Gene expression profiling and ecophysiological analyses of *Populus euphratica* and *Populus* × *canescens* in response to salt stress

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Cuvillier Verlag Göttingen
Internationaler wissenschaftlicher Fachverlag

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Dissertation

In Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy (Ph.D.)
of the Faculty of Forest Sciences and Forest Ecology
Georg-August-University of Göttingen

Submitted by
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Born in Hamburg, Germany

Göttingen, May 2010

Bibliografische Information der Deutschen Nationalbibliothek
Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über http://dnb.d-nb.de abrufbar.
1. Aufl. - Göttingen : Cuvillier, 2011
Zugl.: Göttingen, Univ., Diss., 2010

978-3-86955-710-6

Referee: Prof. Dr. Andrea Polle

Co-referee: Prof. Dr. Reiner Finkeldey

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www.cuvillier.de

1. Auflage, 2011
Gedruckt auf säurefreiem Papier

978-3-86955-710-6

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List of Abbreviations

ABA	abscisic acid
ADP	adenosine diphosphate
AGI	*Arabidopsis* Genome Initiative
AGP	arabinogalactan protein
AMDIS	automated mass spectral deconvolution and identification system
ANOVA	analysis of variance
API	application programming interface
ATHB	*Arabidopsis thaliana* homeobox
BASS	bile acid/sodium symporter
bHLH	basic helix-loop-helix
bp	base pairs
BP	biological process
CaM	calmodulin
CBL	calcineurin B-like protein
CBP	calmodulin-binding proteins
CC	cellular component
cDNA	complementary DNA
CHX	cation/H^+ exchanger
CSH	cross-species hybridization
DNA	deoxyribonucleic acid
e.g.	*exempli gratia* (‘for the sake of example’)
EC	Enzyme Commission
EMBL	European Molecular Biology Laboratory
EST	expressed sequence tag
et al.	*et alii* (‘and others’)
FAE	formaldehyde/acetic acid/ethanol
FLA	fasciclin-like arabinogalactan-protein
FT-ICR/MS	Fourier transform - ion cyclotron resonance mass spectrometry
FTIR-ATR	Fourier transform infrared spectroscopy - attenuated total reflection
GA	gibberellic acid
GAST	gibberellic acid stimulated
GC/MS	gas chromatography - mass spectrometry
GO	gene ontology
GST	glutathione-S-transferase
GTP	guanosine triphosphate

HHP1	heptahelical transmembrane protein
HPLC	high-performance liquid chromatography
i.e.	*id est* (‘that is’)
IAA	indole-3-acetic acid
ICP-OES	inductively coupled plasma - optical emission spectrometry
IQL	interquartile length
JA	jasmonic acid
JGI	Joint Genome Institute
KEGG	Kyoto Encyclopedia of Genes and Genomes
LA	Long Ashton
m/z	mass-to-charge ratio
MAS	Microarray Suite
MATE	multidrug and toxic compound extrusion
mAU	milli absorbance units
MF	molecular function
MRP	multidrug resistance protein
MSTFA	N-methyl-N-trimethylsilyltrifluoroacetamide
Mya	million years
NAC	NAM, ATAF and CUC
NAD	nicotinamide adenine dinucleotide
NAM	no apical meristem
NH	Na^+/H^+
Oliv.	Olivier
ORF	open reading frame
P.	*Populus*
PAR	photosynthetic active radiation
PCD	programmed cell-death
PCR	polymerase chain reaction
PK	protein kinase
PP2C	protein phosphatase 2C
PPFD	photosynthetic photon flux density
ppm	parts per million
PVPP	polyvinylpolypyrrolidone
qRT-PCR	quantitative real-time PCR
REST	relative expression software tool
RMA	robust multi-array average
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxigen species
SA	salicylic acid
SAM	significance analysis of microarrays
SCL	SCARECROW-like

SD	standard deviation
SE	standard error
Sm.	Smith
SOS	salt overly sensitive
SRO	similar to radical induced cell death one
TAIR	The *Arabidopsis* Information Reserve
TCA	tricarboxylic acid
TF	transcription factor
Torr.	Torrey
WOX	WUSCHEL related homeobox

Zusammenfassung

Weltweit stellt Bodenversalzung ein gravierendes Problem dar. In näherer Zukunft werden Land- und Forstwirtschaft, und somit Nahrungs- und Energieversorgung, zunehmend davon betroffen sein. Das Verständnis von Salztoleranzmechanismen in Pflanzen gewinnt dadurch immer mehr an Wichtigkeit.

Der Fokus dieser Arbeit liegt auf der Erforschung der Funktionsweise von Salztoleranzmechanismen in Bäumen. Als Studienobjekte wurden zwei Arten der Gattung *Populus* herangezogen, *P. euphratica* und *P* × *canescens*. Bei *P. euphratica* handelt es sich um eine Pappelart, die für ihre Fähigkeit bekannt ist, Salzstress weit besser als die meisten anderen Pappelarten zu tolerieren, darunter die Graupappel *P.* × *canescens*. Um zu ermitteln, welche Effekte Salzstress auf die zwei Pappelarten hat, wurden *Transcription Profiling*, Metabolom Analysen sowie anatomische und physiologische Messungen durchgeführt.

Ziel der vorliegenden Arbeit war es, 1) die Entwicklung des Xylems beider Arten unter Langzeitsalzstress zu vergleichen, 2) die molekulare Anpassung von *P. euphratica* zur Vermeidung von Salzstress zu untersuchen, und 3) Informationen über präventive Strategien durch den Vergleich von *P. euphratica* und *P.* × *canescens* mittels Expressions- und Metabolomanalysen zu erhalten.

Unter Langzeitsalzstress war das Wachstum beider Pappelarten verlangsamt. Die Holzanatomie war allerdings nur in *P.* × *canescens* betroffen, wobei eine Abnahme der Gefäßdurchmesser und eine Zunahme der Gefäßwandstärken beobachtet wurde. Diese Unterschiede konnten darauf zurückgeführt werden, dass die Salzaufnahme in *P. euphratica* wesentlich

geringer war. Folglich traten salzinduzierte Umstrukturierungen des Transkriptoms nur in *P.* × *canescens* auf. Die Gene, die in *P.* × *canescens* unter Salzstress stärker exprimiert wurden, stehen besonders mit der Erzeugung von Antioxidantien, der Entgiftung von Substanzen, die durch oxidative Schäden erzeugt wurden, und Phytohormon-gesteuerten Signalen in Zusammenhang. Nennenswert waren außerdem 11 verschiedene, mutmaßlich die Holzanatomie beeinflussende Fasciclin-ähnliche Arabinogalactane (*fascilin-like arabinogalactans*, FLAs), die derselben Untergruppe angehörten und deren Expression in *P.* × *canescens* unter Salzstress zurückging; zwei dieser FLAs zeigten ebenfalls in *P. euphratica* reduzierte Expression.

Daraus folgt, dass die Reaktion von *Populus* auf internen Salzstress in zwei unterschiedliche Komponenten gegliedert ist. Einerseits wird durch die anatomische Umstrukturierung den osmotischen Effekten entgegengewirkt, andererseits werden die toxischen Auswirkungen der erhöhten Salzkonzentration vermindert. Die meisten dieser Maßnahmen werden in *P. euphratica* auf Grund der Fähigkeit, die Salzkonzentration im Inneren der Pflanze niedrig zu halten, nicht benötigt. Die teilweise Übereinstimmung der Regulation der FLA-Gene deutet jedoch darauf hin, dass die Reaktion auf Salzstress in *P. euphratica* grundsätzlich ähnlich abläuft.

Wie bereits früher gezeigt, resultierte langfristige Salzexposition von *P. euphratica* in der Ausbildung von verdickten Wurzeln (*cob roots*). Hier wurde erstmals geprüft, welche transkriptionellen Veränderungen mit dieser morphologischen Veränderung einhergingen. Die Transkriptionsanalyse deutete darauf hin, dass in den *cob roots* ein wichtiger Signalweg für den Ausschluss von Natrium, die *salt overly sensitive* (SOS) Signalkette, induziert war, wodurch die Na^+-Aufnahme reduziert werden konnte. Des weiteren wiesen *ATHB12*, *WOX13* und *SCL5*, drei Transkriptionsfaktoren, die bereits

mit Wurzelentwicklung und -morphologie in Verbindung gebracht worden waren, erhöte Transkriptionslevel auf.

Dies zeigt, dass *P. euphratica* unter salinen Bedingungen aktiv die Salzaufnahme durch Kontrolle von Ionenkanälen reduziert, um internen Salzstress zu vermeiden. Die Regulation wichtiger Transkriptionsfaktoren deutet ausserdem darauf hin, dass zusätzlich die Morphologie verändert wird, um die physiologischen Effekte zu unterstützen.

Um die Hypothese zu testen, dass in *P. euphratica* Salztoleranz-relevante Gene inhärent höhere Expressionslevel haben, wurden die Transkriptome und Metabolome von Blättern von unter Kontrollbedingungen angezogenen Pflanzen beider Pappelarten direkt miteinander verglichen. Eine generell höhere Expression von stressrelevanten Genen in *P. euphratica* verglichen mit *P.* × *canescens* konnte jedoch nicht festgestellt werden. Unter den Genen, die stärker in *P. euphratica* als in *P.* × *canescens* exprimiert waren, waren Gene aus dem Sekundär-, Zucker- und Energiestoffwechsel überrepräsentiert. Eine Metabolomanalyse deutete darauf hin, dass dies zu einer Anreicherung von Zuckern und einer schnelleren Umsetzung von Zwischenprodukten des Sekundärstoffwechsels in *P. euphratica* verglichen mit *P.* × *canescens* führte. Diese Indizien wurden durch biochemische Analysen von Kohlenhydraten und phenolischen Inhaltstoffen bekräftigt.

Betrachtet man diese Ergebnisse im Zusammenhang, zeichnet sich folgendes Bild ab: zum größten Teil vermeidet *P. euphratica* Salzstress durch den Ausschluss von Salz auf Wurzelebene. Ausserdem scheint *P. euphratica* Salzstress zu antizipieren: Verschiedene Natriumtransporter waren stärker in *P. euphratica* als in der salzsensitiven *P.* × *canescens* exprimiert und können Natrium vermutlich sofort kompartimentieren, wenn es in die Pflanze gelangt. Zucker, die selbst ohne Salzstress angereichert werden, schützen wahrscheinlich gegen plötzlich auftretenden Salz- oder osmotischen Stress.

Diese Strategie, Stressbedingungen zu widerstehen, führt folglich zu einem höheren zellulären Energiebedarf von *P. euphratica*, der in erhöhten Atmungsraten sichtbar wurde.

Im Gegensatz dazu treten Schutzmechanismen in der salzsensitiven *P.* × *canescens* erst in Kraft, nachdem das Salz in die Pflanze gelangt ist. Beispielsweise können die beobachteten Änderungen der Xylemanatomie bei *P.* × *canescens* eine Anpassung an Salzstress durch Schutz vor Kavitation darstellen. Weitere wichtige Reaktionen auf Salzstress bei *P.* × *canescens* scheinen die Erzeugung von Antioxidantien und die Entgiftung von Zerfallsprodukten oxidativer Schäden zu sein; komplexe, energieaufwändige Mechanismen, die sich bei *P. euphratica* durch verbesserte Schutzmaßnahmen erübrigen.

Abstract

Soil salinization is a serious worldwide problem. In the future, it will affect agriculture and forestry on a larger scale than today and thus influence both food and energy supply. It is therefore important to understand salt tolerance mechanisms in plants.

In this work, I focus on research of salt tolerance mechanisms in trees. As objects of study, I employed two species of the model tree genus *Populus*, *P. euphratica* and *P* × *canescens*. *P. euphratica* is a poplar species well known for its capability to tolerate salt stress far better than most other poplar species, including the grey poplar, *P* × *canescens*. Transcription profiling, metabolome analysis, anatomical and physiological measurements were carried out to evaluate the effects salt stress had on the two poplar species.

Aim of this work was 1) the comparison of xylem development in both species under long-term salt stress, 2) to investigate the molecular adaption of *P. euphratica* to avoid salt stress, and 3) to gaim insight into preventive strategies by comparing *P. euphratica* and *P.* × *canescens* by means of expression and metabolome analyses.

During long-term salt stress, both poplar species showed reduced growth. However, wood anatomy was only affected in *P.* × *canescens*, where vessels showed a decrease in size and an increase in wall strength. This difference could be attributed to the fact that salt uptake in *P. euphratica* was significantly lower. Accordingly, salt-induced changes in the transcriptome occurred only in *P.* × *canescens*. Genes that showed higher expression in *P.* × *canescens* under salt stress focused on antioxidants, detoxification of products of oxidative damage and phytohormone-influenced signaling. Noteworthy were 11 different, presumably wood anatomy influencing fasciclin-like arabinogalactans (FLAs)

belonging to the same subgroup that were downregulated under salt stress in *P.* × *canescens*; two of these were also downregulated in *P. euphratica*.

From this it follows that the reaction of *Populus* to intern salt stress is devided into two different components. On the one hand, the anatomical restructuring counteracts osmotic stress, on the other hand, toxic effects of increased salt concentrations are reduced. In *P. euphratica*, most of these measures are not needed due to its ability to keep intern salt levels low. However, the partially matching regulation of FLA genes suggests that reactions to salt stress in *P. euphratica* are similar.

As formerly shown, long-term salt exposure of *P.euphratica* results in development of thickened roots (cob roots). Here, the transcriptional changes that concur with these morphologic changes where investigated for the first time. The transcription analysis indicates that an important signalling pathway for sodium exclusion, the Salt Overly Sensitive (SOS) pathway, was induced in cob roots, thus decreasing Na^+ uptake. Furthermore, *ATHB12*, *WOX13* and *SCL5*, three transcription factors that have been associated with root development and morphology, had increased expression levels.

This shows that during saline conditions, *P. euphratica* is able to actively reduce salt uptake by controlling ion channels to avoid internal salt stress. Furthermore, the regulation of important transcription factors indicates that the morphology is being altered to support the physiological effects.

The transcriptomes and metabolomes of leaves of both poplar species grown under control conditions were directly compared to test the hypothesis whether *P. euphratica* has innately higher expression levels of genes relevant to salt tolerance. However, a generally higher expression of stress relevant genes in *P. euphratica* than in *P.* × *canescens* was not detected. Genes involved in secondary, sucrose and energy metabolisms as well as transport systems were overrepresented among the genes higher expressed in *P. euphratica* than in *P.*

× *canescens*. Metabolome analysis implied a resulting accumulation of sugars and a faster turnover of secondary metabolism intermediate products in *P. euphratica* compared to *P.* × *canescens*. These indications were affirmed by biochemical analyses of carbohydrates and phenolic compounds.

Considering these results as a whole, the following context emerges. Largely, *P. euphratica* avoids salt stress by excluding salt at root level. Furthermore, *P. euphratica* seems to anticipates salt stress, since a diversity of sodium transporters were higher expressed in *P. euphratica* than in the salt sensitive *P.* × *canescens* and are probably able to compartmentalize sodium when it enters the plant. Sugars that are accumulated even in the absence of salt stress presumably guard against sudden osmotic and salt stress. Accordingly, this approach of *P. euphratica* to withstand stress conditions leads to a higher energy requirement of cellular metabolism, discernible in increased respiration rates.

In contrast, protective mechanisms of the salt sensitive poplar take effect after the salt has entered the plant. For example, the observed changes in the xylem anatomy of *P.* × *canescens* can be an adaptation to salt stress by protecting against cavitation. Further important responses to salt stress in *P.* × *canescens* seem to be the generation of antioxidants and detoxification of products of oxidative damage, complex, energy consuming mechanisms that are made superfluous in *P. euphratica* by its protective abilities.

1. Introduction

Soil salinization is one of the major threats for agriculture worldwide. Currently, a total of 397 million ha, nearly 3 % of the total land mass, are covered by salt affected soils (FAO/AGL). Agricultural land accounts for 77 million ha thereof, a fraction that will increase in the coming years because of inappropriate irrigation regimes, increase in evapotranspiration, sea-level rise and decreased ground water recharge (Kundzewicz *et al.* 2007; Tester & Davenport 2003). To be able to cope with this situation in the future, new plant varieties with enhanced ability to grow on salinized soils must be available, generated either by efficient breeding or genetic engineering. Because of the increasing importance of trees as a renewable energy resource, this applies not only to agricultural crops, but also to woody plants.

An alternative approach to the problem of soil salinization is to counteract the underlying causes. In many cases, irrigation is used on agricultural land where drought-adapted, deep-rooted native vegetation has been replaced by annual crops. In these areas, surfacial irrigation and evapotranspiration will establish a capillary flow, by which often saline water from deep ground water tables is transported to the surface, leading to soil salinization (Salinity stress and its mitigation). To counter this problem, the establishment of a sustainable agricultural management has been proposed. This could include planting of deep-rooted, salt tolerant tree species to lower ground water tables (Pitman & Läuchli 2002).

For the implementation of either of the two concepts, it is essential to increase our knowledge and understanding of mechanisms that are available to trees to tolerate high salinity. So far, research on salt tolerance mechanisms in plants has focused mainly on non-woody species, a circumstance that seems

inattentively, since tree species will play an important part both in the fields of genetic engineering and sustainable agricultural management (Flowers 2004; Turkan & Demiral 2009; Hasegawa *et al.* 2000).

Trees and herbaceous plants differ widely in respect to morphology and physiology. These differences are most apparent in form of the development of wood, *i.e.* secondary xylem. Secondary xylem is also formed in non-woody plants and thus can be investigated using classical model species like *Arabidopsis*. However, wood development is an immensely complex process. Since herbaceous and woody plants differ widely in their physiology and morphology (Demura & Fukuda 2007; Hertzberg *et al.* 2001; Taylor 2002), the influence of salt stress on secondary xylem formation will ultimately have to be studied in trees, because findings that have been made for herbaceous plants may only be transferred to a certain extent and with great care to tree species. In this work, I therefore focused on salt tolerance mechanisms in woody plants.

For this purpose, it is of practical interest to have one specific model organism to work on. Among herbaceous plants, *Arabidopsis thaliana* has been established as one of the most important model species to answer a multitude of questions. Reasons for this were amongst others a short life cycle, a wide ecological tolerance spectrum, availability of techniques for genetic manipulation, and a relatively small genome, making sequencing of the whole genome possible.

For similar reasons, *Populus* has now been firmly established as a model species for trees (Bradshaw *et al.* 2000; Brunner *et al.* 2004; Jansson & Douglas 2007; Taylor 2002; Tuskan *et al.* 2006). The genus *Populus* is formed by a group of closely related tree species. *Populus* has, compared to other tree species, a relatively small genome, and protocols for genetic manipulation are available. But all tree species have life cycles that require several years, which complicates their use as model organisms. Poplars however compensate this

disadvantage by a rapid growth rate and by being easily multipliable by vegetative propagation, thus allowing experiments to be conducted on genetically identical plants rather than inbred plant lines. Finally, while individual poplar species are not necessarily generalists, the many different, closely related species among the genus *Populus* cover a wide ecological range. In addition, poplars are of great commercial value, especially to the pulp and paper industry.

Thellungiella halophila, a salt tolerant plant closely related to *Arabidopsis*, is often used as a model to research salt tolerance in herbaceous plants (Bartels & Sunkar 2005; Taji *et al.* 2004; Wang *et al.* 2004). Among poplars, *Populus euphratica* OLIV. meets the claims for this task. *P. euphratica* grows naturally in areas with saline soil and ground water, ranging from western Europe to China (Feng *et al.* 2001). It is well known for tolerating higher salt concentrations than other poplar species (Watanabe *et al.* 2000; Bolu & Polle 2004; Sixto *et al.* 2005). Under saline conditions, *P. euphratica* is able to maintain higher growth rates and higher photosynthetic rates than salt-sensitive poplar species (Wang *et al.* 2007; Chen *et al.* 2003b). Many works have analyzed the physiological responses of *P. euphratica* to salt stress, and compared them to different salt sensitive poplar species (Wang *et al.* 2007; Chang *et al.* 2006; Hukin *et al.* 2005; Fayyaz 2007; Fung *et al.* 1998; Chen *et al.* 2003b). In single studies, the transcriptomic response of *P. euphratica* to salt and drought stress was even examined on microarrays, and typical genes have been found to be upregulated (Gu *et al.* 2004; Brosché *et al.* 2005; Bogeat-Triboulot *et al.* 2007). However, since no comparison of the salt stress response transcriptome of *P. euphratica* with a salt sensitive poplar exists, it is still unknown which genes or mechanisms are particularly important for the salt tolerance.

The lack of data in this field might be due to the fact that only spotted cDNA microarrays were available. These are made from species-specific cDNA libraries, and therefore should only be used for the respective species; comparing the microarrays' source-species with a different species was not possible. But the acceptance of *Populus* as a model tree now has led to a sound informational basis. Recently, the whole genome of *Populus trichocarpa* TORR. & GRAY has become available through a sequencing project, and annotation of genes has begun (Tuskan *et al.* 2006; JGI *Populus trichocarpa* genome release 1.1). This in turn allowed the development of a whole genome microarray by Affymetrix, the GeneChip Poplar Genome Array, for gene expression analyses of different poplar species (Affymetrix GeneChip Poplar Genome Array Data Sheet). Affymetrix GeneChips feature a new technology; probes are no longer spotted cDNA fragments as in earlier microarray generations, but are oligonucleotides that are synthesized *in situ* on the microarray (Thomas & Burke 1998). This leads to a high reproducibility between separate microarrays, rendering the use of two colors and technical reproductions obsolete (Hardiman 2004).

Thus, with an adequate model tree and new techniques for high-throughput analyses of gene expression, it is now possible to compare the transcriptome of the salt tolerant *P. euphratica* to that of a salt sensitive poplar. For this, I employed *P.* × *canescens* (AITON) SM., a salt sensitive poplar native to Europe whose natural range is in the temperate zone (Bolu & Polle 2004; Hawighorst 2007). In this study, the following topics were addressed:

In Chapter II, the effects of salt stress on the anatomy of trees were studied by comparing the transcriptomes and the morphology of *Populus* × *canescens* and *P. euphratica* xylem formed under long-term salt stress. The aim of this chapter is to gain more insight into mechanisms of stress adaption and stress tolerance in wood.

Chapter III comprises the work on roots of *P. euphratica*, which display a distinct thickening when formed under saline conditions. Here, the transcriptional changes that concur with these morphologic changes where analysed to investigate the molecular adaption of *P. euphratica* to salt stress.

In Chapter IV, the transcriptomes and metabolomes of *P. euphratica* and *P.* × *canescens* leaves formed in the absence of salt stress were compared. Hereby, I address the questions whether *P. euphratica* uses preventive strategies to anticipate salt stress, and which stress tolerance mechanisms are employed.

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2. Transcription profiling of developing xylem of *Populus* × *canescens* and *Populus euphratica* under salt stress to study mechanisms of stress adaption and stress tolerance in wood

2.1. Introduction

Salt stress can be distinguished in short- and long-term effects and evokes different reactions in plants (Munns & Tester 2008). Short-term effects are mostly based on osmotic stress and affect all species; long-term effects are caused by accumulation of salt, which leads to high, toxic levels of Na^+-and Cl^--ions inside the plant. Many salt tolerant species avoid excessive salt accumulation by exclusion (Munns 2005). Because trees have a much longer lifespan than herbaceous plants, time periods like 'short-term' and 'long-term' have different meanings for woody and non-woody plant, and thus reactions and mechanisms to cope with these stresses might be quite divergent. This is especially true when considering the impact on secondary growth and the development of wood tissue.

A short term effect of salt stress in plants is the general reduction of growth. This has been ascribed to varying mechanisms, but as Munns pointed out in reference to the general growth reduction of plants under salt stress, it has not yet been resolved whether water status, hormonal regulation or supply of photosynthate exerts the dominant control over growth of plants in dry or saline soil (2002).

A long term consequence of salt stress is an effect on xylem anatomy. In trees, decreases of vessel diameters and increase of vessel frequency per cross-section area are frequently observed (e.g. Baum *et al.* 2000; Junghans *et al.*

2006; Turco *et al.* 2002). Similar findings exist for drought and freezing stress (Arend & Fromm 2007; Hacke & Sperry 2001; Hacke *et al.* 2001; Abrams 1990). It has been reasoned that plants benefit from these changes in xylem anatomy due to lowered risks of occurrence of cavitation and implosion incidents (Pittermann *et al.* 2006; Chen *et al.* 2009b). However, the mechanisms leading to decreased diameters of vessels under salt are unclear. Several physiological changes associated with salt stress have been proposed to be the cause for a growth reduction of the plant in general and of vessel cells in particular: the decrease of photosynthetic activity, leading to a lack of photosynthates (Kozlowski 1997; Escalante-Perez *et al.* 2009); the decrease of the K^+/Na^+-ratio, leading to a lack of potassium (Escalante-Perez *et al.* 2009; Langer *et al.* 2002); and changes in hormone levels, especially lower auxin and higher abscisic acid concentrations, leading to a lack of growth promotors or an abundance of growth inhibitors, respectively (Junghans *et al.* 2004; Kozlowski 1997). But the differentiation of xylem precursor cells to tracheary elements is a fine-tuned process that demands a complex signaling network to effect the formation of secondary walls and programmed cell death (Fukuda 2004). It is therefore also conceivable that it is influenced by several of these factors interacting with each other.

The aim of this chapter is to gain more insight into the effects of salt stress on the anatomy of trees. Therefore, we conducted a comparative study of control and salt stressed plants of the salt sensitive *P.* × *canescens* and the salt tolerant *P. euphratica*. The reactions to salt stress of the two poplar species were compared regarding transcriptional profiles of developing xylem tissue, wood anatomy and composition, element content, osmotic potential, assimilation rates and sugar content.

2.2. Results

2.2.1. Sodium and osmolyte content in *P.* × *canescens* and *P. euphratica* under salt stress

To evaluate the impact of salt stress on the two different poplar species on the whole-plant level, we measured the osmotic potential and Na^+-content in roots, stem, developing xylem and leaves of *P.* × *canescens* and *P. euphratica* controls and plants adapted to 25 mM NaCl and 100 mM NaCl, respectively. In control plants the decrease in the osmotic potential between roots and leaves was approximately Δ = 7.5 MPa in both *P.* × *canescens* and *P. euphratica*. With increasing NaCl in the nutrient medium, the osmotic potential decreased by 0.8 to 1 MPa in all organs of *P.* × *canescens*, resulting in an almost constant internal allocation gradient that was only slightly increased to 0.9 and 1 MPa at 25 and 100 mM NaCl, respectively. The strongest decrease in osmotic potential was observed in developing xylem of *P.* × *canescens* (Figure 2.1). Contrary to *P.* × *canescens*, a significant decrease of the osmotic potential with increasing NaCl concentration in the nutrient medium did not occur, suggesting that *P. euphratica* is better able to adjust its osmotic equilibrium than *P.* × *canescens* (Figure 2.1).

As expected from a treatment with NaCl, sodium concentrations inside the plants increased strongly in both species with increasing NaCl-content in the outside nutrient medium (Figure 2.2). Fold-changes ranged from 8-fold in developing xylem of *P. euphratica* up to 170-fold in the stem of *P.* × *canescens*. In the 100 mM NaCl-treatment groups, Na^+-content in the stem was higher in *P.* × *canescens* than in *P. euphratica*. This observation was to be expected, since salt tolerant species prevent the uptake of NaCl and the accumulation to toxic levels inside the plant (Munns 2002; Sun *et al.* 2009).

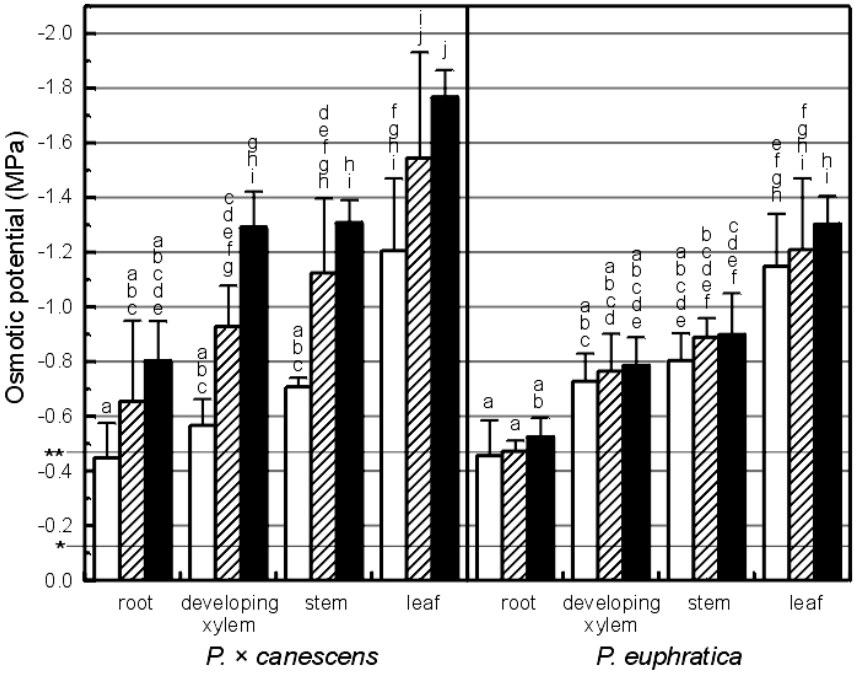

Figure 2.1 - Changes in osmotic potential in *P.* × *canescens* and *P. euphratica* to increasing NaCl-concentrations in the nutrient solution

Plants were harvested under control conditions (white), after two weeks of salt stress with 25 mM NaCl (striped), and after two additional weeks of salt stress with 100 mM NaCl (black). Osmotic potential was measured in roots, developing xylem, stem segments and leaves of *P.* × *canescens* and *P. euphratica*. * indicates the osmotic potential of the nutrient solution with 25 mM NaCl, ** with 100 mM NaCl. Osmotic potential of nutrient solution without added NaCl was -0.01 MPa. Homogeneous subsets are based on an ANOVA followed by a multiple comparison test at p=0.05 (Tukey's Honestly Significant Difference (HSD); n=6, mean±SD).

Differences between the two species (*P.* × *canescens*/*P. euphratica*) amounted to 4-fold in the developing xylem and 10-fold in the stem. Noticeably, in both species the highest concentrations were accumulated in the roots and the lowest in the developing xylem, suggesting that in both *P.* × *canescens* and *P. euphratica*, NaCl uptake into the plant is impeded by the roots and growing tissues are protected.

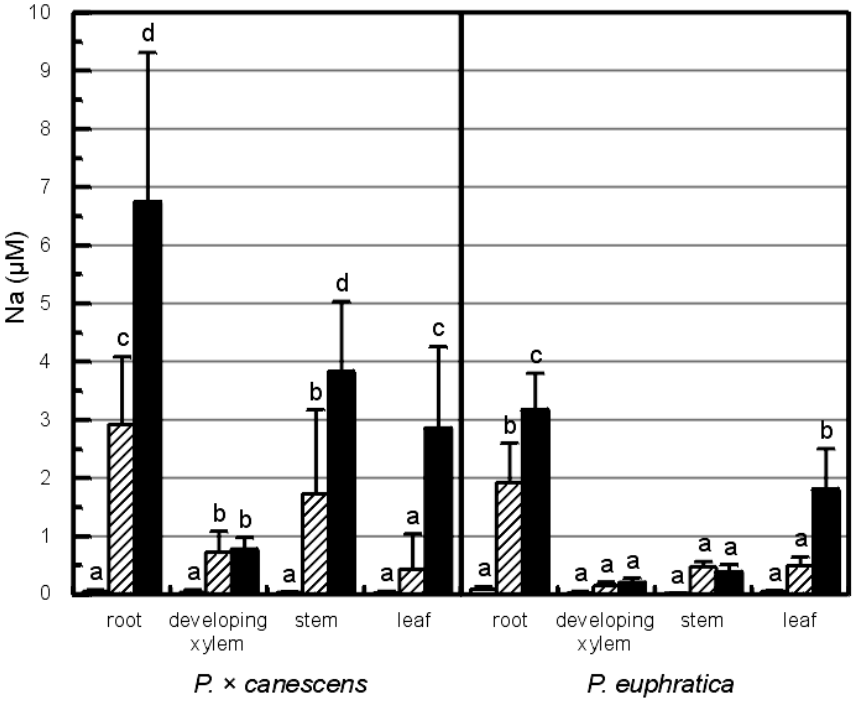

Figure 2.2 - Changes in sodium concentration in *P.* × *canescens* and *P. euphratica* to increasing sodium concentrations in the nutrient solution

Plants were harvested under control conditions (white), after two weeks of salt stress with 25 mM NaCl (striped), and after two additional weeks of salt stress with 100 mM NaCl (black). Sodium content was measured in dried material of roots, developing xylem, stem segments and leaves of *P.* × *canescens* and *P. euphratica* and referred to the water content of the respective sample. Homogeneous subsets are based on an ANOVA followed by a multiple comparison test at p=0.05 (Tukey-Kramer; n=3, respective n=5 for developing xylem, mean±SD).

2.2.2. Growth rates, assimilation and sugar content of *P.* × *canescens* and *P.* euphratica in response to salt stress

Height growth was reduced by salt stress in both species to a similar extent (Figure 2.3). Only *P.* × *canescens* showed further stress symptoms like leaf senescence and a reduction of assimilation rates. NaCl concentrations of 25 mM in the nutrient solution did not affect height growth, whereas 100 mM

NaCl caused growth decreases in both species. However, *P. euphratica* plants did not show visual stress symptoms, while *P.* × *canescens* plants showed typical symptoms of salt injury like dying and shedding of older leaves (Figure 2.4).

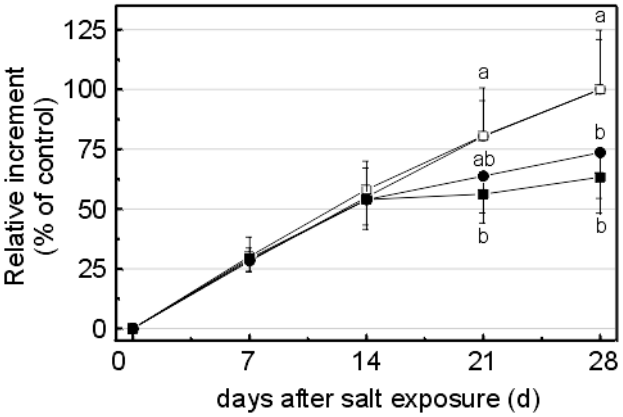

Figure 2.3 - Height growth of *P.* × *canescens* and *P. euphratica* during salt treatment

Relative height increment of *P.* × *canescens* (circles) and *P. euphratica* (squares) during a four week period of increasing salt stress as percentage of the total increment of control plants (white symbols). Salt treated plants (black symbols) were exposed to 25 mM NaCl for the first two and 100 mM NaCl for the last two weeks (n=12, mean±SD).

CO2 assimilation rates of P. × canescens plants stressed with 100 mM NaCl amounted to approx. 30 % of the controls, whereas no significant change was detected in P. euphratica (Figure 2.5). However, despite the decrease in assimilation rate, sugar contents increased under salt stress in phloem sap of P.× canescens (Figure 2.6). Significantly higher levels of glucose and sucrose were measured after 2 weeks of salt stress with 25 mM NaCl and after 2 additional weeks with 100 mM NaCl. Fructose contents were only higher after

Figure 2.4 - Plant performance under salt stress

P. × *canescens* - A: control; B: after two weeks of salt stress with 25 mM NaCl; C: after two additional weeks of salt stress with 100 mM NaCl. *P. euphratica* - D: control; E: after two weeks of salt stress with 25 mM NaCl; F: after two additional weeks of salt stress with 100 mM NaCl.

the first two weeks of salt stress, no significantly higher levels could be detected after the last two weeks. In P. euphratica, no significant changes could be detected for any of the three sugars. In the developing xylem, almost no significant differences were detected in sugar contents of the two poplar species. Only sucrose levels in P. × canescens after two and four weeks of salt stress were lower than control levels (Figure 2.7).

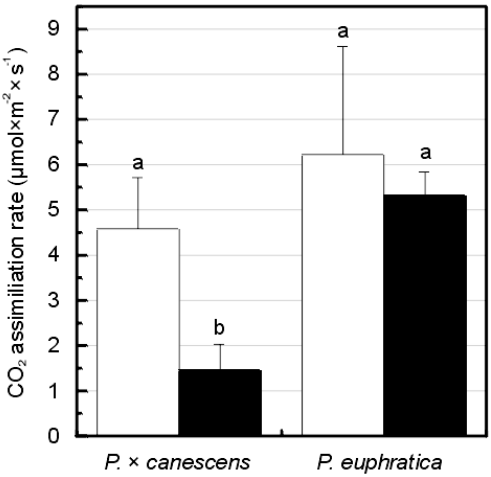

Figure 2.5 - Effect of salt stress on assimilation

Net photosynthetic CO_2 assimilation of *P. × canescens* and *P. euphratica* under control conditions (white) and after a four week period of increasing salt stress (black) with a final salt concentration of 100 mM NaCl. Assimilation was measured using mature leaves (9th or 10th below the apex) at a leaf temperature of 30°C and a PPFD (photosynthetic photon flux density) of 1000 µmol photons m^{-2} sec^{-1} (n=4, mean±SD).

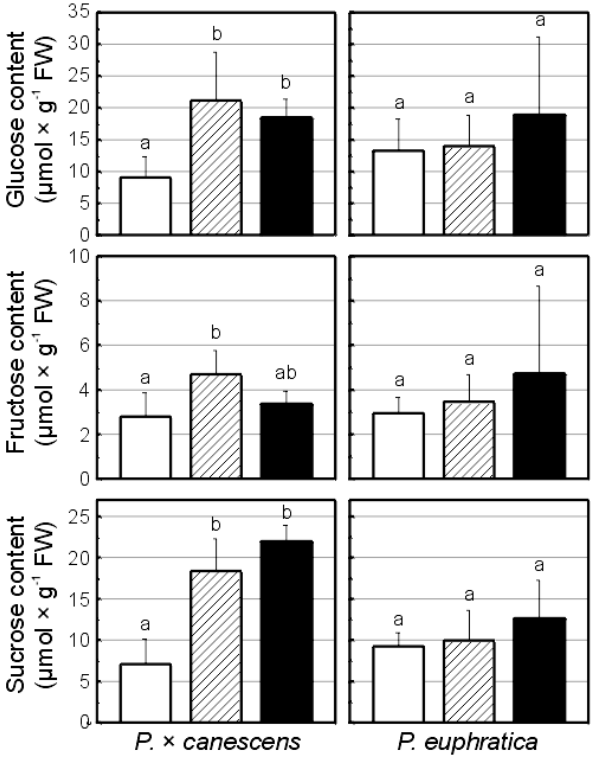

Figure 2.6 - Effect of salt stress on phloem sugar content

Sugar content in phloem sap of *P.* × *canescens* and *P. euphratica* under control conditions (white), after two weeks of salt stress with 25 mM NaCl (striped), and after two additional weeks of salt stress with 100 mM NaCl (black). Measurements were conducted by HPLC on phloem sap exudates. Homogeneous subsets are based on an ANOVA followed by a multiple comparison test at p=0.05 (n=3, mean±SD).

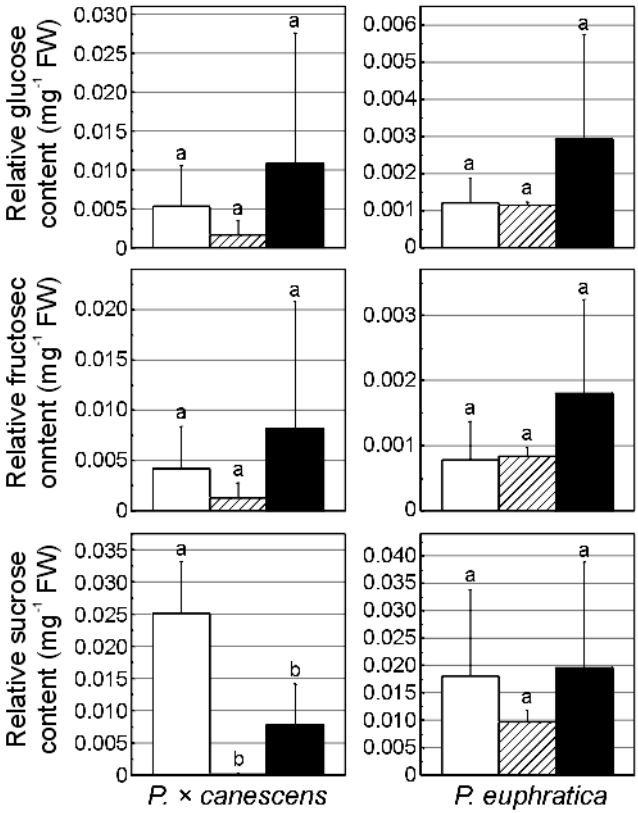

Figure 2.7 - Effect of salt stress on sugar content in developing xylem

Relative sugar content in developing xylem of *P. × canescens* and *P. euphratica* under control conditions (white), after two weeks of salt stress with 25 mM NaCl (striped), and after two additional weeks of salt stress with 100 mM NaCl (black). Measurements were conducted by GC/MS on fine powder of frozen developing xylem. Units are peak area ratios between sample and internal standards (n=3, mean±SD).

2.2.3. Effects of salt stress on xylem anatomy of *P.* × *canescens* and *P. euphratica*

Salt stress causes a decrease in osmotic pressure, and thus a stronger force is exerted on the vessel walls. The strength of the vessels to withstand this force is determined by wall thickness to vessel diameter ratio (Hacke *et al.* 2001). To assess the changes in wall strength, wall thickness and vessel lumina sizes was measured in xylem tissue of plants that had been subjected to either two weeks of 25 mM NaCl, or to two weeks of 25 mM and additional two weeks of 100 mM NaCl. To assess the hydraulic conductivity, the number of vessels per area was measured.

In *P.* × *canescens*, the number of vessels per area increased and the mean vessel size decreased with increasing salt in the nutrient medium. In *P. euphratica*, no changes could be observed for either of the two traits under salt stress. (Figure 2.8 a and b). However, the predicted hydraulic conductivity computed from vessel frequency and individual sizes of vessel lumina stayed constant in both species (Figure 2.8 c). No significant change in wall thickness could be detected in either of the two species (data not shown); accordingly, the wall strength computed from wall thickness and vessel diameters increased in *P.* × *canescens* and stayed constant in *P. euphratica* (Figure 2.8 d).

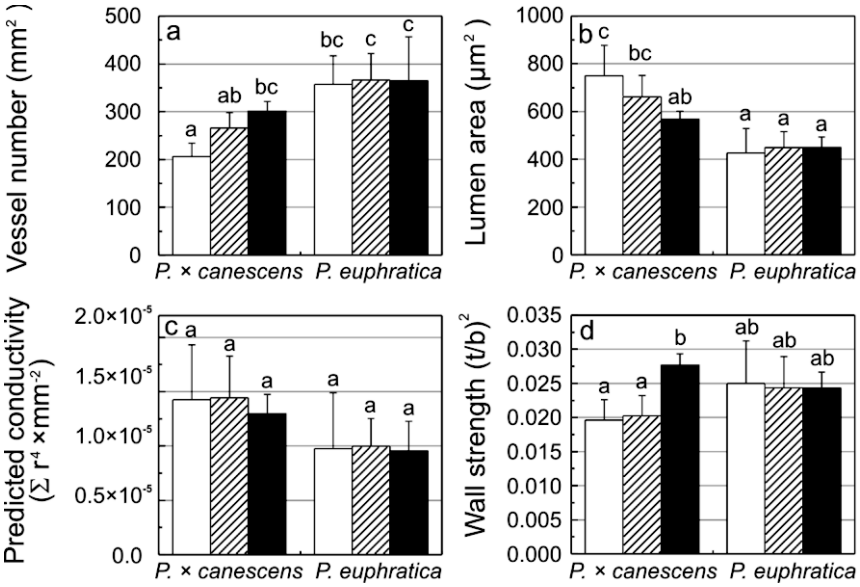

Figure 2.8 - Changes in xylem anatomy of *P.* × *canescens* and *P. euphratica* in response to salt stress

Plants were harvested under control conditions (white), after two weeks of salt stress with 25 mM NaCl (striped), and after two additional weeks of salt stress with 100 mM NaCl (black). a) Number of vessels per cross-sectional area in mm^2, b) mean lumen area of individual vessels in μm^2, c) predicted conductivity, given as the sum of the fourth power radii per area of xylem (Σr^4 mm^{-2}), d) wall strength of vessels, given as $(t/b)^2$ (t: thickness of double cell wall, b: span of cell wall). Homogeneous subsets are based on an ANOVA followed by a multiple comparison test (Tukey's HSD; n=5, mean ± SD).

2.2.4. Transcriptome analysis of developing xylem in *P.* × *canescens* and *P. euphratica* in response to salt stress

To gain insight into the molecular events associated with the anatomical changes in *P.* × *canescens* under salt stress, transcriptional profiles of developing xylem of plants exposed to 100 mM NaCl were analyzed on whole genome arrays. Genes with significantly changed transcript levels in response to salt were determined separately for each species. The response of the two species differed strongly. In *P.* × *canescens*, a total of 457 genes were significantly changed, but only 27 changed genes were identified in *P. euphratica*. Furthermore, 198 of the genes in *P.* × *canescens* were up- and 259 were downregulated, while in *P. euphratica* only one gene was upregulated (Supplemental Tables S1, S2 and S3). Only four genes were commonly regulated in both species: two fasciclin-like arabinogalactan-protein genes, PopFLA8 and PopFLA9, 9-cis-epoxycarotenoid dioxygenase and glycosyl hydrolase family 14 (beta-amylase).

To comprehend the fundamental patterns underlying the transcriptional changes in *P.* × *canescens*, overrepresented Gene Ontology (GO) categories were determined. For each of the lists of up- and downregulated genes, a GO term enrichment analysis was conducted (Table 2.1).

Analysis of the downregulated genes revealed five significantly enriched GO categories after Bonferroni-correction. Only 82 of the 259 downregulated genes, approximately 30%, were annotated by one of these overrepresented GO terms. Dominating the enriched categories were 11 different fasciclin-like arabinogalactan-protein genes (FLAs) with the annotations ‘cell adhesion’ and ‘biological adhesion’ (Table 2.2). All 11 FLAs were also included in the other three overrepresented membrane-related categories, and therefore contributed

Table 2.1 - Gene Ontology (GO) terms significantly enriched* in differentially expressed genes in developing xylem of *P.* × *canescens* under salt stress

GO number	Gene Ontology	number of terms in population	number of terms in study set	adjusted p-value	name
GO terms enriched in upregulated genes:					
GO:0042221	BP	675	31	8.01E-06	response to chemical stimulus
GO:0019748	BP	208	16	4.74E-05	secondary metabolic process
GO:0016491	MF	744	30	2.52E-04	oxidoreductase activity
GO:0050896	BP	1436	44	8.02E-04	response to stimulus
GO:0003824	MF	4478	96	0.0017	catalytic activity
GO:0009404	BP	17	5	0.0028	toxin metabolic process
GO:0009407	BP	17	5	0.0028	toxin catabolic process
GO:0004364	MF	20	5	0.0067	glutathione transferase activity
GO:0009636	BP	36	6	0.0098	response to toxin
GO:0006725	BP	195	12	0.0224	aromatic compound metabolic process
GO:0009813	BP	44	6	0.0323	flavonoid biosynthetic process
GO:0009699	BP	66	7	0.0383	phenylpropanoid biosynthetic process
GO:0009751	BP	67	7	0.0423	response to salicylic acid stimulus
GO:0009812	BP	47	6	0.0474	flavonoid metabolic process
GO terms enriched in downregulated genes:					
GO:0007155	BP	35	11	1.36E-08	cell adhesion
GO:0022610	BP	35	11	1.36E-08	biological adhesion
GO:0031225	CC	104	14	4.65E-06	anchored to membrane
GO:0031224	CC	395	20	0.0306	intrinsic to membrane
GO:0016020	CC	3039	82	0.0477	membrane

* at $p < 0.05$ after Bonferroni-correction.
The whole population (gene universe) consists of 13367, the study sets of 193 (upregulated) and 244 (downregulated) GO terms. BP, Biological Process; MF, Molecular Function; CC, Cellular Component.

to their significant overrepresentation. FLAs have been found to be massively downregulated in wheat by abiotic stresses and are thought to be important during plant development, cell-cell communication and in response to abiotic stress (Johnson *et al.* 2003; Faik *et al.* 2006). The 21 FLAs present in *Arabidopsis* form 4 subgroups, A – D (Johnson *et al.* 2003). In *Populus*, several hundred FLA ESTs were identified by Andersson-Gunnerås *et al.*, of which over 200 belonged to subgroup A (Andersson-Gunnerås *et al.* 2006). A phylogenetic analysis showed that in comparison to *Arabidopsis*, the FLA-genes downregulated in *P.* × *canescens* formed an own distinct group within subgroup A, AtFLA12 being the closest *Arabidopsis* homolog to all 11 poplar FLAs (Figure 2.9). Notably, of the four genes also downregulated in *P. euphratica*, two were homologs of PopFLA8 and PopFLA9.

A heatmap of the 18 *Arabidopsis* FLAs interrogated on the *Arabidopsis* ATH1 genome array and thus available in the Genevestigator database shows their response to different stress conditions (Figure 2.10). An overall mean of

Table 2.2 - Fasciclin-like arabinogalactan-proteins downregulated in *P.* × *canescens* under salt stress

Gene	JGI gene model	fold-change
PopFLA4.1	eugene3.00131210	0.05
PopFLA9	eugene3.00660250	0.06
PopFLA8	estExt_Genewise1_v1.C_LG_IX4802	0.07
PopFLA7.1	gw1.XV.397.1	0.09
PopFLA10	eugene3.00091518	0.09
PopFLA4.2	gw1.684.7.1	0.14
PopFLA6	eugene3.00131208	0.18
PopFLA14	eugene3.00012224	0.21
PopFLA15	estExt_Genewise1_v1.C_LG_XII0499	0.22
PopFLA7.2	estExt_Genewise1_v1.C_LG_XII0103	0.22
PopFLA7.3	grail3.0031016901	0.27

Numbering of poplar FLAs was adopted from (Lafarguette *et al.* 2004).

-0.564 of all fold-changes displayed indicates that *Arabidopsis* FLAs tend to be downregulated under drought, osmotic and salt stress, with group A FLAs stronger downregulated (mean fold-change: -0.805) than the remaining FLAs (mean fold-change: -0.443; p-value t-test: 0.003). Furthermore, the two *Arabidopsis* homologs closest to the 11 downregulated poplar FLAs, AtFLA 11 and 12, are the highest expressed FLAs in *Arabidopsis* stems (Figure 2.11).

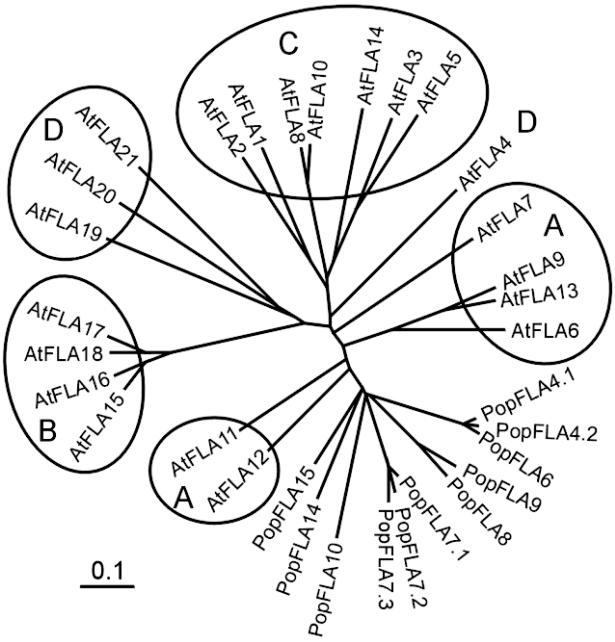

Figure 2.9 - Phylogenetic tree of fasciclin-like arabinogalactan proteins (FLAs)

Shown are all FLAs downregulated in *P.* × *canescens* under salt stress and the 21 FLA-genes present in *Arabidopsis*. JGI gene models and fold-changes under salt stress are listed in Table 2.2. Numbering of poplar FLAs was adopted from (2004). Numbering, group labels and AGI locus identifiers for *Arabidopsis* FLAs were obtained from (2003). Protein sequences were deduced from gene models and aligned with ClustalW2 (ClustalW2). The unrooted tree was generated using Tree View (Zhai *et al.* 2002).

Analysis of the upregulated genes delivered noticeably different results. 14 different GO categories were significantly enriched after Bonferroni-correction, encompassing 116 of the 198 genes, approximately 60 %. Since many of the genes were annotated by multiple GO annotations, an overview is necessary to understand the results of the GO term enrichment analysis. Upregulated genes

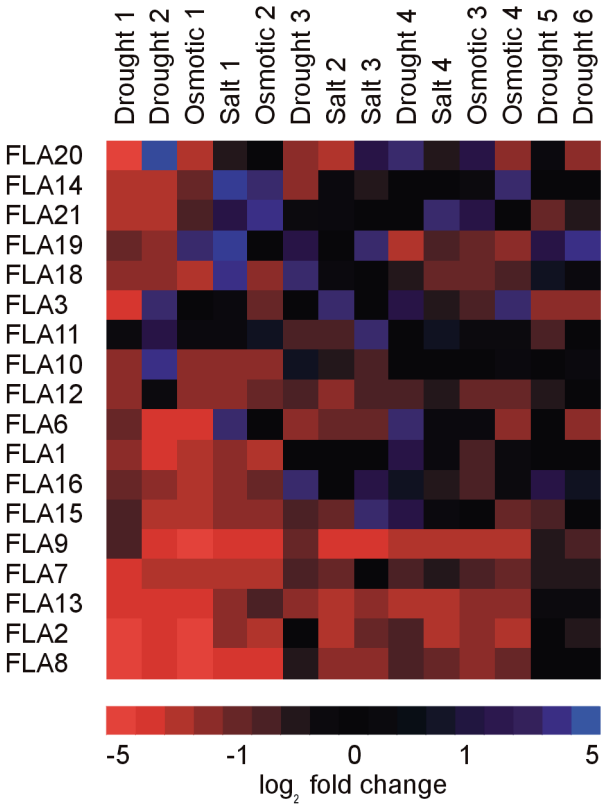

Figure 2.10 - Heatmap of expression values of 18 *Arabidopsis* FLA genes under drought, osmotic and salt stress conditions

Displayed are fold-changes for the 18 *Arabidopsis* FLA genes for which data were available in the Genevestigator database (Hruz *et al.* 2008). AGI locus identifiers for FLAs were taken from (2003). For Genevestigator experiment IDs and experiment description see Table 2.3.

Table 2.3 - Genevestigator experiment IDs and experiment descriptions used for heatmaps

Column	Genevestigator experiment ID	Experiment description
Experiment descriptions for Figure 2.10:		
Drought 1	AT-290	leaves of plants deprived of irrigation for 10 d
Drought 2	AT-292	leaves of plants deprived of irrigation for 7 d
Drought 3	AT-120	roots of plants grown in hydroponics, dried for 15 min in an air stream and harvested after further 0.5–3 h in the liquid medium
Drought 4	AT-120	leaves of plants grown in hydroponics, dried for 15 min in an air stream and harvested after further 0.5–3 h in the liquid medium
Drought 5	AT-120	roots of plants grown in hydroponics, dried for 15 min in an air stream and harvested after further 6–24 h in the liquid medium
Drought 6	AT-120	leaves of plants grown in hydroponics, dried for 15 min in an air stream and harvested after further 6–24 h in the liquid medium
Osmotic 1	AT-120	leaves of plants stressed with 300 mM Mannitol for 6–24 h
Osmotic 2	AT-120	roots of plants stressed with 300 mM Mannitol for 6–24 h
Osmotic 3	AT-120	leaves of plants stressed with 300 mM Mannitol for 0.5–3 h
Osmotic 4	AT-120	roots of plants stressed with 300 mM Mannitol for 0.5–3 h
Salt 1	AT-120	roots of plants stressed with 150 mM NaCl for 6–24 h
Salt 2	AT-120	roots of plants stressed with 150 mM NaCl for 0.5–3 h
Salt 3	AT-120	leaves of plants stressed with 150 mM NaCl for 6–24 h
Salt 4	AT-120	leaves of plants stressed with 150 mM NaCl for 0.5–3 h
Experiment descriptions for Figure 2.12:		
ABA1	AT-110	seedlings sprayed with 10µM ABA and harvested after 1h
ABA2	AT-218	seedlings grown for 24h on medium containing 20 µM ABA
ABA3	AT-231	seedlings grown for 48h on medium containing 0.5 µM ABA
ABA6	AT-241	seedlings sprayed with 50µM ABA and harvested after 4h
JA2	AT-321	cell suspension treated with 50 µM methyl jasmonate for 0.5 h
JA3	AT-321	cell suspension treated with 50 µM methyl jasmonate for 2 h
JA4	AT-321	cell suspension treated with 50 µM methyl jasmonate for 6 h
JA5	AT-110	seedlings sprayed with 10µM methyl jasmonate and harvested after 1h
SA	AT-113	seedlings sprayed with 10µM salicylic acid and harvested after 3h

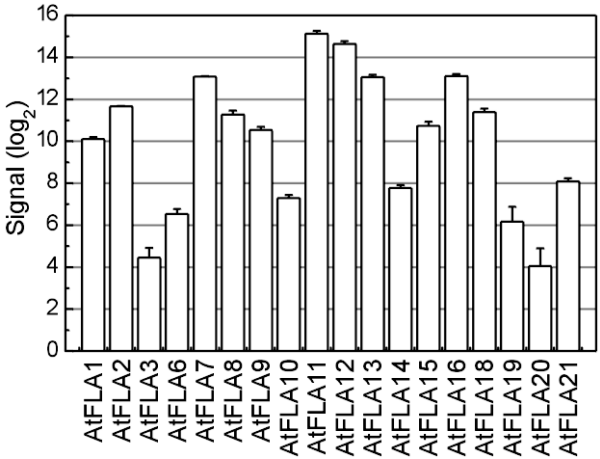

Figure 2.11 - Expression of FLAs in *Arabidopsis* stem

Displayed are signal values for the 18 *Arabidopsis* FLA genes available on the *Arabidopsis* ATH1 genome array. Expression was analyzed in *Arabidopsis* stems (Brown *et al.* 2005). Log_2 signal values and standard errors were obtained from the Genevestigator database (Hruz *et al.* 2008), experiment ID AT-327. Numbering of FLAs was adopted from (2003).

covered by the analysis were therefore manually clustered by corresponding GO terms (Table 2.4). Several groups of genes may thus be differentiated that concentrate on distinct biological processes; these processes therefore seem to play important roles in response to salt stress.

The first group (G1) is composed of genes with a 'response to toxin' annotation and consists almost entirely of glutathione-*S*-transferases (GSTs). GSTs are typically involved in detoxification of xenobiotics, as well as protection of the cell from toxic compounds that are generated by reactive oxigen species (ROS) produced under various stress conditions, amongst others salt stress (Marrs 1996).

Table 2.4 - Genes upregulated in developing xylem of *P.* × *canescens* under salt stress annotated by significantly enriched GO terms

				Annotated by													
	JGI gene model	Description	fold-change	catalytic activity (ca)	oxidoreductase activity (oa)	response to stimulus (rs)	response to chemical stimulus (rcs)	response to salicylic acid stimulus (rsas)	response to toxin (rt)	toxin catabolic process (tcp)	toxin metabolic process (tmp)	glutathione transferase activity (gta)	secondary metabolic process (smp)	aromatic compound metabolic process (acmp)	phenylpropanoid biosynthetic process (phbp)	flavonoid biosynthetic process (fbp)	flavonoid metabolic process (fmp)
G1	estExt_fgenesh4_pg.C_LG_VIII1530	Glutathione transferase	6.89	+	·	+	+	+	+	+	+	+	+	·	·	·	·
	estExt_Genewise1_v1.C_LG_X4725	Glutathione transferase	4.2	+	·	+	+	·	+	+	+	+	+	·	·	·	·
	estExt_Genewise1_v1.C_LG_XIV0250	Glutathione transferase	5.23	+	·	+	+	·	+	+	+	+	+	·	·	·	·
	grail3.0036009801	Glutathione transferase	3.3	+	·	+	+	·	+	+	+	+	+	·	·	·	·
	gw1.XIV.257.1	Glutathione transferase	4.55	+	·	+	+	·	+	+	+	+	+	·	·	·	·
	fgenesh4_pm.C_scaffold_44000008	Glycerol kinase, putative	1.95	+	·	+	+	·	+	·	·	·	·	·	·	·	·
G2a	estExt_fgenesh4_pg.C_LG_XVI1289	WRKY family transcription factor, WRKY4	5.04	·	·	+	+	+	·	·	·	·	+	+	·	·	·
	grail3.0023037401	WRKY family transcription factor, WRKY4	18.18	·	·	+	+	+	·	·	·	·	+	+	·	·	·
	gw1.40.601.1	MYB family transcription factor, MYB73	6.07	·	·	+	+	+	·	·	·	·	·	·	·	·	·
	estExt_fgenesh4_pm.C_LG_VI0283	MYB family transcription factor, putative	2.72	·	·	+	+	+	·	·	·	·	·	·	·	·	·
	estExt_fgenesh4_pm.C_LG_XI0348	Polyubiquitin UBQ10	2.44	·	·	+	+	+	·	·	·	·	·	·	·	·	·
	grail3.0007034202	WRKY family transcription factor	12.78	·	·	+	+	+	·	·	·	·	·	·	·	·	·
G2	estExt_fgenesh4_pg.C_LG_VIII1798	Dienelactone hydrolase	3.26	+	·	+	+	·	·	·	·	·	·	+	·	·	·
	estExt_Genewise1_v1.C_LG_VII0401	Allyl alcohol dehydrogenase	4.8	+	+	+	+	·	·	·	·	·	·	·	·	·	·
	gw1.IX.5030.1	Copper:zinc superoxide dismutase (CSD2)	2.82	+	+	+	+	·	·	·	·	·	·	·	·	·	·
	estExt_fgenesh4_pm.C_LG_VIII0652	Lipoxygenase	2.43	+	+	+	+	·	·	·	·	·	·	·	·	·	·
	estExt_fgenesh4_pg.C_570040	Peroxidase	5.21	+	+	+	+	·	·	·	·	·	·	·	·	·	·
	estExt_Genewise1_v1.C_LG_V0405	Peroxidase	3.06	+	+	+	+	·	·	·	·	·	·	·	·	·	·

Table 2.4 continued

	JGI gene model	Description	fold-change	ca	oa	rs	rcs	rsas	f	tcp	tmp	gta	smp	acmp	phbp	fbp	fmp
G2	estExt_Genewise1_v1.C_LG_VII1188	Nicotinamidase 1, Isochorismatase hydrolase	5.45	+	+	+	+	.	.	.	.	.	.	.	.	.	.
	estExt_fgenesh4_pm.C_LG_VII0202	Myo-inositol 1-phosphate synthase, putative	34.36	+	.	+	+	.	.	.	.	.	.	.	.	.	.
	gw1.XII.1331.1	Protein phosphatase 2C (PP2C)	10.91	+	.	+	+	.	.	.	.	.	.	.	.	.	.
	fgenesh4_pg.C_LG_IX001205	Protein phosphatase 2C (PP2C), ABA induced	11.47	+	.	+	+	.	.	.	.	.	.	.	.	.	.
	fgenesh4_pm.C_LG_IV000044	Serine/threonine kinase-like protein	4.83	+	.	+	+	.	.	.	.	.	.	.	.	.	.
	estExt_Genewise1_v1.C_LG_XIII1173	Two-component phosphorelay mediator	9.66	+	.	+	+	.	.	.	.	.	.	.	.	.	.
	grail3.0002064201	Auxin response transcription factor	3.29	.	.	+	+	.	.	.	.	.	.	.	.	.	.
	estExt_Genewise1_v1.C_LG_XIV2782	Gibberellin receptor	6.3	.	.	+	+	.	.	.	.	.	.	.	.	.	.
	estExt_Genewise1_v1.C_LG_XV1366	Gibberellin-regulated family protein	5.46	.	.	+	+	.	.	.	.	.	.	.	.	.	.
	eugene3.00140486	Homeobox-leucine zipper protein ATHB12	3.71	.	.	+	+	.	.	.	.	.	.	.	.	.	.
	estExt_Genewise1_v1.C_LG_II1841	Late embryogenesis abundant protein 5	3.01	.	.	+	+	.	.	.	.	.	.	.	.	.	.
	gw1.XIX.2946.1	Thylakoid formation1	2.71	.	.	+	+	.	.	.	.	.	.	.	.	.	.
	estExt_fgenesh4_pm.C_LG_XVI0323	Transcription factor-like protein, auxin response factor 4 (ARF4)	2.75	.	.	+	+	.	.	.	.	.	.	.	.	.	.
	gw1.VII.2995.1	Heat shock transcription factor HSF24	3.46	.	.	+	.	.	.	.	.	.	.	.	.	.	.
	estExt_Genewise1_v1.C_LG_IX1952	Hemoglobin II	2.43	.	.	+	.	.	.	.	.	.	.	.	.	.	.
	eugene3.01710026	MATE efflux family protein	2.58	.	.	+	.	.	.	.	.	.	.	.	.	.	.
	fgenesh4_pg.C_LG_X001039	Multi-copper oxidase type I family protein	2.53	.	.	+	.	.	.	.	.	.	.	.	.	.	.
	eugene3.00050086	NAC domain protein	5.31	.	.	+	.	.	.	.	.	.	.	.	.	.	.
	grail3.0020019002	Osmotin precursor	2.85	.	.	+	.	.	.	.	.	.	.	.	.	.	.
	grail3.0002061802	Cysteine proteinase RD19A	2.26	+	.	+	.	.	.	.	.	.	.	.	.	.	.
	gw1.XIX.381.1	Patatin	15.49	+	.	+	.	.	.	.	.	.	.	.	.	.	.
	gw1.VI.1016.1	Protein kinase, putatve	2.58	+	.	+	.	.	.	.	.	.	.	.	.	.	.
	eugene3.00050281	RNA helicase-like	2.83	+	.	+	.	.	.	.	.	.	.	.	.	.	.
	estExt_fgenesh4_pg.C_LG_II0181	Serine/threonine protein kinase	2.93	+	.	+	.	.	.	.	.	.	.	.	.	.	.
	estExt_Genewise1_v1.C_LG_I1784	9-cis-epoxycarotenoid dioxygenase	3.79	+	+	+	.	.	.	.	.	.	+	.	.	.	.
G3	gw1.158.12.1	Flavonoid 3'-hydroxylase (F3'H)	3.58	+	+	+	.	.	.	.	.	.	.	+	+	+	+
	estExt_Genewise1_v1.C_LG_XV0083	Flavanone 3'-hydroxylase-like protein	5.12	+	+	.	.	.	.	.	.	.	.	+	+	+	+
	eugene3.00440024	Flavonol synthase	2.69	+	+	.	.	.	.	.	.	.	.	+	+	+	+
	gw1.1215.4.1	Flavonol synthase	2.5	+	+	.	.	.	.	.	.	.	.	+	+	+	+
	estExt_Genewise1_v1.C_LG_XIV1942	Flavonol 3'-O-methyltransferase 1	2.5	+	.	.	.	.	.	.	.	.	.	+	+	+	+
	gw1.VI.124.1	Cytochrome p450 family protein	2.59	.	.	.	.	.	.	.	.	.	.	+	+	+	+
	estExt_fgenesh4_pg.C_LG_XVIII0872	Cinnamoyl CoA reductase, putative	3.16	+	+	.	.	.	.	.	.	.	.	+	+	+	.

Table 2.4 continued

JGI gene model	Description	fold-change	ca	oa	rs	rcs	rsas	rt	tcp	tmp	gta	smp	acmp	phbp	fbp	fmp
grail3.0016020901	Amidase AMI1	2.6	+	·	·	·	·	·	·	·	·	·	+	·	·	·
gw1.XIV.2533.1	Phospholipase, putative	3.57	+	·	·	·	·	·	·	·	·	·	+	·	·	·
gw1.IV.3981.1	Beta-carotene hydroxylase	3.72	+	+	·	·	·	·	·	·	·	+	·	·	·	·
gw1.II.421.1	Aldo/keto reductase, auxin-induced atb2	4.67	+	+	·	·	·	·	·	·	·	·	·	·	·	·
gw1.XIII.1471.1	Aldo/keto reductase, auxin-induced atb2	3.28	+	+	·	·	·	·	·	·	·	·	·	·	·	·
estExt_Genewise1_v1.C_860386	Carbonyl reductase (NADPH)	7.61	+	+	·	·	·	·	·	·	·	·	·	·	·	·
grail3.0091006302	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	2.86	+	+	·	·	·	·	·	·	·	·	·	·	·	·
eugene3.01270057	Flavin-containing monooxygenase 3	2.97	+	+	·	·	·	·	·	·	·	·	·	·	·	·
estExt_fgenesh4_pg.C_LG_XVIII0354	Flavoprotein monooxygenase	2.59	+	+	·	·	·	·	·	·	·	·	·	·	·	·
gw1.III.1067.1	Glycolate oxidase	3.13	+	+	·	·	·	·	·	·	·	·	·	·	·	·
eugene3.00090496	Isoflavone reductase	3.06	+	+	·	·	·	·	·	·	·	·	·	·	·	·
eugene3.00700243	Lipoxygenase, putative	4.56	+	+	·	·	·	·	·	·	·	·	·	·	·	·
grail3.0039027401	Metal ion binding / oxidoreductase	2.37	+	+	·	·	·	·	·	·	·	·	·	·	·	·
eugene3.00081881	Methionine sulfoxide reductase	2.67	+	+	·	·	·	·	·	·	·	·	·	·	·	·
eugene3.00131159	NADH dehydrogenase, putative	1.88	+	+	·	·	·	·	·	·	·	·	·	·	·	·
gw1.XVIII.1835.1	Glyceraldehyde-3-phosphate dehydrogenase, putative	4.56	+	+	·	·	·	·	·	·	·	·	·	·	·	·
fgenesh4_pg.C_LG_VIII000310	Protein disulfide-isomerase-like protein	3.35	+	+	·	·	·	·	·	·	·	·	·	·	·	·
estExt_fgenesh4_pm.C_860049	Quinone oxidoreductase	4.11	+	+	·	·	·	·	·	·	·	·	·	·	·	·
estExt_fgenesh4_pg.C_LG_V1578	Thioredoxin	3	+	+	·	·	·	·	·	·	·	·	·	·	·	·
eugene3.00100431	Thioredoxin	11.6	+	+	·	·	·	·	·	·	·	·	·	·	·	·
gw1.X.4817.1	Thioredoxin	8.67	+	+	·	·	·	·	·	·	·	·	·	·	·	·
estExt_fgenesh4_pg.C_LG_VI0598	20S proteasome beta subunit A (PBA1)	2.72	+	·	·	·	·	·	·	·	·	·	·	·	·	·
estExt_Genewise1_v1.C_53080003	ABC transporter family protein	11.16	+	·	·	·	·	·	·	·	·	·	·	·	·	·
fgenesh4_pm.C_LG_I001151	ABC transporter family protein	3.25	+	·	·	·	·	·	·	·	·	·	·	·	·	·
gw1.VI.655.1	ABC transporter family protein	3.89	+	·	·	·	·	·	·	·	·	·	·	·	·	·
fgenesh4_pm.C_LG_I000875	Aldose 1-epimerase	2.06	+	·	·	·	·	·	·	·	·	·	·	·	·	·
eugene3.00070657	Alpha-amino-epsilon-caprolactam racemase	1.98	+	·	·	·	·	·	·	·	·	·	·	·	·	·
estExt_fgenesh4_pg.C_640031	Aminopeptidase-like protein	4.75	+	·	·	·	·	·	·	·	·	·	·	·	·	·
gw1.X.4548.1	Cyclopropane-fatty-acyl-phospholipid synthase	6.68	+	·	·	·	·	·	·	·	·	·	·	·	·	·
eugene3.00040664	DegP protease	2.55	+	·	·	·	·	·	·	·	·	·	·	·	·	·
gw1.I.1992.1	Dihydropyrimidinase	3.67	+	·	·	·	·	·	·	·	·	·	·	·	·	·
grail3.0066013503	Esterase/lipase/thioesterase family protein	2.53	+	·	·	·	·	·	·	·	·	·	·	·	·	·
estExt_fgenesh4_pg.C_LG_V0589	Formamidase-like protein	9.29	+	·	·	·	·	·	·	·	·	·	·	·	·	·

Table 2.4 continued

JGI gene model	Description	fold-change	ca	oa	rs	rcs	rsas	rt	tcp	tmp	gta	smp	acmp	phbp	fbp	fmp
gw1.VII.115.1	GCN5-related N-acetyltransferase (GNAT) family protein	3.18	+	·	·	·	·	·	·	·	·	·	·	·	·	·
grail3.0045003902	Glucan endo-1,3-beta-glucosidase	2.95	+	·	·	·	·	·	·	·	·	·	·	·	·	·
gw1.IX.301.1	Glucan endo-1,3-beta-glucosidase	2.47	+	·	·	·	·	·	·	·	·	·	·	·	·	·
estExt_Genewise1_v1.C_LG_IX1717	Glucose-1-phosphate adenylyltransferase (APL3)	4.75	+	·	·	·	·	·	·	·	·	·	·	·	·	·
gw1.II.3341.1	Gr1 protein	2.01	+	·	·	·	·	·	·	·	·	·	·	·	·	·
fgenesh4_pg.C_LG_I002046	Hookless1-like protein	5.45	+	·	·	·	·	·	·	·	·	·	·	·	·	·
estExt_fgenesh4_pg.C_LG_XII0735	Lipase, EDS1-like protein	2.65	+	·	·	·	·	·	·	·	·	·	·	·	·	·
gw1.XIII.1296.1	Matrixin family protein, metallopeptidase	14.45	+	·	·	·	·	·	·	·	·	·	·	·	·	·
estExt_Genewise1_v1.C_LG_I8346	Methionine/cystathionine gamma lyase	4.9	+	·	·	·	·	·	·	·	·	·	·	·	·	·
gw1.XV.3454.1	Methyltransferase MT-A70 family protein	5.94	+	·	·	·	·	·	·	·	·	·	·	·	·	·
grail3.0047000902	Molybdenum cofactor sulfurase	2.1	+	·	·	·	·	·	·	·	·	·	·	·	·	·
grail3.0032010003	MutT domain protein-like	2.15	+	·	·	·	·	·	·	·	·	·	·	·	·	·
gw1.V.2886.1	MutT domain protein-like	2.03	+	·	·	·	·	·	·	·	·	·	·	·	·	·
estExt_fgenesh4_pm.C_LG_V0078	Myo-inositol-1-phosphate synthase	16.69	+	·	·	·	·	·	·	·	·	·	·	·	·	·
gw1.129.19.1	Nuclear ribonuclease Z (RNase Z)	5.98	+	·	·	·	·	·	·	·	·	·	·	·	·	·
grail3.1005000101	O-methyltransferase	5.42	+	·	·	·	·	·	·	·	·	·	·	·	·	·
grail3.0049003701	OTU-like cysteine protease family protein	7.25	+	·	·	·	·	·	·	·	·	·	·	·	·	·
gw1.I.4198.1	Phosphatidylinositol 3-kinase	17.67	+	·	·	·	·	·	·	·	·	·	·	·	·	·
eugene3.00150051	Receptor lectin kinase-like protein	2.71	+	·	·	·	·	·	·	·	·	·	·	·	·	·
gw1.1018.1.1	Receptor serine/threonine kinase PR5K	5.78	+	·	·	·	·	·	·	·	·	·	·	·	·	·
grail3.0010053201	Receptor-like kinase	2.15	+	·	·	·	·	·	·	·	·	·	·	·	·	·
gw1.XVII.1438.1	Receptor-like protein kinase	5.32	+	·	·	·	·	·	·	·	·	·	·	·	·	·
fgenesh4_pg.C_scaffold_782000001	Receptor-like serine/threonine kinase	15.35	+	·	·	·	·	·	·	·	·	·	·	·	·	·
eugene3.00180810	RuBisCO small subunit 2b	3.08	+	·	·	·	·	·	·	·	·	·	·	·	·	·
gw1.6229.2.1	Serine/threonine protein kinase	2.76	+	·	·	·	·	·	·	·	·	·	·	·	·	·
gw1.XI.1271.1	Serine/threonine protein kinase	4.51	+	·	·	·	·	·	·	·	·	·	·	·	·	·
gw1.XVI.239.1	Serine/threonine protein kinase	2.4	+	·	·	·	·	·	·	·	·	·	·	·	·	·
estExt_Genewise1_v1.C_1570104	Storage protein	2.79	+	·	·	·	·	·	·	·	·	·	·	·	·	·
eugene3.00181112	Sucrose-phosphate synthase	3.54	+	·	·	·	·	·	·	·	·	·	·	·	·	·
gw1.II.1542.1	Trehalose-phosphatase	3.21	+	·	·	·	·	·	·	·	·	·	·	·	·	·
fgenesh4_pg.C_scaffold_164000053	tRNA pseudouridine synthase	3.13	+	·	·	·	·	·	·	·	·	·	·	·	·	·
gw1.XVIII.2839.1	Xyloglucan endotransglycosylase (XTR-6)	9.59	+	·	·	·	·	·	·	·	·	·	·	·	·	·
gw1.I.4198.1	Phosphatidylinositol 3-kinase	17.67	+	·	·	·	·	·	·	·	·	·	·	·	·	·

Genes in the second group (G2) are annotated by one or two of the GO terms 'response to stimulus' and 'response to chemical stimulus'. A small subgroup (G2a) of six genes has a further 'response to salicylic acid stimulus' annotation in common. For a better understanding of the role of phytohormones in this regulatory process, all 18 upregulated genes from the GO category 'response to stimulus' that were annotated by response to stimulus of a phytohormone have been selected for a detailed examination of possible impact of phytohormones on changes in xylem anatomy (Table 2.5).

Of the seven GO terms indicating response to a phytohormone, only 'response to salicylic acid stimulus' was significantly overrepresented when Bonferroni-correction was applied to all 861 different GO terms available for upregulated genes. Restricting Bonferroni-correction to the seven phytohormone-related GO terms, 'response to stimulus' of abscisic acid (ABA), jasmonic acid (JA) and gibberellin were also significantly enriched at $p < 0.05$, whereas response to stimulus by auxin, ethylene and cytokinin GO terms were not overrepresented. In contrast, only 7 phytohormone-related annotations were present among the downregulated transcripts, and no annotation for response to a single phytohormone was significantly enriched, even if restricting the analysis to phytohormone-related GO term annotations.

SA, ABA and JA are well known to be involved in salt stress responses (Moons *et al.* 1997; Janda *et al.* 2007). Gibberellin is thought to play an important role in salinity tolerance by enhancing plant growth (Siddiqui *et al.* 2008). Notably, multiple genes among the upregulated ABA responsive genes had been associated with the downregulation of FLAs: the ABA-induced protein phosphatase 2C (PP2C), ABI1, and the ABA-induced homeobox leucine-zipper protein ATHB-12, whose upregulation is dependent on ABI1 (Olsson *et al.* 2004; Johnson *et al.* 2003; Merlot *et al.* 2001).

Table 2.5 - Genes upregulated in developing xylem of *P.* × *canescens* under salt stress annotated by a 'response to phytohormone stimulus'-GO term

			Response to stimulus of						
Description	closest *Arabidopsis* ortholog	fold-change	ABA	SA	JA	G	A	E	C
MYB family transcription factor, MYB73	At4g37260	6.07	+	+	+	+	+	+	
Lipoxygenase	At1g55020	2.43	+		+				
Nicotinamidase 1, Isochorismatase hydrolase	At2g22570	5.45	+						
Homeobox leucine-zipper protein ATHB-12	At3g61890	3.71	+						
Protein phosphatase 2C (PP2C), ABI1	At3g11410	10.91	+						
Protein phosphatase 2C (PP2C), ABA induced	At5g59220	11.47	+						
Serine/threonine kinase-like protein	At4g21410	4.83	+						
MYB family transcription factor, putative	At2g37630	2.72		+	+	+	+		
WRKY family transcription factor, WRKY4	At3g56400	5.04		+	+				
WRKY family transcription factor, WRKY4	At3g56400	18.18		+	+				
Glutathione transferase	At2g29420	6.89		+					
Polyubiquitin UBQ10	At4g05320	2.44		+					
WRKY family transcription factor	At1g80840	12.78		+					
Gibberellin receptor	At3g63010	6.3				+			
Gibberellin-regulated family protein	At5g14920	5.46				+			
Transcription factor-like protein, auxin response factor 4 (ARF4)	At4g30080	2.75					+		
Auxin response transcription factor	At1g19850	3.29					+		
Two-component phosphorelay mediator	At3g21510	9.66							+

'+' indicates the phytohormones to which the respective gene responds. ABA, abscisic acid; SA, salicylic acid; JA, jasmonic acid; G, gibberellin; A, auxin; E, ethylene; C, cytokinin.

Of the phytohormone-response genes, 5 had annotations for response to two or more different phytohormones, stressing the importance of cross-talk between the hormones. Furthermore, the fact that 8 of the 18 genes are transcription factors and 5 are involved in signal transduction emphasizes the high level at which phytohormones influence gene expression.

The third group (G3, Table 2.4) consists of genes involved in flavonoid and phenylpropanoid biosynthetic processes: flavonoid 3'-hydroxylase (EC

1.14.13.21), flavanone 3'-hydroxylase (EC 1.14.11.9), flavonol synthase (EC 1.14.11.23) and flavonol 3'-O-methyltransferase (EC 2.1.1.76). These genes coded almost exclusively for enzymes present in two related pathways, flavonoid biosynthesis (KEGG pathway ec00941) and flavonone biosynthesis (KEGG pathway ec00944). These pathways are located upstream from biosynthesis of anthocyans, compounds which are active as ROS scavengers (Wang *et al.* 1997; Deboo *et al.* 1995).

These three groups account for 50 of the 116 upregulated genes that are annotated by at least one significantly enriched GO term. Most of the remaining 66 genes were only annotated by the significantly enriched GO terms 'catalytic activity' and/or 'oxireductase activity'; since these two terms belong to the 'molecular function' ontology, they provide no information value in respect of biological processes important in salt stress response.

To gain more information on the possible role of phytohormones in changes in xylem anatomy, gene expression data from hormone treatment experiments in *Arabidopsis* were examined. For this analysis, all genes upregulated in *P.* × *canescens* under salt stress annotated by GO terms that have a high relevance for the formation of vessels, *i.e.* 'cell communication', 'cell development', 'cell differentiation', 'programmed cell death', 'cell division', 'pattern specification process', 'cell wall' and 'cell cycle', were chosen. The best matches of *Arabidopsis* orthologs were used to obtain expression data from the Genevestigator database (Hruz *et al.* 2008). In this analysis, we included all available experiments employing SA, ABA and JA, since annotations for response to one of these phytohormones were the most frequent in this study, an observation which is in concurrence with current biological models which consider these three phytohormones to be the principally involved effectors in plant responses to water and salt stress (Thaler & Bostock 2004).

To illustrate the resulting gene expression matrix, the $\log_2$ fold changes for the single genes in the different experiments are displayed as a heatmap (Figure 2.12). The most striking feature of the expression matrix is its irregularity. In none of the experiments in *Arabidopsis*, more than half of the genes were significantly upregulated, and only individual genes were consistently up- or downregulated by one phytohormone. The protein phosphatase 2C (PP2C), ABI1 is the only gene that was continuously strong upregulated, *i.e.* by a fold-change of more than 1.5, in all four ABA treatment experiments. Noticeably, ABA exerts the strongest control on transcript abundance, whereas the JA experiments delivered more inconsistent results for single genes. Referring to individual experiments, more genes are upregulated by ABA and SA than by JA, an observation that reflects the frequencies of the annotations for responses to the different phytohormones (Table 2.5). Furthermore, an upregulation by SA is usually accompanied by a downregulation by ABA, which can be explained by hormonal crosstalk, since ABA has been considered to be an antagonist to SA induced responses (Thaler & Bostock 2004).

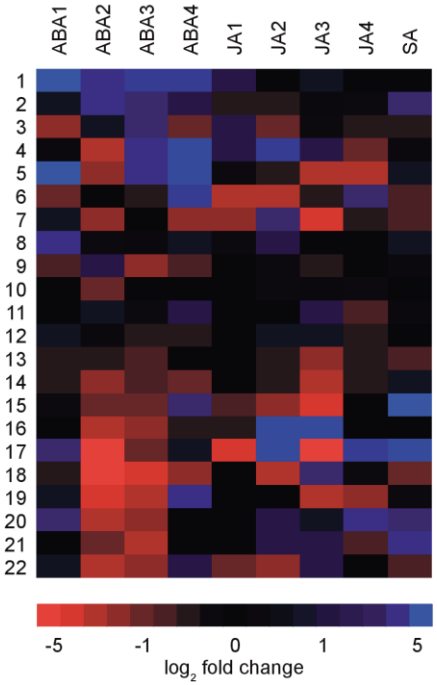

Figure 2.12 - Heatmap of expression values of 22 *Arabidopsis* genes from different phytohormone treatment experiments

Genes displayed are the best matches of *Arabidopsis* orthologs of genes upregulated in *P.* × *canescens* under salt stress that were annotated by one of the GO terms 'cell communication', 'cell development', 'cell differentiation', 'programmed cell death', 'cell division', 'pattern specification process', 'cell wall' and 'cell cycle'. Fold-change values were obtained from the Genevestigator database (Hruz *et al.* 2008). For Genevestigator experiment IDs and experiment description see Table 2.3.

Rows with corresponding gene descriptions and AGI locus identifiers: 1, Protein phosphatase 2C (PP2C; ABI1), At3g11410; 2, Quinone oxidoreductase, At5g61510; 3, Transcription factor-like protein, At4g30080; 4, Late embryogenesis abundant protein 5, At4g02380; 5, Esterase/lipase/thioesterase family protein, At5g22460; 6, Disease resistance protein-like, At5g49290, ; 7, MYB family transcription factor1, At2g37630; 8, Serine/threonine protein kinase, At5g58380; 9, ATP-binding family protein, At5g22370; 10, Thylakoid formation, At2g20890; 11, MYB family transcription factor, At1g14350; 12, Auxin response transcription factor, At1g19850; 13, Receptor-like protein kinase, At3g51550; 14, Similar to mitotic cyclin a2-type, At1g44110; 15, WRKY family transcription factor,At3g56400 ; 16, Peroxidase, At1g71695; 17, Patatin, At2g26560; 18, Peroxidase, At5g42180; 19, Xyloglucan endotransglycosylase (XTR-6), At4g25810; 20, Gibberellin receptor, At3g63010; 21, Multi-copper oxidase type I family protein, At1g71040; 22, Two-component phosphorelay mediator, At3g21510.

2.2.5. Validation of microarray data by qRT-PCR

To validate the microarray results for *P.* × *canescens*, quantitative real-time PCR (qRT-PCR) was employed. The expression patterns of five genes, four identified as being upregulated and one as being downregulated after four weeks of increasing salt stress, were analyzed. A significant linear correlation exists between the $\log_2$ expression ratios of the qRT-PCR analysis and the $\log_2$ signal ratios of the microarrays (Figure 2.13), confirming the accuracy of the microarray data.

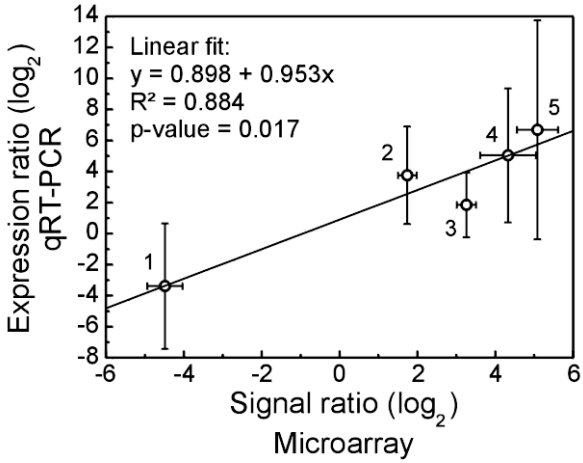

Figure 2.13 - Comparison of qRT-PCR expression and microarray signal ratios

For qRT-PCR, expression ratios for *P.* × *canescens* salt treated/control were calculated from threshold cycle (Ct) values normalized against an actin standard using the Relative Expression Software Tool (REST). Signal ratios for the microarray analysis were taken as calculated by the SAM algorithm (n=3, mean±SE for qRT-PCR data, as calculated by REST, and mean±SD for microarray data, as calculated by SAM).

Transcript description, Affymetrix probe set ID and JGI gene model for analyzed genes: 1 glycosyl hydrolase family 32, PtpAffx.36845.1.S1_s_at, estExt_fgenesh4_pg.C_LG_III0902; 2 stress related protein, PtpAffx.36446.1.A1_at, estExt_fgenesh4_pm.C_LG_VII0202; 3, glycoside hydrolase family 16, PtpAffx.112384.1.S1_s_at, gw1.XVIII.2839.1; 4, WRKY family transcription factor, Ptp.3143.1.S1_at, grail3.0023037401; 5, myo-inositol-1-phosphate synthase, PtpAffx.36446.1.A1_at, estExt_fgenesh4_pm.C_LG_VII0202.

2.2.6. Influence of salt stress on the chemical composition of developing xylem of *P. × canescens* and *P. euphratica*

To assess the magnitude and tendencies of differences in chemical composition of the developing xylem that is formed in *P. × canescens* and *P. euphratica* under control conditions and under salt stress, FTIR-ATR (Fourier transform infrared spectroscopy - attenuated total reflection) measurements were conducted. FTIR spectra have found extensive use in the study of wood chemistry (e.g. Faix *et al.* 1991; Pandey 1999). Per treatment group, five biological replicates were analyzed.

All FTIR spectra showed the same prominent peaks in the fingerprint region of the wavenumbers from 1800 to 600 cm^{-1} as beech wood (Rana *et al.* 2008). Of special interest was the region of the wavenumbers from 1750 to 1200 cm^{-1} (Figure 2.14). In this region, peaks represent major cell wall components, such as cellulose, hemicelluloses, and lignin (Table 2.6). Here, differences between the spectra were most prominent, and a cluster analysis permitted grouping of the FTIR spectra according to their species and treatment groups, *i.e. P. × canescens*/*P. euphratica* and control/100 mM NaCl (Figure 2.15). The analysis showed that the spectra were primarily clustered according to the species, and subclustered according to control and salt treatment.

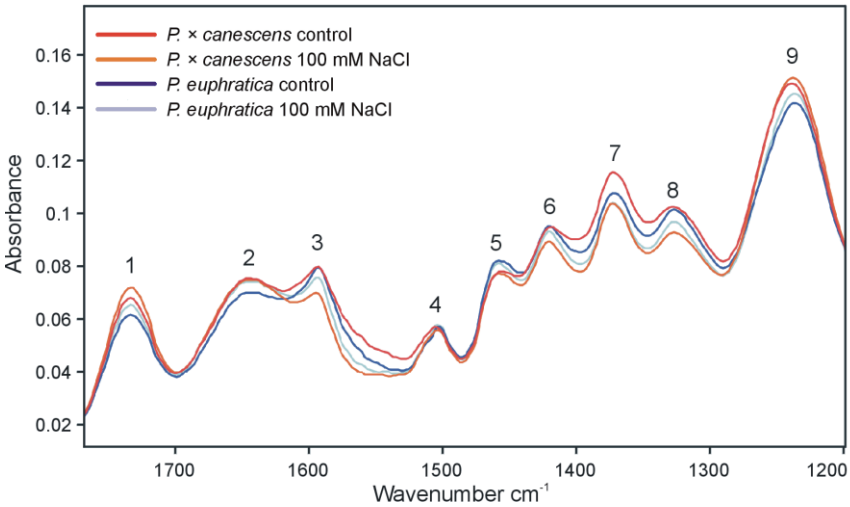

Figure 2.14 - FTIR analysis of developing xylem from *Populus*

Mean FTIR spectra for the region of wavenumbers from 1200 to 1750 m^{-1} of developing xylem from *P.* × *canescens* and *P. euphratica* under control conditions and after a four week period of increasing salt stress with a final salt concentration of 100 mM NaCl. Spectra are means of 5 biological replicates. Peak numbering refers to Table 2.6.

Differences in peak heights between samples of control and salt stressed plants showed similar tendencies for *P.* × *canescens* and *P. euphratica* (Figure 2.14). Absorbance values of peaks 1 and 9 increased equally in both species under salt stress, whereas they decreased for peaks 3, 6, 7 and 8, a shift that was more pronounced in *P.* × *canescens*. Salt stress had no effect on peaks 4 and 5. Peak 2 was an exception insofar as it was only influenced in *P. euphratica*, where the absorbance was stronger in developing xylem of salt stressed plants. The differences in the spectra between control and salt stressed plants indicate a change in chemical composition and suggest a lowered lignin content.

Table 2.6 - Assigned FTIR-bands of developing xylem of Poplar

Peak no.	Wave number (cm^{-1})	Band origin
1	1738	C=O stretch in unconjugated ketones, carbonyls and ester groups in xylans (hemicellulose)
2	1650	Absorbed O-H and conjugated C=O
3	1596	Aromatic skeletal vibrations in lignin plus C=O stretch
4	1505	same as peak no. 3
5	1462	C-H deformation; asymmetric in $-CH_3$ and $-CH_2-$; lignin and carbohydrates
6	1425	Aromatic skeletal vibrations combined with C-H plane deformation; lignin and carbohydrates
7	1375	C-H deformation in cellulose and hemicellulose
8	1330	Syringyl ring plus guaiacyl ring condensed
9	1235	Syringyl nuclei deformation combined with deformation of cellulose

Band assignements in the region of wavenumbers from 1200 to 1750 m^{-1} were taken from (Rana *et al.* 2008).

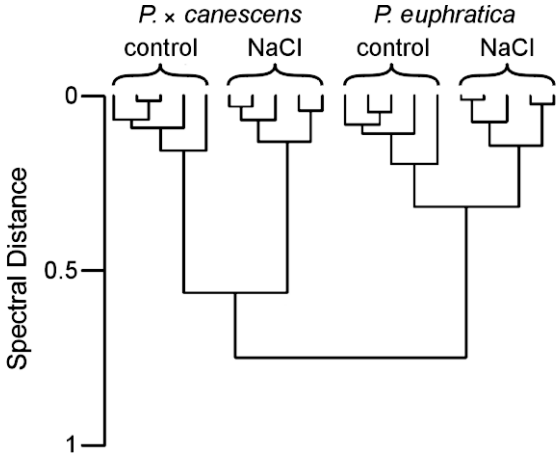

Figure 2.15 - Cluster analysis of FTIR spectra from developing xylem from *Populus*

Developing xylem was harvested from *P.* × *canescens* and *P. euphratica* under control conditions and after a four week period of increasing salt stress with a final salt concentration of 100 mM NaCl. FTIR spectra were processed using a spectroscopy software (OPUS version 6.5, Bruker, Ettlingen, Germany). First derivates of spectra after base-line correction were used for cluster analysis using Ward's algorithm and correlation coefficient as distance metric.

2.3. Discussion

2.3.1. *P. euphratica* is unaffected by salt concentrations that cause stress effects in *P.* × *canescens*

Salt tolerance is based on three principles: homeostasis, detoxification and growth control. Plants that by evolution have become adapted to soil salinity mostly avoid toxic salt levels inside the plant, and thus having to cope with detoxification. They achieve this by concentrating on the principles of homeostasis and growth control; by a decreased biomass production, energy for excluding sodium at the roots or transporting it into vacuoles is made available (Munns 2002; Zhu 2001b). In this study, the differences these mechanisms effectuate in physiology between a salt sensitive and a salt tolerant species become apparent. Although both *P.* × *canescens* and *P. euphratica* were grown for the same time under the same stress conditions, salt accumulation and stress symptoms were only observed in *P.* × *canescens*, while *P. euphratica* was obviously able to maintain ion homeostasis and thus avoid salt stress on a cellular level. This data emphasizes the magnitude of the physiological variation that is possible between two closely related species in response to a distinct abiotic stress.

2.3.2. Changes in xylem anatomy in *P.* × canescens are an adaptation to hydraulic stress

The osmotic stress inside plants that is effectuated by salt stress causes strong negative xylem pressures which can lead to cavitation and subsequently to conduit collapse (Hacke & Sperry 2001). To prevent this collapse, an increase in wall strength is required, where wall strength is given as the wall thickness to vessel diameter ratio (Hacke *et al.* 2001). The increase in strength

can therefore be achieved either by generating thicker walls or narrower vessels. In poplar, narrower xylem vessels and a higher wall strength have been previously observed under salt stress (Junghans *et al.* 2006).

Further observations to changes in xylem anatomy have been made in mangrove trees, plants that are well adapted to salinity. Vessel diameters have been observed to be considerably smaller in mangrove species compared to non-mangrove species of the same genus (Janssonius 1950). Simultaneously, the vessel density, *i.e.* the number of vessels per area, increases. The same changes in vessel size and density occur in early and late wood of *Rhizophora mucronata* (Verheyden *et al.* 2005). In the rainy season, fewer and larger vessels are produced, whereas in the dry season, when soil salinity increases due to higher evaporation and lower precipitation, a higher number of smaller vessels are produced. Verheyden *et al.* (2005) reasoned that this alteration of the xylem anatomy increases the plants hydraulic safety under water stress conditions, because single cavitation incidents pose a lower threat when a high number of alternate conduits are available, a well established hypothesis (Tyree & Ewers 1991; Zimmermann 1983).

In this study, under control conditions, the salt sensitive species has fewer but larger vessels than the salt tolerant species (Figure 2.8 a and b). Under salt exposure, vessel sizes and density, the predicted conductivity and the wall strength remain at a constant level in *P. euphratica*. In *P.* × *canescens*, vessels become smaller and more numerous and have an increased wall strength, while the predicted conductivity is simultaneously maintained. The wood structure of *P.* × *canescens* therefore changes to a state with an increased hydraulic safety, where implosion incidents are less likely to occur. Noticeably, wood anatomy of *P.* × *canescens* is more similar to wood anatomy of *P. euphratica* when formed under salt stress. This suggests that a) xylem formed under salt stress in

P. × *canescens* is better adjusted to hydraulic stress, and b) xylem of *P. euphratica* is already pre-adapted to hydraulic stress under control conditions.

2.3.3. Carbohydrates are not the limiting factor in xylem development

Sugars are known to accumulate in plants under various abiotic stresses despite a decrease in photosynthates. This phenomenon has been associated with osmolyte accumulation and signaling of leaf senescence, and is often observed in leaves (Wingler & Roitsch 2008; Hare *et al.* 1998). However, in this study sugars accumulated in the phloem of *P.* × *canescens* under salt stress despite a decrease of photosynthetic activity. This might be explained by an increased need for energy in roots, since under water stress conditions, higher root:shoot ratios are needed to maintain sufficient rates of water uptake, which necessitates the allocation of a higher fraction of available sugars to the roots (Kozlowski & Pallardy 2002). Consequently, phloem unloading in the stem would decrease, and sugar levels would increase in phloem sap.

In our study, this is reflected by a decrease of sucrose, the primary transport sugar, in the developing xylem under salt stress in *P* × *canescens*. However, glucose and fructose levels do not decrease under these circumstances. Therefore, it is unlikely that a deficit of carbohydrates leads to a change in xylem anatomy, as proposed by Escalante-Pérez *et al.* (Escalante-Perez *et al.* 2009). Presumably, a different signal causes a change of the growth pattern in order to reduce the energetic requirements of the developing xylem. A possible mediator is a hormonal system involving ABA that regulates shoot growth under water stress, as proposed by Munns and Termaat (in Kozlowski 1997).

2.3.4. The transcriptomical response of developing xylem to salt stress in poplar

In this study, we conducted a comparative microarray analysis to gain information about the molecular mechanisms that lead to salt adaptation. Since *P. euphratica* showed no visible stress symptoms and salt accumulation in developing xylem was considerably lower than in *P.* × *canescens*, the extremely low number of differentially regulated genes in *P. euphratica* did not surprise, it in fact confirmed the unstressed state. Studies on other closely related salt sensitive and salt tolerant species showed similar results. In a salt tolerant and a salt sensitive rice (*Oryza sativa*) genotype, salinity stress effectuated differential expression of 330 and 465 genes, respectively, with only 10 genes present in both transcriptomes (Walia *et al.* 2005). In a comparison of *Arabidopsis thaliana* with its close relative *Thellungiella halophila*, 40 respective 6 genes were differentially expressed, with 2 common genes (Taji *et al.* 2004). In both studies, NaCl uptake was lower in the salt tolerant species. The tendency of salt tolerant species to rely on salt avoidance rather than to develop a manipulable transcriptome seems prevalent, and the occurring molecular responses therefore deviate from closely related salt sensitive species.

2.3.5. Salt stress on a cellular level induces antioxidants and detoxification-involved genes

Various biotic and abiotic stress conditions such as pathogen attack or salt, light or temperature stress induce the production of reactive oxygen species (ROS) in plants, a phenomenon that is known as the oxidative burst (Sairam & Tyagi 2004; Shetty *et al.* 2008). The plants' response is an increase in ROS scavengers and antioxidants to control the level of these hazardous molecules

(Zhu 2001b). Flavonoids are known to accumulate under oxidative stress and to be able to act as antioxidants, although their exact role in the antioxidative defense system is still discussed (Hernandez *et al.* 2009). In the salt sensitive *P.* × *canescens*, but not in *P. euphratica*, a disproportional number of genes belonging to the flavonoid biosynthesis pathway were upregulated under salt stress. Similar findings have been made for other plant species: Walia *et al.* found that several genes coding for important enzymes from the flavonoid biosynthetic pathway were induced by salt stress in a salt sensitive, but not in a salt tolerant rice cultivar (Walia *et al.* 2005), and Paolacci *et al.* reported the induction of the flavonoid biosynthetic pathway under ozone stress in an ozone sensitive but not in a resistant bean genotype (Paolacci *et al.* 2001). Based on these and further data, Walia *et al.* suggested that the induction of the flavonoid pathway under salt stress is caused by the coinciding oxidative stress.

Furthermore, in both *P.* × *canescens* and the salt sensitive rice cultivar, salt stress induced glutathione-*S*-transferase (GST) genes (Walia *et al.* 2005). A stress induced subgroup of GSTs in plants is considered to be involved in various stress responses, including oxidative stress (Marrs 1996). Katsuhara *et al.* showed that in transgenic *Arabidopsis*, overexpression of a tobacco GST could reduce lipid peroxidation caused by oxidative stress, which in turn was evoked by salt stress.

The fact that two clusters of the GO term enrichment analysis consist of genes that seem to be involved in oxidative stress response indicates the impact of ROS on *P.* × *canescens* under salt stress. However, an increased level of ROS is needed under stress conditions, as ROS themselves act as signals and influence developmental and defense pathways (Mittler *et al.* 2004). In this regard, apart from avoiding having to deal with ion toxicity and osmotic balance, *P. euphratica* gains an additional energetic advantage by excluding salt; the regulation of genes controlling the complicated network of ROS

production and scavenging, as well as detoxification of products of oxidative damage becomes superfluous.

ROS are also involved in the regulation of cell growth and programmed cell death and might therefore be involved in the observed changes in xylem anatomy (Foreman *et al.* 2003; Overmyer *et al.* 2003). However, a decrease in vessel sizes has been observed in plants under different water stress conditions, *i.e.* salt, drought and freezing stress (Baum *et al.* 2000; Junghans *et al.* 2006; Turco *et al.* 2002; Arend & Fromm 2007; Hacke & Sperry 2001; Hacke *et al.* 2001; Abrams 1990), but to our knowledge not in plants under pathogen attack. But since pathogen attacks also induce the oxidative burst, it is unlikely that the ROS signaling network causes the changes in xylem anatomy (Shetty *et al.* 2008).

2.3.6. Fasciclin-like arabinogalactans are downregulated under salt stress

The GO category 'cell adhesion' consisted of fasciclin-like arabinogalactan-proteins (FLAs) which form a distinct subgroup of arabinogalactan proteins (AGPs). AGPs are thought to be involved in various processes regarding xylem differentiation; Seifert & Roberts summarized previous work on AGPs that suggested roles in cell-cell signalling, cell division and programmed cell-death (PCD), properties that play important roles in vascular pattern formation (2007). Shi *et al.* demonstrated that the *Arabidopsis* Salt Overly Sensitive 5 (SOS5) protein, a synonym for FLA4 [Swiss-Prot:Q9SNC3], is required for controlled cell expansion (2003a). FLA genes have been found to be upregulated in tension wood, a tissue type with an increased cellulose content and a decreased content of lignins and hemicelluloses (Andersson-Gunnerås *et al.* 2006; Lafarguette *et al.* 2004).

However, the function of FLAs in these processes is still unknown. Since FLAs are massively downregulated in *P.* × *canescens* under salt stress, these findings suggest that FLAs in poplar might have an influence on the changes in xylem anatomy and the chemical composition of wood.

The distinct FLAs downregulated in this study form a group of orthologs which have no true homologs in *Arabidopsis*; this observation has also been made for FLAs massively upregulated during the formation of tension wood (Andersson-Gunnerås *et al.* 2006). While in *Arabidopsis* only 20 FLAs are known, several hundreds can be found in Populus (Johnson *et al.* 2003; Andersson-Gunnerås *et al.* 2006). Tree-specific FLAs therefore seem to fulfill functions not required in herbaceous plants. In this study, *P.* × *canescens* and *P. euphratica* showed very few common reactions towards salt stress; one was the downregulation of PopFLA8 and PopFLA9, and one the changes in chemical composition of the wood as measured by FTIR. Only 27 genes were differentially expressed in *P. euphratica*, very few compared to the 457 differentially expressed genes in *P.* × *canescens*. One might therefore assume that the two common genes shown to be involved in cell differentiation and expansion play a role in the changes in wood composition that are similar in the two species.

In *Arabidopsis*, the FLA1, FLA2 and FLA8 genes have been shown to be downregulated by ABA (Johnson *et al.* 2003). Analysis of the loss-of-function mutant *abi1* suggested that the suppression was mediated *via* a pathway including the protein phosphatase 2C (PP2C), ABI1. Data showing that ABI1 and ABI2 are upregulated by ABA and act as negative regulators support this hypothesis (Merlot *et al.* 2001). Olsson *et al.* proposed further that ATHB-12, a homeodomain leucine-zipper gene, acts as a negative growth regulator under water deficit conditions whose upregulation is dependant on ABI1 and ABI2 (Olsson *et al.* 2004). Since ABA levels are well known to increase under salt

stress and ATHB-12, ABI1 and a second ABA-induced PP2C were upregulated in *P.* × *canescens* under salt stress, FLAs were presumably suppressed *via* this signaling pathway. Although the mechanisms by which FLAs exert their influence on wood characteristics are not yet disclosed, our results suggest that they are involved in the underlying process and should be treated as interesting candidate genes to convey salt tolerance by RNAi constructs.

2.3.7. The potential influence of phytohormones on xylem development under salt stress

Phytohormones are involved in plant stress responses. Outstanding in this respect is abscisic acid (ABA), which is involved in stomatal closure and regulation of growth under salt stress (Munns 2002; Tallman 2004). But also jasmonic acid (JA) and salicylic acid (SA) are well known for their roles in abiotic stress response (Wasternack 2007; Janda *et al.* 2007). However, relatively little specific effects have been disclosed, since phytohormones form an intricate network, and many of the phytohormones interact differently at varying concentrations in plants (Sairam & Tyagi 2004).

Regarding only annotations of response to a phytohormone, response to ABA, SA, JA and gibberellin annotations are significantly overrepresented among genes upregulated in *P.* × *canescens* under salt stress; therefore these four phytohormones apparently exert the most influence on the transcriptome during salinity stress (Table 2.4). Interestingly, Fukuda lists auxin, cytokinin and brassinosteroids as the three most important phytohormones for vascular pattern formation and xylem cell differentiation (Fukuda 2004). However, in our study, annotations for responses to these phytohormones were not significantly enriched, or even nonexistent in the case of brassinosteroids. This suggests that the main process of cell development and differentiation is not

affected by salt stress, but fine-tuned by phytohormones not involved in these processes under normal conditions.

The 'hormonal cross-talk' becomes even more obvious when considering data from *Arabidopsis*, where a subset of genes uniformly upregulated in this study was reported to respond differently to treatments with single phytohormones. This might partly be explained by differing functionalities of homolog genes in *Arabidopsis*, especially if one considers that under ordinary conditions, wood development does not occur in *Arabidopsis*. However, one should keep in mind that in the experiments, *Arabidopsis* was treated with single phytohormones, and thus influences from other hormones did not change.

The complicated interaction of phytohormones is the reason why the controlling mechanisms are hard to comprehend. For example, SA causes an increase in ABA and auxin levels, promotes growth and protects against abiotic stress effects (Shakirova 2007). Auxin is supposed to have a considerable impact on this growth stimulation, but especially in poplar, auxin is known to decrease under salt stress (Junghans *et al.* 2006). SA and JA both react to abiotic stresses and pathogens, and are involved in the control of ROS scavengers (Halim *et al.* 2006; Sairam & Tyagi 2004); but ROS are involved in signaling themselves (Foreman *et al.* 2003). ABA and JA have been shown to induce the regulation of salt stress induced genes, but depending on the gene, they can also act as antagonists (Moons *et al.* 1997).

Despite the complicated interaction of phytohormones, some general patterns emerge. ABA and SA seem to exert the strongest influence on gene expression under salt stress. SA is known to cause an increase in ABA levels and to be involved in ROS controlling mechanisms, which are highly upregulated and are known to effect cell growth and programmed cell death. ABA is also known for its involvement in growth control, and has been shown

to suppress FLAs. FLAs are massively downregulated, supposedly by an ABA-induced mechanism. ABA, SA and ROS may therefore all have important effects on changes in xylem anatomy under salt stress, presumably in an interactive manner. Further information about the genetic background of stress effects in wood is desperately needed; since *Arabidopsis* as a model plant cannot be employed for this, RNAi and overexpression experiments in trees are necessary.

2.4. Conclusions

In this study, we showed that *P.* × *canescens* and *P. euphratica* are differently affected by identical salt treatment conditions. *P. euphratica* shows reduction of growth and a relative small increase of internal salt concentrations, but no further stress symptoms. Aside from growth reduction, *P.* × *canescens* is stressed on the whole plant level and shows changes in xylem anatomy which lead to a wood structure resembling that of *P. euphratica*. We suggest the changes to be an adaptation to hydraulic stress that is actively induced. A lack of photosynthates as cause for the changes is ruled out.

We conducted a microarray experiment to gain insight into the molecular mechanisms responsible for the changes in xylem anatomy. Changes in gene expression in *P. euphratica* were nearly non-existent, emphasizing the unstressed state, whereas *P.* × *canescens* exhibits a massive rearrangement of its transcriptome. Upregulated genes in *P.* × *canescens* focused on antioxidants, detoxification of products of oxidative damage and phytohormone-influenced signaling. Most noteworthy among the downregulated genes in *P.* × *canescens* were 11 different FLAs belonging to the same subgroup, two of which were also downregulated in *P. euphratica*. The microarray data suggest that of phytohormones, abscisic acid and salicylic acid exert the most influence on salt

stressed xylem, and we propose an ABA-controlled pathway involving two PP2Cs and the homeodomain leucin-zipper gene ATHB12 that might be responsible for the downregulation of FLAs and in consequence the observed changes in xylem anatomy.

2.5. Materials and methods

2.5.1. Plant material, growth conditions and stress treatment

Plantlets from *P.* × *canescens* (*P. alba* × *P. tremula*) clone INRA717 1-B4 (Leplé *et al.* 1992) and *P. euphratica* clone B2 from the Ein Avdat valley in Israel (Brosché *et al.* 2005) were multiplied by *in vitro* micropropagation (Rutledge & Douglas 1988) and kept in aerated hydroponics using Long Ashton (LA) nutrient solution (Hewitt & Smith 1975) which was changed on a weekly basis. Plants were grown for 12 weeks in a climatized chamber at 26 °C, with a relative air humidity of 60 % and a 16 h/8 h light/dark rhythm with a photosynthetic active radiation (PAR) of 150 $\mu mol \times m^{-2} \times s^{-1}$ (neon lamps: L58W/25 and 58W/840, Osram, Munich, Germany, and TLD 58W/840 Philips, Amsterdam, Netherlands). For analyzing salt stress, two treatment groups were employed. Plants in the first group were exposed to 25 mM NaCl after 10 weeks, plants in the second group were first exposed to 25 mM NaCl after 8 weeks and to a final concentration of 100 mM NaCl after 10 weeks. All plants were harvested at the end of the 12 week period.

2.5.2. Harvest of plant material

Mature leaves were sampled from the middle third of the plant, *i.e.* from 20 to 40 cm (*P.* × *canescens*) respective 30 to 60 cm (*P. euphratica*). Stem samples were taken from a height of 5 cm. The developing xylem was defined

as all extraxylary tissue obtained by peeling off the bark and scraping the surface of the xylem with a razor as described in (1995). Samples were directly frozen in liquid nitrogen and kept at -80 °C until use. For anatomical analysis, samples were fixed in FAE (2% formaldehyde, 5% acetic acid, 63% ethanol). For dry weight, osmolyte content, FTIR spectroscopy and element analysis, samples were dried in a drying oven (Memmert, Schwabach, Germany) at 70 °C for 7 days, respective 105°C for 5 days (osmolyte content).

2.5.3. Anatomy

30 µm-thick stem cross sections were made using a sliding microtome. Cuttings were stained at 60°C for 6 min with toluidine blue (0.1% toluidine [w/v] in 0.1% [w/v] BORAX, sodium tetra borate) and mounted on glass slides. Slices were viewed under a light microscope (Axioskop, Zeiss, Germany) using magnifications of 253 and 2003, respectively. Photographs were taken with a digital camera (Axiocam, Zeiss, Germany). Morphometric measurements (cell lumina and cell number) were carried out in newly formed wood tissue, the outer 150 µm of the xylem, of stem cross sections using the software ImageJ (Abramoff *et al.* 2004)

2.5.4. Osmolyte content and element analysis

Osmolyte content was measured in samples of leaves, stem, developing xylem and roots. Dried samples were pulverized in a ball mill (Retsch, Haan, Germany), and 50 µg powder was incubated in 2 ml reaction tubes with 1.5 ml H_2O at 55°C overnight. Samples were centrifuged at 1000 rpm for 25 min, and 50 µl of the supernatant was analysed in a cryoscopic osmometer (Osmomat 030, Gonotec, Berlin, Germany). Using dry and fresh weight data, osmolyte concentration for fresh tissue was back-calculated. Osmotic potential was

estimated using the van't Hoff law $\Psi = -RTc$, where Ψ is the osmotic potential in Pa, R = 8.314 $JK^{-1}mol^{-1}$ is the universal gas constant, T the absolute temperature in K and c the solution's molar concentration.

For element analysis, plant tissue was dried at 70°C and subsequently digested by using the nitric acid pressure system according to Heinrichs *et al.* (1986). Elemental quantification was carried out by inductively coupled plasma-optical emission spectrometry (ICP-OES; Spectro Analytical Instruments) at λ = 559 nm.

2.5.5. Photosynthetic gas exchange

To determine the assimilation rates of the two poplar species, CO_2-gas exchange rates of four samples of each control and 100 mM NaCl-treatment group were measured using a portable gas exchange fluorescence system (GFS-3000 Walz, Effeltrich, Germany). Before each measurement, the empty cuvette was run for 20 min to adjust the CO_2 and H_2O channels of the infra-red gas analyzer. The measurements were conducted at a leaf temperature of 30°C using mature leaves (9th or 10th below the apex), with a 30 min dark phase followed by a 30 min light phase with a PPFD (photosynthetic photon flux density) of 1000 µmol photons m^{-2} sec^{-1}; gas exchange parameters from the last 5 min of these phases were averaged and used for comparison of data statistical testing.

2.5.6. Carbohydrate content of phloem exudates by HPLC

Phloem exudates of bark pieces were collected as described in (Rennenberg *et al.* 1996). 1 ml of phloem exudate was mixed with 20 mg of polyvinylpolypyrrolidone (PVPP) and shaken continuously for 1 h at 4 °C to remove polyphenols which may interfere with separation of the sugar

compounds during the subsequent HPLC analysis. PVPP and suspended matter were removed by centrifugation (1200 g, 10 min, 4 ºC). The supernatant was diluted with one volume deionized water. A 700 µl aliquot of the diluted supernatant was transferred into a HPLC vial. Instrumental conditions were chosen as described in (Keitel *et al.* 2003). Sucrose, fructose and glucose were quantified by external standards.

2.5.7. Carbohydrate content of developing xylem by GC-MS

Shock-frozen tissue of developing xylem was ground in a ball mill (Retsch, Haan, Germany). For extraction, 1.5 ml 87 % (v/v) methanol were added to approximately 50 mg of frozen developing xylem powder. Additionally, 10 µl 0.3 % (w/v) ribitol in H_2O were added as an internal standard. Samples were shaken for 15 min at 70 °C and centrifuged (5 min, 12000 g, 4 °C). 500 µl aliquots of the supernatant were vacuum dried, dissolved in 25 µl 2 % (w/v) methoxamine hydrochloride in pyridine and shaken at 30 °C for 90 min. Subsequently, 40 µl N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) were added and samples were incubated at 37 °C for 30 min for derivatization of polar functional groups. The derivatized samples were stored at room temperature for 2 hours before injection into a GC-quadrupole MS system (GC: 7890A; MS: 5975C; Agilent Technologies, Waldbronn, Germany) operating in electron impact ionisation mode. 1 µL of the extract was injected in splitless mode with an injector temperature of 230 °C. Separation of metabolites was performed on a fused silica capillary column (HP-5ms; Agilent Technologies Waldbronn, Germany) coated with a 0.25 µm (5%-phenyl)-methylpolysiloxane stationary phase with temperature gradient starting from 80 °C and increasing by 5 °C per min to 320 °C. A mass-to-charge ratio range of 70 to 500 was scanned with the quadrupole mass detector at a rate of 12 scans

per second. For data deconvolution, peak identification and peak area determination, the 'Automated Mass Spectral Deconvolution and Identification System' (AMDIS) software (AMIDS download page) and the 'Golm Metabolome Database' (The Golm Metabolome Database) were used. To account for differences in derivatization efficiency, peak areas of identified compounds were related to the internal standard peak area. The peak area ratio was subsequently related to the amount of plant material used for extraction.

2.5.8. FTIR-ATR spectroscopy

FTIR-ATR (Fourier transform infrared spectroscopy - attenuated total reflection) spectra of developing xylem were recorded with an FTIR spectrometer (Equinox 55, Bruker Optics, Ettlingen, Germany) with a deuterium trigylcine sulfate detector and an attached ATR unit (DuraSamplIR, SensIR Europe, Warrington, UK) at a resolution of 4 cm^{-1} in the range from 600 to 4000 cm^{-1}.

The bark was peeled off of wood samples prior to drying so that the upmost layer of the dried wood consisted of developing xylem. After drying (at 70°C for 7 days) and acclimatization to the measuring room for 24 h, the wood samples were pressed against the diamond crystal of the ATR device; uniform pressure application was ensured using a torque knob. Individual analyses consisted of 32 scans which were averaged to give one spectrum. Each sample was analysed 5 times in different places, and the five spectra were averaged again, resulting in one mean spectrum per sample. Background scanning and correction was carried out regularly after 10–15 min.

Mean spectra for individual plants were processed using a spectroscopy software (OPUS version 6.5, Bruker, Ettlingen, Germany). A cluster analysis was conducted for the range from 1750 to 1200 cm^{-1} after calculation of first

derivatives with 9 smoothing points and vector normalization. Compilation of a dendrogram was done by implementing Ward's algorithm.

2.5.9. RNA extraction

Per poplar species, three biological replicates were analyzed for control and 150 mM NaCl treatment groups. Shock-frozen tissue of developing xylem was ground in a ball mill (Retsch, Haan, Germany). Total RNA was extracted from 500 mg according to Chang *et al.* (1993) with minor modifications: No spermidine was applied in the extraction buffer, and 2% β-mercaptoethanol was used. RNA was additionally purified using an RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA yield and purity were determined spectrophotometrically (BioPhotometer, Eppendorf, Hamburg, Germany) at A_{260} and A_{280}. RNA integrity was assessed on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) at the Microarray Facility Tübingen.

2.5.10. Microarray analysis

For both poplar species, 3 biological RNA replicates were analyzed of each treatment group on the GeneChip® Poplar Genome Array (Affymetrix, Santa Clara, CA). Synthesis of one-cycle cDNA and biotin-labelled cRNA, fragmenting of cRNA, hybridization to the Poplar Genome Array, washing, staining and scanning was performed as stated by Affymetrix (GeneChip® Expression Analysis Technical Manual) at the Microarray Facility Tübingen. Raw and normalized data is available at the ArrayExpress-database [EMBL:E-MEXP-2031].

Statistical analysis of the raw signal intensity data was conducted using the following functions from packages released by the bioconductor project (Gentleman *et al.* 2004), implemented in R (The R project for statistical

computing). Background correction, quantile normalization and summarization of the Affymetrix CEL output files was computed using the rma algorithm from the affy package (Irizarry *et al.* 2003) resulting in a raw list of normalized probe set values. Probe sets referring to genes that were not expressed in the developing xylem were removed by calculating Affimetrix' MAS 5.0 change calls using the mas5calls function from the affy package; only probe sets with three 'present' calls in one of the two treatments were regarded. Also, probe sets for which no annotation was available *via* the PopArray-Database (PopARRAY) were removed. Statistical testing for differentially expressed genes was performed on this filtered list of normalized probe set values using the sam function from the siggenes package (Tusher *et al.* 2001a). Finally, in cases where multiple probe sets corresponded to one gene, duplicates were removed from the list of significant genes by using the JGI *P. trichocarpa* preferred gene model as a unique identifier; probe sets with the lowest p-value were kept for further analysis.

2.5.11. GO term enrichment analysis

For statistical analysis of overrepresentated Gene Ontology (GO) terms, a GO term enrichment analysis was conducted using 'The Ontologizer' (Bauer *et al.* 2008). For this analysis, a gene universe has to be defined that represents the complete set of genes from which the significant genes are drawn. For this gene universe, all duplicate genes were removed from the filtered list of normalized probe set values. A gene ontology file for *Populus* was adapted by linking the JGI *Populus trichocarpa* gene models represented on the Affymetrix GeneChip to the GO identifier of their closest *Arabidopsis* matches as listed by TAIR (The *Arabidopsis* Information Resource). The 'population' of the Ontologizer conforms with our gene universe, whereas the two lists of genes

higher expressed in one of the poplar species each correspond to a 'study set'. As parameter settings, term-for-term analysis with Bonferroni correction was used.

2.5.12. Phylogenetic analysis

For the phylogenetic analysis of the FLA-genes, sequences for open reading frames for *Populus* and *Arabidopsis* were obtained from the JGI *Populus trichocarpa* 1.1 database (JGI *Populus trichocarpa* genome release 1.1) and from The Arabidopsis Information Resource (TAIR) (The *Arabidopsis* Information Resource). Open reading frames were translated into protein code by GeneDoc (GeneDoc Homepage) and the resulting amino acid sequences were aligned using ClustalW2 (ClustalW2). The unrooted tree was generated using the Tree View (TV) program (Zhai *et al*. 2002).

2.5.13. qRT-PCR

For quantitative Real-Time PCR (qRT-PCR), total RNA was DNAse treated with a Turbo DNA-free kit (Ambion, Austin, TX) and transcribed to cDNA with a RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). The qRT-PCR was performed on an iCycler (Bio-Rad, Hercules, CA) using ABsolute qPCR SYBR Green Fluorescein Mix (ABgene, Surrey, UK). Primer design for the qRT-PCR was performed with the Oligo Explorer, and suitable primers were tested for similar melting temperature (T_m), primer dimers and primer loops by Oligo Analyzer (both Gene Link, Hawthorne, NY). qRT-PCR output was analyzed using the MyiQ software (Bio-Rad, Hercules, CA). Statistical analysis was conducted using the Pair Wise Fixed Reallocation Randomisation Test (Pfaffl 2001) implemented in

Excel with the Relative Expression Software Tool (REST) – 384 (Pfaffl *et al.* 2002).

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3. Transcription profiling of salt adapted *Populus euphratica* roots reveals regulation of SOS genes

3.1. Introduction

For most plants, Na^+ is not an essential nutrient, although it often improves plant growth in small amounts, presumably because it acts as an osmoticum and reduces the plant's need for potassium (Marschner 2009). In excess, it has an osmotic and an ion specific effect: it interferes with the plant's ability to take up water on the outside, and it becomes toxic in high concentrations inside the cells (Munns 2005). Osmotic stress caused by high soil salinity will lead to reduced water uptake and thus to water stress. Consequences are a decrease in turgor pressure, leading to a decline in cell expansion (Xiong & Zhu 2002), a decrease in photosynthesis due to a decrease in stomatal conductance (Munns 1993), and inhibition of nutrient uptake (Tester & Davenport 2003). Toxic effects of sodium are not yet fully understood (Munns & Tester 2008). Damage caused by high Na^+ concentrations can partly be explained by disturbance of protein synthesis (Blaha *et al.* 2000), or by Na^+/K^+ interaction, leading to a decrease in K^+/Na^+ ratios and thus inhibiting enzyme activity (Bhandal & Malik 1988). One strategy to avoid the accumulation of toxic sodium levels inside the plant is the prevention of uptake of external Na^+. This occurs by active export at the root epidermis, since Na^+ influx into the root occurs mainly passive by means of non-selective cation channels and the high-affinity K^+ transporter HKT1 (Zhu 2003; Apse & Blumwald 2007; Amtmann & Sanders 1999). An important transporter for Na^+ export in *Arabidopsis* is AtSOS1, a plasma membrane Na^+/H^+ antiporter (Shi *et al.* 2003b).

The Salt Overly Sensitive (SOS) pathway is a cytoplasmic Ca^{2+} signaling cascade activated by rising cytosolic Na^+ concentrations (Zhu 2000). It is a typical example for Ca^{2+} signaling during stress acclimation: Environmental stress triggers the activation of Ca^{2+} channels, which leads to increasing cytosolic free Ca^{2+} concentrations (Zhu 2001a). Free cytosolic Ca^{2+} acts through Ca^{2+}-modulated proteins known as calmodulins (CaM) or calcineurin B-like proteins (CBLs) (Bush 1995; Knight & Knight 2001). In plants, calcium is therefore maintained at very low concentrations in the cytoplasm (Marschner 2009). Because of tight Na^+/Ca^{2+} interactions, presumable a result of their similar crystal ionic radii, excess Na^+ can outcompete Ca^{2+} transport into cells through ion channels that are permeable to both ions (Cramer 2002; White 1998). Therefore, Na^+ uptake increases and Ca^{2+} uptake decreases in response to rising external NaCl concentrations (Rengel 1992; Cramer 2002). Since in many cases, additional Ca^{2+} has an ameliorating effect on Na^+-induced growth reduction, increasing Na^+/Ca^+ ratios have been thought to be responsible for negative effects of salinity stress on plant growth (Cramer 2002). However, optimal Na^+/Ca^{2+} ratios vary strongly between different plant species, and thus effects of Ca^{2+} deficiency must be investigated separately. Besides avoiding toxic concentrations of Na^+, exclusion of Na^+ has therefore the additional advantage of keeping lower Na^+/Ca^{2+} ratios inside the plant and thus minimizing the effect of Na^+ on growth.

Sodium exclusion is especially important for salt tolerant woody plants that may live for several decades or centuries and therefore have to pursue long term strategies. It seems likely that *P. euphratica* has capabilities differing from other poplar species to exclude Na^+, since it accumulates less Na^+ than salt sensitive poplars like *P. tomentosa* or *P.* × *canescens* under high salinity (Chen *et al.* 2003a).

Under salt stress, *P. euphratica* produces cob-like root structures which cannot be observed in the close relative, *P.* × *canescens* (Hawighorst 2007). Ca^{2+} deficit, caused by sodium stress as excess Na^+ reduces uptake and internal levels of Ca^{2+}, has been ruled out as the cause (Hawighorst 2007; Rengel 1992). The development of cob-roots is therefore an ion-specific effect of high sodium levels in *P. euphratica*. Cob-roots were shown to play a role in reducing Na^+-uptake and to enhance the NaCl resistance under saline conditions.

Although a homolog of *AtSOS1* has recently been characterized in *P. euphratica*, expression studies have only been conducted in leaves, but not in roots (Wu *et al.* 2007). Therefore, little is known about the transcriptional responses to salinity in roots of *P. euphratica*, the primary site of NaCl uptake, and about the genetic mechanisms that might cause the morphological changes under high Na^+ levels.

3.2. Results

3.2.1. Validation of microarray data from the Affymetrix GeneChip Poplar Genome Array by qRT-PCR

The results from the Affymetrix GeneChip were validated by quantitative real-time PCR. Transcript abundance was analyzed for five genes, of which four had been identified as being higher expressed in salt adapted cob roots, and one in control root of *P. euphratica*. A significant linear correlation at $p = 0.05$ exists between the $\log_2$ expression ratios of the qRT-PCR analysis and the $\log_2$ signal ratios of the microarrays, confirming the accuracy of the microarray data (Figure 3.1).

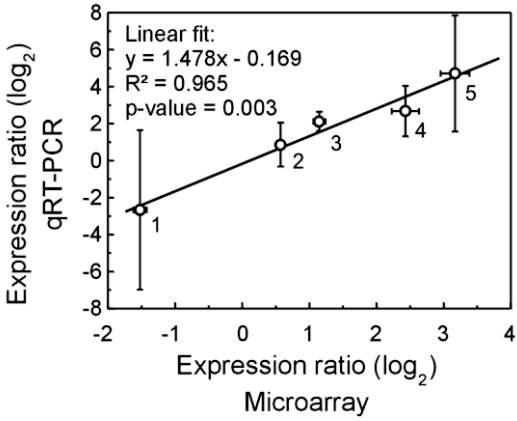

Figure 3.1 - Comparison of qRT-PCR expression and microarray signal ratios

To validate the microarray results, expression of five genes was verified by qRT-PCR. Expression ratios adapted/control roots of *P. euphratica* were calculated from threshold cycle (Ct) values normalized against an actin standard using the Relative Expression Software Tool (REST); signal ratios for the microarray analysis were taken as calculated by the SAM algorithm (n=3, mean±SE for qRT-PCR data, as calculated by REST, and mean±SD for microarray data, as calculated by SAM). Transcript description, Affymetrix probe set ID and JGI gene model for analyzed genes: 1) Cytochrome p450, PtpAffx.212299.1.S1_at, estExt_fgenesh4_pg.C_LG_XIX0602; 2) Scarecrow, Ptp.3535.1.A1_at, grail3.0024010601; 3) Dehydrin, PtpAffx.249.452.S1_at, grail3.0016050401;4) Glycosyl hydrolase family 18, Ptp.4506.1.S1_s_at, eugene3.01970025; 5) Desiccation related protein, PtpAffx.6328.1.S1_at, estExt_fgenesh4_pg.C_LG_II1501.

3.2.2. Differential expression in *P. euphratica* cob roots

To gain insight into the molecular mechanisms of cob root formation and functionality, RNA was extracted from cob roots of plants adapted to 150 mM of NaCl and analyzed on Affymetrix GeneChip Poplar Genome Arrays. A total of 273 genes were differentially expressed between control and cob roots. 208 genes were significantly up- and 65 downregulated, indicating a strong positive control of the plant over physiological and morphological changes in response to salt adaptation (Supplemental Tables S1 and S2). Of particular interest were genes induced in response to salt stress, genes involved in regulation of cell development and pattern formation, and genes with a putative role in regulating

the transcription of these genes. Therefore, we used GO terms of *Arabidopsis* homologs to identify genes involved in responses to salt and water stress, ion homeostasis and calcium signaling, as well as signal transduction and regulation of transcription (Table 3.1). Of the latter, homeobox genes were of special interest as they control cascades of other genes and are involved in pattern formation.

In the response to stress category, proteins coded by upregulated genes included two proteinkinases from the papain-like (C1A) family, a transmembrane protein, a ribosyltransferase and a glucan phosphatase. Two protein phosphateses and a homebox protein in this category are involved in signaling and regulation of transcription, and a multidrug resistance protein (MRP) is involved in ion homeostasis. The cysteine proteinases RD19A and RD21A are two proteinkinases that have been associated with protein remobilization during senescence (Beers *et al.* 2004). The heptahelical transmembrane protein 1 (HHP1) is known to be induced by ABA, especially under salt and osmotic stress, and is thought to be a negative regulator of ABA signaling (Chen *et al.* 2009a). SRO5 (similar to radical induced cell death one 5), a NAD^+ ADP-ribosyltransferase, is known for its response to salt stress and control of reactive oxigen species (ROS) (Borsani *et al.* 2005). The plastidic alpha-glucan phosphorylase is a maltooligosaccharide-degrading enzyme that has been associated with salt tolerance in *Arabidopsis* (Zeeman *et al.* 2004). The *Arabidopsis* protein phosphatase 2CA (PP2CA), ortholog of the two protein phosphatases, has been shown to be involved in negative ABA signaling (Kuhn *et al.* 2006), and has been suggested to influence transcript levels of the homeobox leucine-zipper protein ATHB12, which is induced by ABA and is presumed to mediate growth response during water deficit conditions (Olsson *et al.* 2004). MRP14 is an ABC-transporter that has been associated with detoxification processes (Kolukisaoglu *et al.* 2002). Because of

the strong sequence homology, it might also share functions with MRP5, which is involved in Na^+/K^+ homeostasis (Lee *et al.* 2004; Martinoia *et al.* 2002).

Aside from *MRP14*, only two other genes coded for proteins that were involved in sodium transport and ion homeostasis: A bile acid/sodium symporter (BASS) family protein and a calcineurin B-like protein. BASS proteins form a large family of transporters whose biological functions are poorly characterized (Mansour *et al.* 2007). The calcineurin B-like protein is well known under its synonym Salt Overly Sensitive 3 (SOS3), an important protein from the SOS-pathway, a well established salt tolerance mediating pathway activated by Ca^{2+} (Zhu 2003). Since *SOS1* and *SOS2*, the genes coding for the next two downstream proteins in the SOS pathway, were not among the significantly upregulated genes, we examined the normalized microarray data for all *SOS1*, *SOS2* and *SOS3* orthologs in poplar (Figure 3.2, Table 3.2). The data shows that besides *SOS3*, *SOS1* and *SOS2* were also slightly induced in salt adapted roots of *P. euphratica*. Datamining showed furthermore that of *SOS2* and *SOS3*, two orthologs exist in *Populus*, thus representing examples for presumably the last gene duplication event in poplar (Tuskan *et al.* 2006).

Table 3.1 - Selected genes significantly upregulated in cob roots of *P. euphratica*

Genes involved in responses to salt and water stress, ion homeostasis and calcium signaling, signal transduction and regulation of transcription were selected by GO term annotation of their closest *Arabidopsis* orthologs.

Description	JGI gene model	response to salt stress	response to osmotic stress	response to water deprivation	sodium ion transport	cellular potassium ion homeostasis	hypotonic salinity response	calmodulin binding	protein serine/threonine phosphatase activity	transmembrane receptor protein, tyrosine kinase signaling pathway	protein serine/threonine kinase activity	kinase activity	signal transduction	transcription factor activity
Cysteine proteinase RD21A	fgenesh4_pg.C_scaffold_40000333	+	.	.	.	.	.	.	.	.	.	.	.	.
Cysteine proteinase RD19A	grail3.0002061802	+	+	+	.	.	.	.	.	.	.	.	.	.
Heptahelical transmembrane protein 1 (HHP1)	gw1.XVIII.65.1	+	.	.	.	.	.	.	.	.	.	.	.	.
Similar to RCD1 5 (Radical induced Cell Death)	gw1.XII.81.1	+	.	.	.	.	.	.	.	.	.	.	.	.
Plastidic alpha-glucan phosphorylase	estExt_fgenesh4_pg.C_LG_IX1158	.	.	+	.	.	.	.	.	.	.	.	.	.
Protein phosphatase 2CA (PP2CA)	estExt_Genewise1_v1.C_LG_VIII0367	.	.	+	.	.	.	.	+	.	.	.	.	.
Protein phosphatase 2CA (PP2CA)	gw1.XII.1331.1	.	.	+	.	.	.	.	+	.	.	.	.	.
Homeobox-leucine zipper protein ATHB12	eugene3.00140486	+	+	+	.	.	.	.	.	.	.	.	.	+
Multidrug Resistance Protein 14	gw1.XII.639.1	+	.	.	.	+	.	.	.	.	.	.	.	.
Bile acid/sodium symporter family protein	gw1.III.321.1	.	.	.	+	.	.	.	.	.	.	.	.	.
Calcineurin B-like protein (SOS3)	estExt_fgenesh4_pg.C_LG_XII0203	.	.	.	.	+	+	.	.	.	.	.	.	.
Calmodulin-binding protein	eugene3.00120707	.	.	.	.	.	.	+	.	.	.	.	.	.
Calmodulin-binding protein	estExt_fgenesh4_pg.C_LG_XII0454	.	.	.	.	.	.	+	.	.	.	.	.	.
Calmodulin-binding protein	gw1.XV.769.1	.	.	.	.	.	.	+	.	.	.	.	.	.
Leucine-rich repeat transmembrane protein kinase	fgenesh4_pm.C_LG_VIII000028	.	.	.	.	.	.	.	.	+	+	+	.	.
Leucine-rich repeat transmembrane protein kinase	grail3.0096005001	.	.	.	.	.	.	.	.	+	+	.	.	.
Leucine-rich repeat transmembrane protein kinase	eugene3.00140943	.	.	.	.	.	.	.	.	+	+	+	.	.
Leucine-rich repeat transmembrane protein kinase	eugene3.01470013	.	.	.	.	.	.	.	.	+	+	+	.	.
Leucine-rich repeat transmembrane protein kinase	gw1.184.39.1	.	.	.	.	.	.	.	.	+	+	+	.	.
Serine/threonine protein kinase-like	fgenesh4_pg.C_scaffold_40000078	.	.	.	.	.	.	.	.	.	+	+	.	.
Serine/threonine-specific receptor protein kinase	gw1.XIX.2391.1	.	.	.	.	.	.	.	.	.	+	.	.	.
Receptor-like protein kinase	fgenesh4_pg.C_LG_XI000359	.	.	.	.	.	.	.	.	.	+	+	.	.
Receptor-like protein kinase	gw1.XVII.1438.1	.	.	.	.	.	.	.	.	.	.	+	.	.

Table 3.1 continued

Description	JGI gene model	response to salt stress	response to osmotic stress	response to water deprivation	sodium ion transport	cellular potassium ion homeostasis	hypotonic salinity response	calmodulin binding	protein serine/threonine phosphatase activity	transmembrane receptor protein, tyrosine kinase signaling pathway	protein serine/threonine kinase activity	kinase activity	signal transduction	transcription factor activity
Protein kinase	eugene3.00081111	.	.	.	.	.	.	.	.	.	+	.	.	.
Protein kinase	estExt_fgenesh4_pm.C_LG_XII0279	.	.	.	.	.	.	.	.	.	.	+	.	.
Protein kinase	fgenesh4_pm.C_scaffold_147000033	.	.	.	.	.	.	.	.	.	.	+	.	.
Phosphoribulokinase/uridine kinase family protein	estExt_Genewise1_v1.C_LG_V1093	.	.	.	.	.	.	.	.	.	.	+	.	.
Protein kinase-like	gw1.66.33.1	.	.	.	.	.	.	.	.	.	.	+	.	.
Somatic embryogenesis receptor-like kinase	eugene3.00031358	.	.	.	.	.	.	.	.	.	.	+	.	.
B regulatory subunit of protein phosphatase 2A, putative	gw1.VIII.1929.1	.	.	.	.	.	.	.	.	.	.	.	+	.
WRKY family transcription factor	eugene3.00061944	.	.	.	.	.	.	.	.	.	.	.	.	+
WRKY family transcription factor	gw1.VIII.261.1	.	.	.	.	.	.	.	.	.	.	.	.	+
WRKY family transcription factor	grail3.0007034202	.	.	.	.	.	.	.	.	.	.	.	.	+
bZIP family transcription factor	estExt_fgenesh4_pg.C_LG_V1707	.	.	.	.	.	.	.	.	.	.	.	.	+
WUSCHEL-related homeobox 13 (WOX13)	estExt_fgenesh4_pg.C_LG_II0076	.	.	.	.	.	.	.	.	.	.	.	.	+
Ovule development protein aintegumenta (ANT)	gw1.II.4141.1	.	.	.	.	.	.	.	.	.	.	.	.	+
NF-Y transcription factor	eugene3.00160779	.	.	.	.	.	.	.	.	.	.	.	.	+
SCARECROW-like protein 5 (SCL5)	grail3.0024010601	.	.	.	.	.	.	.	.	.	.	.	.	+
Basic helix-loop-helix (bHLH) family protein	eugene3.00091004	.	.	.	.	.	.	.	.	.	.	.	.	+
Basic helix-loop-helix (bHLH) family protein	grail3.0161002301	.	.	.	.	.	.	.	.	.	.	.	.	+
Basic helix-loop-helix (bHLH) family protein	fgenesh4_pm.C_LG_XIII000116	.	.	.	.	.	.	.	.	.	.	.	.	+
Auxin response factor	fgenesh4_pg.C_LG_II000830	.	.	.	.	.	.	.	.	.	.	.	.	+
Curly leaf protein (polycomb-group)	gw1.II.890.1	.	.	.	.	.	.	.	.	.	.	.	.	+
Floral homeotic gene APETALA1	grail3.0042013901	.	.	.	.	.	.	.	.	.	.	.	.	+
NAC domain protein	estExt_fgenesh4_pm.C_LG_V0368	.	.	.	.	.	.	.	.	.	.	.	.	+
NAM-like protein (no apical meristem)	gw1.XIX.955.1	.	.	.	.	.	.	.	.	.	.	.	.	+

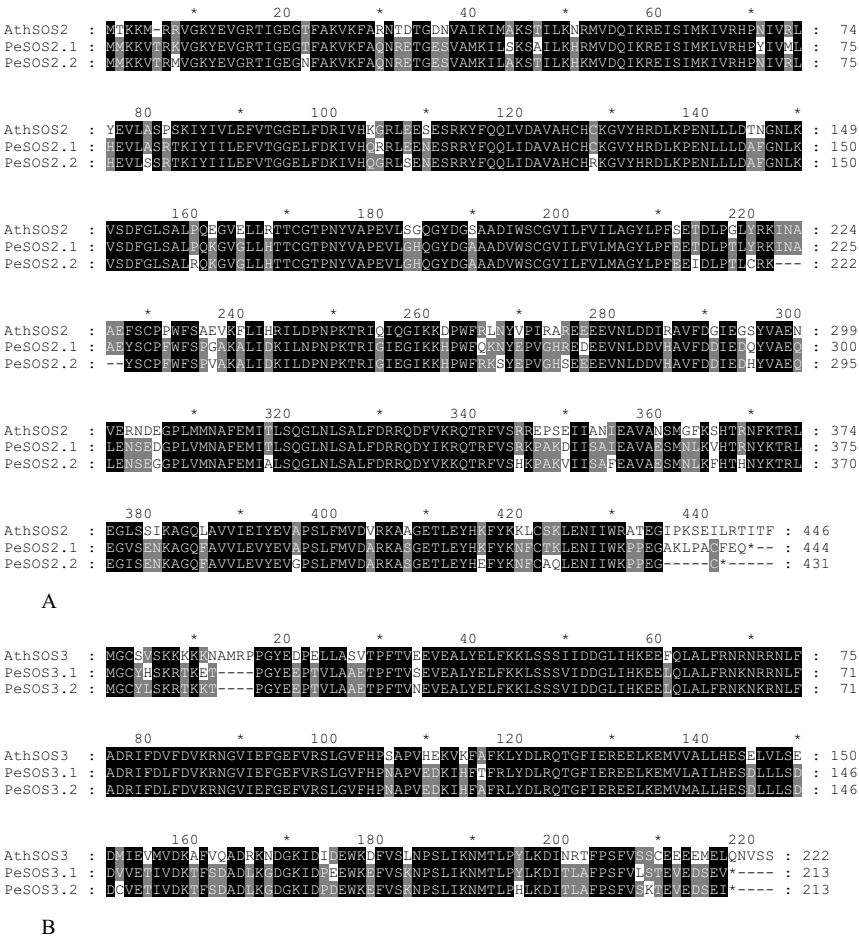

Figure 3.2 - Alignments of *AthSOS2* and *AthSOS3* with their *Populus* orthologs

Two orthologs exist for each of the *AthSOS2* (A) and the *AthSOS3* (B) gene in *Populus*. Sequences for open reading frames for *Arabidopsis* and *Populus* were obtained from the TAIR (The *Arabidopsis* Information Resource) and the JGI *P. trichocarpa* (JGI *Populus trichocarpa* genome release 1.1) databases, respectively. Translating the DNA to amino acid sequences and alignments were conducted with 'GeneDoc' (GeneDoc Homepage). For gene model IDs see Table 3.6.

Table 3.2 - Changes of transcript levels of SOS-genes in *P. euphratica* roots under salt stress

Gene name	fold-change	p-value (unadjusted)	JGI *P. trichocarpa* gene model	Affymetrix probe set ID	Best hit in *Arabidopsis*
PeSOS1	1.077	0.2375	gw1.X.6267.1	Ptp.1715.1.A1_at	At2g01980
PeSOS1	1.317	0.0471	gw1.X.6267.1	PtpAffx.3089.1.S1_at	At2g01980
PeSOS1	1.427	0.0382	gw1.X.6267.1	PtpAffx.64000.1.S1_at	At2g01980
PeSOS1	1.681	0.0067	gw1.X.6267.1	PtpAffx.64000.1.S1_s_at	At2g01980
PtSOS2.1	1.777	0.0042	estExt_Genewise1_ v1.C_LG_XVIII0198	Ptp.7953.1.S1_at	At5g35410
PtSOS2.2	1.115	0.0147	eugene3.00060558	PtpAffx.131490.1.S1_at	At5g35410
PtSOS3.1	4.032	0.0014	estExt_fgenesh4_pg .C_LG_XII0203	PtpAffx.6323.2.S1_a_at	At5g24270
PtSOS3.1	1.298	0.0114	estExt_fgenesh4_pg .C_LG_XII0203	PtpAffx.6323.2.S1_at	At5g24270
PtSOS3.2	2.865	0.0045	estExt_Genewise1_ v1.C_LG_XV0385	PtpAffx.6323.1.S1_at	At5g24270

Changes in transcript levels were determined between control and cob roots of *P. euphratica* by microarray analysis. The p-value is displayed as calculated by the SAM algorithm before correction for multiple testing. *PeSOS1* has been described and characterized by Wu *et al.* (Wu *et al.* 2007). For similarities between *PtSOS2* and *PtSOS3* and their *Arabidopsis* orthologs, see Figure 3.2.

Three different upregulated calmodulin-binding proteins (CBP) with unknown function emphasize the importance of Ca^{2+} signaling in response to salt stress. CBPs are a diverse group of proteins important for calcium-signaling, and are often involved in abiotic stress response (Zhang *et al.* 2008; Kim *et al.* 2009).

Further upregulated genes involved in signal reception and transduction were 15 different genes coding for protein kinases (PK) or proteins with kinase activity whose association to specific pathways was undetermined. 11 of these proteins are potential candidates for reception and transmission of extracellular signals, since they had 'transmembrane receptor protein' or 'plasma membrane' as an additional annotation. These include five leucine-rich transmembrane PKs, one serine/threonine protein kinase-like, two receptor-like PK, two undefined PKs and a somatic embryogenesis receptor-like kinase. Datamining in the Genevestigator database (Hruz *et al.* 2008) showed that three of these

PKs were highly upregulated under salt, drought and osmotic stress in *Arabidopsis* roots, three different forms of abiotic stress that induce an increase in cytosolic Ca^{2+} (Knight & Knight 2001; Sanders *et al.* 2002) (Figure 3.3).

Apart from *ATHB12*, 16 significantly induced genes were annotated by a transcription factor (TF) activity. Most prominently were the basic helix-loop-helix (bHLH) and the WRKY TF families, each being represented by three different genes. Especially WRKY family TFs are known to be involved in response to biotic and abiotic stresses such as wounding, pathogen attack, cold and drought stress (Ulker & Somssich 2004). Of further interest are a NAM-like and a NAC-domain protein, a WUSCHEL-related homeobox (WOX) gene and a SCARECROW-like (SCL) protein. NAM and NAC domain proteins have been associated with regulation of developmental processes, defense and response abiotic stress (Olsen *et al.* 2005). WOX13 is known to affect root and flower development (Deveaux *et al.* 2008). SCL5 is named after the SCARECROW (SCR) transcription factor, which is known for its influence on pattern formation in roots (DiLaurenzio *et al.* 1996; Bolle 2004). SCL5 belongs to the GRAS protein family, in which it forms a subset together with SCL1 and SCL13 (Pysh *et al.* 1999). Datamining in the Genevestigator database (Hruz *et al.* 2008) showed that *SCL1*, *SCL5* and *SCL13* were upregulated in a salt stress time course in *Arabidopsis* roots, both in the early (0.5 to 3 h) and the late (6 to 24 h) response (*SCL1*, At1g21450; *SCL5*, At1g50600; *SCL13*, At4g17230; Genevestigator Experiment AT-120, TAIR-ME00328).

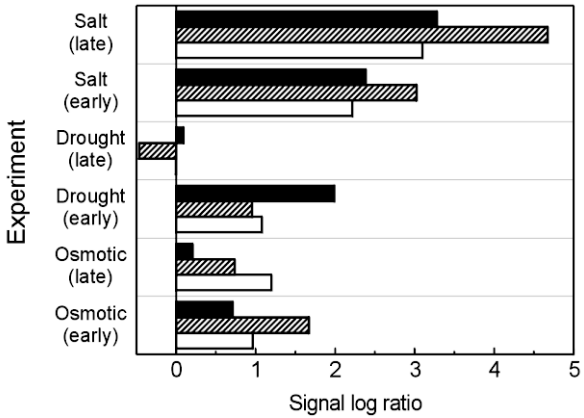

Figure 3.3 - Expression of three proteinkinases (PK) in *Arabidopsis* roots

For three PKs upregulated in cob roots of *P. euphratica*, expression levels of the closest *Arabidopsis* orthologs was assessed. Investigated genes were one serine/threonine PK (At5g47070, white) and two leucine-rich repeat transmembrane PKs (At3g47090, striped; At1g73080, black). Data was obtained from Genevestigator Experiment AT-120 (TAIR-ME00328, salt stress; TAIR-ME00338, drought stress; TAIR-ME00327, osmotic stress).

3.3. Discussion

3.3.1. Functions of cob roots

P. euphratica plants that had developed cob roots can anticipate sudden salt stress and have reduced Na^+ uptake rates (Hawighorst 2007). The influx rate of Na^+ per surface area stays constant in control and cob-roots, but the surface-to-volume ratio is lesser in cob-roots. As a result, the morphological changes lead to a 50 % decrease of Na^+ uptake per time and volume on a purely physical level.

In this work, further functions of adapted roots are disclosed by transcription profiling. The calcineurin B-like protein SOS3 is the sensor protein of the probably best described salt signaling pathway in plants (Zhu 2002). In *Arabidopsis*, SOS3 interacts with SOS2 to activate SOS1 and upregulate the *SOS1* gene. *SOS1* encodes an Na^+/H^+ antiporter that increases Na^+ efflux from root cells and improves salt tolerance when overexpressed (Shi *et al.* 2003b; Chinnusamy *et al.* 2005). The SOS pathway seems to be a conserved pathway that has been shown to be active in plants like rice that, regarding angiosperms, have a large genetic distance to *Arabidopsis* (Martinez-Atienza *et al.* 2007). Recently, *PeSOS1* has been characterized in *P. euphratica* (Wu *et al.* 2007), and homologs for *AthSOS2* and *AthSOS3* were found in the *P. trichocarpa* genome. Therefore the whole SOS pathway is most likely conserved in *Populus*. The upregulation of *SOS3* in *P. euphratica* would therefore suggest the induction of the downstream genes *SOS2* and *SOS1* in cob roots of *P. euphratica*. The microarray data we present here supports this hypothesis, although *SOS2* and *SOS1* are not significantly upregulated when considered on an array wide scale because of multiple-comparison correction, and the increase in transcript abundance is not as high as for *SOS3*. However,

the induction of a pathway that mediates Na^+ efflux from root cells is consistent with the observation that Na^+ uptake in cob roots is decreased.

Two further significantly induced genes coded for membrane-bound transporters with a possible role in salt tolerance, homologs to the ABC-transporter *MRP14* (multi-drug resistance protein 14) and to a *BASS* (bile acid/sodium symporter) family protein in *Arabidopsis*.

Since MRP14 itself has not been characterized, its possible biological function may be deduced from the closely related MRP5 (Martinoia *et al.* 2002). MRP5 may act either as an ion channel or an or ion channel regulator; studies on an *Arabidopsis* MRP5 loss-of-function mutant *atmrp5-2* showed that it is involved in K^+ homeostasis and thus in salt stress response (Lee *et al.* 2004). Because of similarities between *atmrp5-2* and *sos* mutants like hypersensitivity to NaCl, increased Na^+ or reduced K^+ uptake, Lee *et. al* speculated that MRP5 is involved in similar processes as SOS3, SOS2 and SOS1 (Lee *et al.* 2004). Thus, MRP14 might be a second gene upregulated in cob roots of *P. euphratica* that directly or indirectly promotes Na^+ efflux from root cells and improves salt tolerance. However, the available data are only fragmentary, and more work is needed for concrete insights in this matter.

Like MRPs, BASS family proteins have been poorly characterized in plants. *BAT5* (bile acid transporter 5) is to our knowledge the first gene of this family that has been characterized in *Arabidopsis* (Gigolashvili *et al.* 2009). Among the *Arabidopsis BASS* genes, *BAT5* is the closest homolog to *BAT4*, which in turn is the closest homolog in *Arabidopsis* to the *BASS* gene upregulated in cob roots. Thus, it is again possible to speculate on the possible biological function of BAT4. However, BAT5 is located in the chloroplast membrane and involved in the biosynthesis of methionine derived glucosinolates, which protect plants against herbivores and pathogens

(Gigolashvili *et al.* 2009). It is therefore unlikely that the closest homolog should be localized in the plasma membrane or the tonoplast and be involved in a completely different biological process. It can therefore be disregarded in respect to understanding mechanisms for salt tolerance.

3.3.2. Stress signaling in cob roots

The presumed induction of the SOS pathway indicates the significance of Ca^{2+} signaling in cob roots of *P. euphratica*. Ca^{2+} signaling inside plant cells is mediated by increases in cytosolic Ca^{2+} levels. These calcium spikes are generated by activation of calcium channels in the plasma membrane or in endomembranes. Ca^{2+} will then flow along the electrochemical potential into the cytosol, where Ca^{2+} levels are kept low in the unstressed state (Bush 1995; Marschner 2009). This influx onto the cytosol from the vacuole, the endoplasmic reticulum (ER) or the apoplast can be triggered by environmental signals like drought, osmotic or salinity stress (Knight & Knight 2001; Sanders *et al.* 2002). However, the exact method of how, or even where cells sense Na^+ is still speculated upon (Kader *et al.* 2007). Zhu *et al.* proposed that Na^+ is perceived inside the cell, and that SOS1 might act as a sensor for cytosolic Na^+, but this hypothesis is purely speculative and based on the observation that SOS1 has a long cytoplasmic tail similar to those of other proteins with a dual transporter/sensor function (Zhu 2003). Hence, at present no tangible theory exists of how calls sense Na^+.

We therefore tried to identify genes coding for proteins that might assume this task, focusing on membrane-bound protein kinases (PKs) that would be able to sense an extracellular signal and transmit it into the cell. Three promising membrane-bound proteinkinases upregulated in cob roots under salt stress were identified, and datamining showed that *Arabidopsis* homologs were

also upregulated in roots under salt stress. However, these three homologs were also upregulated under drought and osmotic stress, indicating that they do not directly sense Na^+. Admittedly, Na^+ sensors are not necessarily upregulated under salt stress, in which case they are not detectable by applying microarray techniques. But the example of the three stress-induced PKs indicates that upregulation of sensors is a common practice for the amplification of stress signaling in a positive feedback loop. Thus, the three PKs could transduce an extracellular signal different from Na^+ that is common in drought, osmotic and salt stress signaling. This would most likely be either extracellular changes in osmotic potential, or the presumably most important hormonal signal in this regard, abscisic acid (ABA) (Kaur & Gupta 2005; Zhu 2002; Wasilewska *et al.* 2008). They would thereby resemble the heptahelical transmembrane protein 1 (HHP1), relaying information on osmotic stress by ABA signaling (Chen *et al.* 2009a). Hyperosmolarity, like ion-specific signals of Na^+, is thought to be involved in regulation of Na^+ transport, although to a lesser degree (Zhu 2003). Therefore, the three PKs and HHP1 would be interesting candidate genes to investigate signal transduction involved in response to hyperosmotic stress that might mediate salt

3.3.3. Possible molecular mechanisms controlling morphologic changes in *P. euphratica* roots under salt stress

We showed that in cob roots of *P. euphratica*, three important transcription factors were induced by salt stress: ATHB12, WOX13 and SCL5. All three have been associated with regulation of growth and development in roots. Furthermore, ATHB12 and WOX13 are homeodomain-proteins, which are known to influence pattern development by controlling cascades of further

genes, and SCARECROW, the defining member of the SCL gene family, is an essential factor for correct radial patterning of roots (Pysh *et al.* 1999).

The upregulation of *ATHB12* stresses the involvement of ABA in signal transduction in cob roots of *P. euphratica*, as indicated by the upregulation of the genes coding for the three membrane-bound PKs and HHP1. In *Arabidopsis*, upregulation of *ATHB12* is dependant on the serine/threonine phosphatases ABI1 and ABI2 (Olsson *et al.* 2004). In cob roots of *P. euphratica*, two *protein phosphatase 2CAs* (PP2CA) were induced, which are homologs to *AtPP2CA*, a protein phosphatase belonging to the same group of the PP2C family as ABI1 and ABI2 (Kuhn *et al.* 2006). It seems possible that in *P. euphratica*, the two PP2CA fulfill the function of ABI1 and ABI2. Olsson *et al.* showed that ATHB12 influences plant growth in response to water deficit, and that in plants treated with ABA or water deficit, activity of *ATHB12* increased in the differentiation / elongation zone of root tips (Olsson *et al.* 2004). Although root thickening as observed in *P. euphratica* has not been described for *Arabidopsis*, salt stress can cause root thickening by swelling of the outer cell layers, and drought stress can cause the development of short, tuberized *Arabidopsis* roots (Vartanian *et al.* 1994; Burssens *et al.* 2000). The short roots bear some resemblances to *P. euphratica* cob roots, as they are thicker than ordinary roots and occur under water stress conditions, but are caused by cell enlargement. It was shown that ABA influenced formation of these short roots, and that generation of short roots was decreased in *abi1* mutants. The interaction of water stress, ABA signaling, ABI1 involvement and changes in root growth suggests that ATHB12 plays a role in the formation process of short roots. In salt stressed *Arabidopsis* roots, *ATHB12* transcription levels were significantly increased in all layers except the endodermis and the protophloem (Dinneny *et al.* 2008, supplemental material). These data suggest that AtATHB12 is involved in root thickening in *Arabidopsis*. However, it is

therefore very unlikely that ATHB12 is the sole effector for cob root formation of *P. euphratica*, since morphological changes between thickened *Populus* and *Arabidopsis* differ strongly.

But ATHB12 also increased salt tolerance in a different way. This was shown in a salt-sensitive yeast mutant, were expression of *AtATHB12* mediated salt tolerance by promoting Na^+ exclusion (Shin *et al.* 2004). These data suggest that ATHB12 as an important mediator of ABA signaling under osmotic stress has two possible effects on *P. euphratica* roots: it might be involved in growth regulation and the formation of cobroots, and it might induce genes that increase Na^+ efflux. Thus, it would be a very interesting candidate for research of salt tolerance in poplar.

WOX13 is a homedomain protein that is involved in organ development, but in contrast to ATHB12, it is poorly characterized. It has been suggested that it exerts its influence by preventing premature differentiation (Deveaux *et al.* 2008). Expression of WOX 13 is modulated in response to abiotic stresses, but no ameliorating effect on stress tolerance is known. Because of the considerable effects of homeodomain proteins on developmental processes, one might speculate that WOX13 is involved in cob root formation.

SCL5 is a member of the GRAS protein family, named for the three original members GAI, RGA and SCR. GRAS genes, in particular SCARECROW (SCR), are known to be involved in radial patterning during root development (Bolle 2004). The induction of *AtSCL5* and its two closest homologs, *AtSCL1* and *AtSCL13* (Pysh *et al.* 1999) under salt stress in roots suggests a strong influence of this protein family on morphology under these conditions. In thickened salt stressed *Arabidopsis* roots, transcript levels of *SCL5* were only increased the stele and the protophloem, were the swelling of cells that caused root thickening did not occur (Dinneny *et al.* 2008, supplemental material; Burssens *et al.* 2000). It would therefore be possible that

SCL5 inhibits cellular swelling under salt stress, and that root thickening in *P. euphratica* is a result of interaction of SCL5, ATHB12 and WOX13.

3.4. Conclusion

When grown at high NaCl concentrations that are toxic for salt-sensitive poplars, *P. euphratica* shows no typical symptoms of salt stress. Instead, *P. euphratica* develops morphologically changed roots that display a diameter increase, termed cob-roots. We showed that in cob-roots, genes from the SOS pathway, one of the best described signaling cascades mediating salt tolerance by promoting Na^+ efflux from root cells, were induced.

Three transcription factors known to influence growth and development of roots, *ATHB12*, *WOX13* and *SCL5*, showed increased transcript levels in cob roots. Presumably, one or more of these transcription factors are involved in the formation of cob roots. However, whether one of these genes plays a major role, or interaction between two or even all three genes is necessary for the development of cob roots, remains unknown and is a goal for further studies.

3.5. Material and methods

3.5.1. Plant material and growth conditions

Plantlets from *P. euphratica* clone B2 from the Ein Avdat valley in Israel (Brosché *et al.* 2005) were multiplied by *in vitro* micropropagation (Rutledge & Douglas 1988) and afterwards kept in aerated hydroponics using Long Ashton (LA) nutrient solution (Hewitt & Smith 1975) which was changed on a weekly basis. Plants were grown for 12 weeks in a climatized chamber at 26 °C, with a relative air humidity of 60 % and a 16 h/8 h light/dark rhythm with a photosynthetic active radiation (PAR) of 150 $\mu mol \times m^{-2} \times s^{-1}$ (neon lamps: L58W/25 and 58W/840, Osram, Munich, Germany, and TLD 58W/840 Philips, Amsterdam, Netherlands). For adaptation of the plants and the development of cob-root, the NaCl concentration in the nutrient solution was increased to 25, 100 and finally 150 mM in weekly steps. To gain enough root material for studies, plants were grown at 150 mM NaCl for further 12 weeks.

3.5.2. RNA extraction for microarray analysis

For a microarray analysis, roots of control plants were compared to salt adapted cob-roots. Root tips of control and salt adapted plants were harvested, shock-frozen in liquid nitrogen and stored at -80 °C until use. Frozen root tops were ground in a ball mill (Retsch, Haan, Germany). Total RNA was extracted from 500 mg according to Chang *et al.* (1993) with minor modifications: No spermidine was applied in the extraction buffer, and 2% β-mercaptoethanol was used. RNA was additionally purified using an RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA yield and purity were determined spectrophotometrically (BioPhotometer, Eppendorf, Hamburg, Germany) at

A_{260} and A_{280}. RNA integrity was assessed on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) at the Microarray Facility Tübingen.

3.5.3. Microarray analysis

For each of the control and the treatment group, three biological RNA replicates were analyzed on the Affymetrix GeneChip Poplar Genome Array (Affymetrix, Santa Clara, CA). Synthesis of one-cycle cDNA and biotin-labeled cRNA, fragmenting of cRNA, hybridization to the Poplar Genome Array, washing, staining and scanning was performed as stated by Affymetrix (GeneChip Expression Analysis Technical Manual) at the Microarray Facility Tübingen. Raw and normalized data is available at the ArrayExpress-database [EMBL:E-MEXP-2234]. Statistical analysis of the raw signal intensity data was conducted using the following functions from packages released by the bioconductor project (Gentleman *et al.* 2004), implemented in R (The R project for statistical computing). Background correction, quantile normalization and summarization of the Affymetrix CEL output files was computed using the 'rma' algorithm (Irizarry *et al.* 2003) from the 'affy' package resulting in a raw list of normalized probe set values. Probe sets referring to genes that were note expressed in the samples were removed by calculating Affymetrix' MAS 5.0 change calls using the 'mas5calls' function from the 'affy' package; only probe sets with two or three 'present' calls in one of the two treatment groups were regarded. Also, probe sets for which no annotation was available *via* the PopArray-Database (PopARRAY: A database for cross-reference of multiple microarray platforms in *Populus*) were removed. Statistical testing for differentially expressed genes was performed on this filtered list of normalized probe set values using the 'sam' function from the 'siggenes' package. Using the SAM algorithm (Tusher *et al.* 2001a), the

number of significantly differentially regulated genes is dependent on the false discovery rate (FDR) among these genes. Because of a strong decrease of the significant genes to FDR ratio with an FDR converging to 5 % (FDR 6 % ≙ 316 significant genes, FDR 4.9 % ≙ 13 significant genes), we chose an FDR of 5.9 % which is well under the FDR of 12 % used by Tusher *et al.* (2001a). Finally, in cases where multiple probe sets corresponded to one gene, duplicates were removed from the list of significant genes by using the JGI *P. trichocarpa* preferred gene model as a unique identifier; probe sets with the lowest p-value were kept for further analysis.

3.5.4. qRT-PCR

From each of the three biological replicates of the root tips of control and salt adapted roots, three technical replicates were analyzed by quantitative Real-Time PCR (qRT-PCR). Total RNA was DNAse treated with a Turbo DNA-free kit (Ambion, Austin, TX) and transcribed to cDNA with a RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). The qRT-PCR was performed on an iCycler (Bio-Rad, Hercules, CA) using ABsolute qPCR SYBR Green Fluorescein Mix (ABgene, Surrey, UK). Primer design for the qRT-PCR was performed with the Oligo Explorer, and suitable primers were tested for similar melting temperature (T_m), primer dimers and primer loops by Oligo Analyzer (both Gene Link, Hawthorne, NY). The qRT-PCR was performed on an iCycler (Bio-Rad, Hercules, CA). 250 ng cDNA were used in an 25 µl reaction with 1×ABsolute qPCR SYBR Green Fluorescein Mix (ABgene, Surrey, UK; including Thermo-Start DNA Polymerase) and 10 µM primer. First denaturation and activation of the Taq-polymerase occurred at 95°C for 15 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 56°C for 30 s and elongation at 72°C for 30 s.

qRT-PCR output was analyzed using the MyiQ software (Bio-Rad, Hercules, CA). Statistical analysis was conducted using the Pair Wise Fixed Reallocation Randomisation Test (Pfaffl 2001) implemented in Excel with the Relative Expression Software Tool (REST) – 384 (Pfaffl *et al.* 2002).

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4. Comparison of the transcriptome and metabolome of a salt sensitive and a salt tolerant poplar species reveals insight into stress tolerance mechanisms

4.1. Introduction

To gain insight into the molecular basis of the ability to resist salt stress, transcriptional changes in the presence of salt have been investigated (Brosché *et al.* 2005; Ottow 2005; Gu *et al.* 2004). However, comparative studies between salt-sensitive and salt tolerant tree species are still lacking. In this chapter, we compared the transcriptomes of the salt tolerant *P. euphratica* and the salt sensitive *Populus* × *canescens*.

P. × *canescens* and *P. euphratica* are adapted to different environmental conditions, evident from differences in their physiology and morphology. Junghans *et al.* (2006) showed that the xylem anatomy of *P. euphratica* shows resemblances to that of *P.* × *canescens* under salt stress, and physiological and anatomical reactions towards salt stress are comparable when *P.* × *canescens* and *P. euphratica* are treated with 50 and 150 mM NaCl, respectively. Therefore, one would suspect a strong difference in expression and regulation of genes relevant to salt stress tolerance which should be detectable by microarray analysis.

However, when performing a comparison of two different species, there is one important issue, though: cDNA of one species is hybridized to probes of a different organism, which can lead to a bias in hybridization efficiency. This technique which is called cross-species hybridization (CSH) has recently come in frequent use in a variety of species (Bar-Or *et al.* 2007). For example, Taji *et*

al. (2004) compared transcriptomes of the salt sensitive *Arabidopsis thaliana* and its near relative, the salt tolerant salt cress *Thellungiella halophila*, on *Arabidopsis* cDNA microarrays. To avoid hybridization biases due to differences in sequence identity, two approaches were used: The two species were either compared across arrays, *i.e.* controls and treatments of each species were analyzed on separate arrays and the results compared; or, where control samples of both species were directly compared on one array, only genes with an apparent higher expression in *T. halophila* were considered, excluding hybridization bias in favor of *Arabidopsis* due to a higher sequence similarity between samples and microarrays.

Street *et al.* (Street *et al.* 2006) conducted a similar experiment with two poplar species that differ in drought tolerance, *P. trichocarpa* and *P. deltoides*. Drought responses of the two species were compared using spotted cDNA microarrays constructed from EST databases of three poplar species, *P. tremula*, *P. tremula* × *tremuloides* and *P. trichocarpa* (Andersson *et al.* 2004). In accordance with Taji *et al.* (2004), the issue of hybridization bias was avoided by conducting direct comparisons only between controls and treatments within one species; comparisons between the species were performed indirectly across arrays.

Previous microarray analyses conducted with *P. euphratica* revealed surprisingly few salt-responsive genes; Brosché *et al.* (2005) found that under differing natural salt- and drought-stress conditions, only 22 of approximately 6340 genes, roughly 0.35%, were differentially regulated. This led us to hypothesize that through evolution, *P. euphratica* has developed innate mechanisms to tolerate salt stress that require no gene regulation to take effect; plants adapted to stressful environments may possess a preventive stress strategy involving constitutive upregulation of stress related pathways. As a consequence, these genes would not be detected by comparing transcriptomes

of controls and stress exposed plants of the salt tolerant species on microarrays, as done by Taji *et al.* (Taji *et al.* 2004) and Street *et al.* (Street *et al.* 2006). However, by a direct comparison of specimens of the salt tolerant and the salt sensitive species grown under control conditions, such differences should be traceable.

In our study, we used the Affymetrix GeneChip Poplar Genome Array to examine transcriptional differences of *P. euphratica* and *P.* × *canescens*. In contrast to spotted cDNA microarrays, Affymetrix GeneChips do not use cDNA fragments amplified by PCR as probes, but *in situ* synthesized 25-mer oligonucleotides. Because of the small size of a single oligomer, a target sequence is not interrogated by one single probe as on cDNA microarrays, but by a probe set consisting of usually 11 probes (Figure 4.1). The Poplar Genome Array contains 61,251 of these probe sets, interrogating 56,055 transcripts in total. Each probe set is designed based on mRNA or EST sequence data of one of 13 different poplar species, or on predicted genes of *P. trichocarpa*, the latter being the basis for the majority of approximately 60% of all probe sets (Affymetrix GeneChip Poplar Genome Array Data Sheet). Probe sets based on *P. euphratica* and *P.* × *canescens* account for approximately 1% of the array, respectively.

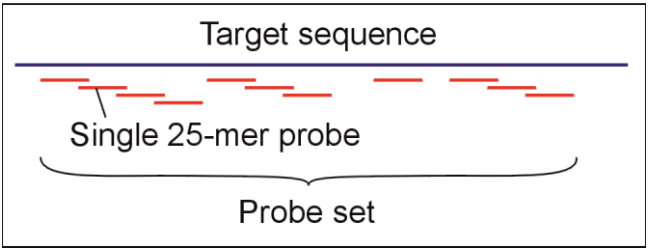

Figure 4.1 - Schematic representation of an Affymetrix probe set

A target sequence, usually a sequenced mRNA, is interrogated by 11 25-mer oligonucleotide probes synthesized in situ on an Affymetrix GeneChip microarray.

The effect of differences in gene regulation on metabolic processes can be investigated by extensive metabolite profiling (Bino *et al.* 2004; Fiehn *et al.* 2000). Fourier transform-ion cyclotron mass spectrometry (FT-ICR MS) is a high-throughput technique for the measurement of thousands of distinct metabolite masses, with an accuracy in the order of magnitude of a few parts per million (ppm) (Schmitt-Kopplin & Hertkorn 2007). It is a nontargeted analytical method that is suitable for a comprehensive metabolomics approach, and tools are available for a global integration of data from FT-ICR MS and microarrays analyses (Suhre & Schmitt-Kopplin 2008; Hirai *et al.* 2004).

Here, we show for the first time that by applying suitable controls and filtering methods on Affymetrix probe sets, a direct comparison of the transcriptomes of *P. euphratica* and *P.* × *canescens* using Affymetrix GeneChip Poplar Genome Arrays can be used to identify differentially expressed pathways. The analysis was validated by combining the results with an FT-ICR/MS-based metabolite analysis to demonstrate the impact of higher expressed pathways on the involved metabolites. We thereby answer the questions: Are stress related genes under control conditions generally higher expressed in *P. euphratica* than in *P.* × *canescens*? What are the differences in the transcriptomes of *P. euphratica* and *P.* × *canescens*, and do they account for different metabolic statuses and an increased salt tolerance of *P. euphratica*?

4.2. Results

4.2.1. *P. euphratica* displays anticipatory prevention against salt stress

Populus × *canescens* and *P. euphratica* were exposed to high salinity by addition of 150 mM NaCl to the hydroponic nutrient solution. Under these conditions, net photosynthesis declined instantaneously from approximately 3

to 1.5 µmol×m^{-2}×s^{-1} in *P. euphratica*. In *P.* × *canescens*, the initial net photosynthetic rate was 4.5 µmol×m^{-2}×s^{-1}, the decrease occurred much slower than in *P. euphratica*, and only very low rates of < 0.5 µmol×m^{-2}×s^{-1} were maintained after 8 h of salt exposure (Figure 4.2 a). The transpiration rates showed similar responses as the net photosynthesis in both species exposed to high salinity. In *P. euphratica*, the transpiration rate decreased instantaneously

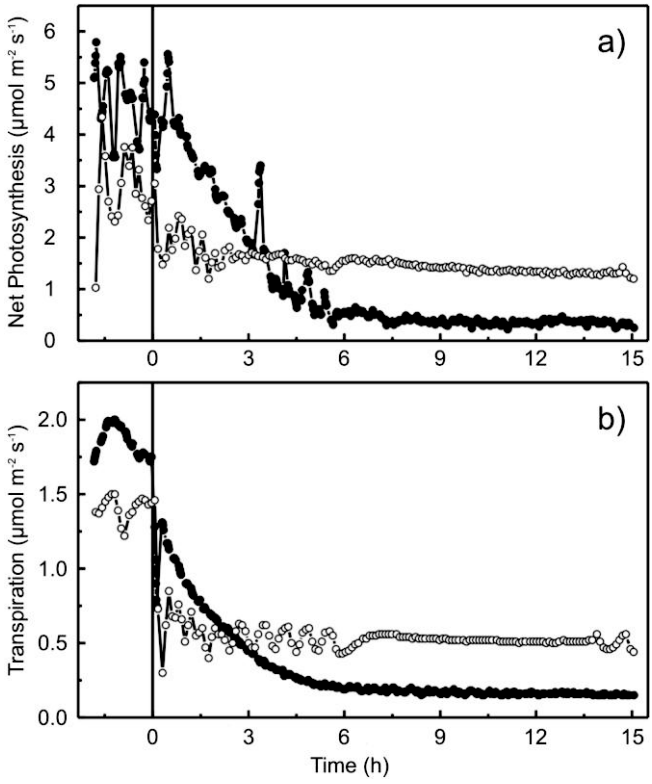

Figure 4.2 - Response of net photosynthesis (a) and transpiration (b) of *P. euphratica* and *P.* × *canescens* to salt addition

NaCl was added to the nutrient solution at t = 0. Gas exchange was measured in *P. euphratica* (open circle) and *P.* × *canescens* (full circle). Transpiration and photosynthesis rates were recorded continuously for 15 h at a PAR of 150 µmol m^{-2} s^{-1}, a temperature of 26 ˚C and an air humidity of 60%. Data are representative for 3 replicates.

from 1.4 to 0.5 µmol×m^{-2}×s^{-1}, whereas in *P.* × *canescens*, it decreased from 1.8 to 0.2 µmol×m^{-2}×s^{-1} in 8 h (Figure 4.2 b). The instantaneous acclimation to high levels of NaCl in the hydroponic nutrient solution and the maintenance of higher net photosynthetic rates in *P. euphratica* compared to *P.* × *canescens* shows that *P. euphratica* is preadapted to high salinity.

4.2.2. Sequence identity of poplar species

A prerequisite for a transcriptional comparison of *P. euphratica* and *P.* × *canescens* is a high sequence identity of their mRNAs with the probe set DNA on the microarray. 35,000 probe sets on the Affymetrix GeneChip Poplar Genome Array are based on *P. trichocarpa*, the most frequently used species for the microarray. Therefore, cDNA sequence data were obtained for *P.* × *canescens*, *P. euphratica* and *P. trichocarpa* for 20 genes present on the microarray to assess the relationship between the three poplar species on the level of mRNA. The percentage of nucleotide identity was calculated separately between each pair (Table 4.1). Identity was consistently high with average values between 95.6 and 97.2%, and no significant differences between the three pairwise comparisons were detected.

4.2.3. Detection and evaluation of transcripts differentially expressed between *P. euphratica* and *P.* × *canescens*

The transcriptomes of leaves of *P. euphratica* and *P.* × *canescens* plants were compared by microarray analysis. Identification of differentially expressed transcripts was conducted by a significance analysis of microarrays (SAM) following normalization of the raw CEL-files by robust multi-array average (RMA) (Irizarry *et al.* 2003; Tusher *et al.* 2001b). The SAM resulted in 4557 differentially expressed transcripts (raw list, Table 4.2). Transcripts that

were not expressed ('absent') were identified by the MAS 5.0 algorithm and removed from the list of significant transcripts (filter 1, Table 4.2).

Table 4.1 - Comparison of sequence data of 20 genes from *P.* × *canescens* (*Pc*), *P. euphratica* (*Pe*) and *P. trichocarpa* (*Pt*)

	Pe/Pt		Pc/Pt		Pe/Pc		Public ID		
gene	ni	al	ni	al	ni	al	*Pe*	*Pc*	*Pt*
AP	97.50%	648	97.00%	668	97.10%	697	AJ777007	CF231430	589502
ATPase	93.80%	145	98.10%	367	93.80%	144	AJ779572	CX655567	821076
BSP	95.50%	334	96.60%	610	94.60%	334	DQ388455	CU233319	687235
FLA12	96.80%	698	95.30%	742	95.20%	665	AJ777975	CF228244	723575
GAST	95.70%	511	96.80%	539	95.50%	508	FJ238511	CF231013	652064
GD	99.10%	645	99.40%	494	96.00%	603	AJ767665	CU223898	832093
GS	99.20%	663	94.10%	642	94.10%	563	AJ768965	DQ855560	565302
Ill3	97.60%	1462	98.40%	1456	96.80%	1443	AJ744952	AJ744953	729069
IPP	97.70%	639	94.10%	422	94.20%	226	AJ774517	CU225654	578868
MADS	99.00%	205	97.90%	573	99.10%	215	AJ780611	CU306852	575376
MCP	97.40%	466	98.00%	556	97.60%	579	AJ774830	CF231502	640384
NdID	93.10%	405	91.60%	419	92.30%	247	AJ774444	CX656537	558763
NhaD1	98.10%	1667	95.00%	220	95.00%	220	AJ561195	*	54522
NifU	97.00%	492	96.70%	492	96.90%	552	AJ775004	CU224355	834330
RGP3	97.90%	438	98.50%	681	97.50%	434	AJ775165	CX659635	673066
RP	95.10%	548	95.80%	618	97.10%	548	AJ775612	CU233448	733792
RPM	99.00%	297	96.70%	426	94.10%	202	AJ770082	CX656156	713972
SIS	98.60%	589	97.30%	559	96.80%	559	FJ238515	FJ238514	560836
TIL	95.80%	671	94.50%	640	96.40%	640	FJ238513	FJ238512	738040
Ubi2	99.00%	312	97.00%	573	96.90%	557	AJ773956	CU233410	664794
Mean±SD	97.20%	±1.8%	96.40%	±1.9%	95.80%	±1.7%			

*partial sequence, published in (Ottow 2005).

Public ID: accession number for GenBank (*Pc* and *Pe*), respective JGI *P. trichocarpa* project protein id (*Pt*); ni: nucleotide identity; al: length of aligned sequence fragments; AP: Aquaporin (tonoplast intrinsic protein gamma); ATPase: H+-transporting ATPase; BSP: Boiling Stable Protein, Chain A;FLA12: Fasciclin-like arabinogalactan-protein; GAST:GAST-like protein;GD: Glycine dehydrogenase; GS: Plastid glutamine synthetase-like; Ill3: IAA-amino acid hydrolase Ill3; IPP: Inorganic pyrophosphatase; MADS: MADS-Box; MCP: Mitochondrial carrier protein; NdID: NAD+ dependent isocitrate dehydrogenase; NhaD1: Na/H antiporter type D; NifU: Nitrogen fixation protein NifU; RGP3: Reversibly glycosylated polypeptide 3; RP: Ribosomal protein L34e; RPM: DNA-directed RNA polymerase; SIS: Salt induced serin-rich; TIL: Temperature induced lipocalin; Ubi2: Ubiquitin.

Table 4.2 - Overview of the different filter steps applied to the list of significant transcripts and the gene universe

Filter criterium	raw list	probe set present/absent	probe set SD	annotation available	origin species of probe set	duplicate gene models
Filter number applied	f0	f1	f2	f3	f4	f5
Number of significant transcripts	4557	4305	4080	3568	2379 (2672*)	2246 (2503*)
Size of gene universe	61413	30887	29343	26118	18326	14254

* including transcripts interrogated by probe sets constructed from *P.* × *canescens* sequences that were higher expressed in *P. euphratica*, and transcripts interrogated by probe sets constructed from *P. euphratica* sequences that were higher expressedin *P.* × *canescens*

Raw lists are the list of significant genes as calculated by SAM and all probe sets on the microarray; filter f1 removed probe sets identified as absent by the MAS 5.0 algorithm; filter f2 removed probe sets with SD in the upper 5% quantile; filter f3 removed probe sets without annotation; filter f4 removed probes sets biased due to the poplar species used for their construction; filter f5 removed duplicate gene models.

Among these remaining significant transcripts, one has to suspect that in single cases, microarray results might be biased in favor of *P. euphratica* or *P.* × *canescens* due to the occurring differences in sequence identity between the two species. This would be expected for transcripts with an exceptional high frequency of sequence differences between *P. euphratica* and *P.* × *canescens* in the DNA sequence interrogated by the respective probe set. Such local sequence differences with a strong effect on hybridization efficiency would not evenly affect a whole probe set, but only single probes (Figure 4.3). The standard deviation (SD) of the single probe signals belonging to one probe set might therefore give an estimate for the similarity of hybridization characteristics, *i.e.* the higher the SD within one probe set (internal probe set SD), the higher is the chance of sequence differences between *P. trichocarpa* and either *P. euphratica* or *P.* × *canescens*, and thus of a falsely detected differential gene expression. Therefore, internal probe set SDs were calculated and averaged over all six arrays to identify probe sets with a high probability of

giving biased data. In order to reduce the number of false positives, transcripts that were interrogated by probe sets with an internal SD in the upper 5% quantile of all 'present' probe set SDs on the microarray were removed from the list of significant transcripts (Figure 4.4; filter 2, Table 4.2).

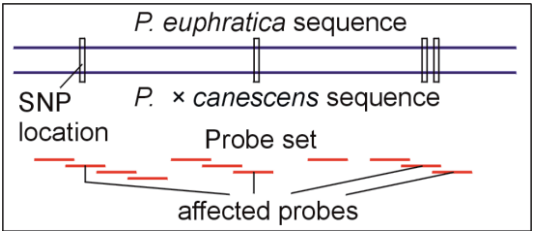

Figure 4.3 - Schematic representation of single nucleotide polymorphisms (SNPs) affecting only individual probes within a set

In case of sequence differences between *P. euphratica* and *P.* × *canescens* (black frames), hybridization efficiency will be affected only of individual probes. The probability of many SNPs influencing the probe signals can therefore be estimeated by calculating the probe signals standard deviation within a probe set.

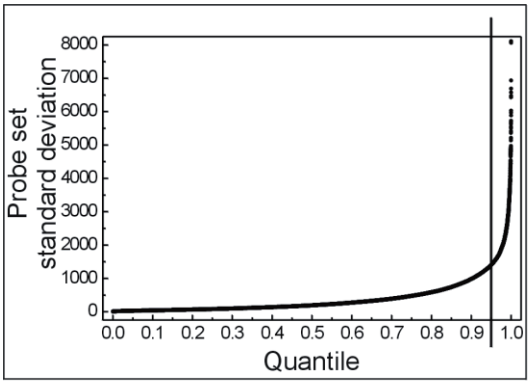

Figure 4.4 - Quantile-SD plot of the standard deviation of the probe signals for each probe set

As an estimate for hybridization efficiency, the standard deviation of the probe signals within each probe set (internal probe set SD) was used to identify probe sets with a high probability to deliver biased data. To reduce the number of false positives, probe sets with the highest 5% of internal probe set SD were excluded from further data analysis (vertical line).

Quantitative real-time PCR (qRT-PCR) was subsequently used to validate the microarray results for differentially expressed transcripts. 10 genes were analyzed, 6 of which were chosen from the list of significant transcripts, and 4 from the excluded transcripts interrogated by probe sets with an internal SD in the upper 5% quantile (Figure 4.5). For five of the six significant transcripts

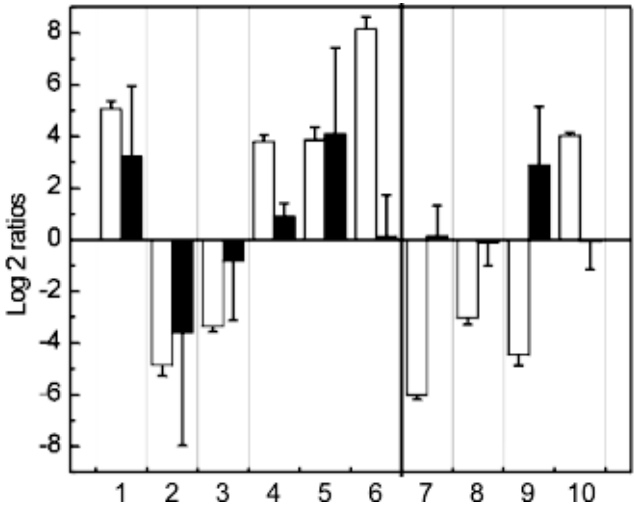

Figure 4.5 - Comparison of qRT-PCR expression and microarray signal ratios

For qRT-PCR (black columns), expression ratios *P. euphratica*/*P.* × *canescens* were calculated from threshold cycle (Ct) values normalized against an actin standard using the Relative Expression Software Tool (REST). Signal ratios for the microarray analysis (white columns) were taken as calculated by the SAM algorithm (n = 3, mean±SE for qRT-PCR data, as calculated by REST, respective mean±SD for microarray data, as calculated by SAM). Transcripts are sorted ascending from left to right by the probe sets SD; transcripts 7 to 10 had probe set SDs in the upper 5% quantile and were therefore excluded from the microarray data analysis. Transcript description, Affymetrix probe set ID and JGI gene model for analyzed genes: 1, Gibberellin regulated protein, Ptp.6252.1.S1_a_at, estExt_Genewise1_v1.C_LG_V1745; 2, MADS-Box protein, Ptp.5993.1.S1_a_at, eugene3.00150771; 3, Mitochondrial carrier protein, Ptp.5103.1.S1_at, grail3.0008039502; 4, Lil3 protein, Ptp.4571.1.S1_at, eugene3.01180096; 5, Aquaporin, Ptp.5700.1.S1_s_at, eugene3.00280238; 6, GTP-binding protein, PtpAffx.25286.1.S1_at, estExt_fgenesh4_pg.C_LG_I1368; 7, Nitrogen fixation protein, PtpAffx.1459.1.A1_s_at, estExt_fgenesh4_pm.C_LG_XII0286; 8, Ubiquitin-like protein, PtpAffx.157059.1.S1_s_at, estExt_fgenesh4_pg.C_LG_XIV1291; 9, 1-Aminocyclopropane-1-carboxylate oxidase, Ptp.5158.1.S1_at, estExt_Genewise1_v1.C_1660131; 10, Glycine dehydrogenase, PtpAffx.19705.1.A1_at, estExt_fgenesh4_pm.C_LG_VI0678.

(MADS-box protein, mitochondrial carrier protein, Lil3 protein, aquaporin and gibberellin regulated protein), a significant higher expression was detected for the same species as by the microarray analysis. Only for one gene (GTP-binding protein), the microarray results could not be validated; notably, this gene was interrogated by the probe set with the highest internal SD of the six genes chosen from the list of significant transcripts. For the four genes taken from the list of transcripts excluded due to a high bias probability (nitrogen fixation protein, ubiquitin-like protein, glycine dehydrogenase and 1-aminocyclopropane-1-carboxylate oxidase), the microarray results could not be validated by qRT-PCR.

Furthermore, since we were not interested in candidate genes but in functional categorization, all transcripts for which no annotation was available were removed from the list of significant transcripts (filter 3, Table 4.2). Of the remaining 3568 transcripts, 1805 had higher expression values in *P. euphratica* than in *P.* × *canescens*, and 1763 had higher expression values in *P.* × *canescens* than in *P. euphratica*.

We examined the expression ratios of these differentially expressed transcripts with regard to the origin species of the respective probe set sequences, as a sequence divergence of as low as 1% can have an influence on hybridization results (Gilad *et al.* 2005) (Gilad *et al.* 2005). Despite the high sequence identity of different poplar species and the removal of transcripts with a high probability of being biased (filter 2), a bias was observed (Figure 4.6). Among the differentially expressed transcripts, the distribution of the expression ratios was strongly influenced by the relationship of the sample species to the origin species of the probe set sequences. Probe sets were grouped in three subsets. The first group was based on sequences from poplar species from the *Tacamahaca* and *Aigeiros* sections, namely *P. trichocarpa*, *P.*

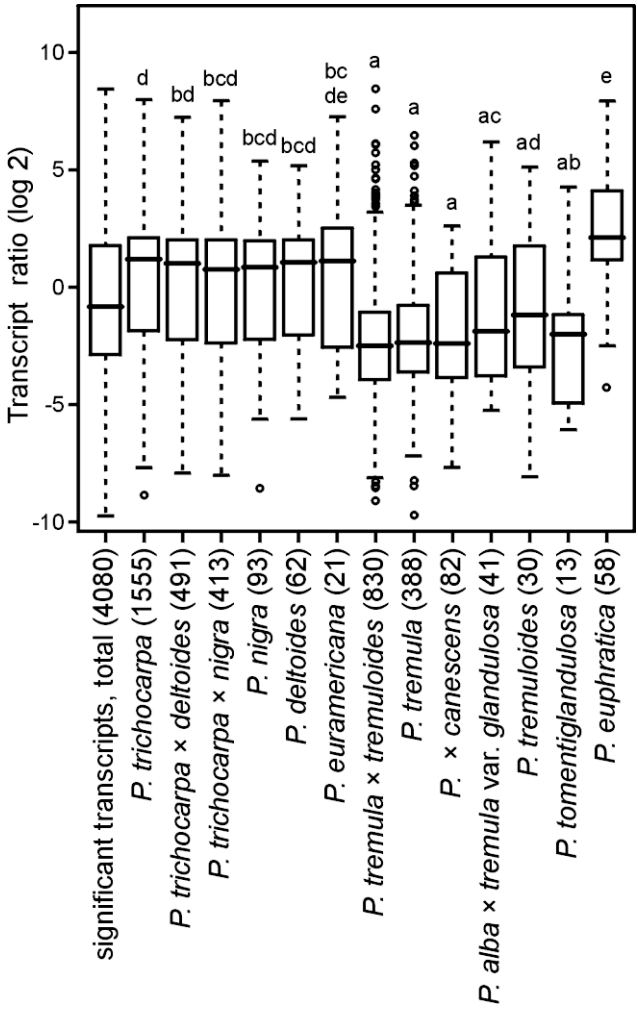

Figure 4.6 - Transcript ratios of *P. euphratica* and *P.* × *canescens* sorted according to the original poplar species used to construct the probe set on the microarray

All transcripts with significantly different signal intensities that were detected as present by the MAS 5.0 algorithm and had probe set SDs in the lower 95% quantile were used (f2,Table 4.2). Signal log ratios were calculated as log-signal$_{(P.\ euphratica)}$ - log-signal$_{(P.\ \times\ canescens)}$, *i.e.* a positive value denotes a higher apparent expression in *P. euphratica*, a negative value correspondingly a higher apparent expression in *P.* × *canescens*. Sample number of significant transcripts (n) is displayed for each species. Boxes represent the interquartile length (IQL) and median, whiskers extend to the most extreme data point with a maximum length of 1.5 times IQL; outliers are shown as circles; significant differences between groups were calculated by an ANOVA followed by a Tukey-Kramer test (Kramer 1956).

trichocarpa × *deltoides*, *P. trichocarpa* × *nigra*, *P. nigra*, *P. deltoides* and *P. euramericana*, and will be further addressed as the *Tacamahaca* group. Expression ratios showed a homogenous distribution, *i.e.* similar numbers of significant genes were identified as being higher expressed in *P.* × *canescens* or in *P. euphratica*, respectively. The second group consisted of probe sets based on the *Leuce* section species *P. tremula* × *tremuloides*, *P. tremula*, *P.* × *canescens* (= *P. tremula* × *P. alba*), *P. alba* × *tremula* var. *glandulosa*, *P. tremuloides* and *P. tomentiglandulosa*. In this group, the distribution of expression values showed a clear shift, more significant genes had been detected as being higher regulated in *P.* × *canescens*. *Vice versa*, in the last group, the distribution of expression values for probe sets based on *P. euphratica* from the *Turanga* section was shifted into the opposite direction: for these probe sets, more genes were detected as being higher expressed in *P. euphratica*.

Because of these shifts of expression ratios towards one species, transcripts of the biased *Leuce* and *Turanga* groups higher expressed in *P.* × *canescens* and *P. euphratica*, respectively, will have an increased rate of false positives. Approximately three quarters of the transcripts in each group belong to this class with an increased rate of false positives, whereas only one quarter of the transcripts have an expression ratio that reflects the true state with a high probability. Presumably, an unbiased distribution would be balanced, with approximately 50% of the transcripts in each group higher expressed in one of the two species. From this shift from 50 to 75% towards the favored species, a false positive rate of approximately 33% would be expected. Therefore, from the *Leuce* group only transcripts higher expressed in *P. euphratica*, and from the *Turanga* group only transcripts higher expressed in *P.* × *canescens* were considered in further analyses; transcripts from the *Tacamahaca* group were

considered unbiased and were not filtered, resulting in a list of 2672 differentially expressed transcripts (filter 4*, Table 4.2).

Finally, in cases where two or more probe sets were annotated by the same gene model, duplicates were removed, thereby preferring probe sets with a higher p-value. This final list of differentially expressed transcripts contained 2503 genes, of which 1645 were higher expressed in *P. euphratica* and 858 in *P.* × *canescens* (filter 5*, Table 4.2).

For enrichment analyses of Gene Ontology (GO) terms and GO slim terms, a slightly different list of significant genes had to be used, which did not include the transcripts from the *Leuce* and the *Turanga* group (Table 4.3). Following filtering step 3, only differentially expressed transcripts from the *Tacamahaca* group were subjected to filter 4 and 5 as described above, resulting in a list of 2246 differentially expressed genes of which 1389 were higher expressed in *P. euphratica* and 857 in *P.* × *canescens* (Table 4.2). The reason for this is that for enrichment analyses, a study set, or 'gene universe', has to be defined that consists of all eligible transcripts on the microarray from which significant genes might be drawn. For transcripts from the *Leuce* and the *Turanga* group, the possibility to be present in the list of differentially expressed genes is not only dependent on differences in expression levels, but also on a high sequence identity, which cannot be determined for the majority of these genes. Therefore, transcripts from the partially biased *Leuce* and *Turanga* groups are not included in the 'gene universe'.

4.2.4. Differences between the transcriptomes of *P. euphratica* and *P. × canescens*

To investigate the differences between the transcriptomes of the two poplar species, we used the Gene Ontology annotations (The Gene Ontology). A 'gene universe' of 14,254 eligible genes was compiled from all 61,413 probe sets on the microarray (Table 4.2). This was done by taking the complete set of probe sets and applying the filtering methods described above: First, probe sets not detected as 'present' by the MAS 5.0 algorithm were removed. Second, probe sets subject to a potential hybridization bias due to an internal SD in the upper 5% quantile of all internal SDs were removed. Third, non-annotated probe sets were removed, as proposed by Falcon and Gentleman (Falcon & Gentleman 2007). Fourth, probe sets subject to a potential hybridization bias due to their target sequence origin species were removed. Finally, all probe sets with a duplicate gene model annotation were removed.

To get an impression of the global transcriptomic differences of *P. euphratica* and *P. × canescens*, we employed the GO slim terms, which represent a reduced, simplified version of the complex GO term hierarchy. Abundances of GO slim term annotations among the 'gene universe' and among the 2246 differentially expressed genes were compared (filter 5, Table 4.2). Significant over- and underrepresentation of GO slim terms among the genes higher expressed in *P. euphratica*, respective *P. × canescens*, were determined by a hypergeometric test (Figure 4.7). The most prominent differences concern underrepresentation of terms associated with transcription and regulation in *P. euphratica*, like 'nucleus', 'DNA or RNA binding', 'transcription factor activity', 'kinase activity' and 'transcription' itself, suggesting that mechanism to react to alterations of external conditions, and to adapt the transcriptome accordingly, are less developed in *P. euphratica* compared to *P. × canescens*. Overrepresented GO slim terms among genes

Table 4.3 - Functional gene categories that were significantly over-represented by genes with a higher expression in *P. euphratica* than in *P.* × *canescens*

GO number	GO	number of terms in gene universe	number of terms in study set	adjusted p-value	GO term
GO:0016020	CC	3124	382	0.0007	membrane
GO:0005739	CC	742	113	0.0013	mitochondrion
GO:0044249	BF	592	89	0.0201	cellular biosynthetic process
GO:0044429	CC	166	33	0.0281	mitochondrial part
GO:0044444	CC	3320	385	0.0281	cytoplasmic part
GO:0005740	CC	141	29	0.0340	mitochondrial envelope
GO:0006082	BF	451	68	0.0427	organic acid metabolic process
GO:0009699	BF	78	19	0.0427	phenylpropanoid biosynthetic process
GO:0019752	BF	451	68	0.0427	carboxylic acid metabolic process
GO:0005623	CC	7583	806	0.0427	cell
GO:0044464	CC	7583	806	0.0427	cell part
GO:0005737	CC	3605	409	0.0427	cytoplasm
GO:0012505	CC	1492	186	0.0427	endomembrane system
GO:0009698	BF	95	21	0.0436	phenylpropanoid metabolic process
GO:0005618	CC	129	26	0.0436	cell wall
GO:0009058	BF	1472	183	0.0451	biosynthetic process
GO:0009813	BF	52	14	0.0462	flavonoid biosynthetic process
GO:0030312	CC	131	26	0.0462	external encapsulating structure
GO:0006519	BF	346	54	0.0474	amino acid and derivative metabolic process
GO:0005386	MF	275	45	0.0475	carrier activity
GO:0016020	CC	3124	382	0.0007	membrane

Significant over-representation is based on a Gene Ontology (GO) term enrichment analysis using the 'Ontologizer' program, calculation method: term-for-term, multiple test correction procedure: Benjamini-Hochberg (Bauer *et al.* 2008). The 'Gene universe' refers to all 14,255 eligible gene models present on the microarray, study sets refer to the 1280 and 777 genes higher expressed in *P. euphratica*, respective *P.* × *canescens*. BP: Biological Process; MF: Molecular Function; CC: Cellular Component.

higher expressed in *P. euphratica* were 'other membranes' (*i.e.* other than plasma membrane), 'mitochondria', 'cell wall', 'hydrolase activity' and 'transporter activity'. In *P.* × *canescens*, the only significant overrepresented GO slim term was 'plasma membrane'. Neither of the species showed an over- or underrepresentation of the GO slim term 'response to stress', contrary to out hypothesis. However, to comprehend the differences in stress tolerance mechanisms between the two poplar species, it is of interest to compare the two lists of genes that were higher expressed in *P. euphratica*, respective *P.* ×

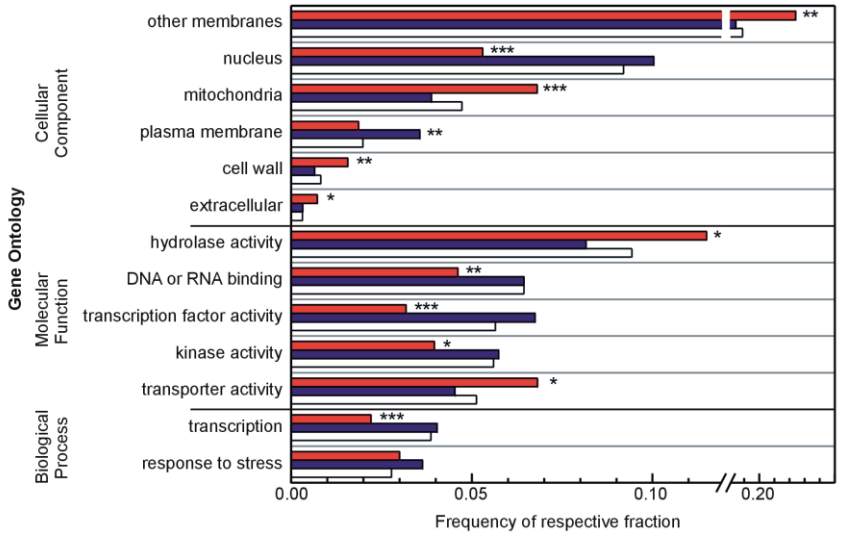

Figure 4.7 - Abundances of selected GO slim categories

Shown are the frequencies of GO slim annotations among the genes significantly higher expressed in *P euphratica* (red), *P.* × *canescens* (blue) and of the 'gene universe' (all eligible gene models present on the microarray; white). All GO slim categories significantly over- or underrepresented as calculated by a hypergeometric test after Benjamini-Hochberg correction are shown except 'unknown biological processes' (underrepresented in *P.* × *canescens*), 'other enzyme activity' (overrepresented in *P. euphratica*) and 'unknown cellular components' (underrepresented in *P. euphratica*). 'response to stress' is the only GO category shown that is not significantly over- or underrepresented in either of the two poplar species. Data is based on 1280 (*P euphratica*), 777 (*P.* × *canescens*) and 14,672 ('gene universe') genes. Significant over- or underrepresentation of categories are indicated by * for p-values ≤ 0.05, ** for p-values ≤ 0.01 and ** for p-values ≤ 0.001.

canescens, and annotated by 'response to stress'. To a certain extent, the lists are similar (Supplemental Table S2). Both lists include stress induced genes like catalase 2 and peroxidases, universal stress proteins, a senescence-associated and a low temperature and salt responsive protein, as well as various heat-shock proteins and protein kinases. Both lists also feature a salt stress induced tubulin beta chain. Most striking differences are three different short chain alcohol dehydrogenases higher expressed in *P.* × *canescens*, and two mitochondrial chaperonins higher expressed in *P. euphratica*.

For a more detailed analysis of the transcriptomic differences, the original, unreduced GO terms were employed. We performed a GO term enrichment analysis for 2057 differentially expressed genes filtered for this purpose using the 'Ontologizer' (Bauer *et al.* 2008). Among the genes higher expressed in *P.*

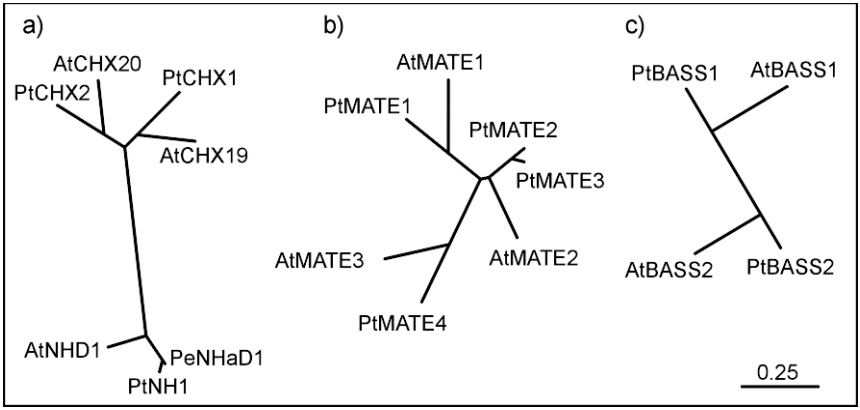

Figure 4.8 - Phylogenetic tree of Na^+-transporters

The unrooted trees represent the genetic relationships of sodium transporters that are higher expressed in leaves of *P. euphratica* than of *P.* × *canescens* during non-stressed state. Included are the *P. trichocarpa* gene models represented on the microarray and their nearest Arabidopsis orthologs, as well as the *P. euphratica* ortholog in case of *PeNHaD1*. The genes belong to the following families: a) Na^+/H^+ antiporter, b) multidrug and toxic compound extrusion (MATE), c) bile acid/sodium symporter (BASS). Descriptions and identifiers are listed in Table 4.4. Sequences were aligned with ClustalW2 (ClustalW2). The unrooted tree was generated using Tree View (Zhai *et al.* 2002).

× *canescens*, no GO terms were significantly enriched after Benjamini-Hochberg correction. Among genes higher expressed in *P. euphratica*, GO terms enriched with the highest significance ratings were ‘membrane’ and 'mitochondrion', the latter suggesting an increased need for energy. Further significantly enriched GO terms were ‘cellular biosynthetic process’, ‘mitochondrial part’, ‘phenylpropanoid biosynthetic process’, ‘cytoplasmic part’, ‘phenylpropanoid metabolic process’, ‘carrier activity’, ‘cell wall’, ‘external encapsulating structure’, ‘mitochondrial envelope’, ‘flavonoid biosynthetic process’ and ‘amino acid and derivative metabolic process’ (Table 4.3). To gain insight into salt tolerance mechanisms, it is of interest to examine the category ‘carrier activity’ more closely (Supplemental Table S3).

Table 4.4 - Sodium transporters that were higher expressed in leaves of *P. euphratica* than of *P.* × *canescens* and their closest *Arabidopsis* orthologs

Gene name/ abbreviation	Description	JGI gene model / AGI locus identifier
PtNH1	Na^+/H^+ antiporter	eugene3.00012381
PtCHX1	Na^+/H^+ antiporter, putative	fgenesh4_pm.C_LG_III000450
PtCHX2	Na^+/H^+ antiporter, putative	fgenesh4_pg.C_LG_VI000456
PeNHaD1	Na^+/H^+ antiporter	AJ561195
AtNHD1	Na^+/H^+ antiporter	At3g19490
AtCHX19	putative Na^+/H^+ antiporter	At3g17630
AtCHX20	putative Na^+/H^+ antiporter	At3g53720
PtMATE1	MATE efflux family	gw1.XIII.2310.1
PtMATE2	MATE efflux family	estExt_fgenesh4_pg.C_LG_II0511
PtMATE3	MATE efflux family	fgenesh4_pg.C_LG_V001238
PtMATE4	MATE efflux family	fgenesh4_pm.C_LG_IV000232
AtMATE1	MATE efflux family	At3g03620
AtMATE2	MATE efflux family	At3g59030
AtMATE3	MATE efflux family	At1g71140
PtBASS1	Bile acid/sodium symporter family	gw1.150.220.1
PtBASS2	Bile acid/sodium symporter family	eugene3.00141255
AtBASS1	Bile acid/sodium symporter family	At2g26900
AtBASS2	Bile acid/sodium symporter family	At3g25410

Most noteworthy among the genes higher expressed in *P. euphratica* were acid/ sodium symporters (BASS), three Na^+/H^+-antiporter (NH and CHX), and four multidrug and toxic compound extrusion (MATE) family proteins (Table 4.4).

4.2.5. Integration of metabolomic and transcriptomic data on nine transporters belonging to four gene families (Figure 4.8): Two bile pathway maps

The metabolites of the two poplar species were analyzed by Fourier transform-ion cyclotron resonance mass spectrometry (FT-ICR/MS) to compare the differences in the two metabolomes with the differences of the transcriptomes. Identification of ions was performed using 'Mass Translator into Pathways' (MassTRIX) (Suhre & Schmitt-Kopplin 2008). 789 ions were found that had significantly higher peak intensities in *P. euphratica* than in *P.* × *canescens*. To 97 of these ions, a bulk chemical formula could be assigned, of which 39 were annotated in poplar. Where metabolites share identical bulk formula, multiple alternative annotations may be possible. Therefore, the 39 ions with different bulk chemical formulas represent 76 possible metabolites with a higher relative abundance in *P.* × *canescens* than in *P. euphratica* (Supplemental Table S4). In *P.* × *canescens*, 983 ions with significantly higher peak intensities than in *P. euphratica* were found. Assignment of a bulk chemical formula was possible for 117 ions, of which 39 were annotated in poplar and could be assigned to 76 possible metabolites with a higher relative abundance in *P. euphratica* than in *P.* × *canescens* (Supplemental Table S4).

Identified and annotated metabolites were automatically mapped to KEGG pathways using MassTrix, simultaneously implementing the transcriptomic data. For this, Enzyme Commission (EC) numbers were obtained for significant genes from the JGI *P. trichocarpa* project (JGI *Populus trichocarpa* genome

release 1.1). Of the differentially expressed genes, 289 genes higher expressed in *P. euphratica* than in *P.* × *canescens* and 132 genes higher expressed in *P.* × *canescens* than in *P. euphratica* could be annotated by EC number. EC numbers were added to the MassTrix pathway analysis query.

Based on the results of the GO term enrichment analysis, pathways were chosen from the 124 KEGG pathways available for poplar. Five pathways were presumed to accentuate important differences between the two species and

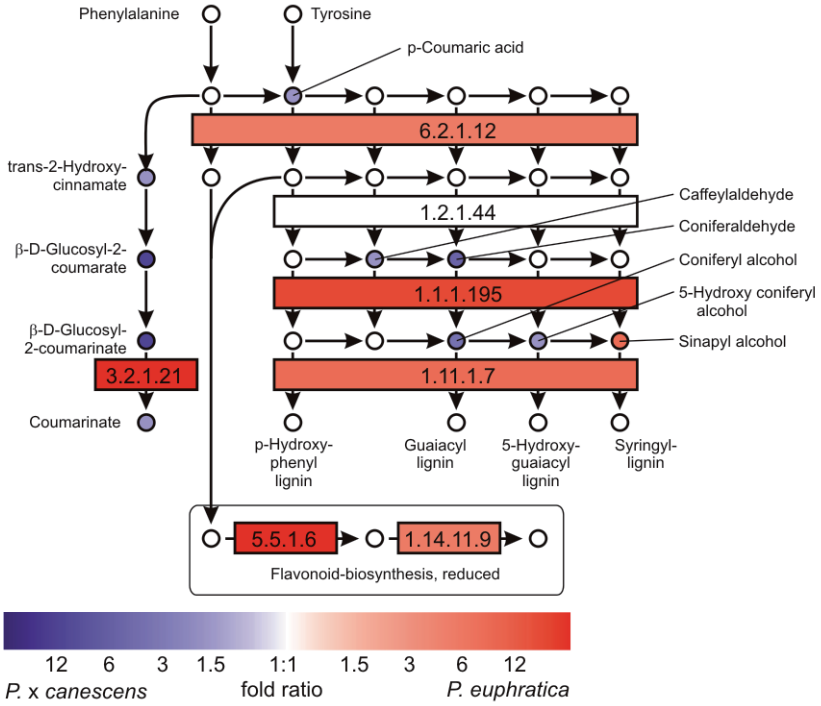

Figure 4.9 - Analysis of pathways related to phenolics metabolism

Map displays selected steps from KEGG pathways dpop00940 'Phenylpropanoid biosynthesis' and dpop00941 'Flavonoid biosynthesis'. Colors indicate significant expression, respective metabolite content ratios between *P. euphratica* and *P.* × *canescens*, red indicates higher relative levels in *P. euphratica*, blue in *P.* × *canescens*. Enzymes are given as EC numbers: 1.1.1.195, cinnamyl-alcohol dehydrogenase; 1.1.1.219, dihydroflavonol 4-reductase; 1.11.1.7, peroxidase; 1.14.11.9, flavanone 3-hydroxylase; 1.2.1.44, cinnamoyl-CoA reductase; 3.2.1.21, beta-glucosidase; 5.5.1.6, chalcone isomerase; 6.2.1.12, 4-coumarate:CoA ligase.

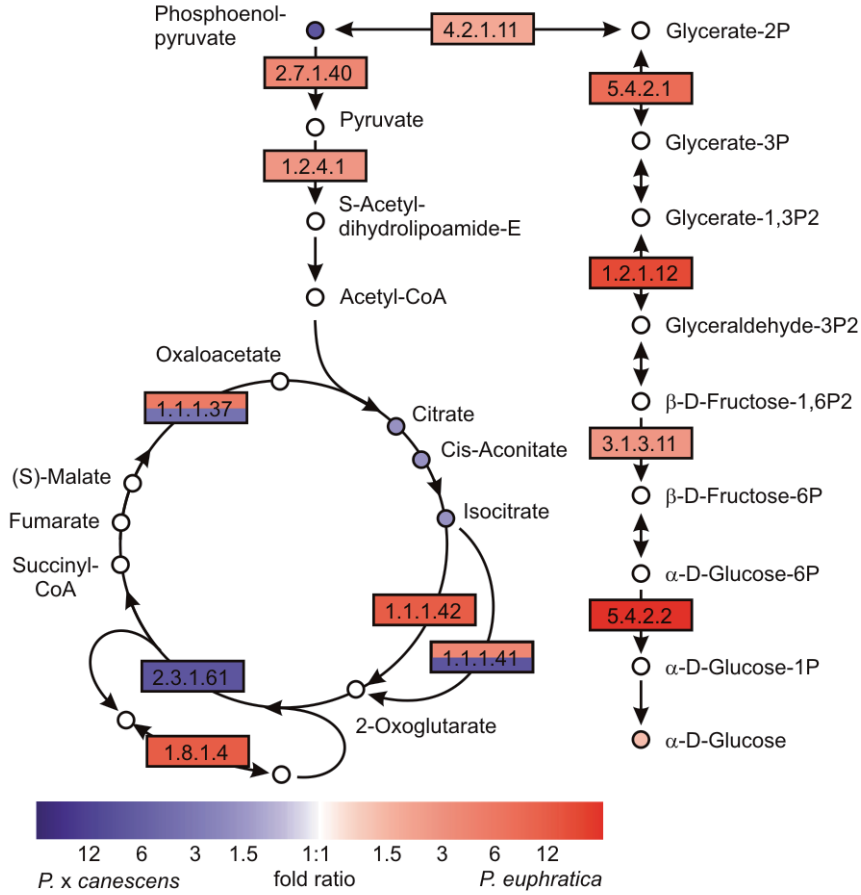

Figure 4.10 - Analysis of pathways related to energy metabolism

Map displays selected steps from KEGG pathways dpop00010 'Glycolysis / Gluconeogenesis' and dpop00020 'Citrate cycle (TCA cycle)'. Colors indicate significant expression, respective metabolite content ratios between *P. euphratica* and *P.* × *canescens*, red indicates higher relative levels in *P. euphratica*, blue in *P.* × *canescens*. Enzymes are given as EC numbers: 1.1.1.37, malate dehydrogenase; 1.1.1.42, isocitrate dehydrogenase; 1.2.1.12, glyceraldehyde-3-phosphate dehydrogenase; 1.2.4.1, pyruvate dehydrogenase; 1.3.5.1, succinate dehydrogenase; 2.3.1.12, dihydrolipoamide S-acetyltransferase; 2.7.1.40, pyruvate kinase; 3.1.3.11, fructose-bisphosphatase; 4.1.2.13, fructose bisphosphate aldolase; 4.2.1.11, 2-phospho-D-glycerate hydrolyase; 5.4.2.1, phosphoglycerate mutase; 5.4.2.2, phosphoglucomutase.

particularly examined. This concerned 'Phenylpropanoid biosynthesis' and 'Flavonoid biosynthesis' (Figure 4.9) and, because of the enriched GO term 'mitochondrion', the pathways 'Citrate cycle' with the connected 'Glycolysis / Gluconeogenesis' (Figure 4.10) and 'Starch and sucrose metabolism' (Figure 4.11).

In all of these pathways, enzymatic chains with many of their elements higher expressed in *P. euphratica* are present. Contrary to that, most metabolites that are contained in differing amounts in the two species were identified in *P.* × *canescens*. Congruence between higher gene expression and higher content of the product can only be observed in the starch and sucrose pathway.

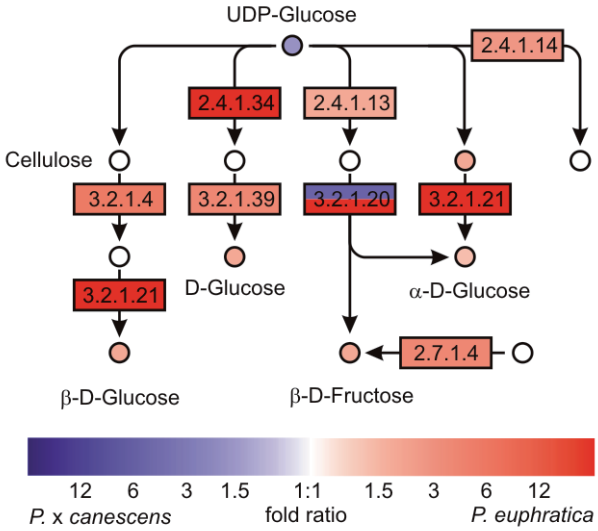

Figure 4.11 - Pathway analysis of starch and sucrose metabolism

Map displays selected steps from KEGG pathway dpop00500 'Starch and Sucrose metabolism'. Colors indicate significant expression, respective metabolite content ratios between *P. euphratica* and *P.* × *canescens*, red indicates higher relative levels in *P. euphratica*, blue in *P.* × *canescens*. Two-colored enzymes can occur where different genes models are annotated by the same enzymatic function. Enzymes are given as EC numbers: 2.4.1.13, sucrose synthase; 2.4.1.18, 1,4-α-glucan branching enzyme; 2.4.1.34, 1,3-β-glucan synthase; 3.2.1.2, β-amylase; 3.2.1.3, glucan 1,4-α-glucosidase; 3.2.1.4, cellulase; 3.2.1.20, α-glucosidase; 3.2.1.21, β-glucosidase; 3.2.1.39, glucan endo-1,3-β-D-glucosidase.

4.2.6. Physiological measurements as proof of concept of the pathway analysis

The informative value and reliability of the GO term enrichment and the subsequent pathway analysis were tested by measuring phenolic compounds, condensed tannins, dark respiration and sugar content. Soluble phenolic compounds were additionally analyzed by high-performance liquid chromatography (HPLC) to determine differences in their composition between *P.* × *canescens* and *P. euphratica.*

Contents of phenolic compounds and tannins were always lower in *P.* × *canescens* than in *P. euphratica.* Content of soluble and non-soluble phenolic compounds, as well as soluble condensed tannins, was approximately 30 to 40%, and content of non-soluble condensed tannins was 60% lower in *P.* × *canescens* than in *P. euphratica* (Figure 4.12 a – d). Dark respiration showed a tendency to be more than two times as high in *P. euphratica* than in *P.* × *canescens* (Figure 4.12 e). Glucose content was also two times as high in *P. euphratica* than in *P.* × *canescens* (Figure 4.12 f). Fructose content showed a tendency to be slightly lower in *P.* × *canescens* than in *P. euphratica* (Figure 4.12 g). Saccharose content was below the detection limit in both species.

The HPLC profiles of soluble phenolic compounds showed that in accordance with the measurements of total soluble phenolic compounds, abundances of individual components were generally higher in *P. euphratica*. However, some components, especially those with a retention time of 25 to 35 min, were more abundant in *P.* × *canescens* (Figure 4.13).

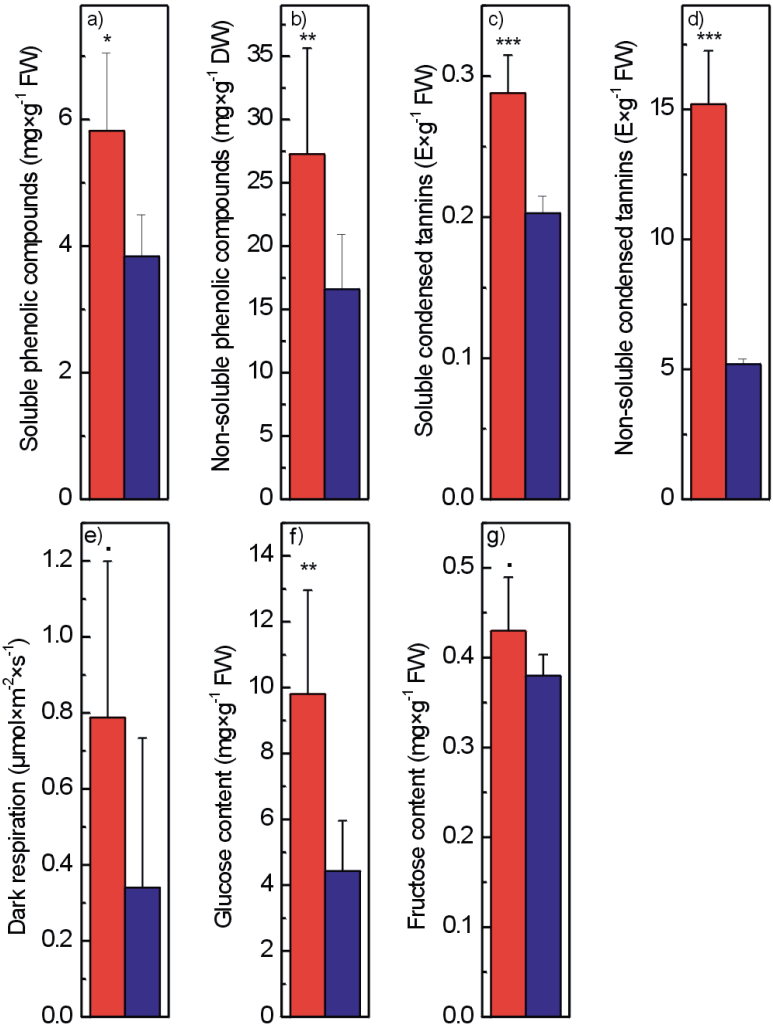

Figure 4.12 - Measurement of total phenolic compounds, condensed tannins, dark respiration, glucose and fructose content as proof of concept

P. euphratica (red) and *P. × canescens* (blue) plants were kept under control conditions in a climatized chamber. a) and b) soluble, respective non-soluble phenolic compounds, expressed as catechin equivalents (n = 5, mean±SD); c) and d) soluble, respective non-soluble condensed tannins, expressed as relative units in Extinction/g FW (n = 5, mean±SD); e) Dark respiration was measured for 5 min after keeping the plants in darkness for 30 minutes (n = 4, mean±SD); f) and g) glucose and fructose content, respectively (n = 5, mean±SD). Statistical significances are indicated by * for p-values ≤ 0.05, ** for p-values ≤ 0.01 and ** for p-values ≤ 0.001; · indicates tendencies with a p-values ≤ 0.1.

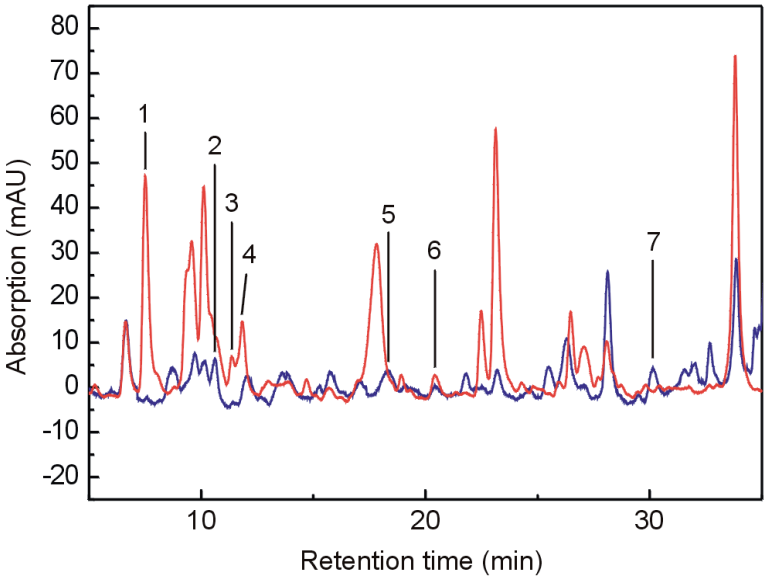

Figure 4.13 - HPLC analysis of soluble phenolic compounds

P. euphratica (red) and *P.* × *canescens* (blue) plants were kept under control conditions in a climatized chamber. Data shown are averages of 5 HPLC chromatographs for each species. Compounds identified by co-elution of internal standards: 1) 3,4-dihydroxybenzoic acid; 2) catechin; 3) 4-hydroxybenzoic acid; 4) chlorogenic acid; 5) cumaric acid; 6) ferulic acid; 7) cinnamic acid (mAU = milli AbsorbanceUnits).

4.3. Discussion

4.3.1. *P. euphratica* is pre-adapted to salt stress

Our salt shock experiment demonstrates that the responses of *P. euphratica* and *P.* × *canescens* to a sudden increase of salt concentration in the nutrient medium regarding photosynthesis are quite dissimilar. Whereas *P.* × *canescens* shows an adaptive phase that quite clearly allows for gene regulation, *P. euphratica* seems to instantly change its physiology. While regulation of single genes might occur quite fast, the alteration of the whole photosynthetic apparatus would necessitate more time. Membrane-bound molecules have to be generated or protected and pools of substances have to be adapted. A change in expression pattern this fast and extensive seems not feasible.

Brosché *et al.* compared the transcriptomes of *P. euphratica* trees growing under differing salt stress conditions on a *P. euphratica* cDNA microarray (2005). Although stress related genes in the EST collection were enriched, the analysis identified less than 0.5% regulated genes. It was pointed out that in *Arabidopsis thaliana*, typically more genes display changes in transcription levels in response to abiotic stress conditions, which seems to apply also for poplar species other than *P. euphratica*. For example, using a microarray containing approximately 10,000 genes, Street *et al.* found 386 regulated genes in *P. trichocarpa* × *P. deltoides* regulated under drought stress (2006). Taking into account the fast response of the photosynthetic apparatus of *P. euphratica* to salt shock, these comparisons suggest that *P. euphratica* possesses a transcriptome that is constitutively pre-adapted to stress.

4.3.2. Direct comparison of microarray expression data of *P. euphratica* and *P. × canescens* requires rigorous filtering of probe set information

In spite of its regular application, correct use of cross-species hybridization is still a much discussed topic. Differences in single nucleotides between probe and target sequences can affect the annealing of single strands. Thus, because of the composition of the Affymetrix probe match-mismatch system, single nucleotide polymorphisms can result in decreased signals of single probes and influence the summarized probe set signal (Rouchka *et al.* 2008; Irizarry *et al.* 2003). This was affirmed by qRT-PCR analysis of genes with differing standard deviations of the single probe signals. Therefore, it is important to consider sequence identity and relations of the employed species.

Analyzing different species of cichlid fish, Renn *et al.* showed that for CSH, consistency of microarray results is higher the more closely related the analyzed species are (Renn *et al.* 2004). For species with a time of genetic divergence of less than 10 million years (Mya) ago, transcription profiling gave most robust results, but divergence times of greater than 65 Mya were still acceptable. Tuskan *et al.* stated that the genus of *Populus* diverged from *Salix* around 60 to 65 Mya, and that evolution in *Populus* proceeds relatively slow, at one-sixth of the rate for *Arabidopsis* (2006). Therefore, we can assume that the relationship between *P. euphratica*, *P. × canescens*, and *P. trichocarpa* as the primary species on the Affymetrix Gene Chip is close enough to allow CSH in principle. This assumption was validated by sequence analysis of 20 genes of *P. × canescens*, *P. euphratica* and *P. trichocarpa*. Between all three species, the degree of sequence identity was equally high.

However, the analysis of signal intensities separated according to the probe set sequences origin species showed that for certain species, the intensity distributions are biased, insofar as hybridization of either *P. euphratica* or *P. ×*

canescens cDNA was favored. Regarding the differently biased groups of probe sets, it becomes obvious that the bias can be explained by the degree of relationship (Figure 4.3). Unbiased probe sets are based on poplar species from the *Aigeiros* and the *Tacamahaca* section: *P. trichocarpa*, *P. nigra*, *P. deltoides* (including their hybrids) and *P. euramericana* (Cervera *et al.* 2005). Probe sets based on species from the *Leuce* section, *P. alba*, *P. tremula*, *P. tremuloides* and *P. tomentiglandulosa* (including their hybrids), are biased in favor of *P.* × *canescens* (Cervera *et al.* 2005; Hong *et al.* 1998). And finally, probe sets biased in favor of *P. euphratica* are all based on *P. euphratica* itself, *P. euphratica* being the sole representative of the *Turanga* section on the microarray (Cervera *et al.* 2005).

Obviously, the bias is caused by higher identity of the probe set sequence to the according sequence of one of the two poplar species, dependent on the degree of relationship. It must therefore be assumed that among the genes that are interrogated by probe sets based on species from the *Leuce* section and which, by the microarray, are found to be higher expressed in *P.* × *canescens*, the fraction of falsely detected genes is increased. *Vice versa*, the same must be true for genes found to be higher expressed in *P. euphratica* by probe sets based on *P. euphratica*. Since it is impossible to control this increased error of the first kind without knowledge of all relevant sequence data of *P. euphratica* and *P.* × *canescens*, probe sets biased in this way must be excluded from further analysis. Thus, of the genes detected to be differentially expressed by probe sets based on poplar species from the *Leuce* section, only those higher expressed in *P. euphratica*, and of the genes detected to be differentially expressed by probe sets based on *P. euphratica*, only those higher expressed in *P.* × *canescens* are considered in the investigation.

This is basically the same approach taken by Taji *et al.*, who did a direct comparison of the transcriptomes of *Arabidopsis thaliana* and its close

relative, *Thellungiella halophila* on *Arabidopsis* cDNA microarray (Taji *et al.* 2004). Only genes that were higher expressed in *Thellungiella* than in *Arabidopsis* were considered, making a comparison to genes higher expressed in *Arabidopsis* than in *Thellungiella* impossible.

A different strategy to handle biased probes was pursued by Ranz *et al.* during the comparison of the transcriptomes of *Drosophila melanogaster* and *D. simulans* on *D. melanogaster* cDNA microarrays (Ranz *et al.* 2003). The two species were reported to have a sequence identity of approximately 96.2%. To analyze the effect of the sequence differences on hybridization results, genomic DNA of both species was hybridized to the microarrays. Despite the fact that hybridization of *D. melanogaster* DNA was on average 4.2% stronger than that of *D. simulans*, the effect was regarded to be 'within the limit of detection of significant differences in gene expression', a correction of the expression data was therefore not conducted.

Compared to these two methods, the use of the multi-species Affymetrix poplar microarray combined with filtering of expression data according to the probe set sequences origin species has several advantages. By using probes based on species different to both of our analyzed species, we are able to directly compare the transcriptomes of *P. euphratica* and *P.* × *canescens*, thereby detecting changes in expression levels in both directions, while simultaneously being able to minimize the error of the first kind by identification and elimination of biased probe sets. Additionally, by utilizing the Affymetrix probe set system and calculating the standard deviation for probe set signals, we are able to identify and exclude probe sets that have a highly increased risk to generate erroneous expression differences between *P. euphratica* and *P.* × *canescens*, as was detected by qRT-PCR. Our analysis of the microarray data shows that special care is necessary when directly

comparing two different species on a microarray, but that through appropriate data filtering, error rates can be minimized.

4.3.3. Transcriptomic data reveal information on stress tolerance mechanism in *P. euphratica* and can be confirmed by integration of metabolomic data

The assignment of EC numbers to the differentially expressed genes allows the integration of transcriptomic and metabolomic data. By mapping enzymes and metabolites to KEGG pathways, the integrity of both data types can be displayed. The GO term enrichment analysis showed that among the genes higher expressed in *P. euphratica*, genes from pathways like phenylpropanoid or flavonoid biosynthesis were enriched. These pathways turn out to be highly activated in *P. euphratica* (Figure 4.6). The majority of identified metabolites in the phenylpropanoid pathway exhibit higher contents in *P.* × *canescens*, though. To understand this seeming contradiction, it is necessary to know that by FT-ICR/MS, only relative concentration levels can be measured. A possible explanation might therefore be that distinct intermediates are faster metabolized in *P. euphratica* due to the higher expressed genes, but that endproducts which are more abundant in *P. euphratica* than in *P.* × *canescens* are not part of the actual pathway. This would explain the relative accumulation of several metabolites in *P.* × *canescens* in this pathway, but higher contents of total phenolic compounds and tannins in *P. euphratica*. The HPLC profile of soluble phenolic compounds illustrates that although total phenolic compounds are more abundant in *P. euphratica*, individual compounds are more abundant in *P.* × *canescens*. Obviously, compounds like these are those present in the phenylpropanoid pathway.

In the starch and sucrose pathway we can observe a more coherent pattern. In *P. euphratica*, hexose sugars seem to be produced and accumulated at a higher rate than in *P.* × *canescens* by enzymes encoded by higher expressed genes (Figure 4.8). Higher contents of glucose and fructose in *P. euphratica* compared to *P.* × *canescens* under non-stressed conditions were confirmed by separate measurements (Figure 4.9 f and g). The higher contents in *P. euphratica* might have two explanations: First, these sugars represent the earliest precursors for phenylpropanoids, flavonoids and other phenolic compounds, and might therefore be necessary as an ample supply for these highly active pathways. Second, they might be preventively produced to help the plant tolerate osmotic stress, and might thus even be one of the reasons why *P. euphratica* is prepared to quickly respond and adapt to salt shock.

More information about the mechanisms of stress tolerance of *P. euphratica* can be deduced from the GO and GO slim term analyses. Underrepresentation of GO slim terms related to signaling and transcription suggest that the whole mechanism responsible for gene regulation and transcription may be neglected. This concurs with the observation that stress response in *P. euphratica* occurs instantaneously and does not seem to necessitate gene regulation, but finding further evidence for this hypothesis might be complicated. Although it is possible to measure the abundance of particular mRNAs, either by microarray analysis or by qRT-PCR, it is not possible to accurately measure the abundance of total mRNA per cell. But taken into account that in *P. euphratica* very few genes seem to be regulated under abiotic stress, we propose that this species may have permanently adapted to stress conditions, thereby abandoning the flexibility and adjustability of its transcriptome and somehow altering its basic state.

Possible preventive mechanisms in *P. euphratica* to tolerate salt stress are the aforementioned accumulation of sugars and an increased control of ion

compartmentation. The latter is indicated by the overrepresentation of GO slim terms 'other membranes' and 'transporter activity', as well as the enrichment of GO terms 'membrane' and 'carrier activity'. Among the 45 genes with carrier activity, nine were sodium transporters and may therefore be involved in salt tolerance mechanisms. Three of the sodium transporters belong to a closely related group of two antiporter gene families consisting of Na^+/H^+ (NH) and cation/H^+ exchanger (CHX) antiporters (Figure 4.8 a). *PeNHaD1*, a member of this group from *P. trichocarpa*, has been characterized and proposed to protect cells from salt stress by translocating Na^+ ions into the vacuole (Ottow *et al.* 2005; Wang *et al.* 2008). Four other sodium transporters belong to the multidrug and toxic compound extrusion (MATE) family (Figure 4.8 b). MATE genes code for antiporters with a variety of functions, amongst others detoxification of secondary metabolites. They are as yet poorly characterized and their actual function remains unclear, but they have in common that they transport organic compounds through H^+ or Na^+ exchange (Omote *et al.* 2006). The last two sodium transporters belong to the bile acid / sodium symporter (BASS) family (Figure 4.8 c). Transporters from the BASS family transport bile salts and other organic anions in combination with Na^+ ions (Mansour *et al.* 2007). Similar to MATE antiporters, their function is not fully understood, since bile acids have not been found in plants; even from BASS5, a better researched member of the family, is only known that it is involved in transporting biosynthetic intermediates of Glucosinolate biosynthesis, but not the exact compound (Sawada *et al.* 2009).

Since the higher expression of BASS and MATE genes in *P. euphratica* coincides with its ability to cope better with salt stress than *P.* × *canescens*, it is possible that members of these two families play similar roles as the NH/CHX antiporters. This would mean that even under non-stressed conditions, *P. euphratica* is prepared to compartmentalize sodium from the cytosol into the

vacuole. The sustentation of these mechanisms could even explain a higher energy demand of *P. euphratica* cells as suggested by the overrepresentation of mitochondrion-related terms. Due to the complexity and the lack of knowledge of the exact functions of these transporter families, it is however only speculation, although more research work and characterization efforts into these gene families as contributors for plant salt tolerance seems promising.

Apart from these indications of *P. euphratica* showing higher expression of genes that might mitigate salt stress, our data shows that stress related genes are not generally overrepresented among genes higher expressed in *P. euphratica* than in *P.* × *canescens*, contrary to our initial hypothesis. Among both our 'gene universe' and genes higher expressed in *P.* × *canescens*, the fractions of stress-response genes are similar to the fraction among genes higher expressed in *P. euphratica*. In a comparable study, Taji *et al.* reported that in the salt tolerant *Thellungiella halophila*, a close relative of *Arabidopsis*, typical salt stress related genes were higher expressed during the absence of stress (Taji *et al.* 2004). However, since the two species were compared on an *Arabidopsis* cDNA microarray, genes higher expressed in *Arabidopsis* could not be considered due to hybridization bias, and thus, a statement about an increased abundance of stress responsive genes in one of the two species was not possible. Here, we show that although a number of typical stress related genes are higher expressed in *P. euphratica*, the same is true for *P.* × *canescens* (Supplemental Table S2). We can therefore conclude that *P. euphratica* does not rely on generally higher expression levels of abiotic stress inducible genes to gain an increased tolerance of salt stress.

4.4. Conclusion

We introduced the method of directly comparing the transcriptomes of a stress sensitive and a stress tolerant tree species using whole genome microarrays to gain insight into stress tolerance mechanisms. We showed that such a direct comparison is actually possible when raw data is sufficiently freed from biases and the results are interpreted on the scale of pathways rather than of specific genes. Our results suggest that tolerance mechanisms in the salt tolerant *P. euphratica* differ strongly from stress response mechanisms in the salt sensitive *P.* × *canescens*; a general higher expression of stress relevant genes in *P. euphratica* could not be determined, and molecular mechanisms for allowing the plant to adapt its transcriptome to stress conditions even seem to be reduced. Instead, genes involved in secondary, sucrose and energy metabolisms as well as membrane systems are highly expressed, suggesting the formation of a wood structure different from that of the salt sensitive *P.* × *canescens*, an accumulation of sugars to tolerate osmotic stress, and a more controlled ion compartmentation, thus giving rise to a high energy requirement.

4.5. Materials and methods

4.5.1. Plant material and salt treatment

Plantlets from *P.* × *canescens* (*P. alba* × *tremula*) clone INRA717 1-B4 (Leplé *et al.* 1992) and *P. euphratica* clone B2 from the Ein Avdat valley in Israel (Brosché *et al.* 2005) were multiplied by *in vitro* micropropagation (Rutledge & Douglas 1988) and kept in aerated hydroponics using Long Ashton (LA) nutrient solution (Hewitt & Smith 1975).

Plants were grown for 3 months in a greenhouse at 20 to 25 °C, with a relative air humidity of 40 to 60 % and additional 16 h of light (neon lamps: L58W/25 and 58W/840, Osram, Munich, Germany, and TLD 58W/840 Philips,

Amsterdam, Netherlands) between 6:00 a.m. and 10:00 p.m. to reach a continuous photosynthetic active radiation (PAR) of 150 µmol $m^{-2}s^{-1}$. Full-grown leaves were harvested from approximately 70 cm (2/3 of the stem height), shock-frozen in liquid nitrogen and kept at -80 °C until further analysis.

For a salt shock treatment and dark respiration measurement, plants were grown for 2 months in a climatized chamber at 26 °C with a relative air humidity of 60 % and a 16 h light, 8 h dark cycle with a PAR of 150 µmol $m^{-2}s^{-1}$ (lamps: L58W/25 and 58W/840, Osram, Munich, Germany, and TLD 58W/840 Philips, Amsterdam, Netherlands). Dark respiration was measured with a portable gas exchange fluorescence system (GSF-3000, Walz, Effeltrich, Germany). Plants were connected to the system and kept for 30 min in darkness. Measurements were performed for 5 min. For salt shock treatment continuous light started two days prior to the salt shock. Single plants were placed in 6 l of LA nutrient solution, to which 1.05 mol of NaCl dissolved in 1 l of LA nutrient solution was added, resulting in a total concentration of 150 mM of NaCl. Gas exchange was measured continuously using a portable gas exchange system (HCM-1000, Walz, Effeltrich, Germany), starting 3 h prior to salt-shock treatment and continuing for 15 h after NaCl addition.

4.5.2. Analysis of phenolic compounds

Frozen leaf tissue was ground in a ball mill (Retsch, Haan, Germany). Fine powder (60 mg) was extracted with 2 ml of 50% methanol in an ultrasonic bath (60 min, 40ºC; Sonorex Super RK 510H, Bandelin electronics, Berlin, Germany). Samples were centrifuged (2500 g, 10 min, 4 ºC) and the supernatant was collected. The pellet was extracted a second time with 2 ml of 50% methanol (10 min, room temperature) and centrifuged as above. The

supernatants were combined and adjusted to 10 ml with distilled H_2O to analyze soluble phenolic compounds.

The pellet was washed and centrifuged (2500 g, 10 min, 4 ºC) twice with 2 ml n-hexane. The pellet was dried at 60 ºC for two days, weighed and homogenized in 2 ml of 1 M NaOH. The suspension was incubated in an ultrasonic bath (60 min, 40 ºC) and centrifuged (2500 g, 10 min, 4 ºC). The supernatant was collected, and the pellet was extracted again with 2 ml of 1 M NaOH for 10 min in the dark at room temperature. The supernatants were combined and adjusted to 10 ml with distilled H_2O to analyze non-soluble phenolic compounds.

5 ml of Folin-Ciocalteus phenolreagent (Merck, Darmstadt, Germany) diluted 1:10 with distilled H_2O were added to 1 ml of the extracts for analysis of soluble and non-soluble phenolic compounds. The mixtures were shaken, and after 3 min at room temperature, 4 ml of 7.5% Na_2CO_3 solution was added. The mixture was vigorously shaken and incubated for 30 min at room temperature. The absorbance was measured at 765 nm (Spectrophotometer DU 640, Beckmann, München, Germany). Absorbance values were compared to a standard curve created with catechin (Sigma-Aldrich, Deisenhofen, Germany). Phenolic concentrations were expressed as catechin equivalents.

To determine the composition of soluble phenolic compounds, 5 ml methanol/H_2O extracts were evaporated (Rotavapor EL 130, Büchl, Switzerland) at 45 ºC and dissolved in 500 µL 50% methanol. The solution was centrifuged twice (15000g, 10 min, 4ºC) and the supernatant used for a high-performance liquid chromatography (HPLC; Beckmann-Coulter, München, Germany) analysis. 50 µL of the solution was separated on a reversed-phase ODS column (250 × 4.6 mm, particle size 5 µm; Ultrasphere, Beckmann, München, Germany) with the following gradient of solvent A (1.5% phosphoric acid (v/v)) and solvent B (water/methanol/acetonitrile Far UV

(1:1:1; v/v/v), pH 2.5) at a flow of 1 ml min^{-1}: 1 min 20% B, 25 min 60% B, 35 min 100% B and 40 min 20% B. Soluble phenolic compounds were detected at 280 nm (Diodenarray-detector 168, Beckmann-Coulter, München, Germany).

4.5.3. Analysis of condensed tannins

Frozen leaf tissue was ground in a ball mill (Retsch, Haan, Germany). Fine powder (200 mg) was extracted twice with 4 ml 50% methanol as described for analysis of phenolic compounds. The supernatants were combined and adjusted to 10 ml with distilled H_2O. 200 µL of the extract was mixed with 800 µl of 100% methanol for analysis of soluble condensed tannins. The pellet was lyophilized, weighed and resuspended in 1 ml of 100% methanol for analysis of non-soluble condensed tannins.

6 ml of acid butanol (5% concentrated HCl in butanol (v/v)) and 0.2 ml of 2% (w/v) $FeNH_4(SO_4)_2 \times 12H_2O$ in 2 M HCl was added to 1 ml of the extracts of soluble and non-soluble condensed tannins in methanol. The solutions were incubated (95°C, 50 min) and then cooled to room temperature in the dark. The absorbance was measured at 550 nm (Spectrophotometer DU 640, Beckmann, München, Germany).

4.5.4. Sequence identity analysis

DNA sequences of twenty genes were compared between *P.* × *canescens*, *P. euphratica* and *P. trichocarpa*. For five genes, full length sequences were available for *P. euphratica* and *P.* × *canescens* cDNA ('IAA-amino acid hydrolase', Ill3, [GenBank:AJ744952], [GenBank:AJ744953] (Junghans *et al.* 2006); 'Na/H antiporter type D', NhaD1, [GenBank:AJ561195] (Ottow 2005); 'salt induced serin-rich' and 'temperature induced lipocalin', SIS and TIL, [GenBank:FJ238515], [GenBank:FJ238514] and [GenBank:FJ238513],

[GenBank:FJ238512] (Fayyaz 2007); 'gibberellin regulated protein', GAST, [GenBank:FJ238511], [GenBank:CF231013]). Criterion for the selection of further genes was their representation by probe sets on the microarray and the availability of sequence data for *P. euphratica* and *P.* × *canescens* in public databases. Probe sets both with and without significantly different signal intensities between *P. euphratica* and *P.* × *canescens* were randomly chosen, and sequence data for the corresponding *P. trichocarpa* gene model retrieved from the *Populus* genome project (Table 4.1) (JGI *Populus trichocarpa* genome release 1.1). Because most sequence data is available from EST datasets and thus as cDNA sequences, open reading frame (ORF) sequences of the *P. trichocarpa* gene models were used to screen the GenBank database for homologs in *P. euphratica* and *P.* × *canescens* by using the megablast algorithm (Benson *et al.* 2009). Pairwise alignments and base identity calculations of the cDNA sequences were generated with the GeneDoc software (GeneDoc Homepage). An ANOVA was conducted to test for significant differences between the sequence identity values at $\alpha = 0.05$.

4.5.5. RNA extraction

Leaf material of 3 plants was pooled. Three pooled biological replicates per poplar species were analyzed. Frozen leaf tissue was ground in a ball mill (Retsch, Haan, Germany). Total RNA was extracted from 500 mg of frozen plant powder according to Chang *et al.* (1993) with minor modifications: No spermidine was applied in the extraction buffer, and 2% β-mercaptoethanol was used. RNA was additionally purified using an RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA yield and purity were determined spectrophotometrically (BioPhotometer, Eppendorf, Hamburg, Germany) at

A_{260} and A_{280}. RNA integrity was assessed on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) at the Microarray Facility Tübingen.

4.5.6. Microarray analysis

Of each species, 3 biological RNA replicates were analyzed on the GeneChip® Poplar Genome Array (Affymetrix, Santa Clara, CA). Synthesis of one-cycle cDNA and biotin-labelled cRNA, fragmenting of cRNA, hybridization to the Poplar Genome Array, washing, staining and scanning was performed as stated by Affymetrix (GeneChip® Expression Analysis Technical Manual) at the Microarray Facility Tübingen. Data were deposited at ArrayExpress [EMBL:E-MEXP-1928].

Statistical analysis of the raw signal intensity data was conducted using the following functions from packages released by the bioconductor project , implemented in R (Gentleman *et al.* 2004; The R project for statistical computing). Background correction, quantile normalization and summarization of the Affymetrix CEL output files resulting in a raw list of normalized genes was computed using the 'rma' function from the 'affy' package (Irizarry *et al.* 2003). Statistical testing for differentially expressed genes was performed on this raw list with the 'sam' function from the 'siggenes' package (Tusher *et al.* 2001a). In the SAM analysis, □ was chosen such as to get an FDR of 0.05. Annotation of the genes was carried out via the PopArray-Database (PopARRAY: A database for cross-reference of multiple microarray platforms in *Populus*). Gene Ontology (GO) terms were matched *via* The Arabidopsis Information Resource (TAIR) (The *Arabidopsis* Information Resource).

Gene lists were subjected to several filtering processes as proposed by Falcon & Gentleman (Falcon & Gentleman 2007): First, probe sets for which no annotation was available were removed. Second, genes that were not expressed were removed by calculating Affymetrix' MAS 5.0 change calls

using the 'mas5calls' function from the 'affy' package; only genes with two or three 'present' calls in any one of the two species were considered. Third, biased probe sets were removed (see results). Fourth, in cases where multiple probe sets were matched to the same JGI *P. trichocarpa* gene model, duplicate probe sets were removed so that each gene model was represented only once. In cases where two differentially expressed probe sets were matched to the same gene model, the probe set with a higher p-value in the SAM analysis was used for further analysis if both genes showed the same tendency in gene expression (*i.e.*, both were detected as being higher expressed in the same poplar species). Differing probe sets annotated by the same gene model with contradicting expression ratios (*i.e.* one higher expressed in each poplar species) were removed. By applying the filtering steps to the list of differentially expressed genes and to the list of all probe sets present on the microarray, we obtained a processed list of significant genes and a 'gene universe' that represents the complete set of genes from which the significant genes are drawn.

For evaluation of over- and underrepresented GO slim terms, the frequencies of annotations within the two lists of significant genes were compared to the frequencies within the defined 'gene universe'. Statistical testing was conducted by calculating the cumulative hypergeometric distribution function, using the 'phyper' function in R. Benjamini-Hochberg correction was applied to the resulting p-values using the 'p.adjust' function.

For statistical analysis of overrepresentation with 'The Ontologizer' (Bauer *et al.* 2008), a gene ontology file for *Populus* was adapted. In this file, all JGI *Populus trichocarpa* gene models represented on the Affymetrix GeneChip were linked to the GO identifier of their closest *Arabidopsis* matches as listed by TAIR (The *Arabidopsis* Information Resource). The 'population' of the Ontologizer conforms with our 'gene universe', whereas the two lists of genes higher expressed in one of the poplar species each correspond to a 'study

set'. As parameter settings, term-for-term analysis with Benjamini-Hochberg correction was used.

4.5.7. qRT-PCR

For quantitative Real-Time PCR (qRT-PCR), primer pairs were designed for actin 9 [GenBank:AJ778775 (*P. euphratica*) and GenBank:CX656348 (*P.* × *canescens*)] as a reference gene and 10 transcripts that were differentially expressed in *P. euphratica* and *P.* × *canescens* (Supplemental Table S5). All primers, respective primer pairs met the following conditions: 1) 100% sequence identity with both *P. euphratica* and *P.* × *canescens* cDNA; 2) fragment lengths between 90 and 180 base pairs (bp); 3) calculated salt-adjusted melting temperatures T_m(salt) between 58 and 62°C. Primer design was performed with the Oligo Explorer, and suitable primers were tested for similar T_m(salt), primer dimers and primer loops by Oligo Analyzer (both Gene Link, Hawthorne, NY, USA).

Total RNA was DNAse treated with a Turbo DNA-free kit (Ambion, Austin, TX) and transcribed to cDNA with a RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). For each gene, three technical repeats were analyzed for three biological samples. The qRT-PCR was performed on an iCycler (Bio-Rad, Hercules, CA). 250 ng cDNA were used in an 25 µl reaction with 1×ABsolute qPCR SYBR Green Fluorescein Mix (ABgene, Surrey, UK; including Thermo-Start DNA Polymerase) and 10 µM primer. First denaturation and activation of the Taq-polymerase occurred at 95°C for 15 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 56°C for 30 s and elongation at 72°C for 30 s.

qRT-PCR output was analyzed using the MyiQ software (Bio-Rad, Hercules, CA). Primer specificity was assessed by melting curve analysis. Statistical analysis of expression ratios and standard error was conducted using

a pairwise fixed reallocation randomization rest implemented in Excel with the Relative Expression Software Tool (REST)-384 (Pfaffl *et al.* 2002; Pfaffl 2001). All primer pairs showed less than 5% differences in PCR efficiency between *P. euphratica* and *P.* × *canescens*.

4.5.8. Phylogenetic analysis

For the phylogenetic analysis of the sodium transporters, sequences for open reading frames for *Populus* and *Arabidopsis* were obtained from the JGI *Populus trichocarpa* 1.1 database (JGI *Populus trichocarpa* genome release 1.1) and from The Arabidopsis Information Resource (TAIR) (The *Arabidopsis* Information Resource). Open reading frames were aligned using ClustalW2 (ClustalW2). The unrooted tree was generated using the Tree View (TV) program (Zhai *et al.* 2002).

4.5.9. FT-ICR/MS measurements

Of each species, 5 biological replicates were measured. Frozen leaf tissue was ground in a ball mill (Retsch, Haan, Germany). Fine powder was diluted in methanol to a methanolic concentration of 70% to give highest ion density inside the electrospray, without eliminating those neutrals which are highly water soluble. High-resolution mass spectra were acquired on a Fourier transform ion cyclotron resonance mass spectrometer (APEX Qe, Bruker, Bremen, Germany) equipped with a 12-T superconducting magnet and an Apollo II Electrospray (ESI) source. The ionization source was run in the negative operation mode to generate mono charged negative ions. Thus, all important multi-functional organic compounds which bear at least one acidic site could be deprotaned to give rise to a detectable anion in the mass spectrometer (Schmitt-Kopplin & Hertkorn 2007). Each sample was introduced

into the ionization source at a flow rate of 2µL×min^{-1} by a microliter pump with a nebulizer gas pressure of 20 psi and a drying gas pressure of 15 psi (heated to 200 °C). Each sample was measured three times and the signal intensities of each detected ion were averaged before running the multi-dimensional statistical data analysis.

Spectra were externally calibrated on clusters of arginine (10 ppm in methanol); calibration errors in the relevant mass range were always below 0.05 ppm. The spectra were acquired with a time domain of 1 Megaword with a mass-to-charge ratio (m/z) range of 146–2000. The spectra were zero filled to a processing size of 2 Megawords. Before Fourier transformation of the time-domain transient, a sine apodization was performed. No fragmentation experiments were performed in this study. Thus, the whole mass range could be scanned, and 300 scans were summed on in each acquisition. The ion accumulation time in the ion source was set to 0.1 s.

FT-ICR spectra were exported to peak lists at a signal to noise ratio (S/N) of two. From those lists, High to Low signal intensity and Low to High signal intensity profiles were obtained by use of the Hierarchical Clustering Explorer HCE Version 3.0 (Seo & Shneiderman 2005). Ions with sharp differences between the acquisitions of the two species were highlighted as High to Low and Low to High signal intensity profiles. The m/z values of all ions were clustered by the use of the average linkage method with similarity/difference measure type: Euclidean distance. The search method is model-based and the used distance measure was Pearson's correlation coefficient of 0.8. For all obtained hits, t-tests were calculated to validate a statistical significance at α = 0.1.

Metabolite identification and annotation of the m/z values was performed *via* the MassTRIX web site with the following parameter settings: 'Scan mode negative ionisation (correct for H^+ loss)', 'Max. error 3.0 ppm', 'Database

KEGG with isotopes', 'Organism *Populus trichocarpa*' (Suhre & Schmitt-Kopplin 2008). Identified compound|s and differentially expressed genes added to the query as EC numbers were automatically mapped to KEGG pathways using KEGG/API (KEGG API) *via* MassTrix. Metabolites with the same total formula could not be distinguished.

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5. Overall conclusion

In this work, I compared the salt tolerant poplar species *Populus euphratica* with the salt sensitive *P.* × *canescens* to address tree specific questions of salt tolerance. For this, we applied transcription profiling as well as various morphological and physiological measurements. One of the most intriguing results of this study was the importance to differentiate between sudden, short termed salt shock and ongoing, long term salt stress. To be prepared against salt shock, plants have to employ the according tolerance mechanisms even during non-stressed state, whereas mechanisms assisting to resist long term salt stress need only be activated by demand.

We showed that under long term salt stress, more genes were regulated in developing xylem of the salt sensitive *P.* × *canescens* than of the salt tolerant *P. euphratica*, many of which were involved in phytohormone-influenced signaling, detoxification of products of oxidative damage, or played a hypothetical role in remodeling cell wall structure. Also, under salt stress *P.* × *canescens* develops xylem that is more similar to xylem of *P. euphratica*. This modified xylem has more and smaller vessels, attributes which might contribute to salt tolerance by lowering cavitation vulnerability. We suggested that many of the signaling and cell wall structure related genes were involved in development of this salt stress adapted xylem. Genes involved in decreasing effects of oxidative damage increase the plants salt tolerance by protecting it against reactive oxigen species (ROS) that are formed during the oxidative burst as part of stress response.

We concluded that under long term salt stress, the transcriptome of developing xylem of *P.* × *canescens* is adjusted to protect the plant both on an anatomical and a physiological level. In contrast, the transcription profile

changed only marginally in *P. euphratica*, leaving both anatomy and physiology nearly unaffected. This was obvioulsy due to the fact that salt levels in the developing xylem of *P. euphratica* remained at much lower levels; presumably, salt tolerance of *P. euphratica* is therefore mostly brought about by salt exclusion.

On one hand, this salt exclusion is caused by a change in root anatomy which leads to a lessened sodium ion uptake on a purely physical basis as shown in an earlier study by P. Hawighorst. On the other hand, expression profiling in this work showed that in *P. euphratica* roots formed under salt stress, genes from the SOS pathway are induced, presumably leading to an active exclusion of salt (see chapter 2).

Nevertheless, we showed that *P. euphratica* is not only able to adapt to long term salt stress, but is also less susceptible to sudden salt shock; it therefore must possess some innate mechanism that are present even in the absence of salt stress. To some extent, the fact that vessel cross sections are generally smaller in *P. euphratica* than in *P.* × *canescens* might contribute to this salt shock resistance by protecting the plant against cavitation.

Moreover, the direct comparison of the transcriptomes and the metabolomes of the two poplar species showed that in *P. euphratica*, two different systems appear to be present which might contribute to salt shock tolerance: First, even under non-stressed state, *P. euphratica* accumulates more sugars in leaves than *P.* × *canescens* and thus possesses an osmotic protection against a sudden increase of external salt levels. And second, ion channels, including several Na^+-channels, appear to be generally higher expressed in *P. euphratica*, suggesting that in case of salt shock, ion compartmentation can commence immediately.

As a final conclusion, we showed that *P. euphratica* possesses several mechanisms for salt tolerance, both for long term stress and for salt shock.

These take effect in the different plant organs, *i.e.* root, stem and leaf. They appear to protect the plant sufficiently against salt, so that mechanisms against inner stress like protection agains ROS as found in *P.* × *canescens* are superfluous in *P. euphratica*. However, many of these protective means include anatomical alterations that are induced by cell development-affecting phytohormones and homeobox transcription factor genes. The complexity of these cause-and-effect relationships are thus that in this study, we can only give initial data for future work on this topic.

Acknowledgements

The completion of this thesis was only made possible by enduring and persistent support and help by a large number of people. First of all, I would like to thank my supervisor Prof. Dr. Andrea Polle for giving me the chance to work on this intriguing and complex topic, for support, guidance and last but not least patience. I am also grateful to the German Science foundation for funding the work on this thesis (DFG projec Poplar research Group Germany, P6 – Expression profiling during salt stress, PO 362/12-3). I thank Prof. Dr. Reiner Finkeldey from the Department of Forest Genetics and Forest Tree Breeding for examining my thesis, and Prof. Dr. Joachim Saborowski from the Department of Ecoinformatics, Biometrics and Forest Growth for agreeing to be my third referee.

I am sincerely thankful to the following people for contributing with huge amounts of data, their interpretation and explaining the methods applied: Henning Wildhagen, Chair of Tree Physiology, Freiburg, for HPLC data of phloem exudates; Prof. Dr. Jörg-Peter Schnitzler and Dr. Katja Behnke, Institute for Meteorology and Climate Research, Garmisch-Partenkirchen, for gas exchange measurements; and Dr. Basem Kanawati, Institute of Ecological Chemistry, München, for FT-ICR/MS data of developing xylem.

Furthermore, I would like to thank the staff of the 'forest botany', for competent help whenever needed and being the best colleagues one can have: Christine Kettner, for providing perfect poplar plantlets in incredible quantities; Sabine Elend, for an unspeakable amount of technical assistance regarding both plant care and laboratory work; Marianne Smiatacz, for tending to the plants and for harvest help; Thomas Klein, for isolation of RNA and running qRT-PCRs, Monika Franke-Klein, for doing the analysis of phenolic compounds and

condensed tannins; Rainer Schulz, for generating HPLC data of phenolic compounds; Dr. Payam Fayyaz, for growing Euphrates poplars with cob-roots; Dr. Andrea Olbrich, for doing flawless microtome cuttings; Dr. Annette Naumann, for introducing me to FTIR-ATR; Gisbert Langer-Kettner, for perfect technical support from his work shop, no matter what had to be done; Dr. Thomas Teichmann and Dr. Urs Fischer, for helping with and explaining the more advanced laboratory techniques; Bernd Kopka, for coffee, borrowed cream and being the acting sysop; and finally the rest of my 'peer group' in Göttingen, who contributed with support, help, advice and discussions, as well as with coffee, cakes, cookies and sweets, both during work and social hours: Peter Hawighorst, Saskia Flörl, Karl Catewicz, Frauke Kleemann, Lara Danielsen, Christine Drübert, Christa Lang and Rodica Pena.

But in the end, my deepest gratitude and love goes to my parents and especially to Silje, who supported me during the whole time and always believed in me, and without whom this thesis would not have been finished. I thank you!

Declaration

The following data shown in this thesis have been provided by colleagues:

Chapter 1: Transcription profiling of developing xylem of *Populus* × *canescens* and *Populus euphratica* under salt stress to study mechanisms of stress adaption and stress tolerance in wood

The analysis and evaluation of HPLC data of phloem exudates were conducted by Henning Wildhagen, Chair of Tree Physiology, Freiburg.

The analysis and evaluation of gas exchange measurements were conducted by Prof. Dr. Jörg-Peter Schnitzler and Dr. Katja Behnke, Institute for Meteorology and Climate Research, Garmisch-Partenkirchen.

Chapter 3: Comparison of the transcriptome and metabolome of a salt sensitive and a salt tolerant poplar species reveals insight into stress tolerance mechanisms

The analysis and evaluation of FT-ICR/MS data of developing xylem were conducted by Dr. Basem Kanawati, Institute of Ecological Chemistry, München.

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