Planta (2009) 229:299–309
DOI 10.1007/s00425-008-0829-7

ORIGINAL ARTICLE

Salt stress affects xylem differentiation of grey poplar (*Populus* × *canescens*)

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Received: 25 June 2008 / Accepted: 11 September 2008 / Published online: 23 October 2008

Abstract In this study the impact of salt stress on the physiology and wood structure of the salt-sensitive *Populus* × *canescens* was investigated. Two weeks of salt stress altered wood anatomy significantly. The xylem differentiation zone was reduced and the resulting vessels exhibited reduced lumina. To understand this phenomenon, ion composition, levels of corresponding transcripts and of the stress hormone ABA were analysed. With increasing sodium and chloride concentrations, a general reduction of potassium was found in roots and shoots, but not in leaves. Consequently, the corresponding K^+ channel transcripts in roots favoured K^+ release. The overall osmolarity in leaves was up to fourfold higher than in roots or shoots. Therefore, adjustment of the K^+/Na^+ balance seemed not to be required in leaves. Sodium increased gradually from roots to shoots and then to leaves indicating that sodium storage took place first in roots, then in shoots, and finally in leaves to protect photosynthesis from salt effects as long as possible. Since leaf abscisic acid levels markedly increased, stomatal closure seemed to limit CO_2 uptake. As a consequence, diminished nutrient supply to the cambium in combination with lowered shoot K^+ content led to decreased vessel lumina, and a reduction of the radial cambium was observed. Thus, xylem differentiation was curtailed and the development of full size vessels was impaired.

Keywords ABA · Cation transport · Poplar · Salt stress · Wood morphology

Abbreviations

ABA	Abscisic acid
PCR	Polymerase chain reaction
TEM	Transmission electron microscopy
EDXA	Energy-dispersive X-ray analysis

Introduction

Plant biomass production is largely affected by abiotic stress factors like drought or salinity. Many attempts to understand the underlying mechanisms of salt tolerance have been made in recent years (for review see Yamaguchi

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Electronic supplementary material The online version of this article (doi:10.1007/s00425-008-0829-7) contains supplementary material, which is available to authorized users.

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and Blumwald 2005; Munns 2005). Research on the molecular basis of salt resistance in plants has mainly focused on herbaceous plants so far (Flowers 2004; Zhang et al. 2004). The main targets of salt impact are vegetative growth, anatomy, flowering, fruit development and seed germination, accompanied by metabolic dysfunctions, including decreased photosynthetic rates, protein and nucleic acid metabolism and enzymatic activity (Kozlowski 1997 and references therein). While sodium enters the plant, the K^+/Na^+ ratio decreases (Rubio et al. 1995). Subsequently both ion and osmotic homeostasis are affected, which in turn leads to water stress and ABA accumulation. In roots, the ABA increase seems to depend on ionic and osmotic changes, while in leaves it is primarily osmotic stress that affects ABA levels (Jia et al. 2002). Finally, many of the observed effects associated with salt stress are probably due to the initial reduction of photosynthesis and the resulting insufficient nutrient supply (Kozlowski 1997 and references therein).

Plants adapt to salinity by exhibiting little effect upon salt uptake (tolerance) and/or by largely excluding uptake of salt (avoidance). Absorbed salts are sequestered in vacuoles to reduce the salt concentration of the cytoplasm and chloroplasts. The cytoplasm often has to build up high concentrations of compatible osmolytes that counterbalance the high salt concentrations in the vacuoles (Kozlowski 1997). An additional mechanism of salt tolerance, the regulation of K^+/Na^+ selectivity by carrier proteins, has been discussed previously (Gorham et al. 1997).

Because of the long life span of woody species, it might be expected that trees could have evolved adaptive strategies to survive long periods of salt stress that are different from those of annual plants like *Arabidopsis*. The physiological basis of genetic variation in salinity tolerance of the genus *Populus* has been studied in recent years (Bolu and Polle 2002; Ottow et al. 2005a, b and references therein). Moreover the role of ABA in salt stress was the focus of several studies (Shinozaki and Yamaguchi-Shinozaki 2000; Chen et al. 2001, 2002; Chang et al. 2006). In addition, effects of salinity on poplar growth, water relations, stomatal conductance and CO_2 assimilation have been explored (Fung et al. 1998).

To understand the salt tolerance mechanisms of trees it is important to explore first how salt exposure interferes with nutrient uptake, growth and wood production of poplar trees on a molecular level. Therefore, in this study a salt-sensitive poplar species was chosen to address the key questions: (1) How does salinity interfere with poplar nutrient uptake and translocation? (2) How are these changes reflected in anatomical differences? In this context, we analysed different physiological and molecular factors associated with leaf physiology and wood morphology of the salt-sensitive grey poplar.

Materials and methods

Plant material and experimental design

Poplar saplings (*Populus* × *canescens,* INRA clone no. 717 1B4) were obtained from Picoplant Pflanzenvertrieb und-verkauf (Oldenburg, Germany), transferred to clay granules and cultivated in a greenhouse at Freiburg University as hydroponic cultures in Hoagland solution (Hoagland and Snijder 1933). After 2 weeks this solution was replaced with Long–Ashton (Hewitt 1966) solution containing 1 mM KNO_3. The media was exchanged every second day. After 10 and 12 weeks, respectively, plants were transferred to Garmisch-Partenkirchen where they were cultivated in hydroponics for an additional week in a greenhouse prior to the transfer into the solar domes (for details see Brüggemann and Schnitzler 2002) at Mt. Wank research station. Before the experiment started, the plants were acclimated to the new environment for 7–9 days. Salt stress was applied to the trees by gradually increasing the NaCl concentration of the hydroponic medium in steps of 25 mM from 0 to 75 mM within 4 days starting at time point 0. Six to 12 plants were cultivated for each treatment with or without NaCl.

A marker was set directly below the apex of each plant to mark the position of leaves which were developed prior and after arrival in Garmisch-Partenkirchen.

Samples were taken at day 0, day 7 and day 14 after salt application. At 1 p.m. MET sampling started with shock freezing (in liquid N_2) of 7–8 fully expanded leaves of each plant directly below the marker. Sampled plants were then sectioned (remaining leaves, shoot without leaves, roots) and harvested for additional analysis.

Quantification of minerals and osmolytes

Cations

Plant material was dried at 65°C for 10 days and digested with HNO_3 (100 mg tissue + 1 ml of 65% suprapur HNO_3 for 10 h at 160–170°C, 10 bar) in a pressure ashing device (Seif, Unterschleißheim, Germany). Samples (10 ml) diluted with 18.2 megaohm H_2O, were analysed by inductively coupled plasma optical emission spectroscopy (ICP-OES; JY 70 Plus, Devision d′Instruments S.A., Jobin Yvon, France). Because they originated in a common experiment, the element data presented in Fig. 3a, b are also presented and discussed in another context in Ehlting et al. (2007).

Anions

Plant material was dried as before. Ground tissue (50 mg) was suspended in 5 ml deionised H_2O, centrifuged at

14,000*g* for 10 min, and boiled at 105°C for 3 min. Insoluble constituents were removed by centrifugation (10,000*g*, for 15 min). The concentration of chloride ions in the supernatant was determined by isocratic anion chromatography and suppressed conductivity detection (Biotronik, Maintal, Germany).

For osmotic analysis, 100 mg of the dried material was diluted in 900 µl H_2O and boiled for 5 min. After centrifugation at 14,000*g*, the osmolarity of the supernatant was determined using a vapor pressure osmometer 5520 (Wescor, Langenfeld, Germany).

Xylem anatomy

Shoot segments were fixed in FAE (2% formaldehyde, 5% acetic acid, 63% ethanol; modified after Sanderson 1994), dehydrated in an ethanol/isopropanol series (modified after Gerlach 1969) and embedded in Rotiplast with Roti-Histol (Roth, Karlsruhe, Germany) as intermedium according to the instructions of the manufacturer. Sections (30 µm) were cut with a sliding microtome (Reichert-Jung, Heidelberg, Germany) and mounted on gelatin-coated slides. The paraffin was removed with xylene, sections were stained for 15 min with Toluidine Blue [0.05% (w/v) in 0.1 M sodium acetate, pH 5.8; Merck, Darmstadt, Germany] and viewed with a microscope (Axioskop, Zeiss, Oberkochen, Germany). Photographs were taken at 400× magnification with a digital camera (Nikon CoolPix 4500, Nikon Corporation, Tokyo, Japan). All cells in an area of 275 µm × 75 µm that were newly formed during the salt stress treatment (selected upon comparison with controls) were considered for analysis. The lumen areas were determined for vessels and fibres using the image processing software analySIS (Soft Imaging System, Muenster, Germany). For analysis of the fibre diameter the average of the 30 largest individual elements in a given area was taken as one replication. Cell wall thickness was estimated as half the distance between the lumina of adjacent cells.

Transmission electron microscopy (TEM)

Small sections of shoot tissue were cut with a razor blade and immediately immersed in a fixation medium containing 1% (w/v) formaldehyde, 1 mM EGTA, 50 mM cacodylate buffer and 5% glutaraldehyde. After 4-h fixation, the tissue was post-fixed with 2% (w/v) osmium tetroxide overnight at room temperature, then stained with 3% (w/v) uranyl acetate in 20% ethanol for 1 h, dehydrated in a graded series of ethanol and embedded in Spurr's epoxy resin (Spurr 1969). Ultra-thin sections were cut with a diamond knife on an ultra microtome (Ultrotome Nova, LKB, Bromma, Sweden), transferred onto formvar-coated copper grids and stained with lead citrate. Sections were examined in a Zeiss EM 10c transmission electron microscope at 80 kV.

X-ray microanalysis (EDXA)

Sections from fine roots were immediately shock-frozen in liquid isopentane. After freeze-drying, the samples were coated with chromium and examined on a scanning electron microscope (AMR 1200, Leitz) equipped with an EDX microanalysis system (KEVEX 4000). Element specific X-ray spectra were obtained from stele and cortex tissue using a reduced scan raster area at 200× magnification. Relative potassium, chloride and sodium concentrations were expressed as peak/background ratios from five recorded spectra.

RNA isolation, cDNA synthesis and real time PCR

Total RNA of leaves was extracted from ground plant material using the Plant RNeasy mini kit, according to the manufacturer's instruction (Qiagen, Hilden, Germany) with modifications. For one RNA extraction, three samples of leaf tissue were used. RLT buffer (900 µl), supplemented with 1% β-mercaptoethanol, 1% PVP and 70 mM K^+-ethylxanthogenate, were added to 30 mg of homogenised leaf tissue and incubated for 30 min at room temperature. After centrifugation the three samples were subsequently transferred to the QIAshredder spin column. The samples were centrifuged for 1 h at 4°C at maximal speed, and further treated according to the protocol. After washing the first sample with 500 µl RPE buffer, the second and third samples were pipetted onto the RNeasy mini column and washed sequentially. The RNA was eluted with 50 µl RNAse-free water. Remaining DNA was digested using RNase-Free DNase (Amersham, Freiburg, Germany) according to the manufacturer's protocol.

First-strand cDNA and real time PCR were performed as described previously (Langer et al. 2002, 2004). Primers used (TIB MOLBIOL, Berlin, Germany) have been designed for *Populus tremula* × *tremuloides* or *Populus trichocarpa* and were therefore tested with *Populus* × *canescens* cDNA prior to real time PCR. Primers are listed in the Supplement (S-table S1).

All quantifications were normalised to actin cDNA fragments amplified by PtACT2fwd and PtACT2rev. These fragments are homologous to the constitutively expressed *Arabidopsis* actins 2 and 8 (for details see An et al. 1996; Szyroki et al. 2001).

ABA analysis

Ground tissue samples were homogenised and extracted in 80% aqueous methanol. Extracts were passed through a Sep

Pak C_{18}-cartridge. Methanol was removed under reduced pressure and the aqueous residue was partitioned three times against ethyl acetate at pH 3.0. The ethyl acetate of the combined organic fractions was removed under reduced pressure. The residue was resuspended in TBS-buffer (Tris buffered saline, 150 mmol/l NaCl, 1 mmol/l $MgCl_2$, and 50 mmol/l Tris at pH 7.8) and subjected to an immunological ABA assay (ELISA) as described previously (Mertens et al. 1985; Peuke et al. 1994). The accuracy of the ELISA has been verified in earlier investigations (Peuke et al. 1994). The immunochemicals were generously supplied by Prof. Weiler, Ruhr Universität Bochum (Germany).

Results

To study the effect of salt stress on growth and wood structure of the salt-sensitive grey poplar *Populus tremula* × *Populus alba (syn. Populus* × *canescens)* we analysed samples from different plant organs (leaves, bark, wood and roots) after 1–2 weeks of salt exposure.

Salt effect on cambial cell patterning and structure

In previous studies we focused on the role of potassium and calcium in wood formation (Langer et al. 2002; Arend et al. 2005; Lautner et al. 2007). Here, we extended this research by assessing the impact of salt treatment on wood structure. After 2 weeks of salt stress leaves exhibited brown lesions and turned a yellow colour, indicating that the photosynthetic apparatus was severely impaired (cf Teuber et al. 2008). In order to detect changes in the wood producing region induced by the diminished nutrient supply, we used TEM analysis to investigate cambial zones of salt stressed poplars. Under control conditions the cambial zone was always comprised of 7–9 radial cell layers with greatly vacuolated cytoplasm (Fig. 1, left), while salt stressed poplars developed only 3–5 radial cell layers (Fig. 1, centre and right). In the wood region, anatomical parameters also changed following 2 weeks of salt exposure. Poplar wood consists of vessels, fibres and rays. The lumina of the vessels were significantly reduced (Fig. 2a–c) while the total number of vessel cells appeared to be increased (Fig. 2a, b, d). These data confirmed the findings of Junghans et al. (2006), obtained from *P. canescens* after 6 weeks of milder salt stress (50 mM NaCl). Fibre lumina and number, in contrast, remained unchanged (Fig. 2e, f). For a detailed compilation and statistics see Supplement Table S2.

Salt affects cell solute concentration and composition

In an attempt to identify the basis for the morphological changes, we investigated the element and metabolite contents in different organs of the stressed trees. In general, all organs showed an increase in their osmolarity, ranging from 61 (±19) to 83 (±35) in shoots, 100 (±12) to 156 (±17) in roots and 230 (±18) to 315 (±39) in leaves (relative values). In the roots, levels peaked after the first week. Shoot osmolyte levels were more elevated after the second week and osmotic changes in the leaves appeared only after the second week (Fig. 3a). As expected from sodium treatment, the Na content in roots, shoots, and leaves rose sharply (Fig. 3b). Roots showed the greatest Na concentrations after the first week, while shoots accumulated sodium continuously. In leaves, markedly higher levels were observed only after the second week. Concomitant with changes in the Na^+ content, potassium levels in roots and shoots dropped uniformly, while potassium in leaves increased by about 50% (Fig. 3c). Like sodium, the chloride content of leaves was elevated during salt stress (Fig. 3d). Absolute Cl^- levels increased from 151 µmol/g DW (±20, T0) to 341 (±102, T1) and 502 (±174, T2), respectively. No changes were observed with phosphate and sulphate (data not shown).

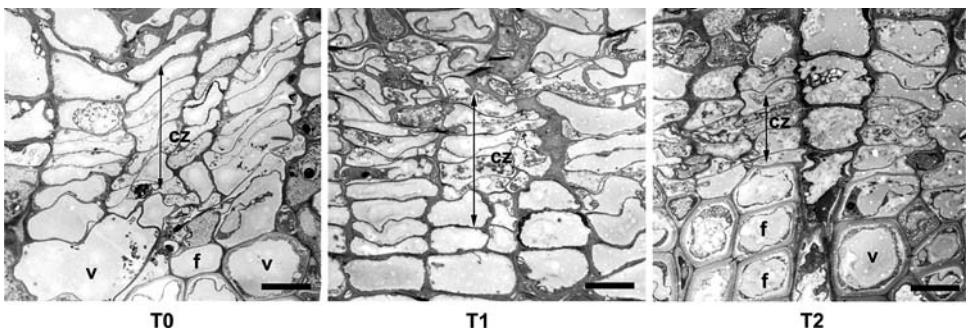

Fig. 1 TEM imaging of cambial zone (*cz*) of grey poplar stems. At the beginning of the experiment (*T*0), under control conditions and after 1 week of salt exposure (*T*1), the cambial zone consisted of 7–9 layers. After the second week (*T*2), the number of cell layers in the cambial zone was reduced. *f* Differentiating fibre, *v* Differentiating vessel. *Bar* represents 10 µm

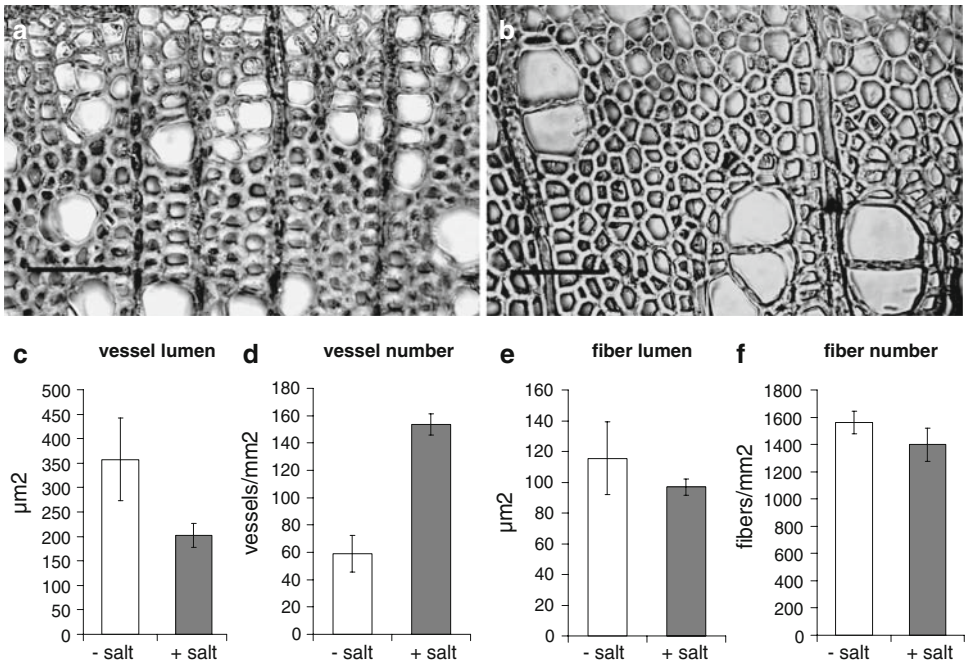

Fig. 2 Anatomical changes of wood structure of grey poplar after 2 weeks of salt stress (**a**) and (**b**, control). **a**, **c** Decreased vessel lumina. **a**, **d** Elevated number of vessels under salt stress. **e**, **f** Fibre lumina and number remained unaffected. Mean values ± SE, $n = 6$, *bars* = 50 µm

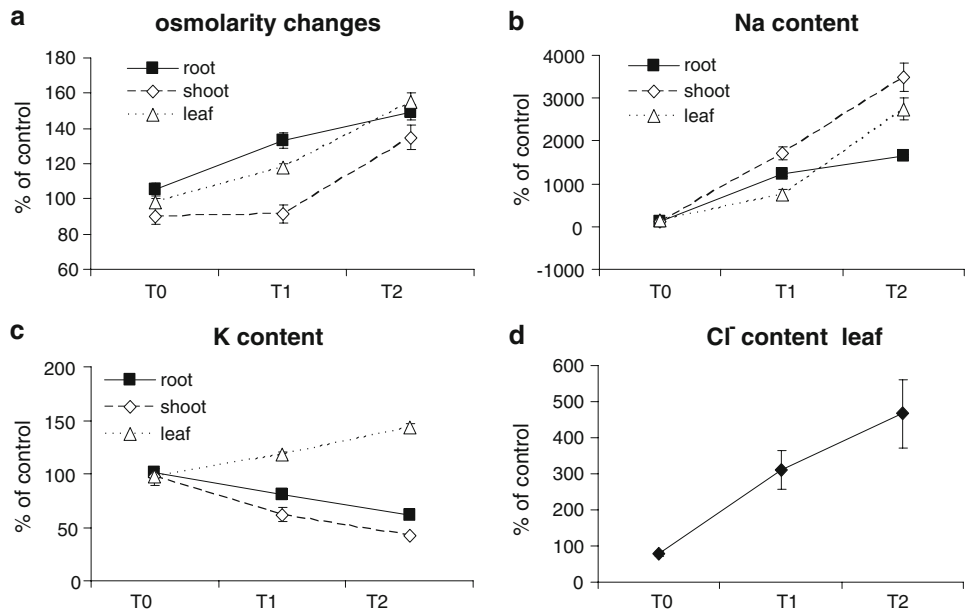

Fig. 3 Element and ion analysis. All values represent mean values ± SE. **a** In all organs the osmolarity was elevated after 2 weeks of salt stress ($n = 12$). **b** The content of Na increased in all organs ($n = 12$). **c** The content of K decreased in root and shoot, but increased in leaves ($n = 12$). **d** The content of chloride of salt stressed leaves was elevated ($n = 3$)

NaCl feeds back on K^+

Roots

To examine spatial changes in ion content in more detail, we applied the EDXA technique to tissues of roots and aerial organs. Element analysis of the fine root cortex and stele revealed increases in Na and Cl in both tissues throughout the experiment (Fig. 4). In cortex and stelar tissues of control plants, sodium was below the detection limit and the Cl-concentration was half of the potassium level at each time-point (data not shown). Under salt stress, the Na and Cl content were elevated, while the potassium content of these cells was reduced (Fig. 4). Hence, these results in fine root cortex and stele confirmed the data obtained by element analysis of whole roots (Fig. 3).

Aerial tissues

The plant tissue that has the earliest contact with NaCl is the root system. When taken up, salt first accumulates within the root, then enters the shoot, followed by the leaves and finally the guard cells. Using shoot cross sections we differentiated between phloem and the developing

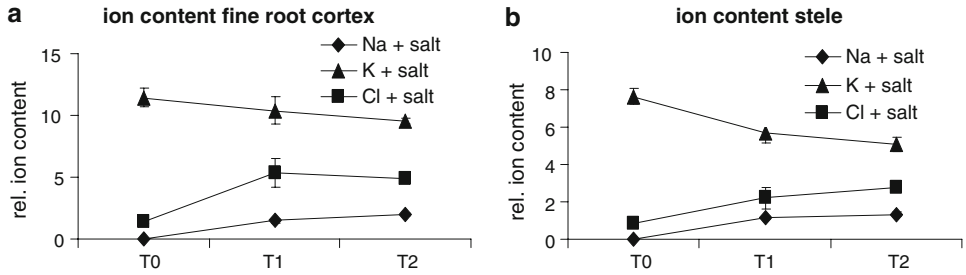

Fig. 4 EDXA: relative Na, Cl and K content in roots of poplars under salt stress at the beginning of the experiment (*T*0), after 1 week (*T*1) and after the second week (*T*2). **a** In the fine root cortex the increase of Na and Cl is high. **b** In the stele, the rise in Na and Cl is less pronounced. K decreased in both organs (relative values reflect peak/background ratios, $n = 5$, mean ± SE)

xylem tissues after 2 weeks of salt stress. In the phloem we found an increase in the Na- and Cl-content, while K decreased (Fig. 5a). In the developing xylem we observed similar trends as in the phloem; however, the absolute concentration of the ions measured was lower than in the phloem (Fig. 5b). With the exception of the sodium content, these results confirmed data obtained by element analysis of whole shoots (Fig. 3). In leaves, guard cells showed a pronounced increase in chloride and potassium, whereas the sodium content increased only marginally (Fig. 5c). This behaviour is in line with K^+ and Cl^- as major osmolytes in guard cell action. Although K^+ nutrition was not altered, the presence of Cl^- seems to trigger K^+ uptake into guard cells.

Salt affects expression of K^+ and Na^+ transporters

In order to study the molecular mechanisms underlying the ion distribution processes we searched for genes involved in the uptake and release of Na^+ and K^+ in the *Populus trichocarpa* genome. Figure 6 shows a phylogenetic tree of all *Arabidopsis* and related poplar shaker like K^+ channels. Of these, K^+ channel transcripts *PKT6*, *PtKC1* and *PtKC2* could not be detected in the *Populus* × *canescens* cDNA samples. In addition, genes well known to be involved in the salt stress responses of other species were monitored. Primers for these transcripts were used for expression analysis in real time PCR experiments. Detailed descriptions of the transcripts analysed, as well as related primers, are listed in the Supplement (Fig. S1 , Table S1).

Expression under salt stress

In order to investigate how the ion concentrations correlate with ion uptake and transport throughout the plant under control and stress conditions, we analysed the expression patterns of several poplar transport proteins that may be involved in ion uptake and distribution. The resulting data are compiled in Fig. 7. Detailed results of the individual measurements (including mean ± SD) can be found in the Supplement (Fig. S1). The genes analysed were classified into three groups according to their expression pattern.

The first group consisted of genes that either did not change, or were not detectable throughout the experiment. These data are consistent with the findings of previous studies showing that poplar *β-tubulin* (*TUB*, EMBL AY353093) and *PtKUP* behave like housekeeping genes (Langer et al. 2002, 2004). These transcripts served as negative controls as their expression remained constant at all stages of the experiment. The Na^+/H^+ antiporter *PcNhaD1* (Ottow et al. 2005a) was detected in all investigated tissues but expression was not salt-sensitive. Transcripts of the putative K^+ uptake channel *PKT1b* have only been detected in roots and leaves where their expression did not change upon the application of salt stress. Like the *Arabidopsis* ortholog SKOR (Gaymard et al. 1998), *PTORK3* expression was restricted to roots, where it is very likely that the channel mediates K^+ export from the xylem parenchyma into the xylem. These data suggest that the allocation of K^+ from root to shoot via the xylem is not affected by salt stress.

The second group responded to stimuli in leaves but remained almost unchanged in other organs. Among them *PtHKT1* (putative high affinity K^+ uptake system), *KPT1* (guard cell K^+ uptake channel, Langer et al. 2004), and *PKT1* (putative K^+ uptake channel) appeared to be down-regulated in leaves upon salt stress while *KPT1* shows a transient, strong up-regulation in the bark.

The third group responded in a more complex manner after the application of salt stress. In leaves, K^+-release-channel transcripts of *PTORK* (Langer et al. 2002) and *PTORK2* were reduced in the same way as previously shown for the uptake transporters *PtHKT1*, *KPT1*, and *PKT1*. *PTORK* was transiently up-regulated in roots, and down-regulated in bark and leaves, while it did not change in shoots. These suggest an early salt stress response of *PTORK* in roots, which are primarily exposed to elevated salt concentrations. In contrast the K^+-release-channel *PTORK2* was up-regulated in roots, shoots and bark but not in leaves and thus paralleled the changes of K^+ concentrations in

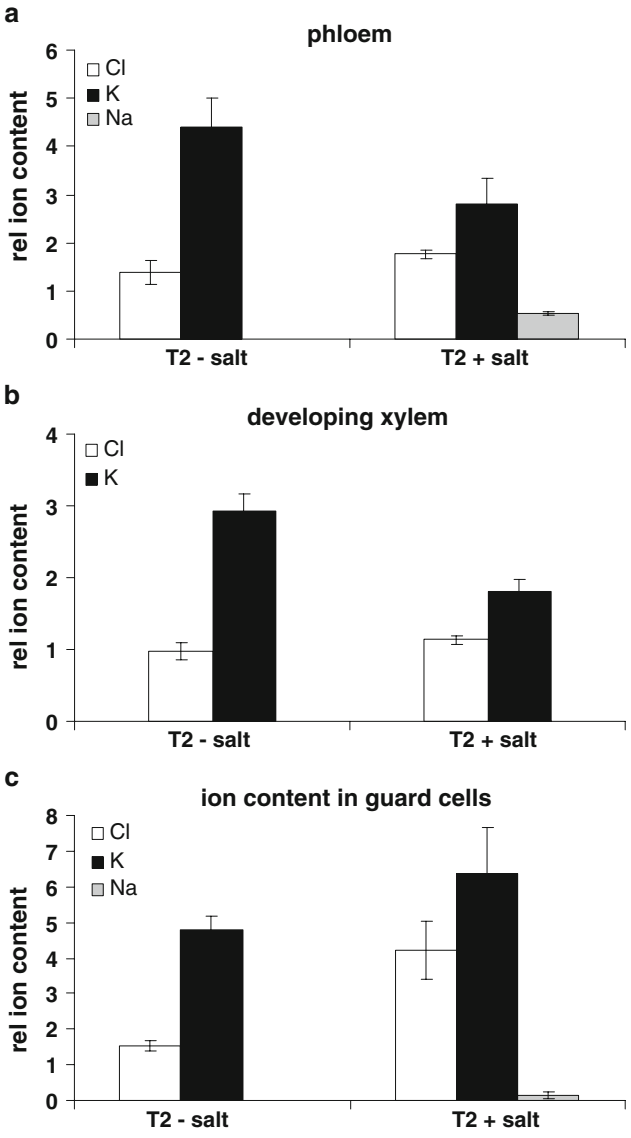

Fig. 5 EDXA: relative Na, Cl and K content of poplar shoot tissues after 2 weeks (*T2*) with and without salt stress. **a** In the phloem, the rise in Na is accompanied by a K decrease. **b** Developing xylem shows reduced K values under salt stress, sodium was below the detection limit. **c** In guard cells, K and Cl values are elevated, whereas little Na is taken up. (relative values reflect peak/background ratios, $n = 5$, mean ± SE). Control plants maintained the same values in all tested tissues as at the beginning of the experiment (not shown)

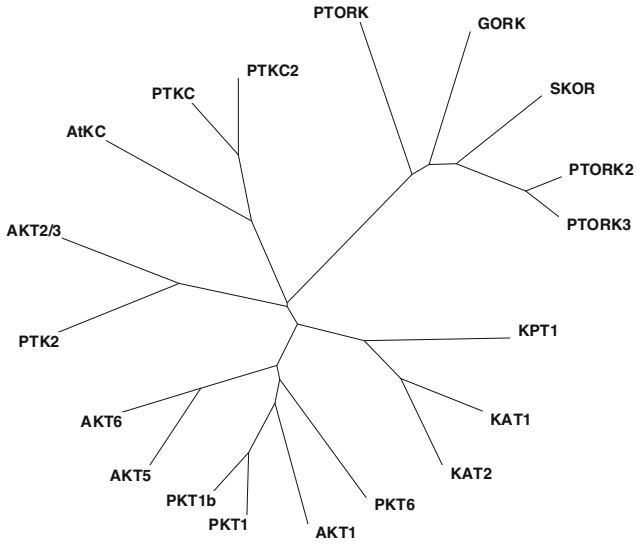

Fig. 6 Phylogenetic tree of *Arabidopsis thaliana* and poplar *Shaker* like K^+ channels. Accession numbers (as per genome version 1.0): PTORK, AJ271446; AtGORK, AJ279009; SKOR, NM_123109; PTK2, AJ271447; AKT2/3, NM_118342; KAT1, NM_123993; KAT2, NM_117939; KPT1, AJ344623; AKT1, NM_128222; SPIK, AC006053; AKT5, AJ249479; AtKC1, U81239. Sequences from the poplar genome have the following protein IDs (http://genome.igi-psf.org): PTORK2, 545510; PTORK3, 343059; PKT1, 705131; PKT1b, 685674; PKT6, 91259; PTKC1, 701980; PTKC2, 344691

these organs. Similar expression patterns were detected with *PTK2*, a channel which is also able to release K^+ (Langer et al. 2002). In roots, shoots and bark, however, an elevated osmotic potential evoked by salt uptake seems to be partially compensated by PTORK2 and PTK2 mediated potassium release. *PtSOS1*, like the *Arabidopsis* ortholog AtSOS1 (Na^+ exporter, Shi et al. 2000), responded to salt stress primarily in roots thus preventing excess Na^+ uptake and thus delivery to shoot and leaves.

AttDT is an *Arabidopsis* vacuolar malate transporter involved in osmoregulation (Emmerlich et al. 2003; Hurth et al. 2005) whose expression is induced by salt exposure (unpublished results, PA and MEP). Expression of the poplar ortholog *PttDT* was elevated upon salt stress in roots and shoots and thus correlated with the osmotic changes in these organs. In bark and leaves, in contrast, no changes in *PttDt* expression were observed (Fig. 7). In these organs nitrate or chloride seems to replace malate in the vacuole. To test if the *PttDT* expression coincides with the amount of its substrate we determined the leaf malate contents. Consistent with the large Cl^- increase we observed under salt stress (Fig. 3d), leaf malate was substantially reduced (Fig. 8), pointing to chloride rather than malate as the prominent anionic osmotic substance in leaves.

Salt stress is accompanied by increased ABA levels (Shinozaki and Yamaguchi-Shinozaki 2000). The *Arabidopsis KIN2* gene (also known as *COR6.6*, cold regulated) is strongly induced following ABA application, cold or salt stress (Kurkela and Borg-Franck 1992; Wang et al. 1995; Webb et al. 1996; Cheong et al. 2002). Also *PtKIN2*, the poplar ortholog, is also ABA inducible (unpublished data, PA and MEP) and thus was used as a marker for gene induction by ABA. Upon salt treatment, *PtKIN2* transcript numbers were highly elevated in roots and shoots, whereas in leaves expression was not altered. To correlate salt stress to changes in the ABA level, we determined the ABA content in root and leaves. As expected, during ongoing salt exposure root ABA increased continuously (up to fivefold)

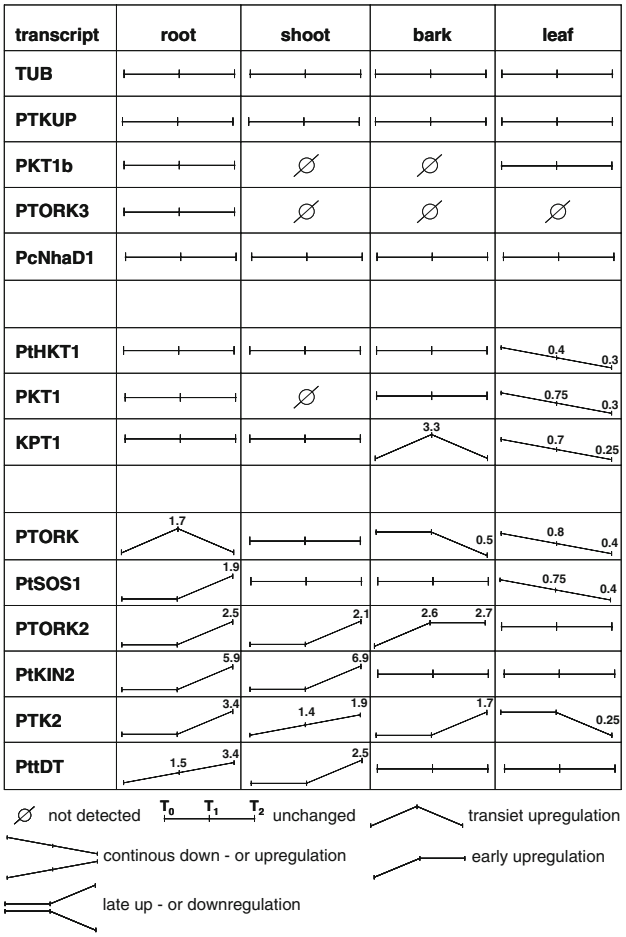

Fig. 7 Expression levels of marker transcripts at the beginning of the experiment (*T*0), after 1 week (*T*1) and 2 weeks (*T*2) of salt stress in different organs of *Populus* × *canescens*. Gene expression upon salt stress (*upper part* of the figure) was not altered; genes in the middle almost exclusively react by down regulation in leaves, whereas genes with different reactions in different organs are depicted in the *lower part*. *Numerical values* indicate mean fold changes of expression ratios (treated to untreated)

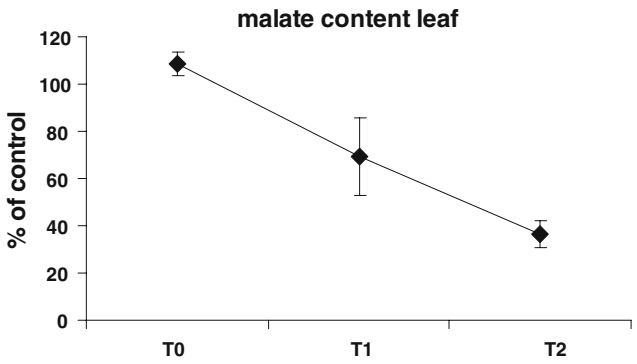

Fig. 8 Leaf malate content is reduced upon salt stress. After 2 weeks of salt exposure malate levels reached less than 50% of controls ($n = 6$; mean values ± SE)

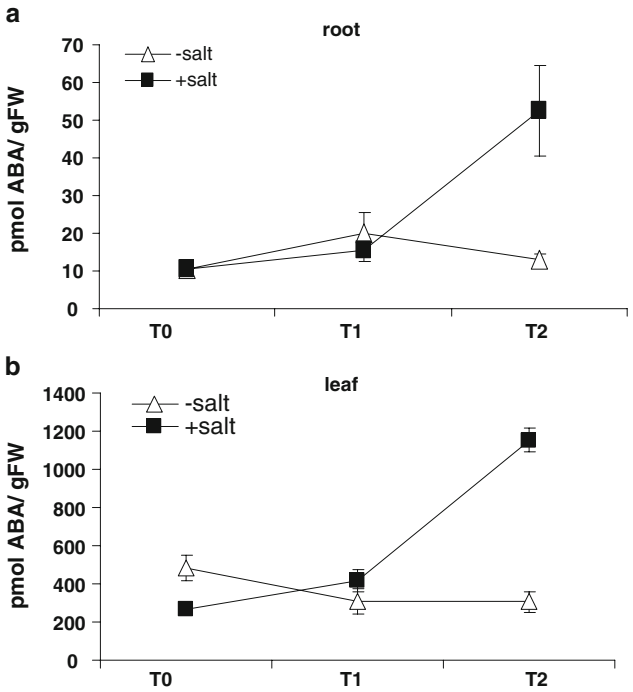

Fig. 9 Varying ABA content of roots and leaves upon salt stress. In both organs ABA levels strongly increased during the second week of salt treatment. In leaves ABA background levels from the beginning of the experiment were at least 20-fold higher than in roots ($n = 6$, mean values ± SE)

and thus coincided with *PtKIN2* expression (Figs. 7, 9a). To our surprise and in contrast to the salt insensitive *PtKIN2* expression in leaves, ABA levels in these organs were also elevated as much as fivefold under salt stress (Fig. 9b). The background ABA levels in leaves of either control or salt stressed plants, however, were 20- to 30-fold higher than those of roots (Fig. 9), indicating already high (ABA induced) leaf *PtKIN2*-expression even under control conditions.

Discussion

In the present study we examined the feedback of salt stress on morphological changes in correlation to ion status and expression patterns of selected transcripts in different organs of grey poplar. Particular attention was given to salt-dependent differences in the wood differentiation zone.

Ion content

Total ion content in leaves and roots of salt resistant *P. euphratica* subjected to NaCl stress have been investigated previously, e.g. by Chen et al. (2001) and Ma et al. (1996). According to these authors, NaCl stress increased the content of Na^+ and Cl^- in roots and leaves. Salt

treatment reduced K^+ levels in both tissues of *P. euphratica*, whereas leaf K^+ content of salt-sensitive species was less affected. No K^+ decrease was observed in roots (Chen et al. 2001). In our studies, salt-sensitive grey poplar plants under salt stress conditions exhibited a shift in ion concentration towards increasing Cl^- and Na^+ levels in almost all tissues examined (Figs. 3, 4, 5). Salt increase was accompanied by a reduction of the relative K-concentration in all root and shoot tissues, which points to a Na^+/K^+ antagonism to balance osmotic changes in poplar cells. In roots a substantial increase in sodium content (up to 40-fold) was observed within 2 weeks, accompanied by a 60% decrease of potassium (Fig. 3). Na^+ increase and K^+ decrease in shoots appeared to be delayed by 1 week (Fig. 3). According to our previous studies, a reduction of K^+ in the wood region is a possible reason for altered wood morphology (Langer et al. 2002). In leaves and in particular in guard cells, the overall osmolarity and the potassium basal levels were remarkably higher than in the other organs. Although Na^+ increased strongly, the maximum Na concentration reached only about 75% of root and shoot values and a K^+ decrease was not detected (Fig. 3). Thus osmotic Na/K balance seems not to be required in leaves. Peak sodium levels were already found in roots after the first week; increases in sodium level were retarded in shoots, and peak values were not reached in leaves until the second week, where the maximum concentration was about 25% below roots and shoots. It seems that storage compartments in roots and shoots are first "filled" with sodium, in order to delay severe salt impact on photosynthesis. This hypothesis was confirmed by the fact that in roots the overall osmolarity rose continuously, whereas shoot and leaf osmolarity were significantly elevated only after the second week of salt exposure (Fig. 3).

Expression

Transcript numbers are not necessarily identical to protein abundance or function but are indicative of gene regulation and hence of possible physiological adaptation. The expression analyses of poplar ion transporters under salt stress were consistent with our initial results regarding K decrease in roots, shoots and bark. In particular ion channels able to release K^+ (PTORK, PTORK2 and PTK2), showed remarkable up-regulation during salt stress in these organs (Fig. 7). Of these *PTORK* was transiently elevated after the first week, pointing to an early K^+ release in response to elevated sodium levels. Thus, in roots and shoots but not in leaves an elevated osmotic potential evoked by salt uptake is at least partly compensated by potassium release. Indeed none of the transcripts tested was up-regulated in leaves, pointing to a general decrease of cation transporter expression activity within this organ under salt stress (Fig. 7).

AtHKT1 is a low affinity Na^+ transporter that mediates Na^+ entry into *Arabidopsis* roots under salt stress and has been discussed as an important factor in Na^+/K^+ homeostasis (Rubio et al. 1995; Uozumi et al. 2000; Liu et al. 2001; Mahajan and Tuteja 2005). According to the proposed mechanism, AtHKT1 loads Na^+ into the shoot phloem to enable Na^+ recirculation to the root. This function seemed to be independent of the salt overly sensitive (SOS) signal pathway, where a salt stress signal activates the plasma membrane SOS1 Na^+/H^+ antiporter, which in turn mediates Na^+ efflux (Rus et al. 2004 and references therein). While PtHKT1 remained unaffected *PtSOS1*expression was twofold up-regulated in roots after 2 weeks of salt stress, pointing to a reduced sodium uptake at the primary entry site. It is therefore, very likely that both transporters are regulated via activity rather than at a transcriptional level (for a review of the effect of K^+ content on K^+ transporter activity cf. Hedrich and Kudla 2006).

Organic acids and polyols are involved in osmoregulation as well as in the plant response to salt stress (Emmerlich et al. 2003; Hu et al. 2005). Typical candidates are free amino acids, which are determined in another study of the same experiment (Dluzniewska et al. 2007). In fact, the amount of several amino acids, in particular serine, appeared to be elevated and might therefore, contribute to the osmotic balance under salt stress. The calculated total amount of the elevated free amino acids, however, was in no way sufficient to counterbalance the increased Cl^-. In line with an osmotic stabilisation mechanism, PttDT, a putative vacuolar malate transporter, is able to release malate from the vacuole when Cl^- enters. Under salt stress *PttDT* induction in poplar roots and shoots coincided with the osmolarity changes and with Cl^- uptake of these organs. In leaves, osmotic stress by salt uptake is much weaker than in roots and shoots. Thus, in the presence of access anions such as NO_3^- PttDT transcript levels are constant.

Transcripts of the bark reacted in general like a mixture of shoot and leaf. This reflects the bark morphology, which is partly related to the shoot (e.g. rays) and partly related to leaves (e.g. chloroplasts, photosynthetic active cells).

Role of ABA

Salinity in general causes increased ABA biosynthesis and accumulation, which in turn modulates alterations in plant physiology (Karmoker and van Steveninck 1979; Behl et al. 1981; Chen et al. 2001; Chang et al. 2006). With 50 mM NaCl in the roots of the salt-tolerant *Populus euphratica*, ABA synthesis is increased (Chen et al. 2001, 2002). Furthermore, in contrast to salt-sensitive species, *P. euphratica* maintains higher ABA concentrations with long-term increasing salinity (Chen et al. 2001). In our

studies with the salt-sensitive grey poplar, the ratio of salt stress-related ABA increase was similar in roots and leaves. Basal ABA levels in leaves, however, were about 20- to 30-fold higher than in roots (Fig. 9). In contrast to the salt-sensitive poplar investigated previously (Chang et al. 2006), grey poplar throughout the experiment exhibited similar ABA values in leaves to those measured in the salt tolerant *P. euphratica* (Chang et al. 2006). These high levels in leaves were maintained for 1 week and further increased (fourfold) during the second week. This observation with the salt-sensitive grey poplar is not consistent with the hypothesis by Chang et al. (2006) predicting that continuously high ABA levels are important for salt resistance of *P. euphratica*.

PtKIN2 expression is, like *AtKIN2,* strongly induced upon ABA stimulation (unpublished data, PA and MEP). In roots *PtKIN2* induction coincided with rising ABA concentrations; however in leaves *PtKIN2* expression was not altered (Fig. 7) even when ABA levels were highly elevated (Fig. 8). An explanation for this contradiction might be that from the beginning of the experiment ABA levels were already above the threshold of *PtKIN2* induction, which is probably true for most of the ABA induced genes. Further induction of ABA synthesis might therefore, have an impact on physiological processes such as stomatal closure, but not on gene induction. We thus conclude that in grey poplar a gene regulating long-term (2 weeks and longer) salt stress signal is not transmitted to the photosynthetic tissue by means of changes in ABA concentration. ABA induced permanent stomatal closure, in contrast, leads to reduced CO_2 uptake and is thus another possible reason for reduced wood formation.

Future experiments including microarray analyses will provide further insight into the complex salt stress response of the grey poplar.

Acknowledgments We thank Ute Neef for technical assistance and Duncan Cameron (University of Sheffield, UK) and Lois Banta (Haverford College, PA, USA) for carefully reading of the manuscript and helpful comments. The study was supported financially by the German Science Foundation (DFG; Ache: AC97/4-2, Schnitzler: SCHN653/4, Nehls: Ne332/9-1, Fromm: Fr955/10-2, and Polle: PO362/13) within the German joint research group 'Poplar—A model to address tree-specific questions'.

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