

Differential Regulation of Sorbitol and Sucrose Loading into the Phloem of *Plantago major* in Response to Salt Stress¹[OA]

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Several plant families generate polyols, the reduced form of monosaccharides, as one of their primary photosynthetic products. Together with sucrose (Suc) or raffinose, these polyols are used for long-distance allocation of photosynthetically fixed carbon in the phloem. Many species from these families accumulate these polyols under salt or drought stress, and the underlying regulation of polyol biosynthetic or oxidizing enzymes has been studied in detail. Here, we present results on the differential regulation of genes that encode transport proteins involved in phloem loading with sorbitol and Suc under salt stress. In the Suc- and sorbitol-translocating species *Plantago major*, the mRNA levels of the vascular sorbitol transporters PmPLT1 and PmPLT2 are rapidly up-regulated in response to salt treatment. In contrast, mRNA levels for the phloem Suc transporter PmSUC2 stay constant during the initial phase of salt treatment and are down-regulated after 24 h of salt stress. This adaptation in phloem loading is paralleled by a down-regulation of mRNA levels for a predicted sorbitol dehydrogenase (PmSDH1) in the entire leaf and of mRNA levels for a predicted Suc phosphate synthase (PmSPS1) in the vasculature. Analyses of Suc and sorbitol concentrations in leaves, in enriched vascular tissue, and in phloem exudates of detached leaves revealed an accumulation of sorbitol and, to a lesser extent, of Suc within the leaves of salt-stressed plants, a reduced rate of phloem sap exudation after NaCl treatment, and an increased sorbitol-to-Suc ratio within the phloem sap. Thus, the up-regulation of *PmPLT1* and *PmPLT2* expression upon salt stress results in a preferred loading of sorbitol into the phloem of *P. major*.

Higher plant species from different families translocate Suc or raffinose together with one of three different linear polyols in their phloem (Zimmermann and Ziegler, 1975). These polyols are sorbitol [= glucitol; translocated in Rosaceae (e.g. sour cherry, *Prunus cerasus*) and in Plantaginaceae (e.g. *Plantago major*)], mannitol [translocated in Apiaceae (e.g. celery, *Apium graveolens*), in Oleaceae (e.g. olive, *Olea europaea*), and in Rubiaceae (e.g. coffee, *Coffea arabica*)], and dulcitol [= galactitol; translocated in Celastraceae (e.g. *Euonymus* spp.)]. Polyol translocation in the phloem provides several advantages, such as a more efficient use of carbon (Pharr et al., 1995b; Stoop et al., 1996), better protection against hydroxyl radicals (Smirnov and Cumbers, 1989; Shen et al., 1997), or improved boron mobility (based on the formation of boron/polyol complexes; Bellaloui et al., 1999; Lehto et al., 2004). In addition, linear polyols are efficient osmolytes, and their accumulation results in an improved tolerance to salinity

or drought stress (Everard et al., 1994; Stoop and Pharr, 1994; Hu et al., 2005; Rejskova et al., 2007). For several members of the genus *Plantago* (e.g. *P. coronopus*, *P. lanceolata*, *P. major*, *P. maritima*, *P. media*), an increase of sorbitol concentrations after salt treatment has been described (Ahmad et al., 1979; Lambers et al., 1981; Königshofer, 1983; Smekens and Tienderen, 2001).

Other plants, such as *Mesembryanthemum crystallinum*, do not translocate significant amounts of polyols in their phloem under normal, unstressed growth conditions. However, these plants can increase the phloem sap concentrations of cyclic polyols in response to NaCl treatment (myoinositol and ononitol; Nelson et al., 1998). As for linear polyols, it is assumed that myoinositol is accumulated to improve water uptake under unfavorable conditions (Adams et al., 1992) and/or to stabilize cellular proteins under high salt (Ghosh et al., 2001).

Linear polyols are synthesized from hexose-6-Phs by NADPH-dependent reductases and by polyol-phosphate phosphatases. Their oxidation is catalyzed by NAD⁺-dependent dehydrogenases. The influence of increased environmental salt concentrations on the expression of the respective genes has been studied in detail (Stoop and Pharr, 1994; Zamski et al., 2001), and expression of some of these genes was shown to increase the salinity tolerance of transgenic plants (Tarczynski et al., 1992, 1993; Hu et al., 2005).

In contrast, little is known about the regulation of polyol transporter genes under conditions of high salt.

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Since the identification of the first cDNA of a plant mannitol transporter from celery (*AgMAT1*; Noiraud et al., 2001), several cDNAs or genes have been cloned from other higher plant species, including sour cherry (Gao et al., 2003), *P. major* (Ramsperger-Gleixner et al., 2004), *Malus domestica* (Watari et al., 2004), and *Arabidopsis thaliana* (Klepek et al., 2005). For two *Plantago* polyol transporters (*PmPLT1* and *PmPLT2*), the cellular localization was determined and both proteins were localized to the phloem companion cells (Ramsperger-Gleixner et al., 2004). This localization and their functional characterization in yeast and *Xenopus laevis* oocytes (Ramsperger-Gleixner et al., 2004) suggest a role of *PmPLT1* and *PmPLT2* in sorbitol loading into the *Plantago* phloem. The possible physiological role of *PmPLT1* and *PmPLT2* (or of any other higher plant polyol transporter) in salt-stressed plants, however, has not been analyzed. A possible effect of NaCl treatment on phloem loading has been published for celery (Noiraud et al., 2000). Prolonged salt treatment (300 mM NaCl for 4 weeks) caused a strong reduction in *AgSUT1* mRNA levels. *AgSUT1* encodes a Suc transporter that is thought to be involved in phloem loading in celery (Noiraud et al., 2000). To our knowledge, a possible effect on mannitol loading has not been studied in celery.

In *M. crystallinum*, Chauhan et al. (2000) identified sequences of putative myoinositol transporters (*MITR1* and *MITR2*) and showed that their expression was up-regulated (in roots, stems, and leaves) or down-regulated (in seedlings) after treatment with NaCl. In the same paper, these authors published that homologous genes are also regulated by salt stress in the glycophyte *Arabidopsis*. Only recently one of these *Arabidopsis* transporters (*AtINT4*) was characterized as H⁺-inositol symporter (Schneider et al., 2006).

In this article, we report on the differential regulation by salt treatment of polyol and Suc transporter genes in the companion cells of *P. major* (*PmPLT1* and *PmPLT2*; Ramsperger-Gleixner et al., 2004; *PmSUC2*; Stadler et al., 1995). In addition, expression levels of selected sorbitol and Suc metabolic enzymes were studied, and analyses of sugar and sugar alcohol concentrations in the mesophyll, in the vascular tissue, and in phloem exudates of NaCl-stressed *Plantago* plants and of water-treated controls are presented. Finally, the cellular pattern of transporter gene expression was analyzed in salt-treated plants.

RESULTS

Analysis of Salt-Responsive Transporter Gene Expression on Macroarrays and by Reverse Transcription-PCR

Vascular bundles (up to 10 cm and longer) are easily and rapidly pulled out from *P. major* leaves and petioles due to the presence of an endodermis that surrounds the entire vascular tissue. The Casparian stripes

within this endodermis are ruptured during extraction of the bundles (Gahrtz et al., 1994), leaving only the tips of the vascular tissue (including the minor veins) in the remaining leaf tissue. The extracted bundles show a bicollateral phloem anatomy with a central xylem and an abaxial and an adaxial phloem (Gahrtz et al., 1994). Only recently, more than 5,800 ESTs were sequenced from a cDNA library generated from *Plantago* vascular tissue that had been isolated using this technique (Pommerrenig et al., 2006). Of these sequences, 150 ESTs represented mRNAs of 87 different transporter genes (Pommerrenig et al., 2006).

Many *Plantago* species, including *P. major*, are known to be salt tolerant. To detect possible changes in vascular-specific transporter gene expression after salt treatment, macroarrays with all transporter sequences (spotted in duplicate) were hybridized to radiolabeled probes derived from mRNAs that had been isolated from vascular tissue of salt-treated plants or from water-treated control plants ($n = 3$). The expression of only seven transporter genes turned out to be up-regulated in response to salt treatment, and only for three transporter genes a slight down-regulation was detected. Two of the up-regulated genes encoded amino acid permeases (2.7-fold and 2.9-fold up-regulated; data not shown), and one gene encoded a MATE-efflux transporter (2.5-fold up-regulated; data not shown). These three genes are not in the focus of this article and will not be discussed further.

Figure 1 demonstrates that based on macroarray analyses, the expression levels of *PmPLT1* (2.9-fold up-regulated), *PmPLT2* (3.3-fold up-regulated), *PmAQP1* (= *AQUAPORIN1*; accession no. AJ843991; 1.7-fold up-regulated), and *PmPMA2* (*H⁺-ATPase2*; accession no. AM392363; 4.7-fold up-regulated) increased under high salt. During the same treatment, however, no change in expression was seen for the Suc transporter gene *PmSUC2*. This indicates that (1) polyol transporters are regulated in a salt-responsive manner and that (2) polyol and Suc transport may be differentially regulated.

To independently confirm these array-derived data on the salt-dependent up-regulation of *PmPLT1* and *PmPLT2* after 24 h, quantitative reverse transcription (qRT)-PCRs were performed with mRNAs isolated from plant material that had been salt stressed for 24 or 48 h (Fig. 2A). In addition, RT-PCRs were performed with primers specific for *PmSUC2* and for *PmUBQ1* (ubiquitin = control). Macroarray analyses (data not shown) and PCR analyses (Pommerrenig et al., 2006) had demonstrated that *PmUBQ1* expression is not influenced by salt treatment. The data presented in Figure 2A confirmed the salt-responsive induction for *PmPLT1* and *PmPLT2* expression shown in Figure 1. Moreover, the data showed a down-regulation of *PmSUC2* mRNA levels 24 and 48 h after salt treatment that had not been detected on the macroarray (Fig. 1).

A possible salt-responsive regulation of gene expression at time points earlier than 24 h was studied with mRNAs isolated 2 or 6 h after treatment with 400 mM NaCl. In fact, increased mRNA levels were observed

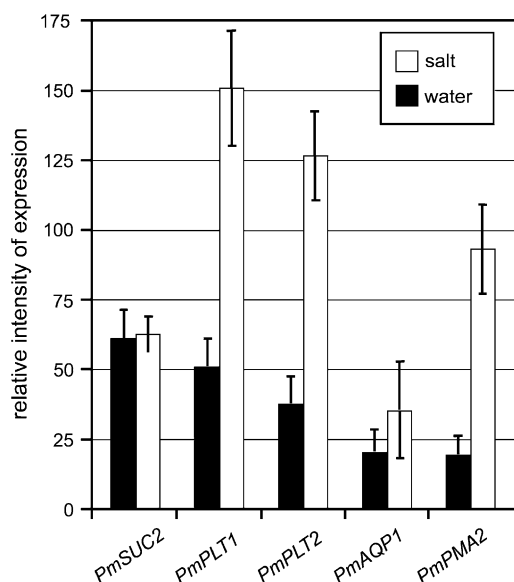


Figure 1. Macroarray analyses of the salt responsiveness of selected vascular transporter genes from *P. major*. Filters with PCR fragments of 87 *Plantago* transporter mRNAs were spotted on nylon filters and hybridized to radiolabeled probes generated from RNA of salt-treated *Plantago* or from water-treated control plants (plants had been watered with 40 mL of water or with 40 mL of 400 mM NaCl; vascular bundles were isolated after 24 h; $n = 3$). Only selected genes are presented.

already 2 h (*PmPLT2* only) and 6 h (both polyol transporter genes) after the salt treatment (Fig. 2B). However, at none of these time points was down-regulation of *PmSUC2* expression detected.

Quantification of Changes in Gene Expression

The observed changes in transporter mRNA levels were now quantified and compared between isolated vascular tissue and enriched mesophyll (= leaf blades from which the vascular bundles had been extracted). qRT-PCRs were performed with primers for *PmPLT1*, *PmPLT2*, and *PmSUC2* mRNAs and for mRNAs of SUCROSE PHOSPHATE SYNTHASE1 (*PmSPS1*; accession no. AJ843125) and SORBITOL DEHYDROGENASE1 (*PmSDH1*; accession no. AM393877). In Figure 3, relative mRNA levels are presented for control plants (0- and 10-h water treated) and for plants that had been treated with NaCl for 10 h. Moreover, absolute qRT-PCR values are given for FRUCTOSE-1,6-BISPHOSPHATE ALDOLASE1 (*PmFBPA1*; accession no. AM384903). As for *PmUBQ1*, macroarray analyses had shown constant expression for *PmFBPA1* in vascular and mesophyll tissue and in controls and salt-treated plants (data not shown). Due to its lower expression levels, *PmFBPA1* seemed to be a better control for qRT-PCRs than *PmUBQ1* (the names of some of the mRNAs studied in the presented RT-PCRs [*PmSPS1*, *PmSDH1*, *PmFBPA1*, and *PmUBQ1*] are not based on functional analyses of the respective proteins but rather were predicted from sequence homologies).

Figure 3 demonstrates very low *PmPLT1* mRNA levels in the enriched mesophyll and about 5-fold higher

PmPLT1 levels in the vasculature from 0-h control plants. During the 10-h water treatment, these mRNA levels remained constant. A 10-h treatment with NaCl, however, caused a 5-fold induction of *PmPLT1* expression that was restricted to the vascular tissue and that resulted in 25-fold higher *PmPLT1* levels in the vasculature than in the enriched mesophyll. An even stronger salt-dependent induction in the vasculature was observed for *PmPLT2* (about 15-fold; Fig. 3). Also, the relative transcript numbers of *PmPLT2* were higher than those of *PmPLT1* (10,000 versus 2,500). In contrast to *PmPLT1*, expression of *PmPLT2* was also induced in the enriched mesophyll fraction of salt-treated plants, resulting in only 5-fold higher *PmPLT2* levels

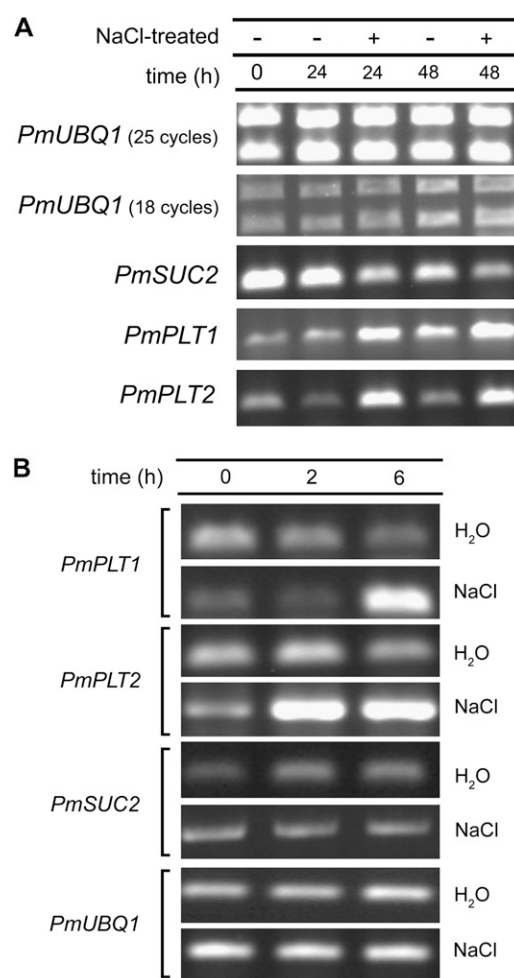
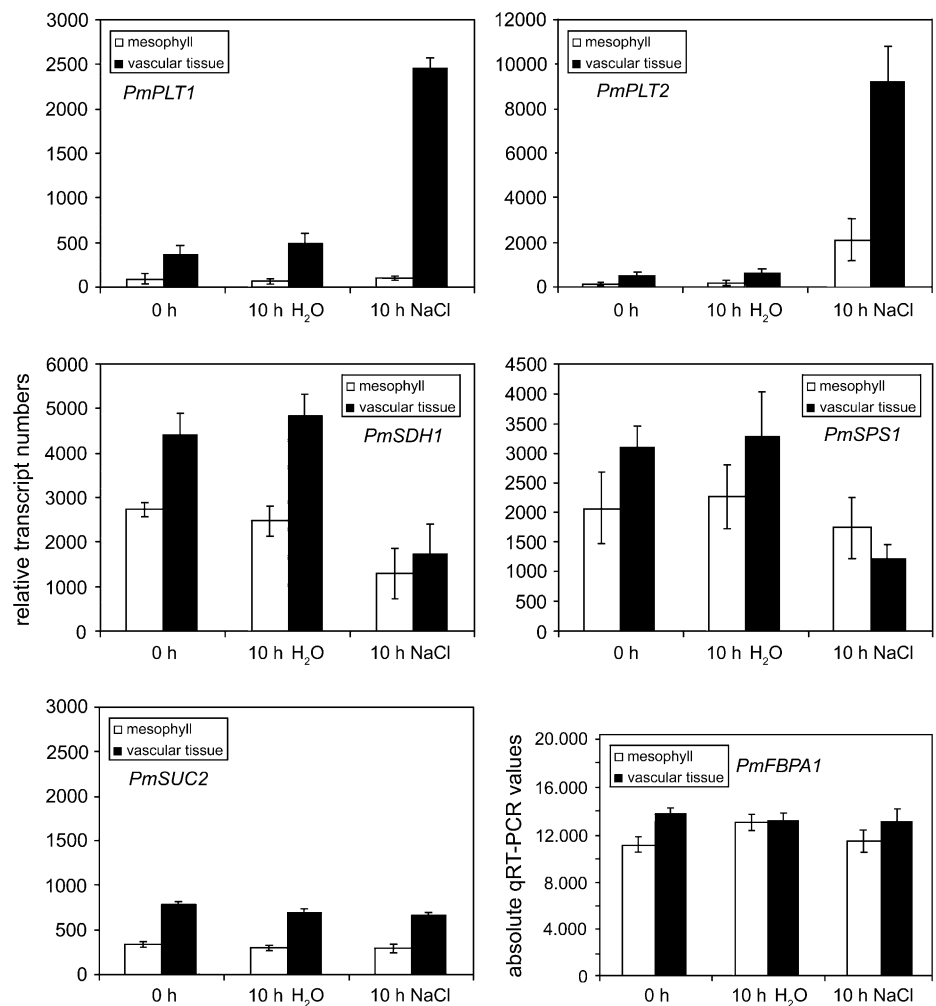


Figure 2. Analysis of the salt responsiveness of *PmPLT1*, *PmPLT2*, and *PmSUC2* by RT-PCR. A, *Plantago* plants (one plant per pot; approximately 320 mL soil volume) were watered either with 40 mL of water or with 40 mL of 400 mM NaCl. Total RNA was isolated from vascular bundles of 0-h control plants or of water-treated or salt-treated plants at the indicated times and used for RT-PCRs. Reactions for *PmUBQ1* were performed with 50% less template cDNA and with fewer cycles than the other reactions. B, *Plantago* plants were watered with water or with 400 mM NaCl and total RNA was isolated after the indicated times. For all RT-PCR reactions, 25 cycles were performed.

Figure 3. Analysis of the salt responsiveness of selected genes by qRT-PCR. Relative transcript numbers of the *Plantago* genes *PmPLT1*, *PmPLT2*, *PmSUC2*, *PmSPS1*, and *PmSDH1* were quantified by qRT-PCR as described in "Materials and Methods" ($n = 3$). Based on microarray analyses (not shown), *PmFBPA1* was selected as control that revealed almost identical expression levels in the vascular tissue and the mesophyll and that is not influenced by salt treatment. The presented *PmFBPA1* values show the absolute numbers of the qRT-PCRs as calculated from *PmTUA2* reference dilutions.



(vasculature versus enriched mesophyll). This increase in the mesophyll fraction is likely to reflect the strong induction of *PmPLT2* (Fig. 2B) in the minor veins that cannot be removed from this fraction. In the same experiment, the *PmSUC2* mRNA levels stayed constant and were not at all influenced by the NaCl treatment. These data confirmed the results of the RT-PCRs shown in Figure 2B.

Parallel analyses of the influence of increased NaCl concentrations on carbon metabolism revealed that *PmSDH1* and *PmSPS1* mRNA levels were down-regulated in response to high salt (Fig. 3). Interestingly, the reduction of the *PmSPS1* mRNA levels (about 3-fold) was restricted to the vasculature and no significant reduction was seen in the enriched mesophyll. In contrast, *PmSDH1* mRNA levels were reduced in the vascular tissue plus in the enriched mesophyll, pointing toward a more general reduction of sorbitol oxidation in the leaf by *PmSDH1*. The absolute qRT-PCR values for *PmFBPA1* are presented to demonstrate that expression of this gene is in fact not altered by NaCl or control treatments and that *PmFBPA1* levels can be used for reference.

Analysis of Sugar and Carbohydrate Levels in the Vasculature of NaCl-Treated and Control Plants

The combination of increased *PmPLT1* and *PmPLT2* expression in the vasculature after salt treatment (Figs. 2, A and B, and 3) and of an initially unchanged (Figs. 2B and 3) and later on reduced expression of *PmSUC2* (Fig. 2A) should result in a rise of sorbitol concentrations in the phloem and in increased sorbitol-to-Suc ratios in the vasculature. The reduced oxidation of sorbitol (putatively by *PmSDH1*) should support this effect.

To study possible changes in sorbitol and Suc concentrations, extracts were prepared from vascular tissue and from enriched mesophyll, and analyzed for Glc, Suc, and sorbitol contents by HPLC. The extracts were generated from leaf tissue of the very plants that had also been used for the qRT-PCR analyses shown in Figure 3. HPLC data from three independent analyses are presented in Figure 4.

As expected, sorbitol concentrations increased in the vasculature of NaCl-treated plants (more than 5-fold), whereas no change in sorbitol concentration was observed in the vasculature of water-treated control plants.

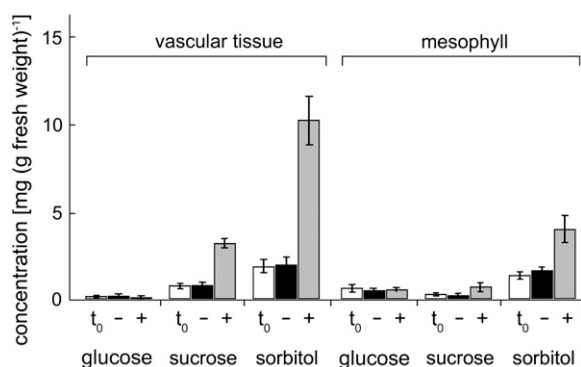


Figure 4. Analysis of sugar (Glc and Suc) and sorbitol concentrations in enriched vascular tissue and in vascular-depleted mesophyll of *Plantago* plants. Bars show the concentrations of the indicated sugar or sugar alcohol and represent the average of four independent analyses (\pm SD). Data are presented for 0-h (t_0) and 10-h (-) control plants and for 10-h NaCl-treated plants (+). For each analysis 100 mg of plant material was extracted and filtered, and the equivalent of 2 mg of fresh weight was analyzed by HPLC.

A smaller increase of sorbitol concentrations (about 2.5-fold) was also observed in the enriched mesophyll of NaCl-treated plants. This may point toward increased sorbitol biosynthesis and/or reduced sorbitol oxidation. Unexpectedly, a salt-responsive increase was also observed in vascular Suc concentrations (about 3.5-fold). Nevertheless, the sorbitol-to-Suc ratio in the vasculature increased during the 10-h NaCl treatment from about 2.0 in the water controls to 3.2. Under all conditions analyzed, no significant changes were observed for Glc, which was always higher in the mesophyll than in the vasculature.

Analysis of Sugar and Carbohydrate Levels in Phloem Exudates of NaCl-Treated and Control Plants

Suc, monosaccharide (Glc and Fru), and polyol (sorbitol or mannitol) concentrations have previously been determined in several subcellular compartments of polyol-translocating plants, including *P. major* (G. Lohaus, personal communication). In these analyses, Suc and polyol concentrations were determined in *P. major* phloem sap exuding from severed aphid stylets (*Myzus persicae*). In all analyses, cytosolic, vacuolar, and apoplastic sorbitol concentrations of the mesophyll were much higher than the Suc concentrations in the respective compartments. In contrast, Suc concentrations in the phloem sap exceeded those of sorbitol by a factor of 2 to 3 (G. Lohaus, personal communication).

This suggests that the data presented in Figure 4 (more sorbitol than Suc) do show primarily responses in sugar and sugar alcohol concentrations of nonphloem tissue, and an inverse ratio (more Suc than sorbitol) is expected for the phloem sap.

To test this possibility, we determined sorbitol, Suc, Glc, and Fru concentrations in phloem exudates of

NaCl-stressed plants and in water-treated control plants using the EDTA method as described previously (King and Zeevaert, 1974; Lejeune et al., 1988; Corbesier et al., 1998, 2003). Figure 5 demonstrates that, in fact, phloem exudates collected from leaves of water-treated *Plantago* showed high Suc and sorbitol contents with significantly higher Suc than sorbitol concentrations (Suc:sorbitol = 3.7). The concentrations of Glc and Fru in the collected exudates were low. In contrast, watering of the plants with 400 mM NaCl had two consequences. It resulted (1) in a reduced exudation (the total of Suc plus sorbitol is only about 50% of control plants) and (2) in an altered Suc-to-sorbitol ratio in the exudates.

These data demonstrate that the concentration ratios (Suc to sorbitol) in phloem exudates from *P. major* obtained with the EDTA method are inverse to those of intact mesophyll or vascular tissue (Fig. 4) and comparable to those described for phloem sap obtained with the aphid technique (G. Lohaus, personal communication). Moreover, they demonstrate a reduced Suc-to-sorbitol ratio (about 1.8) in the phloem exudates from leaves of NaCl-treated plants and support the up-regulation of sorbitol loading under salt stress that was predicted from the altered mRNA levels shown in Figure 2.

The results were confirmed in experiments where phloem exudates were collected for 240 min already 4 h after the NaCl treatment. However, at this early stage, phloem sap exudation from petioles of NaCl-treated plants was reduced to less than 20% (data not shown).

Localization of PmPLT1 and PmPLT2 Proteins in the Vasculature of NaCl-Treated *Plantago*

In a final approach, we wanted to see if the observed induction of *PmPLT1* and *PmPLT2* expression is restricted

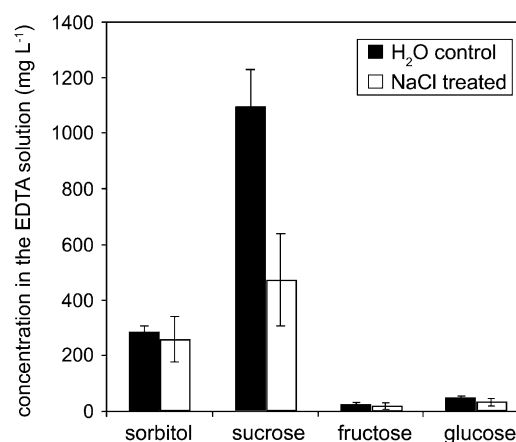


Figure 5. Analysis of sorbitol, Suc, Glc, and Fru concentrations in *Plantago* phloem exudates. *Plantago* plants were watered with 40 mL of water or with 40 mL of 400 mM NaCl. After 10 h, fully developed source leaves were detached from the rosettes (one leaf per treated plant), petioles were dipped into 750 μ L of EDTA solution (20 mM EDTA, pH 8.0), and phloem exudates were collected for 10 h. Carbohydrate concentrations in this solution are given ($n = 12$).

to the phloem companion cells, the only cell type that expresses *PmPLT1* and *PmPLT2* in the vasculature of unstressed *Plantago*. This had to be analyzed, as the observed up-regulation of *PmPLT1* and *PmPLT2* mRNA levels (Fig. 2) could also result from induction in other cells of the vasculature (e.g. phloem or xylem parenchyma). To discriminate between these possibilities, immunolocalization studies were performed with anti-*PmPLT1* and anti-*PmPLT2* antisera. These antisera had previously been used to confirm the companion cell-specific localization of both *Plantago* polyol transporters and are known to specifically recognize only *PmPLT1* or *PmPLT2* (Ramsperger-Gleixner et al., 2004).

Cross sections of leaves from *Plantago* plants that had been treated with 400 mM NaCl for 10 h were incubated with anti-*PmPLT1* or anti-*PmPLT2* antisera. Antibody binding was detected with anti-rabbit IgG-fluorescein isothiocyanate (FITC)-isomer 1-conjugate 2nd antibody. FITC fluorescence was visualized under a confocal laser-scanning microscope.

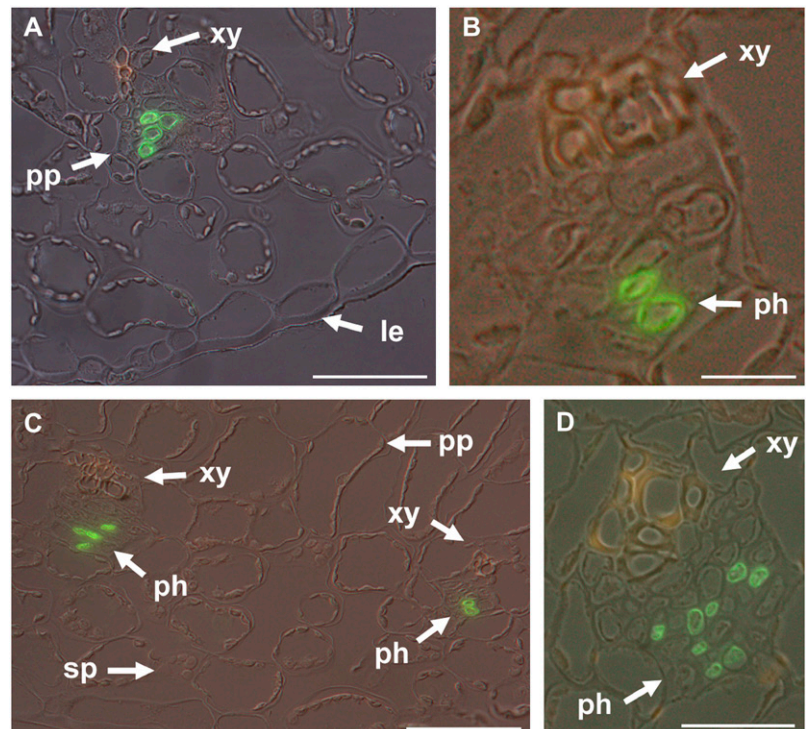
Two typical sections of anti-*PmPLT1*-labeled and of anti-*PmPLT2*-labeled vascular bundles are presented in Figure 6. The antisera label specifically only the phloem companion cells (anti-*PmPLT1*: Fig. 6, A and B; anti-*PmPLT2*: Fig. 6, C and D). This shows that salt-responsive induction of *PmPLT1* and *PmPLT2* expression is restricted to the same cells that mediate phloem loading with sorbitol also in unstressed *Plantago* plants, i.e. to the companion cells.

DISCUSSION

Sorbitol is an important component of the phloem sap in Plantaginaceae. For *P. major* and *P. maritima*, sorbitol concentrations in phloem sap collected from cut aphid stylets have previously been determined (*M. persicae* Sulz.; G. Lohaus, personal communication). The concentrations were between 400 and 500 mM for *P. major* and between 300 and 400 mM for *P. maritima*. These concentrations were significantly higher (40-fold to 80-fold) than those found in the leaf apoplast of the same plants (about 10 mM in *P. maritima* and about 6 mM in *P. major*; G. Lohaus, personal communication). This demonstrates that in both species sorbitol is loaded into the phloem by active transport systems. In *P. major*, the companion cell-localized sorbitol H⁺-symporters *PmPLT1* and *PmPLT2* are known to catalyze this loading step under normal, unstressed growth conditions (Ramsperger-Gleixner et al., 2004). Similarly, phloem loading of Suc in *P. major* is mediated by the companion cell-specific Suc H⁺-symporter *PmSUC2* (Stadler et al., 1995) that accumulates Suc in the phloem sap to concentrations that are more than 2,000-fold higher than in the apoplast (G. Lohaus, personal communication).

Ramsperger-Gleixner et al. (2004) had shown that in the smallest veins and during the early stages of *Plantago* vascular development, only *PmSUC2* is expressed, whereas expression of *PmPLT1* and *PmPLT2* is low or missing. Polyol transporter gene expression starts at a later developmental stage. In fact, this observation fits well to results obtained in celery by Davis

Figure 6. Immunolocalization of *PmPLT1* and *PmPLT2* in leaf sections from *Plantago* plants that had been salt treated for 10 h. A and B, Immunolocalization of *PmPLT1* in cross sections of *Plantago* leaf blades after 10 h of salt stress. A, Overview of a leaf section containing a minor vein. B, Single vein at higher magnification. C and D, Immunolocalization of *PmPLT2* in the cross section of a *Plantago* leaf after 10 h of salt stress. C, Overview of a leaf section containing two minor veins. D, Medium-sized vein at higher magnification. Green labeling shows detection of *PmPLT1* (A and B) or *PmPLT2* (C and D) with specific antibodies; yellowish staining of xylem vessels results from the autofluorescence of phenolic compounds (le = lower epidermis, ph = phloem, pp = palisade parenchyma, sp = spongy parenchyma, xy = xylem). Scale bars are 50 μ m in A, 10 μ m in B, 50 μ m in C, and 20 μ m in D.



et al. (1988), who showed that Suc is produced in all photosynthetically active cells, whereas mannitol is synthesized primarily in mature leaves.

***PmPLT1* and *PmPLT2* Expression Is Up-Regulated upon Salt Treatment**

This article describes a differential regulation of phloem loading with sorbitol and Suc in *P. major* plants that were exposed to salt stress. Based on macroarray and RT-PCR analyses, it is shown that expression of the two polyol transporter genes, *PmPLT1* and *PmPLT2*, is enhanced under conditions of salt stress. The observed salt-responsive induction of gene expression is rapid (Fig. 2B), and *PmPLT1* and *PmPLT2* mRNA levels stay at these elevated levels when the salt stress persists (Fig. 2A). This demonstrates that *Plantago* does adapt its carbon partitioning to changing environmental conditions by persistently enhancing polyol transporter gene expression.

Interestingly, the induction of *PmPLT1* and of *PmPLT2* differs in two important aspects. First, induction of *PmPLT2* is stronger than that of *PmPLT1* (15-fold versus 5-fold). Because the initial transcript numbers in control plants are similar for *PmPLT1* and *PmPLT2* (Fig. 3), this stronger induction results in significantly higher *PmPLT2* transcript numbers in salt-stressed plants. Secondly, *PmPLT2* induction is faster than that of *PmPLT1*. Up-regulation of *PmPLT2* is seen already after 2 h, at a time when *PmPLT1* mRNA levels have not yet responded (Fig. 2B). Taken together, these results suggest that *PmPLT2* may play a more important role in the phloem loading of salt-stressed plants than *PmPLT1*.

This interpretation is supported by the stronger induction of *PmPLT2* that is observed also in the enriched mesophyll fraction (Fig. 3). This fraction contains torn-off tips of the extracted bundles plus minor veins that are not extracted at all during the tissue preparation. A strong, salt-responsive induction of *PmPLT2* also in minor veins will, therefore, result in a visible increase of *PmPLT2* mRNA levels in the enriched mesophyll fraction. This increased expression, which is not detected for *PmPLT1* and may be taken as a measure for the "minor vein contamination" of the mesophyll fraction, underlines the primary role of *PmPLT2* in salt-induced phloem loading with sorbitol.

***PmSUC2* Expression Responds with a Delay and Is Reduced**

During the first hours after salt treatment (2 and 6 h in Fig. 2B and 10 h in Fig. 3), at a time when the stress signal has already reached the leaf and has caused elevated polyol transporter mRNA levels, expression of *PmSUC2* stays constant. Only 1 and 2 d after the start of the salt stress, however, *PmSUC2* mRNA levels start to decrease. This suggests that treatment with NaCl does not trigger an immediate active degradation of already existing *PmSUC2* mRNA. It rather seems that the activity of the *PmSUC2* promoter may be reduced

and that the observed slow reduction in *PmSUC2* mRNA results from the turnover of the protein.

A 30% to 50% down-regulation after salt treatment has already been described for the celery *AgSUT1* Suc transporter gene (Noiraud et al., 2000). However, in these analyses plants had been treated with 300 mM NaCl for 4 weeks and fast responses to NaCl have not been analyzed.

Treatment with NaCl Results in Increased Suc and Sorbitol Concentrations in *Plantago* Leaf Tissue

An increase in polyol concentrations in response to salt treatment has previously been described for mannitol in celery (Everard et al., 1992; Stoop and Pharr, 1994). Stoop and Pharr (1994) showed a negative correlation between the observed mannitol concentrations and the activity the mannitol dehydrogenase (MTD). Moreover, they demonstrated that mannitol accumulation in osmotically stressed celery is regulated by diminished catabolism. Finally, they reported that the activity of Suc-metabolizing enzymes (including SPS) changed only little in response to increasing salt concentrations. Pharr et al. (1995a) showed that regulation of *AgMTD* expression is an important control point in determining whether mannitol is to be used in metabolism for the production of carbon skeletons for assimilation and energy or whether it will accumulate for use as an osmoprotectant. The *AgMTD* protein of celery was analyzed in detail and described as a cytoplasmic protein that is localized in young, expanding leaves and in the cambium and phloem (companion cells and sieve elements) of the vascular tissue (Zamski et al., 1996). Little *AgMTD* was found in mature, fully developed leaves of celery.

Our analyses of the *PmSDH1* mRNA levels (Fig. 3) show a similar situation in *P. major*. The activity of the *PmSDH1* gene is reduced after salt treatment both in the mesophyll and in the vascular tissue, and the predicted reduction in sorbitol utilization is likely to contribute to the increased sorbitol concentrations in *Plantago* leaf tissue (mesophyll and intact vascular tissue; Fig. 4). Unexpectedly, however, in both tissues this increase in sorbitol concentrations is paralleled by a smaller but significant increase in Suc concentrations (Fig. 4). The observed reduction in *PmSPS1* mRNA levels (Fig. 3) makes it unlikely that this increase results from enhanced Suc biosynthesis. It rather seems to be the consequence of reduced assimilate export from NaCl-stressed leaves. In fact, a comparison of phloem exudates of water-treated plants and NaCl-treated plants (Fig. 5) demonstrates that exudation from leaves of salt-stressed plants is reduced to 50% (Suc plus sorbitol) of the controls. This may be explained by the massive osmotic stress within these leaves and by the reduced availability of water.

Treatment with NaCl Results in an Altered Suc-to-Sorbitol Ratio in the Phloem Exudate

A comparison of sorbitol and Suc concentrations in phloem exudates (Fig. 5) and in mesophyll or vascular

tissues (Fig. 4) revealed inverse Suc-to-sorbitol ratios. Whereas sorbitol is always higher than Suc in the mesophyll or in the complete vascular tissue (both in water- and NaCl-treated plants), Suc is the primary compound in phloem exudates. This is in agreement with data from aphid analyses (G. Lohaus, personal communication) that found higher sorbitol than Suc concentrations in *P. major* leaf cells and higher Suc than sorbitol concentrations in *P. major* phloem sap. It therefore demonstrates that results obtained from phloem exudates of *Plantago* by the EDTA method do reflect the solute composition of the phloem sap and are not blurred by solutes leaking from other wounded tissues.

Analyses of the relative percentages of Suc and sorbitol (Suc plus sorbitol = 100%) in phloem exudates from NaCl-treated and control plants show that the percentage of sorbitol doubles from about 20% in controls to almost 40% in salt-stressed plants (Fig. 5). Most importantly, this increase in the sorbitol-to-Suc ratio of the phloem exudate was seen already 10 h after the salt treatment, at a time when *PmPLT1* and *PmPLT2* mRNA levels were shown to be up-regulated (Figs. 1 and 2). This suggests a preferred loading of sorbitol into the phloem of NaCl-treated *P. major* plants by these transporters.

In combination with the rapid, salinity-dependent up-regulation of *PmPLT1* and *PmPLT2* mRNA levels (Fig. 2) and with the cell-specific immunodetection of the respective proteins in the companion cells of the *Plantago* phloem (Fig. 6, A–D), our data demonstrate that these H⁺-sorbitol transporters are responsible for the observed increase in sorbitol-to-Suc ratios in the *P. major* phloem sap after salt treatment.

Within the family of Plantaginaceae, there are other species that are even more tolerant to salt and drought than *P. major*. *P. maritima* and *P. coronopus*, for example, are growing along the splash zones of ocean coast lines and in arid areas, and for both species accumulation of sorbitol as an osmolyte has been described (Ahmad et al., 1979; Lambers et al., 1981). Maybe the observed capacity to quickly respond to environmental changes in salt concentrations or more general in water availability is a widely spread property within this plant family allowing the colonization of arid, hot, and salty habitats.

What Is the Nature of the Signal That Triggers the Observed Responses?

A central question in all studies of plant responses to increased salinity stress is that after the nature of the signal that is perceived by the plant and that triggers the observed responses. Since salinity reduces the ability of plants to take up water, responses observed after NaCl treatment may result either from water (osmotic) stress or from altered ion homeostasis (ionic stress). Several analyses suggested that rapid responses are more likely to be triggered by rapidly occurring changes in water relations (Passioura and Munns, 2000; Munns, 2002). Other analyses showed rapid induction of abscisic

acid biosynthetic genes both after ionic and osmotic stress, and, moreover, many salt- or drought-induced genes respond to treatments with abscisic acid and often even to cold (for review, see Zhu, 2002). Knowing this complex interplay of several pathways of stress signaling, it is not possible to make a prediction on the pathway (ionic or osmotic stress) that is responsible for *PmPLT1*, *PmPLT2*, and *PmSUC2* regulation without further analyses.

MATERIALS AND METHODS

Strains and Growth Conditions

Plantago major plants were grown under long-day conditions (16 h light and 8 h dark) at 20°C in growth chambers on potting soil. Plant material was harvested from 8-week-old plants before (0 h) and after (10 h) watering each pot with 50 mL of NaCl (400 mM) or with 50 mL of water. For all analyses at least two independent NaCl treatments and two independent water treatments were performed. Plant material was separated into vascular tissue and the remaining tissue (= enriched mesophyll). Vascular tissue was pulled out by hand ($t < 5$ s) and immediately transferred into liquid nitrogen. This material was used for both HPLC analysis and RNA isolation.

RNA Isolation, cDNA Synthesis, and Quantitative PCR

A total of 200 mg of *Plantago* vascular tissue or enriched mesophyll was used for RNA preparation. The tissue was homogenized under liquid nitrogen and total RNA was extracted using the Trizol reagent of Invitrogen. After treatment with DNase I, cDNA was synthesized from 2 µg of total RNA in a volume of 20 µL using the RevertAid H Minus First Strand cDNA synthesis kit of Fermentas.

From these reactions, 1 µL was used as template for PCRs with the following gene-specific primers: *PmSPS*-fwd (5'-AATGTGATCCCTGTCTGC-3') and *PmSPS*-rev (5'-CTCTTGCCAACACCCAATT-3') for amplification of *PmSPS1*, *PmSuc2*-fwd (5'-TCAGTGTCCATTGCTCTG-3') and *PmSuc2*-rev (5'-CCAGCCCACTCAGGTTCTT-3') for amplification of *PmSUC2*, *PmUdq1*-fwd (5'-AAGGAGGGTTATCCACCAGA-3') and *PmUdq1*-rev (5'-TCCATCAAAGTGTCAAACA-3') for amplification of *PmUBQ1*, *PmSDH1*-fwd (5'-AAGCATCGTTACGAGCCCTA-3') and *PmSDH1*-rev (5'-TTTTCGCTACTCCCTTTT-3') for amplification of *PmSDH1*, *PmPLT1*-fwd (5'-ACGAGGATCGACTCAGTGGT-3') and *PmPLT1*-rev (5'-GTCGGGATCGGGTCTATTA-3') for the amplification of *PmPLT1*, *PmPLT2c* + 924f (5'-CCACTCTTTCAGCAGGGTATC-3') and *PmPLT2c* + 1273r (5'-CTATAGTGCCCAATCCCATGA-3') for amplification of *PmPLT2*, and *PmAldP1*-fwd (5'-TCCAAGGGTTCAGAGT-3') and *PmAldP1*-rev (5'-TTCCTGCTGTTTTTG-3') for amplification of *PmFBPA1*. All obtained PCR fragments had a length between 250 bp and 300 bp.

Quantitative PCR was performed with a Rotor Gene 2000 (Corbett Life Science). Real-time PCR reactions were performed with the QuantiTect SYBR Green PCR kit from Qiagen.

Copies per reaction were calculated from the amplification of standard dilutions (ranging from 10¹–10⁶ copies per reaction) of a pBluescript SK- vector containing a *PmTUA2* cDNA (*TUBULIN-A 2*; accession no. AM392362; primers: *PmTUA*-5 5'-CATCTGCCGTAATCTTGATAT-3' and *PmTUA*-3 5'-AGCAACAGAAAGCTGCTCATGGTA-3'). *PmFBPA1* was revealed to be expressed equally in all cDNA samples. Average *PmFBPA1* copy numbers from 16 qRT-PCR runs were used to adjust the copy numbers for the other mRNAs. Three independent runs with duplicates were performed for each tested mRNA.

Collection of *Plantago* Phloem Exudate

For isolation of phloem exudates from *P. major* source leaves, plants (one plant per pot) were watered with 40 mL of 400 mM NaCl or with 40 mL of water and incubated for the indicated times under long-day conditions in a growth chamber. Leaf exudates were collected using the EDTA method (King and Zeevaart, 1974) as described previously by Corbesier et al. (2003) for

Arabidopsis (*Arabidopsis thaliana*). Briefly, after the NaCl or water treatments, leaves (one leaf per plant) were detached from the rosettes. After a second cut with a scalpel that removed between 0.5 cm and 1 cm of each petiole end while the petioles were submerged in 20 mM EDTA, pH 8.0, each leaf was placed immediately in a 1.5-mL reaction tube containing 400 μ L of 20 mM EDTA, pH 8.0. Tubes containing the detached leaves were incubated for the indicated times in an airtight chamber containing water-soaked paper at the bottom to ensure maximum humidity.

Macroarrays

For macroarray analyses, cDNA fragments, cloned into a pBluescript SK- vector, were used as templates for PCR amplification by oligonucleotides directed to flanking vector sites. Fragments were purified and spotted onto Hybond N⁺ nylon membranes (Pharmacia Amersham) in duplicate (Micro-Grid II; BioRobotics). Spotting efficiency was tested by hybridizing each array with ³³P-labeled oligonucleotide 5'-TAATACGACTCACTATAGGG-3' that recognized the flanking vector sequences of the PCR products.

For hybridizations, 200 ng of poly(A⁺) RNA, separated from total RNA with the Oligotex mRNA separation kit (Qiagen), were labeled with 2.1 MBq [α -³³P]dATP by oligo(dT)-primed RT using the RevertAid H Minus First Strand cDNA synthesis kit of Fermentas. Hybridizations and membrane washes were performed according to standard protocols. Data were collected from three independent biological replicates. Images were processed using ArrayVision software (Imaging Research). The intensity ratios between the signals of both arrays were normalized to account for systematic errors using the Lowess fitting (Yang et al., 2002). Differentially expressed genes were identified using the HT-self method (Vêncio and Koide, 2005), which combines the use of nonparametric estimation techniques with intensity-dependent cutoffs generated from self-selection experiments. This approach provides higher statistical credibility for low replication experiments. Genes with at least 65% of the replicate points outside the cutoff limits (defined with $P = 0.99$) were considered differentially expressed.

HPLC Analyses

For sample preparations ($n = 4$), 10 leaves were used from each NaCl or water treatment (two leaves from each of five different plants). Combined vascular tissues and combined enriched mesophyll were homogenized in liquid nitrogen in a mortar. One hundred milligrams of powdered tissue was used for ethanol extraction and HPLC analyses.

Sugars and sugar alcohols were extracted from the powdered material by incubation in 1 mL of 80% ethanol at 80°C for 4 h. After centrifugation at 20,800g for 15 min, 800 μ L of the ethanolic supernatant was transferred into a new tube and ethanol was completely evaporated under vacuum. Dry material was resolved in 500 μ L of double-distilled water, filtered through a sterile polystyrene filter, and analyzed with an ICS-3000 ion chromatography system from Dionex. Chromeleon Chromatography Management System (Dionex) was used for HPLC control and data analysis. A CarboPac Ma1 column (Dionex) was used for carbohydrate separation. Standard dilutions with known concentrations of sorbitol, Glc, and Suc (0.2 mg/L, 2 mg/L, 20 mg/L, and 200 mg/L) were used as references.

Immunohistochemistry

Sectioning, fixation, and embedding of plant material as well as the incubations with anti-PmPLT1 or anti-PmPLT2 antisera were performed essentially as described by Ramsperger-Gleixner et al. (2004). Binding of anti-PmPLT1 or anti-PmPLT2 antisera was detected with anti-rabbit IgG-FITC-isomer 1-conjugate 2nd antibody (Sigma-Aldrich).

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