cell layers) was observed, which suggests that during the experiment as poplars grew in the same pots, the size of the pot appeared to be smaller for the big plants and this led to difficulties in controlling treatment conditions (Fig. 1). This resulted in a situation that both controls and drought-treated plants behaved in the same way during the five-week of drought treatment.

Among the transcript abundance of the cytokinin related genes analysed in the transcriptome of the developing xylem, drought reduced the transcript levels of two of the type-A *RR* genes (*RR7* and *RR9*) and increased that of a cytokinin degradation gene (*CKX6*). This suggests a reduced cytokinin signaling and increased degradation under drought. Interestingly, there was no significant variation in the transcript levels of cytokinin biosynthesis genes under drought. The reduced signaling and increased degradation of cytokinins due to *CKX6* noted in the developing xylem under drought, may suggest that the drought-induced wood anatomical changes might have been related to reduced cytokinin signaling and increased cytokinin degradation. This may have resulted in the reduction of number of cambial cell layers observed under drought. Cytokinins are shown to be the main regulators of cambial activity (Nieminen et al., 2008; Matsumoto-Kitano et al., 2008). Reduction of cambial activity under drought is important as a mechanism to cope up with the available resources and result in wood anatomical changes required for plant survival under drought.

5.5. Conclusions

During the five-week drought treatment, radial growth, relative width of developing xylem, number of cambial cell layers and lumen area per fibre were significantly reduced when

compared to the control plants. The other anatomical traits analyzed did not show a significant effect of drought. Regression analyses revealed significant positive correlations between radial growth and number of cambial cell layers, radial growth and relative width of the developing xylem, radial growth and lumen area per fibre, number of cambial cell layers and relative width of the developing xylem. The analysis of transcript abundance of cytokinin related genes showed reduced cytokinin signalling and increased degradation under drought, which suggested that the wood anatomical changes might have been related to reduced cytokinin signalling and increased degradation.

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5.7 Declaration

I conducted the wood anatomical analyses, analysis on transcript abundance of cytokinin related genes and the related statistical analyses.

Vendramin V (IGA Technology Services, Udine, Italy) and Cattonaro F (IGA Technology Services, Udine, Italy) conducted the RNA-sequencing.

Wildhagen H (Forest Botany and Tree Physiology, Georg-August University of Goettingen, Goettingen, Germany; Present address: HAWK University of Applied Sciences and Arts, Faculty of Resource Management, Goettingen, Germany) conducted bioinformatic and statistical analyses based on the RNA sequencing data.

The data on daily water consumption per plant and air temperature inside the greenhouse presented here were provided by Bogeat-Triboulot MB (UMR EEF, INRA, Université de Lorraine, Champenoux, France).

Chapter 6

Overall conclusion and outlook

6.1. Conclusion

In this thesis, factors such as cytokinin activity and intraspecific variation under drought which affects wood growth and biomass production have been a main focus. Cytokinin activity fluctuations during summer and winter were also analyzed. This work has implications in improving wood and biofuel production of poplars.

Transgenic poplars transformed with *ARR5::GUS* reporter construct introduced in this study has helped to throw light on tissue- and cellular-level cytokinin activity in poplar which was not known before. In this thesis, *ARR5::GUS* poplar reporter lines grown outdoors and under controlled conditions in a climate chamber were used. Despite the environmental and age-related differences, the tissue and cellular cytokinin activity pattern was similar for apical bud base, pith of stem top, bark of stem middle and bottom, even though there were some differences in the intensity and extent of localization area under various environmental conditions studied. Differences in cytokinin activity due to changes in environmental conditions were mainly observed in the fine root tips, cambial cells and parts of xylem rays associated with vessels. Among these tissues, fine roots are of great importance as they deliver water and nutrients to plants and help in maintaining growth and survival especially under stress conditions like drought (Brunner et al., 2015). Drought plants often show increased fine root production. As cytokinins are negative regulators of root growth (Werner et al., 2003), a reduction in the cytokinin activity noted in the root tips of drought-treated

plants compared to that of well-watered plants, is in agreement with the increased fine root production under drought. Cambial activity promotes secondary growth in plants. But under drought, this activity has to be reduced in order to cope up with the available resources. As cytokinins have positive effect on cambial activity (Nieminen et al., 2008; Matsumoto-Kitano et al., 2008), a reduced *ARR5::GUS* activity in the cambial zone of drought-treated plants was as expected. Regarding the *ARR5::GUS* activity observed in the parts of xylem rays associated with vessels, "the biological significance of high cytokinin activity close to the vessels is unknown. However, root-derived cytokinin that are transported with the xylem sap through the vessel, are likely to be supplied by this route to the rays" (Paul et al., 2016). *ARR5::GUS* activity in the parts of xylem rays associated with vessels was observed only in the case of poplar lines grown outdoors. This may be either due to the age differences in the plants grown under outdoor and climate chamber conditions or due to the reason that the plants grown outdoors possess stronger lateral conduction system through rays.

Unlike in the case of drought, under dormancy, lack of *ARR5::GUS* activity in root tips, stronger *ARR5::GUS* activity in cambial zone and parts of xylem rays associated with vessels were observed. However, these results are not in line with the absence of growth observed in winter, based on the known functions of cytokinins for growth as mentioned above. This suggests that under the conditions of reduced temperature and day length, the functions of phytohormones including cytokinins may have a different behavioural pattern whose significance for plant performance is still unclear.

In both the experiments conducted using *ARR5::GUS* reporter poplars, active cytokinin localization was not observed in the developing xylem cells. But greater intensity and localization area of active cytokinins were observed in the veins of well-watered poplar leaves than in drought-stressed plants, which resulted in greater leaf area in poplar under well-watered conditions. Greater leaf area is correlated with greater stem biomass production (Ridge et al., 1986, Barigah et al., 1994; Bartelink, 1997). Therefore, cytokinin also plays an indirect role in determining wood production by regulating the leaf area. Thus *ARR5::GUS* poplar reporter lines may serve as a main tool to strengthen our understanding on the production of woody biomass.

The results from this study also give insight into the intraspecific variation in drought-induced wood anatomical changes and the molecular responses underlying them. Therefore it

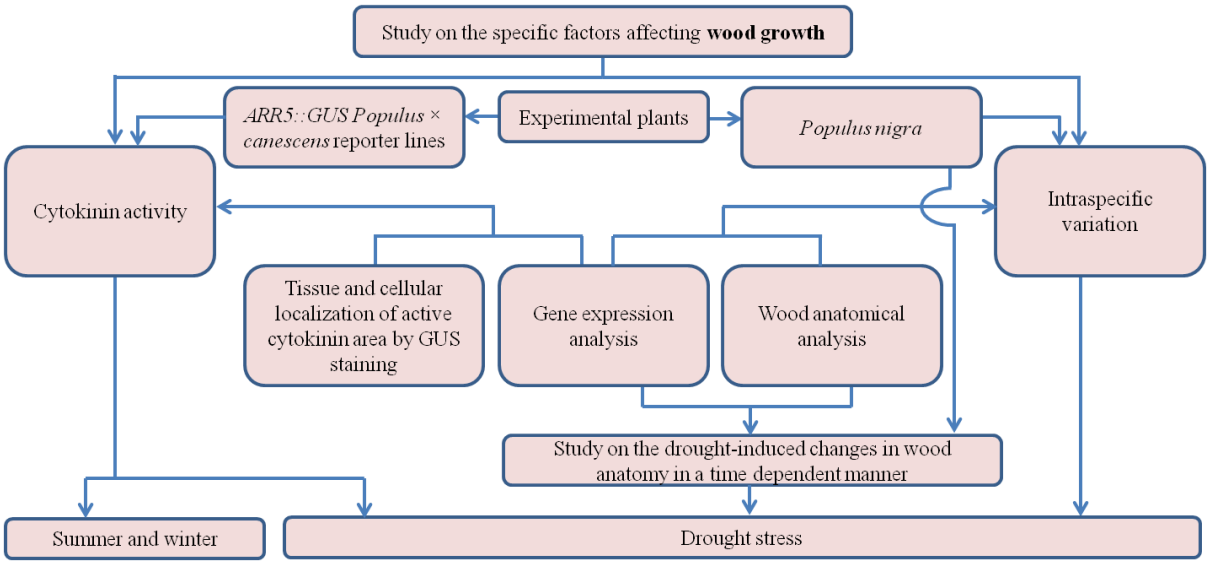

Figure 1. An overall view of the work presented in this thesis.

is apparent that the present results can lead to a better understanding on the selection of drought tolerant genotypes. The time-dependent progress of drought induced significant wood anatomical changes like reduction in number of cambial cell layers, relative radial width of developing xylem and lumen area per fibre. The relation between these changes and transcript abundance of cytokinin related genes suggested that these drought induced wood anatomical changes might have been related to reduced cytokinin signaling and increased cytokinin degradation. The present study suggests how trees maintain productivity under drought and eventually survive the drought conditions involving cytokinin action. A scheme showing an overall view of the study carried out in this thesis is provided in Fig. 1.

6.2 Outlook

It is evident that the *ARR5::GUS* reporter lines that have been introduced in this work can be used as a tool to study the cytokinin responsiveness and localization pattern in poplar and thereby pave way to new advances in cytokinin research related to wood formation, development and production under various environmental stress conditions. In the future, the correlation between endogenous active cytokinin content and intensity of *ARR5::GUS* activity in different tissues of poplar has to be investigated, which can be done using liquid chromatography-mass spectroscopy (LC-MS). As trans-zeatin (tZ) and isopentenyladenine type (iP) cytokinins indicate root-derived and shoot-derived cytokinins (Aloni et al., 2005; Bishopp et al., 2011), respectively, the xylem and phloem sap has to be examined for the abundance of tZ and iP in order to understand the cytokinin synthesis and transport under

various environmental conditions in poplar. Sites of cytokinin biosynthesis in shoot of poplars remain unclear. For this, poplars can be transformed with cytokinin biosynthetic genes (IPTs), under the control of *GUS* reporter. Analyzing *ARR5::GUS* activity in combination with *IPT::GUS* poplar reporter lines can throw more light on relation between site of action and site of biosynthesis of cytokinin in poplars. From this work, pith was observed to be an important tissue regarding cytokinin activity. As the role of pith in relation to hormone activity has not been extensively studied, a research in this direction has to be carried out.

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List of Publications

Publications

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