

# Linking the Salt Transcriptome with Physiological Responses of a Salt-Resistant *Populus* Species as a Strategy to Identify Genes Important for Stress Acclimation<sup>1[W][OA]</sup>

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To investigate early salt acclimation mechanisms in a salt-tolerant poplar species (*Populus euphratica*), the kinetics of molecular, metabolic, and physiological changes during a 24-h salt exposure were measured. Three distinct phases of salt stress were identified by analyses of the osmotic pressure and the shoot water potential: dehydration, salt accumulation, and osmotic restoration associated with ionic stress. The duration and intensity of these phases differed between leaves and roots. Transcriptome analysis using *P. euphratica*-specific microarrays revealed clusters of coexpressed genes in these phases, with only 3% overlapping salt-responsive genes in leaves and roots. Acclimation of cellular metabolism to high salt concentrations involved remodeling of amino acid and protein biosynthesis and increased expression of molecular chaperones (dehydrins, osmotin). Leaves suffered initially from dehydration, which resulted in changes in transcript levels of mitochondrial and photosynthetic genes, indicating adjustment of energy metabolism. Initially, decreases in stress-related genes were found, whereas increases occurred only when leaves had restored the osmotic balance by salt accumulation. Comparative in silico analysis of the poplar stress regulon with *Arabidopsis* (*Arabidopsis thaliana*) orthologs was used as a strategy to reduce the number of candidate genes for functional analysis. Analysis of *Arabidopsis* knockout lines identified a lipocalin-like gene (*AtTIL*) and a gene encoding a protein with previously unknown functions (*AtSIS*) to play roles in salt tolerance. In conclusion, by dissecting the stress transcriptome of tolerant species, novel genes important for salt endurance can be identified.

Excess sodium in soil is a widespread and common stress in natural and agricultural ecosystems (FAO, 2008). High salt concentrations decrease the osmotic potential of the soil solution, thereby lowering the availability of water to plants. Therefore, salinity causes dehydration stress, at least in nonacclimated plants. Furthermore, sodium competes with other

cations, thereby affecting plant nutrition and ion homeostasis (Zhu, 2003; Munns and Tester, 2008; Shabala and Cuin, 2008). Since most plant species have only very limited capacities to cope with excess sodium, productivity on saline soil is strongly diminished and plant growth may even become impossible. Globally, salinity is an important environmental problem, because the total area of salt-affected soils, including saline and sodic soils of currently about 831 million ha, is continually increasing (Martinez-Beltran and Manzur, 2005).

The elucidation of salt tolerance mechanisms, therefore, is an important issue and has attracted considerable interest in recent years (Munns, 2005; Vinocur and Altman, 2005; Yamaguchi and Blumwald, 2005; Ma et al., 2006; Munns and Tester, 2008; Chen and Polle, 2010). Comparative analysis of transcriptional profiles of *Arabidopsis* (*Arabidopsis thaliana*) and salt cress (*Thellungiella halophila*) suggested that salt-sensitive and salt-tolerant species have a common set of salt responses whose regulation differs (Taji et al., 2004).

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Upon sudden salt exposure, both species activated genes involved in ribosomal functions, photosynthesis, cell growth, osmolyte production, and transport activities (Gong et al., 2005), whereas in salt-acclimated *T. halophila* plants, only a few genes were differentially regulated (Wong et al., 2006).

An intriguing question is how trees, which have a long life span and, therefore, must be able to cope with excess salt for extended periods of time, adapt to high salinity. *Populus euphratica* is a model for salt tolerance in trees (Chen and Polle, 2010). It withstands sodium concentrations up to 450 mM in nutrient solution (Watanabe et al., 2001). It accumulates high sodium concentrations in leaves, which develop succulence after long-term exposure (Ottow et al., 2005). In a preceding study, a small microarray with 315 cDNAs of transcripts obtained by suppression subtractive hybridization from *P. euphratica* was used to analyze salt responses, but clear tissues-specific patterns were not found (Gu et al., 2004). Brosché et al. (2005) constructed *P. euphratica* EST-based microarrays containing 7,342 different ESTs corresponding to about 6,300 different genes. The ESTs were captured from 17 different cDNA libraries of different tissues (roots, leaves, stems) from stress-exposed trees (drought, salt, flooding, cold, ozone). Using microarrays with this collection of stress-responsive genes, Brosché et al. (2005) found that leaves of mature desert-grown *P. euphratica* trees acclimated to high salinity show only a few differentially expressed genes compared with tap-watered control trees. Salt-acclimated Euphrat poplars show less gene regulation than salt-sensitive poplar species (Ding et al., 2010), probably because transporters important to control excessive salt accumulation and the flavonoid/phenylpropanoid pathway, which may counteract negative salt effects by controlling reactive oxygen species, are constitutively up-regulated in the tolerant poplar species (Janz et al., 2010). However, it is still unclear which early molecular responses are required to adjust the metabolism to life at a new level of osmotic balance and ionic stress.

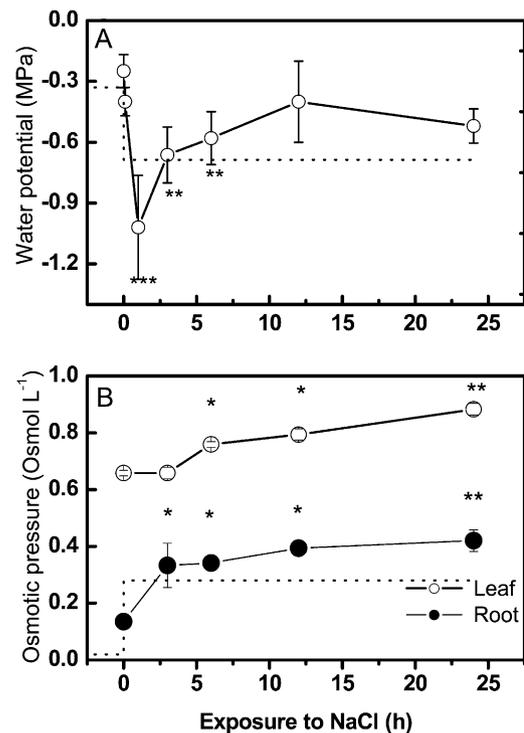
To obtain insights into the initial events taking place in response to exposure to high salinity in a salt-tolerant tree species, we exposed *P. euphratica* to salt stress and followed early changes in gene expression, metabolic profiles, and physiological responses to Na<sup>+</sup> accumulation and changes in plant water relations. The kinetics of salt accumulation and osmotic adjustment differed between roots and leaves displaying a phase of initial dehydration stress, followed by osmotic adjustment and finally a new level of "high-salinity" homeostasis. These different stages were associated with distinct clusters of coexpressed genes. Comparative in silico analysis with Arabidopsis orthologs was used as a strategy to reduce the number of candidate genes for functional analysis. Using Arabidopsis knockout lines, a lipocalin-like gene (*AtTIL*) and a gene encoding a protein with previously unknown functions (*AtSIS*) were shown to play roles in salt tolerance.

## RESULTS

### The Kinetics of Sodium Accumulation and Recovery of Water Potential Show Distinct Phases in Roots and Leaves of *P. euphratica*

Exposure of plants to elevated salinity imposes stress by a decrease in water potential in the root medium. When young *P. euphratica* trees were exposed to 150 mM NaCl in the nutrient solution, which corresponds to a drop in osmotic potential of  $-0.68$  MPa, a severe and rapid decrease in the shoot water potential of  $0.77$  MPa occurred (Fig. 1A). This caused transient wilting of the plants.

The shoot water potential recovered almost completely within 24 h (Fig. 1A). Roots and leaves, however, displayed different time courses of osmotic adjustment (Fig. 1B). In roots, acclimation was completed within 3 h, because the osmotic pressure, which increased to ensure water uptake, was adjusted above the osmotic pressure of saline solution in this period of time. Afterward, it increased only marginally (Fig. 1B). In contrast, the osmotic adjustment of leaves started only after roots had reached their new plateau (Fig. 1B).



**Figure 1.** Water potential (A) and osmotic pressure (B) in *P. euphratica* exposed to salt stress. NaCl at 150 mM or a corresponding water volume was added to the nutrient solution. Water potential was measured on shoots (white circles). Osmotic pressure was measured in pressure sap from roots (black circles) and leaves (white circles). Dotted lines indicate changes in the water potential or osmotic pressure in the nutrient solution. Data indicate means of five plants per treatment and time point  $\pm$  SE. Asterisks indicate significant differences compared with controls.

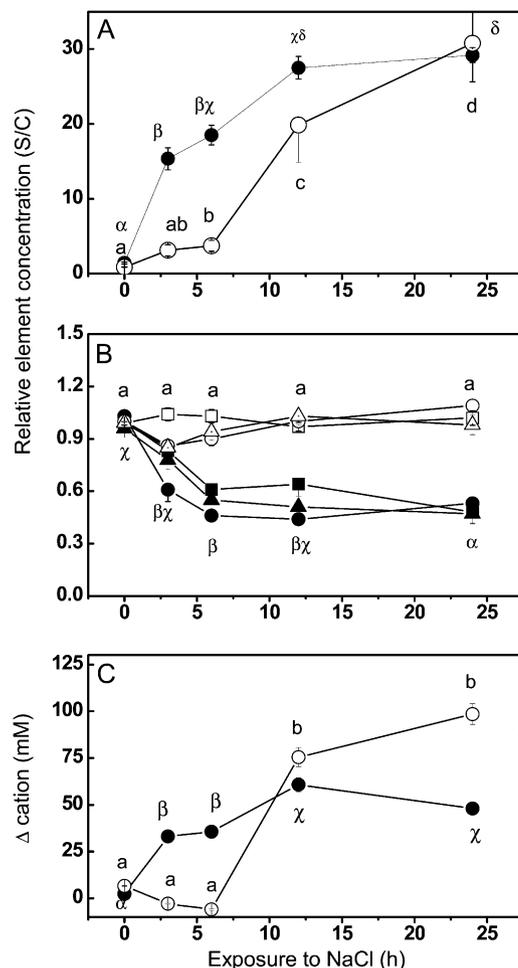
Since changes in osmotic potential are the result of changes in solute concentrations in plant tissues, we determined profiles of nutrient elements and organic metabolites. In roots, salt exposure caused 28-fold increases in  $\text{Na}^+$  concentrations within 12 h and simultaneously about 2-fold decreases in major cations ( $\text{Ca}^{2+}$ ,  $\text{K}^+$ , and  $\text{Mg}^{2+}$ ; Fig. 2, A and B). Afterward, elevated  $\text{Na}^+$  and decreased cation levels were maintained in roots (Fig. 2, A and B). In leaves, an about 30-fold increase in  $\text{Na}^+$  was also observed; however, accumulation started with a delay of about 6 h compared with roots and reached levels similar to those of roots after 24 h of sodium exposure (Fig. 2A), whereas the major cations in leaves remained unaffected (Fig. 2B). Other nutrient elements, such as phosphorus (P) and sulfur (S), were neither influenced in roots nor in leaves of salt-stressed poplar [means across all time points and controls and salt-exposed plants:  $P_{(\text{roots})} = 6.50 \pm 0.27 \text{ mg g}^{-1}$  dry weight,  $S_{(\text{roots})} = 4.45 \pm 0.06 \text{ mg g}^{-1}$  dry weight,  $P_{(\text{leaves})} = 7.12 \pm 0.10 \text{ mg g}^{-1}$  dry weight,  $S_{(\text{leaves})} = 7.97 \pm 0.05 \text{ mg g}^{-1}$  dry weight].

Differences between sodium uptake by roots and leaves affected the overall pattern of cation accumulation, leading to earlier but less pronounced increases in total cation concentrations in roots compared with leaves (Fig. 2C). The changes in water contents were small,  $-3.2\%$  and  $-1.2\%$  in leaves (control, 85.4%) and roots (control, 94.9%), respectively.

Metabolite profiling was conducted for soluble carbohydrates, sugar alcohols, organic acids, and amino acids in leaves and did not show significant changes for most compounds analyzed (Supplemental Table S1). Exceptions were Asn, whose levels were decreased after 12 h, and Val and Ile, which showed significant increases after 24 h of salt exposure (Fig. 3). However, the estimated concentrations of Val and Ile of  $7.4$  and  $3.3 \mu\text{g g}^{-1}$  dry weight contributed only  $0.03\%$  and  $0.01\%$ , respectively, to the osmotic potential imposed by sodium. Thus, the changes of these amino acids were too low to affect the total osmotic potential of leaves significantly. Other compounds such as Thr and citric acid showed only transient changes after 12 and 3 h, respectively. Overall, osmotic recovery in *P. euphratica* was thus mainly achieved by sodium accumulation.

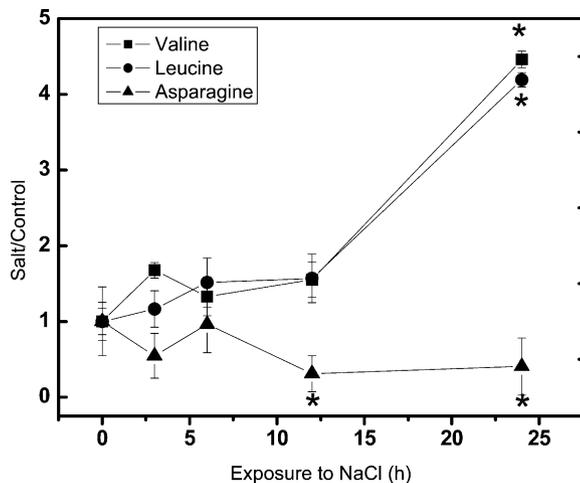
### Transcriptional Profiling Reveals Groups of Coregulated Genes

As changes in the ion balance were completed in roots (0–12 h) before adjustment in leaves started (12–24 h), we took advantage of these distinct phases to dissect transcriptional responses related to water deficit and/or high sodium concentrations in leaves and roots, respectively. We used previously developed microarrays containing ESTs of about 6,340 genes from *P. euphratica* (Brosché et al., 2005). To identify tightly stress-responsive genes, stringent settings of statistical analysis of microarrays (SAM;  $\Delta = 0.189\text{--}0.269$ ) were applied to obtain low false discovery rates (0–0.1).



**Figure 2.** Salt-induced changes in element concentrations in leaves and roots of *P. euphratica*. Element concentrations of leaves (white symbols) or roots (black symbols) after salt treatment (150 mM) were divided by mean concentrations of controls (S/C). A, Sodium. B, Magnesium (triangles), potassium (circles), and calcium (squares). C, Change in cation concentrations (mM). Data indicate means of 15 plants  $\pm$  SE. Different letters indicate significant differences at  $P \leq 0.05$ , with Greek letters for roots and Roman letters for leaves. Control plants contained the following ion concentrations ( $\text{mg g}^{-1}$  dry mass  $\pm$  SE) in roots: sodium,  $1.43 \pm 0.37$ ; potassium,  $1.00 \pm 0.11$ ; calcium,  $1.03 \pm 0.07$ ; and magnesium,  $0.96 \pm 0.05$ . Control plants contained the following ion concentrations ( $\text{mg g}^{-1}$  dry mass  $\pm$  SE) in leaves: sodium,  $0.16 \pm 0.01$ ; potassium,  $37.85 \pm 0.98$ ; calcium,  $14.4 \pm 0.91$ ; and magnesium,  $5.75 \pm 0.32$ .

Using these criteria, we identified 56 and 48 ESTs in leaves and roots, respectively, which showed significant changes of transcript levels in response to salinity (Supplemental Table S2). These numbers correspond to  $0.9\%$  and  $0.8\%$  of all ESTs present on the array. About  $22\%$  and  $41\%$  of these transcripts in leaves and roots, respectively, did not show homology with genes of known functions. Only three genes (dehydrin [Dhn1 protein; AJ774829], osmotin-like protein [AJ779386], Suc synthase [AJ778233, AJ778234]) responded with significant changes in transcript abundance in both



**Figure 3.** Salt-induced changes in amino acids in leaves of *P. euphratica*. Data indicate means  $\pm$  SE ( $n = 5$ ). Asterisks indicate significant changes compared with controls.

roots and leaves, respectively. Therefore, the total number of stress-responsive ESTs was 101, corresponding to 1.7% of the ESTs on the array. The total number of unique salt-responsive genes was even lower, because some of the identified transcripts (e.g. those for Rubisco activase, PsbA, and ribosomal RNA) were represented by multiple ESTs on the array (Supplemental Table S2).

At a given time point, all responsive genes had either only increased or decreased expression. For some selected genes, the response was confirmed by quantitative real-time PCR (Supplemental Table S3). Cluster analysis based on the transcript levels across all time points revealed groups of coexpressed genes (Fig. 4). In roots, four clusters were obtained, one for each time point (Fig. 4, RC1–RC4; for genes included in each cluster, see Supplemental Table S2). In leaves, six clusters were obtained, because transcriptional responses after 24 h of salt exposure were more diverse than at other time points (Fig. 4).

#### Root Genes Responsive to Different Phases of Osmotic Stress and Recovery

We exploited the different phases of salinity stress (Figs. 1 and 2) to assign genes forming transcriptional clusters to different physiological stages: dehydration stress, osmotic adjustment, and homeostasis associated with high tissue salt concentrations.

Root cluster 1 (RC1) was associated with the initial dehydration phase. This cluster was small, containing only two members both of the dehydrin family (Fig. 4; Supplemental Table S2).

RC2, which characterized the major phase of osmotic readjustment of roots, was formed by a group of genes with decreased transcript levels (Fig. 4) and contained three putative aquaporins. Furthermore, several genes involved in protein metabolism and

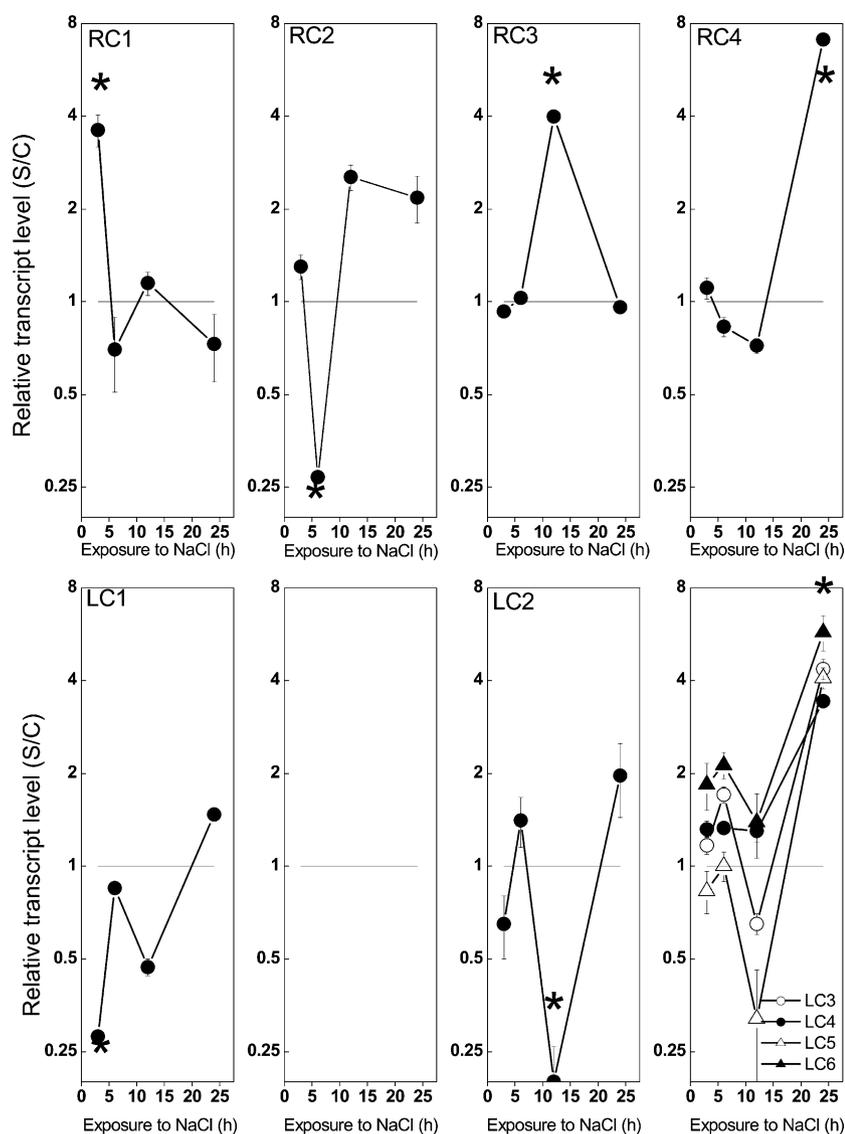
genes that may play roles in stress sensing and signal transduction (a putative Gly-rich RNA-binding protein, a homolog to a metallothionein MT1a, and a Suc synthase; Supplemental Table S2) were also found.

Genes forming RC3 were associated with a physiological stage in which osmotic recovery of the roots was accomplished but in which most likely metabolic adjustment to the new ionic relationships was still ongoing (Fig. 2A). This cluster contained the highest proportion of genes with unknown functions or no hit in the databases (74%). The remaining transcripts indicated cell wall changes (polygalacturonase-like protein), stress and stress signaling (a cyclophilin, a putative PR protein, and a putative Ser/Thr kinase), the involvement of glycolysis (malate dehydrogenase), and remodeling of protein metabolism (tRNA for Gln synthase, Ser hydroxymethyltransferase, and a proteasome AAA-ATPase subunit; Supplemental Table S2). However, salt-induced changes in protein content were not found (Supplemental Fig. S2).

RC4 contained only two genes, both belonging to the osmotin family (Fig. 4; Supplemental Table S2). The different time courses of dehydrins (RC1) and osmotins (RC4) suggest that dehydrins are important for the primary adaptation processes in roots, whereas osmotins seem to be required in the homeostatic phase to maintain cell functions at low osmotic potentials and high ionic stress.

#### Leaf Genes Responsive to Different Phases of Osmotic Stress and Recovery

In leaves, a higher number of genes (leaf cluster 1 [LC1]) were responsive to the initial phase of dehydration stress than in roots (Supplemental Table S2). This was remarkable because at this early time point, leaves were not yet exposed to elevated sodium concentrations (Fig. 2A) but the plants showed wilting, which recovered during the further time course of sodium exposure. LC1 was composed of six transcripts representing Rubisco activase and one of a Clp protease (heat shock protein) with putative chloroplast localization (Supplemental Table S2). This suggests a massive influence of the initial dehydration phase on chloroplast metabolism. To corroborate this assumption, we determined PSII activity by measuring chlorophyll fluorescence. However, at this early time point, PSII activity was not yet affected (Fig. 5), although photosynthetic CO<sub>2</sub> assimilation and stomatal conductance were decreased (Wang et al., 2007). Furthermore, LC1 contained mitochondrial catalase, cytochrome P450 monooxygenase, and glyoxalate oxidase (Supplemental Table S2). Decreased transcript abundance of these genes suggests compromised mitochondrial hydrogen peroxide metabolism and the formation of methylglyoxalates, which are generated as toxic oxidative side products of glycolysis. Suc synthase transcripts were also decreased. However, correlations of transcriptional changes of this gene with carbohydrate concentrations were not found (Supplemental Table



**Figure 4.** Salt-induced changes in transcript levels in roots and leaves of *P. euphratica*. Data indicate mean transcript levels of genes assigned to root clusters (RC) and leaf clusters (LC) according to Supplemental Table S2. Asterisks indicate significant changes compared with controls.

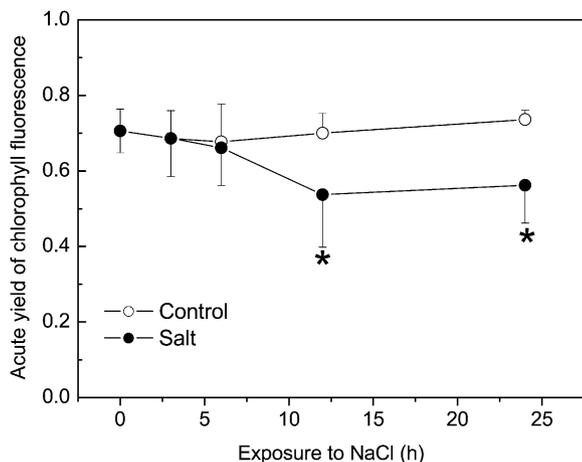
S1). The maintenance of soluble carbohydrate concentrations might have been achieved by the activity of other enzymes whose transcript abundance remained unchanged.

It is notable that in leaves after 6 h of salt exposure, when the initial dehydration stress was relieved (Fig. 1B) but sodium accumulation had not yet occurred (Fig. 2B), no changes in transcripts were found.

After 12 h, at the stage when massive sodium accumulation occurred, only genes with significantly decreased transcript levels (LC2) were identified (Fig. 4). Five members of LC2 were of chloroplast origin (three *psbA*-related proteins, D2 protein, Rubisco large subunit). At this stage, PSII activity was also decreased (Fig. 5). Furthermore, LC2 contained genes for plastidic and nuclear protein synthesis (26S and 60S ribosomal RNA and a translation initiation factor), genes with ill-defined or undefined functions (putative glycosyl transferase, predicted GTP-binding protein, un-

knowns), and genes pointing to glycolysis (oxoglutarate dehydrogenase) and stress (a putative glutathione-S-transferase and COBRA protein precursor; Supplemental Table S2).

Increased transcript levels in leaves were only found after 24 h, when the water potential had been restored and the tissues were exposed intrinsically to high salinity (Figs. 1A, 2A, and 4). Time courses and relative transcript levels differed among each other, yielding four clusters (Fig. 4). Two of these clusters (LC3, LC5) contained genes whose transcript levels showed time courses similar to those of LC2, with a decrease at 12 h of salt exposure, although stronger increases after 24 h than those of LC2. LC3 contained calcineurin-like protein CLB10, which is known to play a role in the response to salinity, and 1-aminocyclopropane-1-carboxylic acid oxidase, suggesting the recruitment of ethylene-related pathways (Ottow et al., 2005). LC5 contained mainly organelle-specific genes (*psbA*, mi-



**Figure 5.** Quantum yield of photochemistry in irradiated leaves of control and salt-exposed *P. euphratica* plants. Data indicate means  $\pm$  SE ( $n = 5$ ). Asterisks indicate significant changes compared with controls.

tochondrial ATPase) and the osmotin-like protein that also had increased expression in roots.

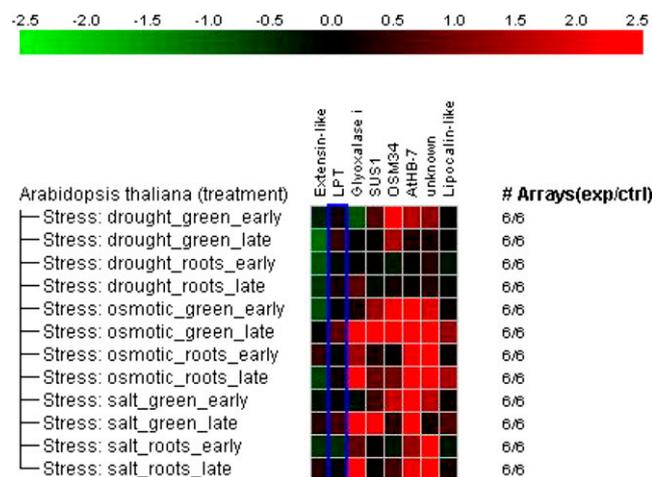
In contrast to LC3 and LC5, transcript levels of genes in clusters LC4 and LC6 did not change during the initial phases of salt exposure, suggesting that these groups must be specific for acclimation to high cellular solute concentrations. Relative transcript levels of LC6 were higher than those of LC4, but otherwise no differences between these two clusters were found. LC4 was composed of a GA-responsive gene (*GASA3*), a dehydrin-like protein, an aldehyde dehydrogenase, a homeodomain transcription factor with homology to *ATHB-7*, a lipocalin-like protein, two metallothioneins (*MT2a*, *MT3*), and genes with unknown functions. LC6 contained genes for a putative Kunitz trypsin inhibitor, Asn synthase, GA-responsive protein, and a metallothionein *MTa*-like protein. Among all clusters, LC4 and LC6 were characterized by the highest abundance of stress-related genes and transcription factors. Since osmotic recovery was almost accomplished at this stage (Fig. 1A), these genes must play roles in the adaptation to high salinity or in maintaining cellular metabolism at a new level of osmotic pressure.

#### Identification of Novel Salt-Resistant Genes by Comparative Analysis of Arabidopsis Orthologs of *P. euphratica* Genes and Functional Characterization of Knockout Mutants

To investigate the significance of the stress-responsive genes identified by transcriptional profiling in *P. euphratica* in a wider context, we BLASTed the poplar ESTs against The Arabidopsis Information Resource database to extract Arabidopsis genes with significant homology. For the leaf set, 39 significant matches, and for the root set, 27 significant matches with Arabidopsis genes were obtained (Supplemental Table S2). Some of these genes were retrieved in both tissues and

some were represented by different ESTs, resulting in a total of 49 different Arabidopsis homologs. This condensed data set was used to search Genevestigator (Hruz et al., 2008) for genes that were silent during normal vegetative development. Applying this criterion, we obtained a short list of eight stress-specific genes out of the total 49 stress-responsive genes (Supplemental Fig. S1). Two of these Arabidopsis orthologs were found in both roots and leaves of *P. euphratica* (osmotin [At4g11650], Suc synthase [At5g20830]), three genes were only responsive in roots (glyoxalase [At1g80160], extensin [At2g27380], seed storage/lipid transfer protein [At5g05960]), and three were only responsive in leaves (*ATBH-7* homeodomain transcription factor [At2g46680], a lipocalin-like gene [At5g58070], a gene with unknown functions [At5g02020]). The latter three genes and osmotin belonged to the group of genes induced in *P. euphratica* leaves after 24 h of salt stress. With the exception of the lipocalin-like gene, all other leaf genes were also responsive to salt, drought, and osmotic stress in Arabidopsis, whereas the lipocalin-like gene was only marginally induced in response to osmotic and salt stress (Fig. 6).

The Arabidopsis lipocalin-like gene (At5g58070) and the gene with unknown functions (At5g02020) have no established roles in salt tolerance. Since the unknown gene was induced by salt in Arabidopsis as well as in poplar (Fig. 6; Supplemental Table S2) and contained an overabundance of Ser residues in its amino acid sequence (Fayyaz, 2008), we termed the gene At5g02020 as Salt-Induced Ser-rich (*AtSIS*). The lipocalin-like gene (At5g58070) belongs to a family of previously annotated temperature-induced lipocalin-like genes (Flower et al., 2000) and, therefore, was termed *AtTIL*. To find out whether these candidate genes contribute to salt resistance, homozygous T-DNA insertion mutants *Attil1-1*, *Attil1-2*, *Atsis1-1*,



**Figure 6.** Analysis of changes in transcript levels in response to salinity, drought, and osmotic stress in Arabidopsis orthologs of eight selected poplar salt-responsive genes. Analysis was conducted with Genevestigator.

and *Atsis1-2* were isolated and exposed to salinity stress. Growth assays in soil supplemented with increasing NaCl concentrations revealed a significantly higher sensitivity of leaf expansion of *Attil1-1* and *Attil1-2* to excess salt than that of the wild type (Fig. 7). *Atsis1-1* and *Atsis1-2* showed intermediate behavior (Fig. 7). All knockout mutants produced significantly less biomass under salt stress than the wild type, showing that these genes play roles in salt tolerance (Fig. 7).

## DISCUSSION

### Different Transcriptional Timetables of Salt Responses in Roots and Leaves Accommodate Different Physiological Needs

In this study, we dissected the physiological responses of the salt-tolerant Euphrat poplar into distinct phases and showed that these phases were characterized by suppression or activation of clusters of coregulated genes. The major events have been compiled in an overview (Fig. 8). By these kinetic analyses, we gained insights into the molecular responses of salt-acclimation processes associated with an initial dehydration phase in leaves, subsequent salt accumulation, and finally restored osmotic homeostasis at a new level of ionic stress. The timing of adaptive responses differed in aboveground and belowground plant tissues, because transport through the stem delays salt accumulation in leaves compared with roots. Furthermore, our study highlights important differences between poplar roots and leaves regarding early salt effects, with little overlap of the genes involved (three of 101). Differences between early

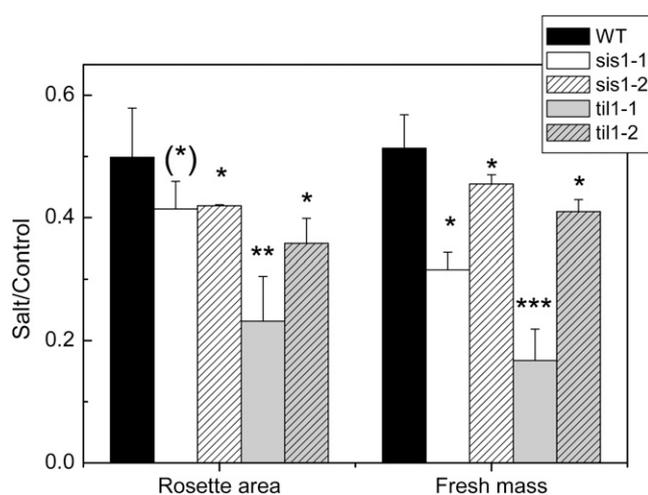
and late transcriptional responses as well as between roots and leaves have also been reported for *Arabidopsis* (Ma et al., 2006). However, in those previous studies, the link to physiological differences in the tissues was lacking. Our study shows that ionic stress is immediately high in roots. In contrast, leaves suffer initially from dehydration stress by water loss but not from shifts in the ion balance. With continued sodium uptake, roots can avoid excessive ion accumulation by compensatory decreases in other cations. The existing leaves must accumulate sodium on top of the cations already present, thereby experiencing the strongest ionic stress (Fig. 2C).

Notably, only two genes, osmotins and dehydrins, were commonly induced in roots and leaves, albeit with different time courses. This finding points to functional differences of chaperones in stress acclimation of Euphrat poplar. Dehydrins that occur immediately in roots but only late in leaves have been shown to maintain a highly disordered status under high solute concentrations where globular proteins would collapse (Mouillon et al., 2006). They may also act as protein cryoprotectants (Hara et al., 2001; Seki et al., 2002) and contribute to protection from drought stress (Rinne et al., 1999; Wang et al., 2004). The time courses observed here suggest a protective function of dehydrins against ionic disorders (Mouillon et al., 2008), whereas the delayed activation of osmotins, another class of well-known stress-activated genes (Shinozaki and Yamaguchi-Shinozaki, 2007), indicates that these proteins must be involved in helping the plant to cope with life at a new level of cellular homeostasis.

Surprisingly, Suc synthase was also salt responsive in both roots and leaves, but its transcript levels decreased under stress (Gu et al., 2004; Bogeat-Triboulot et al., 2007; this study). In contrast, its homolog *AtSUS1* increases under stress (Ma et al., 2006). *AtSUS1* also responds to elevated carbohydrate concentrations (Bieniawska et al., 2007). However, our metabolite profiling did not provide evidence for strong redirecting of carbohydrate metabolism during salt stress. As *PeSUS1* was decreased in the early stage of leaf dehydration stress, it may be responsive to changes in cell turgor that lead to physiological increases in solutes.

### Stress Acclimation Involves a Redirection of Protein Metabolism in Roots and Leaves But Different Stress Pathways

Although there was little overlap between stress-responsive genes in roots and leaves, both tissues showed similar patterns of regulation of pathways involved in protein and amino acid metabolism. When tissues were exposed to an increment in sodium (6 h in roots, 12 h in leaves), decreased transcript abundance of genes for protein biosynthesis suggested that this metabolic path was transiently suppressed in both roots and leaves. The recovery correlated with increased transcript abundance for genes involved in amino acid biosynthesis (12 h in roots, 24 h in leaves).



**Figure 7.** Performance of *Arabidopsis* T-DNA insertion lines *Attil1-1*, *Attil1-2*, *Atsis1-1*, and *Atsis1-2* in response to salinity. Plants were grown in soil and exposed to increasing concentrations of NaCl (25, 50, and 250 mM). Rosette leaf area and biomass were determined 20 d after starting salt exposure ( $n = 7-10$ ; values are means  $\pm$  SE). WT, Wild type.

Roots				Leaves			
Time of Salt Exposure	Phase	Response	Direction	Phase	Response	Direction	
	Salt uptake	Dehydrins	↑	Dehydration	Adjusting mitochondrial and chloroplast metabolism	↓ ↓	
	Osmotic recovery	Limiting water loss, aquaporin	↓	Salt uptake		= =	
		Redirecting protein metabolism	↓				
	Moderate ionic stress		Redirecting protein metabolism	↑	Osmotic recovery	Redirecting protein metabolism	↓
			Adjusting mitochondrial metabolism	↑		Adjusting PS efficiency	↓
			Chaperones, osmotin	↑	Ionic stress	Osmotin and dehydrins	↑
					Stress-related TFs and genes	↑	

**Figure 8.** Overview of the different phases of salt stress in roots and leaves of *P. euphratica* and the molecular responses. Time of salt exposure was 0 to 24 h. TF, Transcription factors.

Indeed, long-term salt stress leads to an accumulation of free amino acids in both salt-sensitive and salt-tolerant poplar species (Ottow et al., 2005; Ehling et al., 2007). It is likely that amino acids, or at least certain amino compounds, have significance for salt stress compensation, because functional links between Gln synthetase and drought and salt tolerance exist (Hoshida et al., 2000; El-Khatib et al., 2004).

Overall, there was surprisingly little overlap between the stress transcriptomes of leaves and roots. Our analysis suggested that the restoration of the water potential in roots involved aquaporins (Supplemental Table S2). These genes are important for the regulation of plant internal water transport (Chaumont et al., 2005) and respond to salt and drought stress in different species (Boursiac et al., 2005; Bogeat-Triboulot et al., 2007) as well as to salt stress in the field (Brosché et al., 2005). Their suppression at early stages may help to prevent water loss. As sodium uptake of roots progressed, cyclophilins were transiently induced. This is further support for the idea that chaperones play a major role in protecting metabolism when plants use  $\text{Na}^+$  as an osmolyte.

A putative Ser/Thr kinase was increased when salt adjustment in roots was almost accomplished. Kinases are involved in signal transduction in response to stress (Zhu, 2002), but the relatively long delay between stimulus and response does not suggest a primary role of this kinase in salt signaling. An involvement in

oxidative stress signaling is possible, because we also observed a transient increase in transcripts for glyoxalases (syn. lactoyl-glutathione lyase) that reduce toxic glyoxals to glycolate (Shangari et al., 2003).

In leaves, the early phase of dehydration stress was characterized by the suppression of transcripts encoding chloroplastic or mitochondrial enzymes, suggesting cross talk between these organelles. Rapid suppression of Rubisco activase and chloroplastic Clp protease, which was also observed under natural salt stress in *P. euphratica* (Brosché et al., 2005), may be required for the down-regulation of photosynthetic  $\text{CO}_2$  assimilation and the stress adaptation of the photosynthetic apparatus, respectively (Zheng et al., 2006; Wang et al., 2007; Stanne et al., 2009). Decreased PSII yield occurred only after the leaves started to accumulate sodium and was probably caused by suppression of the biosynthesis of PSII D1 protein (psbA), Qa protein, and Rubisco large subunit. This indicates the necessity to establish a new level of homeostasis for photosynthetic electron transport. Since the decrease in fluorescence yield was reversible in darkness, the overall electron transport capacity was not injured but down-regulated. This probably reflects an accommodation for decreases in assimilation. Notably, similar responses have also been found in salt stress in response to excess salinity (Wong et al., 2006). Comparisons of stress transcriptomes of *Arabidopsis* also support the notion that down-regulated clusters

mainly contain genes for developmental processes and metabolic adjustment (Ma and Bohnert, 2007).

In contrast to roots, sodium accumulation activated some of the well-known stress and signal transduction pathways in leaves, including transcripts for calcineurin B-like protein and 1-aminocyclopropane carboxylate oxidase, pointing to the involvement of calcium and ethylene. Salt exposure generally leads to increased abscisic acid levels, but in Euphrat poplar more rapidly than in salt-sensitive poplar species (Chen et al., 2001; Luo et al., 2009). Here, activation of a putative homeodomain transcription factor (homolog to ATHB-7) and aldehyde dehydrogenase was observed, which are known to be regulated by abscisic acid and to be responsive to salinity and drought (Söderman et al., 1996; Seki et al., 2002; Olsson et al., 2004; Sunkar et al., 2003; Kotchoni et al., 2006). The late response of these genes, which occurred only when the leaves were exposed to a high salt level, suggests that they are necessary to afford tissue protection from excessive ion accumulation or high osmotic pressure. Notably, transcript levels of aldehyde dehydrogenase, osmotin, metallothioneins, as well as an unknown gene (AJ773239) were also salt affected under field conditions (Brosché et al., 2005). The late response observed here indicates that they are not involved in the primary recognition and signaling of salt stress. As metallothioneins, GA-responsive genes, and several genes with unknown functions were present in the same regulon together with well-established stress-regulated genes, they are likely candidates for mediating salt stress protection.

#### Lipocalin-Like TIL and Ser-Rich Salt-Induced SIS Are Required for Salt Endurance

Transcriptional profiling of salt-tolerant species holds the promise of revealing candidate genes involved in stress adaptation. In our study, about 25% of the salt-responsive genes had unknown functions and, furthermore, about 10% had no significant hit with GenBank, suggesting that we do not yet fully understand the molecular events taking place to adjust the metabolism to excess salt and that *P. euphratica* contains a number of unique salt-responsive genes. By in silico analysis, we identified eight Arabidopsis orthologs among the salt-responsive genes of Euphrat poplar that were silent during most stages of vegetative plant development, but some of them were activated during seed formation or germination (Supplemental Fig. S1). This suggests a role of these genes in osmotic adaptation. However, not all of the eight Arabidopsis genes showed the same responses to salt, drought, or osmotic stress as poplar genes, pointing to differences in stress regulation. Comparative analysis of salt stress and Arabidopsis transcriptomes also supported the idea that the regulation of some key genes may be different in salt-tolerant and salt-sensitive species (Gong et al., 2005; Wong et al., 2006), and our study is strong experimental evidence for this suggestion.

In our study, differences in regulation between poplar and Arabidopsis were particularly obvious for genes encoding a putative lipocalin-like protein (TIL), a lipid transfer protein, and SUS1. We found the lipocalin-like protein particularly intriguing, because it was not regulated in salt-stressed or osmotically stressed Arabidopsis (Kilian et al., 2007), whereas other members of this gene family are stress responsive, especially under low temperatures (Frenette Charron et al., 2005). Lipocalins are most likely localized in the plasma membrane (Kawamura and Uemura, 2003), bind small hydrophobic ligands, act as transporters, and protect plants from oxidative stress (Frenette Charron et al., 2002; Charron et al., 2008). We used loss-of-function mutants of Arabidopsis to test the involvement of TIL and SIS, a salt-regulated unknown gene in salt tolerance. Data mining showed that *SIS* expression was typically regulated by salt treatment (Kilian et al., 2007; Luo et al., 2009). We show that suppression of its expression renders Arabidopsis knockout mutants more salt susceptible. *Attil* mutants were also more salt susceptible than the wild type, although this gene was not identified in previous salt screens. Our results indicate that both AtTIL and AtSIS play roles in salt tolerance, probably by increasing the protection of membranes.

#### CONCLUSION

In this study, we report the molecular timetable of the early salt transcriptome of the salt-tolerant Euphrat poplar. Molecular chaperones, especially dehydrins and osmotin, which help the cell to keep their proteins in an active status, appear to be key factors for coping with elevated salinity and ionic stress. Together with Suc synthase, these two chaperones were the only overlapping salt-responsive genes in roots and leaves. Adjustment of cellular metabolism involved remodeling of protein and amino acid biosynthesis in both roots and leaves, albeit employing different genes. In roots, decreases in aquaporins occurred, which might have limited water loss. In leaves, flexible down-regulation and well-orchestrated adjustment of energy metabolism involving mitochondrial and photosynthetic genes were among the initial responses to salinity and not, as one might have expected, up-regulation of typical stress-related enzymes. Induction of known stress-associated genes occurred only at late stages in leaves, when leaves had accumulated excessive sodium concentrations. Roots and leaves perceived physiologically different stress situations, and accommodation obviously required the activation of different stress transcriptomes.

To identify the stress-specific genes within the salt transcriptome, the Arabidopsis orthologs of poplar genes were used for in silico analyses to extract genes that were silent during vegetative development. This strategy resulted in eight candidates (Suc synthase, osmotin, glyoxalase, extensin, seed storage/lipid transfer protein, homeodomain transcription factor AtHB-7, a lipocalin-like gene [*TIL*], and a gene with unknown functions

[SIS]), among which the lipocalin-like protein (TIL) was unique because it did not show early responses to salt or osmotic stress in *Arabidopsis* and SIS was unique because it was found in all salt experiments analyzed. Knockout mutants of *Arabidopsis* for TIL or SIS showed salt-sensitive phenotypes. In conclusion, the stress regulation of the salt-tolerant Euphrat poplar contains genes previously not known for functions in salt tolerance, such as *TIL* and *SIS*. In a wider context, these findings imply that salt-susceptible plants harbor genes important for salt tolerance that cannot be identified by conventional salt screens relying on differential gene expression but by comparison of sensitive and tolerant species.

## MATERIALS AND METHODS

### Plant Material

*Populus euphratica* (clone B2 from the Ein Avdat Valley in Israel; Hebrew University of Jerusalem) was multiplied by micropropagation (Leplé et al., 1992). Rooted plantlets were transferred to aerated hydroculture with Long Ashton nutrient solution (Hewitt and Smith, 1975) and grown for 3.5 months at 22°C, 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetically active radiation, with a photoperiod of 16 h of light. After reaching an average height of  $0.83 \pm 0.02$  m, the medium was supplemented with 150 mM NaCl. Roots and leaves of six plants per treatment were harvested after 0, 3, 6, 12, and 24 h of salt stress and of nonstress treatment for controls. Plants were removed from the nutrient solution, immersed for 10 s with the roots in distilled water, and divided into roots and shoots. Root and leaf tissues were immediately frozen in liquid nitrogen. During the experimental phase, light was continuously supplied to avoid interference of light/dark transitions. The experiment was performed three times corresponding to three biological replications.

### Ecophysiological Measurements

Chlorophyll fluorescence was measured by a pulse-modulated MINI-PAM (Walz) on adult leaves of stressed plants and controls under ambient light (Maxwell and Johnson, 2000) and used to calculate the actual yield of photochemistry:  $\Phi_{\text{light}} = (F_m' - F_o')/F_m'$ . A separate set of plants was used to measure the fluorescence of dark-adapted leaves to determine the maximum yield of photochemistry:  $\Phi_{\text{dark}} = (F_m - F_o)/F_m$ .

Water potential was determined on 30-cm-long main shoots of plants (measured from the apex) at the indicated time points using a Scholander pressure chamber (Scholander et al., 1965; Cochard et al., 2001).

To determine osmotic pressure, leaves and roots that had been shock frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  were thawed, and pressure sap was gained with a home-built pressing device. The sap was kept on ice and centrifuged (5 min, 5,000g), and the osmolality (osmol  $\text{kg}^{-1}$ ) of the supernatant was determined in a Cryo-osmometer (Osmomat 030; Ganotec). To convert differences in water potential into osmotic pressure, the following equation was used:  $1 \text{ osmol} = 0.00832 \times T_{\text{abs}} \text{ MPa}$ , where  $T_{\text{abs}} = 293.15 \text{ K}$ .

### Element Analysis

Plant tissues were dried at  $70^\circ\text{C}$  and subsequently digested by using the nitric acid pressure system according to Heinrichs et al. (1986). Quantification of elements was carried out by inductively coupled plasma-optical emission spectrometry (Spectro Analytical Instruments) at  $\lambda = 559 \text{ nm}$ .

To determine cation solute concentrations, the measured concentrations of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{Mg}^{2+}$  were summed up and expressed on the basis of the water content of the tissue (mM). The water content was determined as follows: (fresh mass – dry mass)/dry mass ( $\text{mL g}^{-1}$ ).

### Analysis of Leaf Metabolites by Gas Chromatography-Mass Spectrometry

The extraction protocol was modified from Roessner-Tunali et al. (2003). Briefly, frozen leaf tissue powder (100 mg dry mass) was extracted in 1.4 mL of

80% (v/v) aqueous methanol in a 2.0-mL microcentrifuge tube for 2 h by shaking (200 rpm) at ambient temperature. A total of 120  $\mu\text{L}$  of ribitol (0.2 mg  $\text{mL}^{-1}$  water) was added as an internal standard prior to incubation. The mixture was extracted for 15 min at  $70^\circ\text{C}$ . The extract was vigorously mixed with 1,500  $\mu\text{L}$  of water and subsequently centrifuged for 15 min at 4,000 rpm. Aliquots of the methanol/water supernatant (150  $\mu\text{L}$ ) were dried in vacuum overnight. The dry residue was modified for gas chromatography-mass spectrometry analysis according to Fiehn et al. (2000). Residues after reduction were redissolved and derivatized for 90 min at  $37^\circ\text{C}$  (in 60  $\mu\text{L}$  of 30 mg  $\text{mL}^{-1}$  methoxyamine hydrochloride in pyridine) followed by a 30-min treatment with 120  $\mu\text{L}$  of *N*-methyl-*N*-[trimethylsilyl]trifluoroacetamide at  $37^\circ\text{C}$ . A total of 10  $\mu\text{L}$  of a retention time standard mixture (0.029% [v/v] *n*-dodecane, *n*-pentadecane, *n*-nonadecane, *n*-docosane, *n*-octacosane, *n*-dotracontane, and *n*-hexatriacontane dissolved in pyridine) was added before trimethylsilylation. Sample volumes of 1  $\mu\text{L}$  were then injected onto the gas chromatograph column on a splitless mode.

The gas chromatography-mass spectrometry system was composed of a Pal autosampler (Ophir Analytic), a TRACE GC 2000 gas chromatograph, and a TRACE DSQ quadrupole mass spectrometer (Bargal Analytics). The mass spectrometer was tuned according to the manufacturer's recommendations. Gas chromatography was performed on a 30-m Rtx\_5Sil MS column with 0.25- $\mu\text{m}$  film thickness (Restek). The injection temperature was set at  $230^\circ\text{C}$ , the interface at  $250^\circ\text{C}$ , and the ion source adjusted to  $200^\circ\text{C}$ . Helium was used as the carrier gas at a flow rate of  $1 \text{ mL min}^{-1}$ . The analysis was performed under the following temperature program: 5 min of isothermal heating at  $70^\circ\text{C}$ , followed by a  $5^\circ\text{C min}^{-1}$  oven temperature ramp to  $350^\circ\text{C}$ , and a final 5-min heating at  $330^\circ\text{C}$ . The system was then temperature equilibrated for 1 min at  $70^\circ\text{C}$  before injection of the next sample. Mass spectra were recorded at two scans per second with a scanning range of 50 to 600 mass-to-charge ratio. Both chromatograms and mass spectra were evaluated using the XCALIBUR version 1.3 program (ThermoFinnigan). A retention time and mass spectral library for automatic peak quantification of metabolite derivatives was implemented within the XCALIBUR method format. Substances were identified by comparison with authentic standards, as described by Roessner-Tunali et al. (2003).

Identified peak areas were normalized based on the internal standard and dry weight and analyzed statistically with the Tukey multiple comparison algorithm ( $P = 0.05$ ) incorporated into the JMP statistical software (Statistics and Graphics Guide: Version 3; SAS Institute).

### Statistical Analyses of Physiological Data

Data are shown as means  $\pm$  SE. Six plants were analyzed per time point. Statistical analysis (ANOVA) was performed using Statgraphics (Centurion XV). Significant differences at  $P \leq 0.05$  were obtained after ANOVA by a least significant difference test. Asterisks in figures are as follows: \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ .

### Microarray Analyses

RNA was isolated from *P. euphratica* leaves or roots using the method described by Chang et al. (1993). RNA samples from three biological replicates were used; each of the independent biological samples consisted of leaves or roots from six plants. To avoid bias in the microarray evaluation as a consequence of dye-related differences in labeling efficiency and/or differences in recording fluorescence signals, dye labeling for each paired sample was reversed in two individual hybridizations on microarrays containing 7,662 different *Populus* ESTs, among them 6,320 ESTs from *P. euphratica*. The EST libraries were constructed from different tissues and stressed plants and used to produce microarrays on glass slides as described by Brosché et al. (2005). A total of six hybridizations per time point were obtained. The complete protocols for probe labeling and hybridization, normalized data, and raw data files are available from the ArrayExpress database ([www.ebi.ac.uk/arrayexpress/](http://www.ebi.ac.uk/arrayexpress/)) under the accessions of E-MEXP-2095. By the use of *P. euphratica* arrays, problems that may arise by low hybridization efficiencies caused by sequence divergence are avoided (Janz et al., 2010).

Images were analyzed in GenePixPro 5.1 (Axon Instruments). Visually bad spots or areas on the array and low-intensity spots were excluded. Low-intensity spots were determined as spots where less than 55% of the pixels had intensity above the background + 1 SD in either channel. Slides with hybridized microarrays were scanned sequentially for Cy3- and Cy5-labeled probes.

Raw expression data were normalized for sources of systematic variation using a normalization model based on Wolfinger et al. (2001), which is a modified version of the global ANOVA model of Kerr and Churchill (2001). The  $\log_2$ -transformed raw data are represented by  $y$  and subjected to the normalization model  $ygijk = \mu + Ti + Aj + Dk + (TA)ij + (DA)ij + \varepsilon gijk$ , where  $\mu$  represents an overall mean value,  $T$  the fixed treatment effect,  $A$  the random array effect,  $D$  the fixed dye effect,  $TA$  the random interaction effect of arrays and treatments,  $DA$  the random interaction effect of arrays and dyes, and  $\varepsilon$  the residual stochastic error. Normalization was carried out using SAS. For each gene  $g$ , the empirical residuals calculated by subtraction of the fitted effects from the observed  $ygijk$  were used as normalized data in the subsequent significance analysis with SAM (Tusher et al., 2001). The SAM algorithm was obtained as a Microsoft Excel Add-in from <http://www-stat.stanford.edu/~tibs/SAM/>. It uses the false discovery rate and  $q$  value method presented by Storey (2002).

SAM was used with the option "paired data," the  $\Delta$  value was chosen as the false discovery rate became 0, and the limit of fold change was set to 3. The fold changes in gene expression between the control and salt-treated samples for each harvesting time point were estimated after normalization as the ratio of the mean signal intensities.

Six arrays were used for each time point (except for root after 12 h, where two technical replicates were missing) and probed with samples of the three biological replications.

The normalization method described above led to homogenous array means of  $\log_2$ -transformed raw data, and not all effects in the normalization model could be tested because of missing degrees of freedom. Therefore, the simpler normalization model  $ygijk = \mu + Ti + Aj + Dk + \varepsilon gijk$  was tested. In this case, all effects were significant at the 5% error level, but array means were no longer homogenous after normalization. The sets of genes recognized as significant by the SAM algorithm for the normalization model with interactions were very similar to those based on the simpler normalization model. They differed negligibly in the number of significant genes, and they always were subsets of each other. Therefore, we always refer to the results based on the normalization model.

## Expression Analysis by Quantitative Real-Time PCR

The transcript levels of five genes, which showed significant changes in response to salt stress in the microarray experiments, were confirmed by quantitative real-time PCR. RNA was isolated according to the protocol of Chang et al. (1993). DNA was removed with DNase I (Fermentas), and first-strand cDNA was reverse transcribed from 5  $\mu\text{g}$  of RNA using reverse transcriptase (Fermentas) according to the manufacturer's recommendations. Gene-specific primers were designed by using the Primer3 software (Rozen and Skaletsky, 2000). The relative transcript abundance was detected by the iCycler using iQ SYBR Green Supermix (Bio-Rad). The tubulin amplicon was used as an internal control for normalization.

## Analysis of Arabidopsis T-DNA Insertion Lines

Arabidopsis (*Arabidopsis thaliana*) lines with T-DNA insertions in the genes At5g58070.1 (Salk\_136775 = *Attil1-1*, SALK\_150259 = *Attil1-2*) and At5g02020 (Salk\_146631 = *Atsis1-1*, Salk\_064028 = *Atsis1-2*) were obtained from the Nottingham Arabidopsis Stock Center. Seeds of the segregating T3 generation were sown on half-strength Murashige and Skoog medium supplemented with 50  $\mu\text{g mL}^{-1}$  kanamycin as the selectable marker. DNA of kanamycin-resistant plants was isolated with the DNeasy Kit (Qiagen) according to the protocol of the manufacturer. Homozygous lines were identified by PCR analysis using gene-specific primers (RP-TIL, LPTIL, RP-SIS, LP-SIS) and the left border primer LBa1 (Supplemental Table S4). PCR was performed with a 5-min initial denaturation step at 94°C and 40 cycles of 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C. PCR analysis revealed the T-DNA insertion into each target gene. Using the gene-specific primers exp\_RP and exp\_LP did not result in a PCR product, indicating that the selected lines *Attil1-1*, *Attil1-2*, *Atsis1-1*, and *Atsis2-1* were homozygous for the T-DNA insertion.

To test salt responses of soil-grown plants, Arabidopsis wild-type and T-DNA lines were germinated for 10 d on water-agar (16 h of light at 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetically active radiation, 25°C, 60% relative air humidity). Uniform seedlings were planted into soil (T25 Feinerde; Frühstorfer Erde) and grown in a climatized cabinet under short-day conditions (8 h light at 115  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetically active radiation/16 h of darkness, 20°C air temperature, 60% relative air humidity). After 10 d, the plants were

gradually exposed to NaCl (50 mM for 5 d, 100 mM for 5 d, and 200 mM subsequently) and harvested after 10 d of exposure to 200 mM NaCl.

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Genevestigator analyses (development, anatomy) of orthologs in Arabidopsis of *P. euphratica* salt-responsive genes in roots and leaves, respectively.

**Supplemental Figure S2.** Changes in protein content in response to salt in leaves and roots of *P. euphratica*.

**Supplemental Table S1.** Changes in metabolites in response to salt exposure.

**Supplemental Table S2.** Genes with significantly changed transcript abundance in salt-exposed compared with control roots of poplar.

**Supplemental Table S3.** Comparison of quantitative real-time PCR of selected genes with their fold changes on the microarrays, and primers used for quantitative real-time PCR.

**Supplemental Table S4.** Primers used for the analysis of Arabidopsis T-DNA insertion mutants of TIL and SIS.

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