

# Transcriptome analysis of root transporters reveals participation of multiple gene families in the response to cation stress

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## Summary

Plant nutrition critically depends on the activity of membrane transporters that translocate minerals from the soil into the plant and are responsible for their intra- and intercellular distribution. Most plant membrane transporters are encoded by multigene families whose members often exhibit overlapping expression patterns and a high degree of sequence homology. Furthermore, many inorganic nutrients are transported by more than one transporter family. These considerations, coupled with a large number of so-far non-annotated putative transporter genes, hamper our progress in understanding how the activity of specific transporters is integrated into a response to fluctuating conditions. We designed an oligonucleotide microarray representing 1096 *Arabidopsis* transporter genes and analysed the root transporter transcriptome over a 96-h period with respect to 80 mM NaCl, K<sup>+</sup> starvation and Ca<sup>2+</sup> starvation. Our data show that cation stress led to changes in transcript level of many genes across most transporter gene families. Analysis of transcriptionally modulated genes across all functional groups of transporters revealed families such as V-type ATPases and aquaporins that responded to all treatments, and families – which included putative non-selective cation channels for the NaCl treatment and metal transporters for Ca<sup>2+</sup> starvation conditions – that responded to specific ionic environments. Several gene families including primary pumps, antiporters and aquaporins were analysed in detail with respect to the mRNA levels of different isoforms during ion stress. Cluster analysis allowed identification of distinct expression profiles, and several novel putative regulatory motifs were discovered within sets of co-expressed genes.

**Keywords:** *Arabidopsis thaliana*, mineral nutrition, abiotic stress, expression profiling, microarray, membrane transporter.

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## Introduction

Plant mineral nutrition is crucial to plant growth and development and, as a consequence, to agriculture and human health. However, optimal plant growth is rarely achieved in non-agricultural settings because in most soils deficiency for one or more essential minerals leads to nutrient stress. High concentrations of inorganic ions can further compromise plant growth and development: well-known examples include salinity and the presence of toxic levels of heavy metals in soils.

Nevertheless, plants have developed flexible strategies to cope with fluctuations in their environment and thus to minimise the adverse effects of nutrient deficiency and excess toxic ions. Adaptive responses can include a significant alteration in gene expression, particularly of membrane transporters that are responsible for the uptake, efflux, translocation and sequestration of beneficial and toxic minerals.

For example, starvation of essential mineral nutrients results in enhanced uptake capacity, and this is reflected in enhanced transcript abundance for specific uptake systems (e.g. for  $K^+$ , *KUP3* of *Arabidopsis* (Kim *et al.*, 1998); for  $NH_4^+$ , *AMT1;1* of tomato (von Wiren *et al.*, 2000); for  $P_i$ , *PT1* and *PT2* of tomato (Liu *et al.*, 1998); for  $SO_4^{2-}$ , *Sultr1;1* and *Sultr2;1* of *Arabidopsis* (Yoshimoto *et al.*, 2002)). Conversely, for some transporters, gene expression is induced by the presence of substrate rather than de-repressed by its absence: examples include *AMT1;2* for  $NH_4^+$  uptake in tomato (von Wiren *et al.*, 2000) and *NRT2.1* and *NRT2.2* for  $NO_3^-$  uptake in *Arabidopsis* (Zhuo *et al.*, 1999). High, stress-inducing concentrations of some ions can also induce upregulation of transporters involved in efflux of the ion from the cytosol. Thus, 100 mM NaCl induces expression of a vacuolar  $Na^+/H^+$  antiporter in beet that is putatively involved in sequestration of cytosolic  $Na^+$  into the vacuolar lumen (Xia *et al.*, 2002).

Such studies have yielded valuable insights into the mechanisms that might underlie plant adaptation to nutrient availability, on one hand, and into effects of potentially toxic concentrations of ions on the other. Nevertheless, significant questions remain to be addressed. First, it is now apparent from expressed sequenced tag (EST) and genome sequencing projects that the vast majority of transporters in plants are encoded by multigene families whose members often exhibit apparently strongly overlapping expression patterns. Therefore, it is not immediately obvious which members of a family play a crucial role in the adaptive response to a particular ion. Second, and more extensively, many inorganic nutrients are transported by more than one transporter family, and in many cases, the roles of the respective transporters in adaptive responses are not clearly defined. In *Arabidopsis*, for example,  $K^+$  uptake can occur through members of the KUP/HAK/KT transporter family, as well as via KAT and AKT channel

families (Mäser *et al.*, 2001). The possibility also exists of  $K^+$  uptake via other uncharacterised transporters such as those belonging to the KEA family. Third, there remain some putative transporter families (e.g. the MATE family) where there is, currently, no information at all concerning the transported substrate. Fourth, outside the laboratory, plants experience multiple and complex combinations of nutrient supply. In this context, understanding how plants execute a coordinated response to fluctuating conditions becomes a near-impossible goal if studied at the level of single genes.

The availability of complete genome sequences allows many of these outstanding questions to be addressed through a global analysis of gene expression. For the analysis of genes encoding membrane transport systems, we developed an oligonucleotide-based microarray representing all annotated *Arabidopsis* membrane transporters and all non-annotated genes with six or more transmembrane spans.

The *Arabidopsis* membrane transporter (AMT) array was used to analyse the root transporter transcriptome with respect to  $K^+$ ,  $Ca^{2+}$  and  $Na^+$  stress.  $K^+$  and  $Ca^{2+}$  are essential macronutrients and normally the two most abundant cations in plants.  $K^+$  is essential for many metabolic processes and a major contributor to cell turgor, whereas  $Ca^{2+}$  plays a number of roles in stabilising cell walls and membranes and as a second messenger. By contrast,  $Na^+$  is normally a non-essential element that is toxic to many plant species when present in high concentrations. Physiological interactions between the three ions are well documented. For example,  $Na^+$  stress often results in deficiency of  $K^+$  because of the physicochemical similarities between  $Na^+$  and  $K^+$  (e.g. Maathuis and Amtmann, 1999). Thus,  $K^+$  uptake by KUP-type transporters is inhibited by  $Na^+$  (Santa-Maria *et al.*, 1997). In contrast, at low ambient  $K^+$ , low or moderate levels of  $Na^+$  actually promote plant growth (Maathuis *et al.*, 1996) by replacing  $K^+$  in its role as provider of turgor. In similar low  $K^+$  conditions,  $Na^+$  can stimulate high-affinity uptake of  $K^+$  (Spalding *et al.*, 1999), possibly through mechanisms that couple high-affinity  $K^+$  uptake to downhill  $Na^+$  transport, as in the case of HKT1 from wheat (Rubio *et al.*, 1995).

High concentrations of  $Na^+$  can also lead to  $Ca^{2+}$  deficiency by replacing cell wall- and membrane-bound  $Ca^{2+}$  and by reducing root pressure-driven  $Ca^{2+}$  translocation to the shoot. Conversely, elevated levels of ambient  $Ca^{2+}$  have long been known to relieve salinity stress (Marschner, 1995). The latter is believed to originate, at least in part, through a blocking effect of  $Ca^{2+}$  on non-selective ion channels that allow  $Na^+$  influx into the root (Demidchik *et al.*, 2002; Tyerman and Skerret, 1999). Elevated  $Ca^{2+}$  levels also stimulate net uptake of  $K^+$ , possibly by enhancing membrane integrity (Marschner, 1995).

With these multiple interactions in mind, we have deployed the AMT microarrays to investigate the effects

on the *Arabidopsis* transporter transcriptome of deprivation of the key nutrients K<sup>+</sup> and Ca<sup>2+</sup>, and of a high (80 mM) but sublethal concentration of Na<sup>+</sup>. The results yield a comprehensive picture of the transcriptional response of transport systems and serve as a basic platform for further characterisation of gene function and regulation.

**Results and discussion**

*Microarray design and data quality control*

The AMT array comprises 1250 oligonucleotide probes as 50-mers representing 1153 genes, of which 57 are controls. All probes are spotted in replicate on each array. A complete list of genes can be found in the Supplementary Material online. Table 1 summarises different types of transporters represented on the AMT array. Most genes have been assigned to labelled transporter families based either on functional characterisation or, more frequently, on the basis of sequence homology with characterised transporters. Genes in the group labelled 'Other transporters' are homologous with members of transporter families

characterised in non-plant systems, and genes in the group labelled 'Putative transporters' are represented on the array because their high number of predicted membrane-spanning domains indicated that these encode integral membrane proteins potentially mediating transport functions.

Importantly, the use of 50-mers rather than cDNAs allowed the design of gene-specific probes for nearly all genes represented on the array. In only eight cases, gene sequences were too similar to ensure specific hybridisation signals: At1g07810 and At1g07670 (*ECA1/4*, 95.6% homology), At3g28710 and At3g28715 (*VHA-d1/d2*, 99.9% homology), At2g16130 and At2g16120 (*STP31/32*, 92.7% homology), At5g43350 and At5g43370 (*PHT1.1/1.2*, 97.7% homology), At1g26730 and At1g35350 (putative proteins in the DASS family, 88.6% homology), At3g03700 and At3g04440 (hypothetical proteins, 99.8% homology), At1g18010 and At1g18000 (hypothetical proteins, 100% homology), and At4g37680 and At4g38320 (alternative splice forms of putative protein).

Most genes are represented on the AMT array by one probe. However, for 54 ABC transporters, two or three oligonucleotide probes were designed. In several cases, it was not possible to find more than one or two specific

**Table 1** Numbers and percentages of affected transcripts across transporter families represented on the AMT array

Gene family	Probes		Regulated*			Na-specific			Ca-specific			K-specific			Non-specific		
	No.	(%)	No.	(%)	%	No.	(%)	%	No.	(%)	%	No.	(%)	%	No.	(%)	%
ABC	226	(100)	96	(43)	<i>100</i>	23	(10)	24	48	(21)	50	5	(2)	5	20	(9)	21
P-type pump	47	(100)	27	(57)	<i>100</i>	6	(13)	22	11	(23)	41	0	(0)	0	10	(21)	37
V-type pump	32	(100)	22	<b>(69)</b>	<i>100</i>	3	(9)	14	5	(16)	22	0	(0)	0	14	(44)	<b>64</b>
PPase	3	(100)	2	<b>(67)</b>	<i>100</i>	0	(0)	0	0	(0)	0	0	(0)	0	2	(67)	<b>100</b>
Sugar transporter	67	(100)	36	(54)	<i>100</i>	10	(15)	28	16	(24)	44	1	(2)	3	9	(13)	25
Metal transporter	38	(100)	15	(40)	<i>100</i>	1	(3)	7	11	(29)	<b>73</b>	0	(0)	0	3	(8)	20
AA transporter	99	(100)	45	(46)	<i>100</i>	15	(15)	33	26	(26)	58	0	(0)	0	4	(4)	9
Peptide transporter	51	(100)	26	(51)	<i>100</i>	8	(16)	31	12	(24)	46	0	(0)	0	6	(12)	23
Auxin transporter	12	(100)	4	(33)	<i>100</i>	1	(8)	25	1	(8)	25	0	(0)	0	2	(17)	50
Ammonium transporter	6	(100)	3	(50)	<i>100</i>	0	(0)	0	0	(0)	0	2	(33)	<b>67</b>	1	(17)	33
Nitrate transporter	9	(100)	4	(44)	<i>100</i>	1	(11)	25	1	(11)	25	0	(0)	0	2	(22)	50
Phosphate transporter	17	(100)	8	(47)	<i>100</i>	0	(0)	0	4	(24)	50	0	(0)	0	4	(24)	50
Sulphate transporter	14	(100)	10	<b>(71)</b>	<i>100</i>	1	(7)	10	5	(36)	50	0	(0)	0	4	(29)	40
Other anion transporter	13	(100)	5	(39)	<i>100</i>	2	(15)	<b>40</b>	2	(15)	40	0	(0)	0	1	(8)	20
K, Cl, Ca channel	36	(100)	15	(42)	<i>100</i>	3	(8)	20	6	(17)	40	1	(3)	7	5	(14)	33
CNGC	20	(100)	11	(55)	<i>100</i>	5	(25)	<b>46</b>	3	(15)	27	0	(0)	0	3	(15)	27
Glutamate receptor	20	(100)	11	(55)	<i>100</i>	4	(20)	<b>36</b>	5	(25)	46	0	(0)	0	2	(10)	18
K transporter	20	(100)	3	<b>(15)</b>	<i>100</i>	0	(0)	0	3	(15)	<b>100</b>	0	(0)	0	0	(0)	0
Mg transporter	11	(100)	4	(36)	<i>100</i>	0	(0)	0	3	(27)	<b>75</b>	0	(0)	0	1	(9)	25
Antiporter	50	(100)	19	(38)	<i>100</i>	4	(8)	21	10	(20)	53	0	(0)	0	5	(10)	26
Aquaporin	38	(100)	30	<b>(79)</b>	<i>100</i>	3	(8)	10	6	(16)	20	0	(0)	0	21	(55)	<b>70</b>
Other transporter	137	(100)	70	(51)	<i>100</i>	10	(7)	14	43	(31)	61	1	(1)	1	16	(12)	23
Putative transporter	227	(100)	121	(53)	<i>100</i>	27	(12)	22	60	(26)	50	4	(2)	3	30	(13)	25
All	1193	(100)	587	<b>(49)</b>	<i>100</i>	127	(11)	<b>22</b>	281	(23)	<b>48</b>	14	(1)	<b>2</b>	165	(14)	<b>28</b>

Shown are numbers, % of family members (in brackets) and % of regulated family members (in italics). Gene families with significantly higher or lower than average number of regulated members are shown in bold.

\*Genes were considered 'regulated' when hybridisation signals showed greater than twofold difference between control and treatment, in both array replica spots, for at least one time point and treatment.

50-mer probes. Furthermore, subsequent data analysis revealed that, for many of the 54 genes, only one of the probes generated a significant signal, as judged by P-flagging (see Experimental procedures).

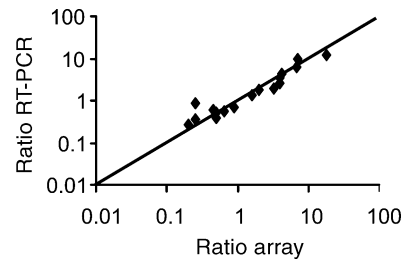
In general, the length of the probes ensured very good hybridisation signals that were highly reproducible, determined either within the array replica spots, or through replicated chips or through RT-PCR experiments (see below). Furthermore, hybridisation signals from our relatively long oligonucleotide probes are unlikely to be affected by single-base-pair mismatches (Kane *et al.*, 2000). This is an advantage because most sequence information originates from the *Arabidopsis* sequencing project and will contain errors. Thus, the AMT probes combine the benefits of high specificity with robustness against odd sequencing mistakes.

To evaluate results from the microarrays, 15 genes exhibiting microarray-reported expression ratios ranging from 0.1 to 10 were selected for independent evaluation by semiquantitative competitive PCR. The subset of genes was selected from different arrays and treatments, and PCR was performed on the same RNA extracts as those used for microarray hybridisation. Figure 1 shows that PCR analysis was in excellent agreement with results derived from array hybridisation with the exception of one gene for which the microarray and PCR data varied by a factor of around 4. These results show that a 50-mer microarray yields reliable gene expression data, as was previously shown by others (e.g. Ozturk *et al.*, 2002).

To test whether biological replicates yielded comparable results, we performed a triplicate experiment for one of the sampling times (5 h NaCl treatment). Assuming a  $\geq$ twofold change in signal ratio as a significant deviation from the median signal ratio, the overall reproducibility was around 70% for the three independent experiments, which included replicates made more than 2 months apart and at different geographical locations.

#### Physiological responses to cation stress

We applied three different conditions – 0 mM  $K^+$ , 0 mM  $Ca^{2+}$  and 80 mM NaCl – to hydroponically grown plants to test the response of transporter gene transcription for 96 h. Addition of NaCl and  $K^+$  deprivation did not lead to any visible damage of the plant tissues during the 4-day period. Prolonged  $Ca^{2+}$  starvation resulted in some mild necrosis at the leaf edges at the longest sampling time (96 h). Thus, variation in transcript level should be mainly because of the ionic composition of the growth medium rather than a general secondary stress response. Figure 2 shows that NaCl treatment led to a rapid increase in tissue  $Na^+$  content of both roots and shoots, whereas  $Ca^{2+}$  starvation induced a decline in shoot and root  $Ca^{2+}$  levels to around 10 and 1 mmol  $kg^{-1}$  FW, respectively. Interestingly, a 4-day period of exposure to a growth medium without added  $K^+$  hardly



**Figure 1.** Verification of array results with semiquantitative, competitive RT-PCR.

Semiquantitative RT-PCR was carried out on a subset of genes ( $n = 15$ ) represented on the AMT array. Transcript ratios determined from microarray hybridisation were plotted against those obtained from RT-PCR analysis. Genes were chosen to represent a wide range of ratios (between 0.1 and 10) obtained in various treatments. The line indicates perfect agreement between microarray and PCR experiments.

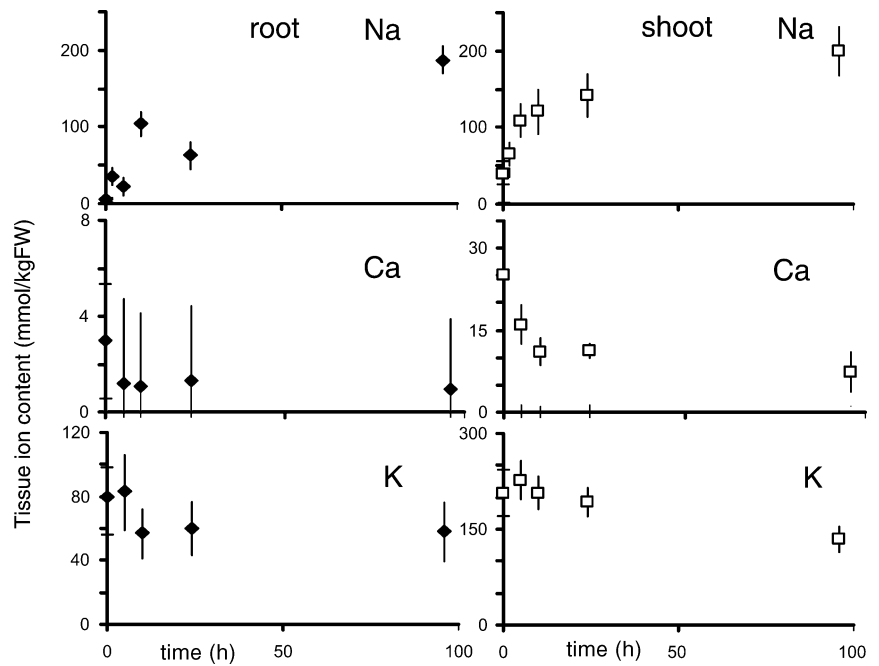
affected root  $K^+$  levels whereas shoot  $K^+$  dropped from around 220 to 150 mmol  $kg^{-1}$  FW. This observation is in contrast to findings by Pilot *et al.* (2003) who measured a drop of root  $K^+$  content by about 50% during  $K^+$  deprivation over a similar period. The main difference between the two studies is the presence (Pilot *et al.*, 2003) or absence (our study) of  $NH_4^+$  in the growth medium.  $NH_4^+$  blocks high-affinity  $K^+$  uptake systems, and its inhibitory effect on plant growth in low  $K^+$  media is well documented (Hirsch *et al.*, 1998; Santa-Maria *et al.*, 2000; Spalding *et al.*, 1999).

Plant exposure to NaCl (80 mM) led to a rapid reduction in tissue water content during the initial phase of the treatment. However, Figure 3 demonstrates that this phenomenon was reversed subsequently during a period of osmotic adjustment. Neither  $K^+$  nor  $Ca^{2+}$  starvation resulted in a significant effect on plant water status (Figure 3).

#### Cation stress affects specific genes in many transporter families

For hybridisation with the AMT array, RNA was extracted at different times during cation stress (2–96 h). To assess the response to  $K^+$ , additional samples were taken after re-supply of  $K^+$  following starvation (see Experimental procedures). In total, transcripts from 32 samples were analysed. Figure 4 shows how the different treatments affected mRNA levels within the total set of AMT genes. Any gene probe that showed a  $\geq$ twofold difference between control and treatment, in both replica spots and for at least one time point, was included in the Venn diagram. A total of 587 genes (49%) out of 1193 met these criteria. Among these, 127 (22%) genes responded specifically to salt stress, 281 (48%) to  $Ca^{2+}$  starvation and 14 (2%) to  $K^+$  starvation. In total, 165 genes (28% of responsive genes) responded to more than one treatment. Protein accession numbers and numerical expression data for all genes in these groups can be found in the Supplementary Material online.

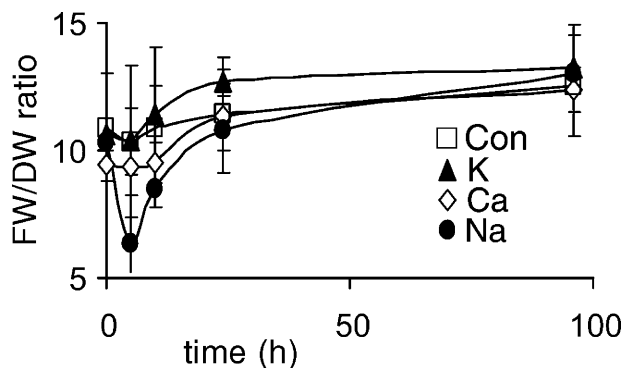
**Figure 2.** Ion contents of root (left,  $\blacklozenge$ ) and shoot (right,  $\square$ ) tissue of plants subjected to NaCl (80 mM, top panel),  $\text{Ca}^{2+}$  deprivation (middle panel) or  $\text{K}^+$  deprivation (lower panel). Ion contents are expressed as  $\text{mmol kg}^{-1}$  FW. Note differences in scaling between root and shoot data for Ca and K.



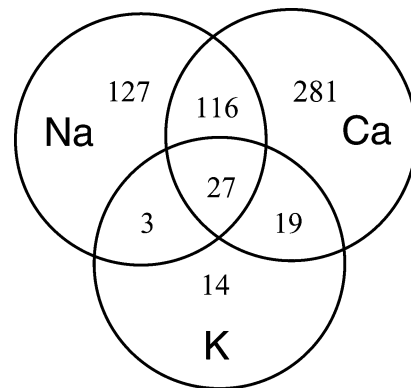
For those genes that responded to cation stress, Table 1 provides an overview of their distribution across functional categories. For most transporter families, the ratio of regulated/non-regulated genes reflected that for the entire set of genes (49%). However, V-type ATPase subunits and aquaporins produced a much higher than average percentage of regulated genes (79 and 69%, respectively). Sulphate transporters were also strongly regulated (71%), whereas regulation amongst  $\text{K}^+$ -transporters of the KUP/HAK family was less than average (15%). Separate analysis of treatment-specific and non-specific genes revealed that both V-type pumps and aquaporins were highly represented in the group of non-specifically responding genes. Thus, V-type pumps and aquaporins were strongly regulated by all three treatments. Proteins encoded by these families are likely to participate in many aspects of cellular homeostasis explain-

ing why their expression levels may alter frequently and in response to many different conditions.

Further tendencies became apparent for other gene families (marked in bold). For example, relatively high numbers of CNGC, glutamate receptor and anion transporter genes were specifically affected by NaCl treatment. It has been postulated that CNGCs and glutamate receptors contribute to  $\text{Na}^+$  uptake in their capacity of non-selective cation channels (Demidchik *et al.*, 2002; Kim *et al.*, 2001; Talke *et al.*, 2003). Thus, their transcriptional regulation might be part of an essential response to salinity stress. Little is known about the mechanism of  $\text{Cl}^-$  uptake during salt stress, but it is likely to involve  $\text{H}^+$ -coupled symport systems (Sanders, 1994) that might be subsumed within the



**Figure 3.** Water status expressed as the FW/DW ratio of control plants ( $\square$ ) and plants subjected to NaCl (80 mM,  $\bullet$ ),  $\text{Ca}^{2+}$  deprivation ( $\diamond$ ) or  $\text{K}^+$  deprivation ( $\blacktriangle$ ).



**Figure 4.** Venn diagram of AMT genes regulated by  $\text{Na}^+$ ,  $\text{Ca}^{2+}$  and  $\text{K}^+$  treatments.

Numbers are based on oligonucleotide probes and relate to more than twofold changes of signal in both replica spots for at least one time point of the respective treatment(s).

(mostly non-characterised) family of anion transporters. The large family of metal transporters (around 40 genes) showed a large proportion of probes responsive to  $\text{Ca}^{2+}$  starvation, suggesting a close relationship between transport of  $\text{Ca}^{2+}$  and that of other metal ions.

#### *Few genes respond to $\text{K}^+$ starvation*

A most striking result was that very few transporter genes responded to  $\text{K}^+$  starvation. The reasons for this are not entirely clear but might be related to the fact that ammonium was lacking from the medium (see above). Tissue analysis shows that the  $\text{K}^+$  content of root tissue was not significantly affected during the 4-day treatment, but that  $\text{K}^+$  levels in shoot tissue declined from around 200 to 150 mmol  $\text{kg}^{-1}$  FW (Figure 2). It can therefore be anticipated that at least some  $\text{K}^+$  redistribution at both the tissue (Marschner *et al.*, 1997) and the subcellular level (Walker *et al.*, 1996) took place. Consequently, regulation of  $\text{K}^+$  transporter activity would be expected. However, our results could indicate that the majority of  $\text{K}^+$  transporters in *Arabidopsis* are regulated post-transcriptionally rather than at the transcript level. Indeed, very few *Arabidopsis* genes encoding  $\text{K}^+$  channels and transporters have been reported to be regulated by external  $\text{K}^+$ , although many of such genes are induced or repressed by other stresses and hormones (Pilot *et al.*, 2003) or in other plant species (Uozumi *et al.*, 2000; Wang *et al.*, 1998).

The stelar outward rectifier SKOR was the only  $\text{K}^+$  channel to be affected by our  $\text{K}^+$  treatment. SKOR is thought to be involved in delivery of  $\text{K}^+$  to the shoot (Gaymard *et al.*, 1998), and the observed downregulation of SKOR during  $\text{K}^+$  starvation might be a means of retaining  $\text{K}^+$  in the root in these conditions. Similar results have been obtained by Pilot *et al.* (2003). More surprisingly, none of the members of the KUP/HAK/KT family exhibited  $\text{K}^+$ -induced changes in transcript abundance. KUP3 transcript was previously shown to be de-repressed during  $\text{K}^+$  starvation (Kim *et al.*, 1998). We confirmed upregulation of KUP3 transcript in  $\text{K}^+$ -starved seedlings with the AMT array (P. Armengaud and A. Amtmann, unpublished results), but this response was absent in roots of mature plants. Loss-of-function mutations in other KUP transporters (*trh1 = KUP4*, Rigas *et al.*, 2001; and *shy3 = KUP2*, Elumalai *et al.*, 2002) produced perturbations in cell growth but had no significant effect on  $\text{K}^+$  homeostasis, indicating that KUP-type transporters could be primarily involved in turgor-driven growth rather than in  $\text{K}^+$  nutrition *per se*. A function of KUP-type transporters in apical growth, which is  $\text{Ca}^{2+}$  dependent (Sanders *et al.*, 2002), might explain why several KUP genes were specifically regulated by  $\text{Ca}^{2+}$  starvation (Table 1).

Although the overall number of genes affected by  $\text{K}^+$  was too low to analyse with respect to their distribution across gene families, it is noteworthy that two out of three regu-

lated ammonium transporter genes specifically responded to  $\text{K}^+$  treatments. Interactions between ammonium and  $\text{K}^+$  nutrition have been reported (e.g. Santa-Maria *et al.*, 2000), but those studies tended to focus on the impact of ammonium nutrition on  $\text{K}^+$  transport. Our results suggest that the converse might also occur, i.e. an effect of  $\text{K}^+$  nutrition on ammonium transporters.

When  $\text{K}^+$  was re-supplied after 24 h, all genes that had previously changed transcript levels during  $\text{K}^+$  starvation returned to control levels. By contrast, when  $\text{K}^+$  was re-supplied after 96 h, only 50% of the genes affected by  $\text{K}^+$  starvation returned to control levels within 5 h. Thus, either longer starvation resulted in irreversible change of transcript level or reversal to control levels took longer than 5 h. Transcript levels of 17 genes that had not responded to  $\text{K}^+$  starvation, were significantly changed after re-supply at 96 h. These were mostly uncharacterised genes (among others, four members of the MATE family, but also including five ABC transporters, two sugar transporters, one ammonium transporter and one aquaporin). We suggest that the role of these genes is linked to the recovery of the plant from secondary effects of long-term  $\text{K}^+$  starvation.

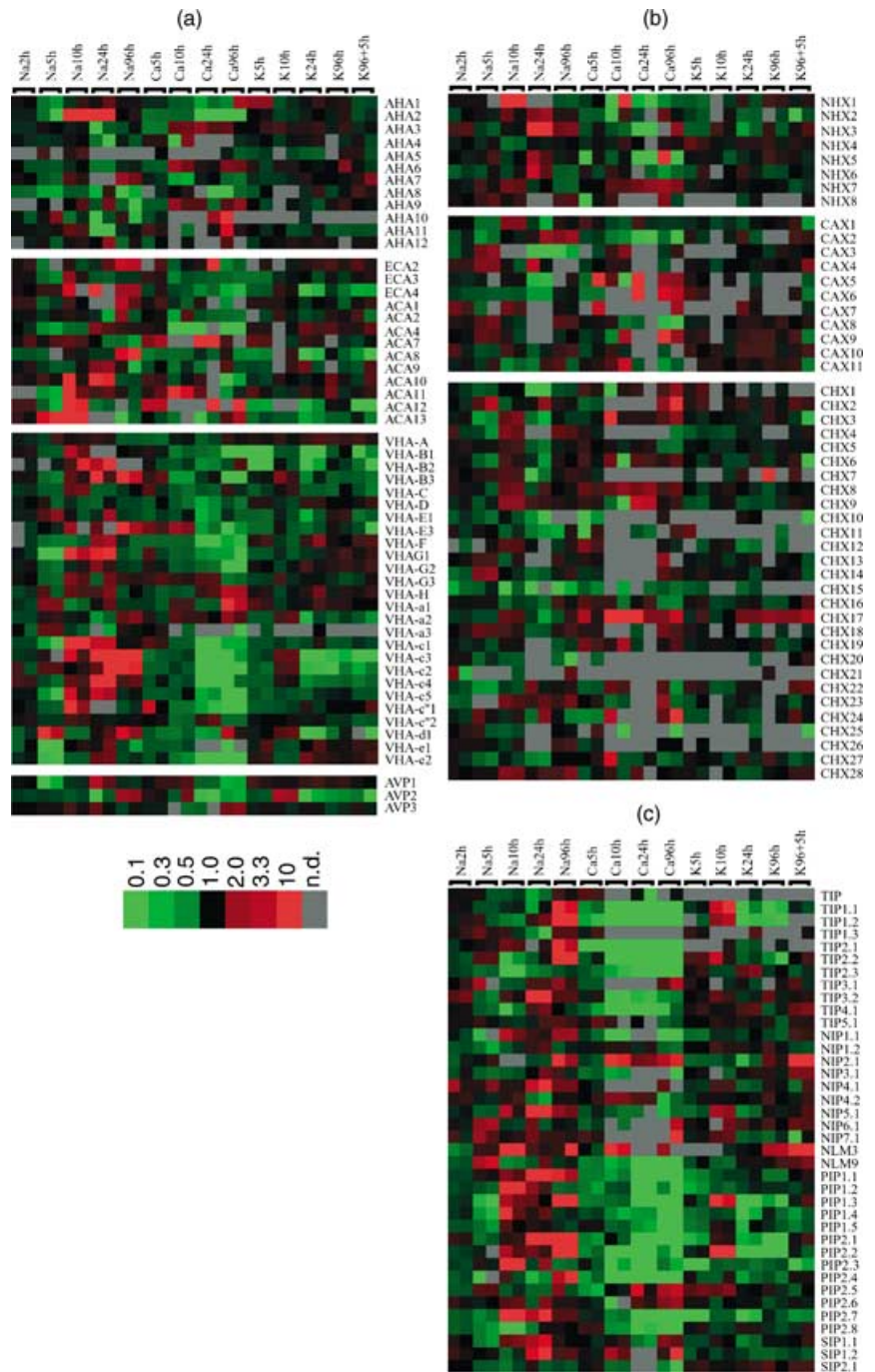
#### *Cation stress evokes specific and non-specific responses in the transcripts of primary pumps*

Many plant transport processes are directly or indirectly energised by the proton motive force (PMF) that is maintained across membranes by  $\text{H}^+$ -pumping ATPases. At the plasma membrane, P-type ATPases encoded by the *AHA* gene family establish a PMF, which can energise the uptake of nutrients including  $\text{K}^+$  via  $\text{H}^+$  symport (Maathuis and Sanders, 1994) and the extrusion of  $\text{Na}^+$  (Apse *et al.*, 1999) and  $\text{Ca}^{2+}$  (Kasai and Muto, 1990) via  $\text{H}^+$  antiport. A second class of P-type ATPase, encoded by the *ACA* and *ECA* gene families, functions in ATP-driven  $\text{Ca}^{2+}$  efflux pumping at plasma and endomembranes to maintain cytoplasmic  $\text{Ca}^{2+}$  concentrations in the nanomolar range (Geisler *et al.*, 2000). V-type  $\text{H}^+$ -pumping ATPases (*VHA* gene family) are major contributors to the acidification of the vacuolar compartment (Ratajczak, 2000) and, alongside  $\text{H}^+$ -pumping pyrophosphatases (*AVP* family), generate a PMF across the vacuolar and organellar membranes. Secondary  $\text{H}^+$ -driven mechanisms at these membranes participate in organellar compartmentation of  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Na}^+$  and other ions (Maeshima, 2000).

Figure 5a shows transcriptional modulation of these primary pumps. Their expression patterns show some general characteristics also observed for other transporters (see below): (i) little transcriptional regulation occurs during the first 2–5 h for all three treatments; (ii) considerably more variation in expression is observed in response to NaCl application and  $\text{Ca}^{2+}$  starvation compared with  $\text{K}^+$  deprivation and (iii) very few genes show similar expression profiles for the three treatments.

**Figure 5.** Transcriptional modulation of genes encoding primary pumps, cation transporters and aquaporins in response to NaCl (80 mM), Ca<sup>2+</sup> starvation and K<sup>+</sup> starvation/re-supply. Results for both replica spots on the array are given for each time point.

(a) Transcriptional regulation of plasma membrane H<sup>+</sup>-ATPases (AHA family), Ca<sup>2+</sup>-ATPases (ACA/ECA family) and tonoplast V-ATPases (VHA) and PPases (AVP).  
 (b) Transcriptional regulation of plasma membrane and tonoplast Na<sup>+</sup>:H<sup>+</sup> antiporters (NHX family), Ca<sup>2+</sup>:H<sup>+</sup> antiporters (CAX family) and general cation:H<sup>+</sup> antiporters (CHX family).  
 (c) Transcriptional regulation of plasma membrane and tonoplast aquaporins (PIP/NIP/NLM/TIP and SIP subfamilies).



The root epidermis will be among the first tissues to sense alterations in the surrounding growth medium. Interestingly, the only isoform belonging to the AHA H<sup>+</sup>-ATPases that was consistently modulated in its expression level was *AHA2*, which is mainly expressed in the root epidermis (Palmgren, 2001). After an initial moderate downregulation, expression of *AHA2* rose to over 10 times the control level during the middle stages of salt treatment, and returned to control levels after 4 days. *AHA2* is likely to play a crucial role in energising Na<sup>+</sup> extrusion at the root-

soil boundary by providing the necessary PMF to drive Na<sup>+</sup>:H<sup>+</sup> antiport. The same isoform is significantly down-regulated when plants are exposed to Ca<sup>2+</sup>-deficient growth medium. The significance of this finding is unclear, but the observation fits into a general pattern of down-regulation of transporter gene expression in response to Ca<sup>2+</sup> deficiency. No significant alteration in *AHA4* transcript was found during either of the applied conditions. *AHA4* is predominantly expressed in the root endodermis and flowers (Vitart *et al.*, 2001), and a disruption of *AHA4* activity led

to increased salt sensitivity, possibly through a dominant negative effect on the activity of other pumps.

Among  $\text{Ca}^{2+}$  pumps, there is, most notably, a general downregulation of expression during  $\text{Ca}^{2+}$  starvation. This response applies to both the ECA and the ACA subfamilies, and is independent of membrane location: ECA1 (formerly known as ACA3) is located at the ER (Liang *et al.*, 1997) as is ACA2 (Hong *et al.*, 1999), and transcripts of both pumps exhibited marked downregulation after 10 h of treatment (note, however, that the ECA1 probe also recognises ECA4; see above). Similarly, transcript levels of *ACA8* (plasma membrane localised; Bonza *et al.*, 2000) and *ACA4* (small vacuoles; Geisler *et al.*, 2000) are both heavily attenuated in the absence of external  $\text{Ca}^{2+}$ . These responses can be interpreted as reflective of the requirement for cytosolic  $\text{Ca}^{2+}$  homeostasis during  $\text{Ca}^{2+}$  starvation, with a reduced requirement to remove  $\text{Ca}^{2+}$  from the cytosol if the external supply is withdrawn. It is interesting that *ACA12* mRNA is rapidly and strongly decreased in response to  $\text{K}^+$  deprivation and *ACA13* is upregulated by NaCl stress: neither the membrane locations nor the functions of these putative  $\text{Ca}^{2+}$  pumps have yet been described, but the observation might point to an involvement in some aspect of  $\text{K}^+$  nutrition.

The *Arabidopsis* genome contains around 30 annotated and putative V-type ATPase subunits (Sze *et al.*, 2002) and three genes encoding PPases. A primary role of these pumps is thought to be the energisation of the tonoplast to mediate  $\text{H}^+$ -coupled transport of ions into the vacuole. Figure 5a shows that many of V-ATPase subunits are transcriptionally regulated in response to cation stress (see also Table 1). During salt treatment, tonoplast energisation is necessary to drive  $\text{Na}^+:\text{H}^+$  antiport-mediated sequestration of  $\text{Na}^+$  in the vacuole. Early biochemical studies (e.g. Braun *et al.*, 1986) showed increased V-ATPase activity in salinised plants, and later studies showed that the basis for increased activity was, at least in part, the result of augmented transcription (Niu *et al.*, 1993). This pattern is reflected in *Arabidopsis* where the majority of subunits from both V1 (soluble: upper case gene name) and V0 (integral membrane: lower case gene name) sectors are upregulated in response to salt, particularly in the later stages (24–96 h) of the treatment. Remarkably, transcripts of the catalytic ATP-binding subunit (*VHA-A*) do not appear to be affected by any of the applied conditions, suggesting differential mRNA turnover for the various subunits.

In conditions of ample nutrient supply, large quantities of  $\text{K}^+$  and  $\text{Ca}^{2+}$  are accumulated in the vacuole (Sanders *et al.*, 2002; Walker *et al.*, 1996), probably through the action of  $\text{K}^+:\text{H}^+$  and  $\text{Ca}^{2+}:\text{H}^+$  antiport. We observed a downregulation of several V-type ATPase subunits during both  $\text{Ca}^{2+}$  and  $\text{K}^+$  starvation.  $\text{Ca}^{2+}$  starvation leads to a consistent downregulation of most subunits apart from the *VHA-H* and *VHA-a1* subunits, which respectively form a peripheral

regulatory V1 subunit, and an integral membrane subunit involved in the assembly of V0 and V1 domains (Sze *et al.*, 2002). Some of the subunits downregulated during  $\text{Ca}^{2+}$  deficiency also showed lower expression levels in response to  $\text{K}^+$  starvation, notably the V1 B subunits and the V0 c and d subunits.

The general pattern of V-type ATPase downregulation in  $\text{K}^+$ - and  $\text{Ca}^{2+}$ -starvation conditions was also observed for PPases, particularly for *AVP2*. Starvation treatments are likely to induce net release of  $\text{Ca}^{2+}$  and  $\text{K}^+$  from the vacuolar compartment to maintain cytoplasmic homeostasis. Thus, downregulation of endomembrane  $\text{H}^+$  pumps might reflect the reduced requirement for PMF-driven  $\text{Ca}^{2+}$  and  $\text{K}^+$  sequestration. Overexpression of the vacuolar  $\text{H}^+$  pump *AVP1*, which resulted in increased accumulation of  $\text{K}^+$  and  $\text{Ca}^{2+}$  in transgenic plants (Gaxiola *et al.*, 2001), supports this notion. Alternatively, our data might indicate a change in vacuolar pH homeostasis upon  $\text{Ca}^{2+}$  and  $\text{K}^+$  depletion.

Seki *et al.* (2002) used a post-genomics approach to study transcript levels in response to ABA, drought, cold and salt. Their study did not show any significant alteration in transcript levels for any primary pump. However, they used growth conditions, material (3-week-old whole plants) and significance criteria that were different from ours. Furthermore, the total number of primary pumps represented on their array is unknown. In a study by Kreps *et al.* (2002), *ACA4* transcript levels were shown to decrease in response to salt treatment, whereas our results show downregulation of *ACA4* in response to  $\text{Ca}^{2+}$  stress but not to NaCl. This might reflect the milder salinity stress that was applied during this study (80 mM NaCl) compared to 100 mM NaCl used by Kreps *et al.* (2002).

#### *Several known and putative cation antiporters respond to $\text{Ca}^{2+}$ starvation and NaCl stress*

Secondary transporters that are driven by the PMF include a large number of cation: $\text{H}^+$  antiporters expressed throughout many plant tissues at both plasma membranes and endomembranes. Figure 5(b) shows expression patterns for NHX-, CAX- and CHX-type transporters, which are respectively annotated as  $\text{Na}^+:\text{H}^+$ ,  $\text{Ca}^{2+}:\text{H}^+$  and cation: $\text{H}^+$  exchange systems. It is worthwhile noting that only in a minority of cases the ionic substrates for these transporters have been experimentally verified and that, especially in the case of the *CHX* family, functional ascription is completely absent (Mäser *et al.*, 2001).

$\text{Na}^+:\text{H}^+$  antiport has been shown to play an important role in sequestration of  $\text{Na}^+$  in the vacuole as well as  $\text{Na}^+$  efflux across the plasma membrane. *NHX1* is expressed at the tonoplast in roots, shoots and flowers, and its role in salt tolerance is well documented (Apse *et al.*, 1999). Salt treatment led to upregulation after 10 h of *NHX1* transcript, but this relaxed back to control levels after 4 days

(no significant hybridisation signal was obtained at 24 h). Thus, *NHX1* upregulation appears to coincide with the intermediate period of salt exposure when root vacuoles rapidly accumulate  $\text{Na}^+$  ions (Figure 2). After 4 days, the root tissue  $\text{Na}^+$  content (around 230 mM on FW basis; see Figure 2) does not significantly increase any further (results not shown), obviating the need for substantial *NHX1* activity. Expression of *NHX3*, which is predominantly expressed in root tissue, did not alter during the first 10 h of salt treatment, as was shown previously for plants subjected to 125 mM NaCl over a period of 12 h (Chauhan *et al.*, 2000). However, at 24 h, *NHX3* expression was induced over 10-fold, with some subsequent relaxation of expression level as was observed for *NHX1*. *NHX7* encodes the plasma membrane expressing SOS1 antiporter whose role in salt tolerance is well documented through forward genetic screens (Zhu, 2001). No change in *SOS1* transcript level was evident in salt-treated plants. *SOS1* expression was reported to increase in response to NaCl (Shi *et al.*, 2000), although this occurred at much higher concentrations (200–300 mM) than the moderate (80 mM) NaCl treatment we applied.

$\text{Ca}^{2+}$  starvation led to some downregulation of several tonoplast expressing *NHX* isoforms, notably *NHX1*, *NHX3* and *NHX5*. This may, to some extent, reflect the observations made for the V-ATPases and indicate a general disturbance of vacuolar pH homeostasis. *NHX* transporters are capable of transporting other monovalent cations (Venema *et al.*, 2002) and it has been postulated that a primary role of these exchangers in non-saline conditions is in pH regulation (e.g. Yamaguchi *et al.*, 2001).

Two *CAX* transcripts encoding  $\text{Ca}^{2+}:\text{H}^+$  antiporter isoforms were significantly downregulated in expression levels: *CAX2* by  $\text{Ca}^{2+}$  starvation and *CAX3* by NaCl treatment. Although the encoded proteins are highly homologous, only *CAX2* is capable of complementing yeast mutants deficient in vacuolar  $\text{Ca}^{2+}$  transport, pointing to potential differences in substrate specificity (Cheng *et al.*, 2002; Shigaki and Hirschi, 2000). Thus, *CAX2* is likely to function as a vacuolar  $\text{Ca}^{2+}$  exchanger, and its downregulation may be related to the rapidly falling levels of tissue  $\text{Ca}^{2+}$  in both roots and shoots (Figure 2) after the initiation of the  $\text{Ca}^{2+}$ -starvation treatment. The yeast complementation studies suggest that *CAX3* might not function as a  $\text{Ca}^{2+}$  exchanger *in planta* and its precise function remains unknown (Cheng *et al.*, 2002).

As discussed above, although *NHX*- and *CAX*-type transporters are generally believed to function in  $\text{Na}^+$  and  $\text{Ca}^{2+}$  transport, there is clear evidence that members of these families can also transport other substrates. However, substrate specificities and membrane locations of members of the large *CHX* family are even less well characterised. For several *CHX* transcripts, little or no hybridisation signal was obtained, suggesting that expression levels in roots may be

low. During salt stress, several *CHX* isoforms are significantly and consistently downregulated, notably *CHX10* and *CHX15*. Saline growth conditions typically result in high levels of  $\text{Na}^+$  sequestration in the vacuolar compartment, often at the expense of vacuolar  $\text{K}^+$ . As  $\text{K}^+$  is normally the prevalent monovalent cation in plants, it might be anticipated that many *CHX* isoforms are involved in movement of  $\text{K}^+$ . Thus, if a major function of *CHX*-type transporters includes uptake of  $\text{K}^+$  into the vacuole to contribute to turgor generation, this process might be downregulated in saline conditions when  $\text{Na}^+$  replaces  $\text{K}^+$ . This would also contribute to the formation of an adequately high cytoplasmic  $\text{K}^+/\text{Na}^+$  ratio. Deprivation of  $\text{K}^+$  did not significantly affect expression of any member of the *CHX* family, which might appear to argue against a major role in  $\text{K}^+$  transport. However, as Figure 2 shows, root  $\text{K}^+$  content was hardly changed during the  $\text{K}^+$ -starvation period, and therefore redistribution of  $\text{K}^+$  within root cells may not have been required at this stage.  $\text{Ca}^{2+}$  starvation led to enhanced expression of *CHX17*. Interestingly, this gene was found by others to be induced by cold, osmotic and salt stress (Kreps *et al.*, 2002). Although upregulation of *CHX17* did not occur in our NaCl treatment, the combined findings reported here and from Kreps *et al.* (2002) might indicate that *CHX17* plays a general role in stress signalling rather than a specific role in  $\text{Ca}^{2+}$  homeostasis.

#### *Many aquaporins are strongly regulated by cation stress*

Water is crucial to plant growth not only because of its involvement in turgor generation, but also because it provides a medium for mass flow translocation of solutes. Movement of water is intricately linked to that of ions. For  $\text{K}^+$ , this is exemplified by the prominent role of this ion as a cellular osmoticum in turgor formation. In the case of salt stress, high external concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  lower the extracellular water potential, which leads to water deficit in the plant tissue.  $\text{Ca}^{2+}$  is relatively immobile, and inadequate transpiration rates frequently lead to  $\text{Ca}^{2+}$  deficiency, particularly in peripheral, fast growing tissues (e.g. Marschner, 1995).

Although water permeability of phospholipid bilayers is substantial, it is now clear that water channels or aquaporins constitute a parallel and regulated pathway for water flux, at both the intracellular and the whole plant level (Chrispeels *et al.*, 2001). Expression of aquaporins is especially prevalent at vacuolar and plasma membranes.

Figure 5(c) shows how transcript levels of aquaporin isoforms are affected by all three conditions applied in this study. It is clear that high concentrations of NaCl, as well as  $\text{Ca}^{2+}$  and  $\text{K}^+$  deprivation, lead to dramatic and divergent responses in aquaporin expression. Salt stress leads to an initial downregulation, which is followed by a substantial increase in mRNA levels of many aquaporin isoforms.  $\text{Ca}^{2+}$

starvation generates a general downregulation of aquaporin expression. This overall pattern of transcriptional regulation of aquaporins by NaCl or Ca<sup>2+</sup> deprivation pertains to major intrinsic proteins (MIPs) and NOD26-like MIPs and NOD26 intrinsic proteins (NLMs/NIPs) – believed to be predominantly plasma membrane expressed – as well as tonoplast intrinsic proteins (TIPs), and small basic intrinsic proteins (SIPs), for which membrane location remains to be determined (Johanson *et al.*, 2001). By contrast, K<sup>+</sup> deprivation specifically affects only a small number of aquaporin isoforms. Members of the PIP and MIP family were downregulated during later stages of K<sup>+</sup> deprivation (after a transient upregulation in some cases). Two NLM/NIP-type aquaporins (*NIP2.1*, *NLM3*) were upregulated in response to re-supply of K<sup>+</sup> after starvation. The same two genes were upregulated by Ca<sup>2+</sup> starvation and thus showed an expression pattern that differed from that of most other aquaporins.

The clear impact of NaCl treatment on plant water status – with a rapid decline, followed by a recovery in tissue water content (Figure 3) – is paralleled to a remarkable extent by changes in aquaporin expression (Figure 5c). During the first 2–5 h, as tissue water is declining, there is, generally, an extensive downregulation of aquaporin gene expression. It seems likely that this rapid response could serve the purpose of limiting initial tissue water loss. Subsequently, and accompanying the recovery of tissue water content, the dramatic upregulation of a range of aquaporin isoforms shown in Figure 5(c) will assist the influx of water as uptake of ions such as Na<sup>+</sup> and Cl<sup>−</sup> and the synthesis of compatible osmolytes lower the cellular water potential. Interestingly, both initial downregulation and the subsequent upregulation of aquaporin gene expression occur earlier for the plasma membrane-expressing isoforms (PIP subfamily) than for the tonoplast-expressing TIP subfamily. Microarray analysis of salt-treated rice (Kawasaki *et al.*, 2001) showed a similar expression profile for at least one aquaporin (initial downregulation and subsequent upregulation). Interestingly, a study using whole *Arabidopsis* plants (Seki *et al.*, 2002) found significant upregulation for only one aquaporin (*PIP2.3*), but in both cases, the subset of aquaporins represented on the arrays is unknown.

In marked contrast to NaCl treatment, Ca<sup>2+</sup> starvation evoked a general and consistent decrease in aquaporin transcripts for many isoforms in all subfamilies. Figure 3 shows that Ca<sup>2+</sup> deprivation did not have a large impact on water relations at the whole-plant level. However, aquaporin activity is post-translationally and negatively regulated by external Ca<sup>2+</sup> (Gerbeau *et al.*, 2002). Thus, the overall transcriptional downregulation at low Ca<sup>2+</sup> might reflect a compensatory response to limit aquaporin activity in these conditions. Alternatively, it has been suggested that trans-root movement of Ca<sup>2+</sup> is principally apoplastic (White, 2001). Therefore, to ensure adequate delivery of

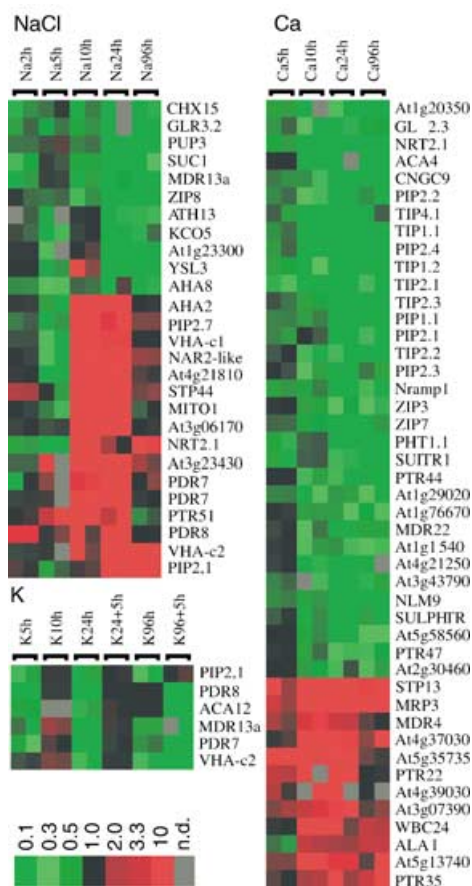
Ca<sup>2+</sup> to the xylem when Ca<sup>2+</sup> is limiting, a shift to apoplastic water movement might be required through a decrease in symplastic water permeability.

Growth on K<sup>+</sup>-free medium affected transcription mainly of plasma membrane-expressing aquaporins. For several isoforms (e.g. *TIP1.1*, *PIP1.3* and *PIP2.2*), the expression profile forms a mirror image of that seen during salt treatment. Regulation of aquaporin expression during K<sup>+</sup> starvation might result from several factors. Movement of K<sup>+</sup> within cells and tissues will be accompanied by the substantial movement of water and thus implicate the activity of aquaporins. Although the overall K<sup>+</sup> tissue content was not significantly affected during short-term K<sup>+</sup> starvation (Figure 2), inter- and intracellular K<sup>+</sup> redistribution could act as a signal for osmotic adjustment. Thus, transcriptional regulation of aquaporins by low external K<sup>+</sup> provides a potential means of preventing osmotic stress during long-term K<sup>+</sup> deprivation.

Thus, all three treatments had considerable effects on aquaporin transcript levels. Other treatments such as low oxygen (Klok *et al.*, 2002), drought and cold stress (Seki *et al.*, 2001) and iron deficiency (Stanford Microarray Database: <http://afgc.stanford.edu>) also affect aquaporin expression levels, indicative of the importance of water channels in many physiological processes.

#### *Identification of novel genes involved in cation stress*

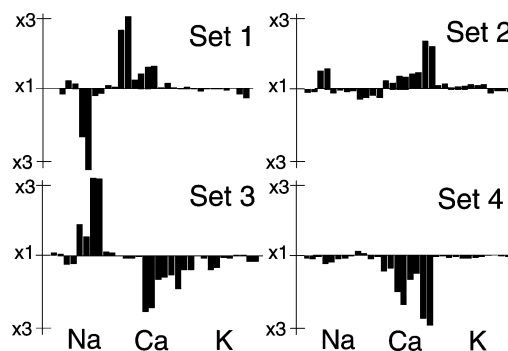
Figure 6 shows the 10% most significantly up- and down-regulated genes for each treatment. These groups include not only members of many gene families discussed above but also members of other transporter families that were not anticipated to have a role in cation stress. In particular, ABC transporters were found to be responsive to all three types of cation stress. ABC transporters form a large group of membrane proteins (129 members; Sanchez-Fernandez *et al.*, 2001), many of which remain to be characterised. We found that transcript levels of several ABC transporters were changed in both Na<sup>+</sup> and K<sup>+</sup> treatments. *MDR13a* transcripts decreased during K<sup>+</sup> and Na<sup>+</sup> treatments, whereas *PDR7* and *PDR8* transcripts were upregulated by salt stress and downregulated during K<sup>+</sup> starvation. Interestingly, a cluster of the ATH subfamily (*ATH13–16*) showed a dramatic downregulation in response to NaCl, especially during the later stages of the treatment (see Supplementary Material, List Na & Ca). Additional ABC transporters were affected by Na<sup>+</sup> or Ca<sup>2+</sup> treatments alone. Although ABC transporters have been shown to play a role in detoxification of herbicides and heavy metals (Rea *et al.*, 1998), our results suggest that they might also be implicated in nutrient transport and salt stress. Indeed, a PDR-type ABC transporter from the aquatic plant *Spirodella polyrrhiza* was found to respond transcriptionally to hormonal and environmental



**Figure 6.** Genes exhibiting strong transcriptional regulation in response to NaCl (80 mM) treatment, Ca<sup>2+</sup> starvation and K<sup>+</sup> starvation. For each treatment, changes in transcript were sorted by the absolute deviation of the normalised ratio from 1. The changes had to occur in at least three time points. The top 10% of each list are shown here. All time points are shown in duplo, gene names are given for annotated genes and accession numbers for non-annotated genes.

treatments such as high levels of NaCl (Smart and Fleming, 1996).

A member of the largely uncharacterised purine permease family (*PUP3*) showed a large decrease in transcript level in response to NaCl. The significance of this observation remains to be revealed but might be related to a general decline in nucleic acid metabolism. Both NaCl and Ca<sup>2+</sup> starvation impacted on nitrate nutrition judging by their considerable downregulation of *NAR2-like* and *NRT2,1* transcripts. An earlier genomics study by Wang *et al.* (2000) showed the converse, wherein the Ca<sup>2+</sup> antiporter *CAX1* transcript level was upregulated by nitrate deficiency. Several members of the PTR peptide transporter family (*PTR22, 35, 44* and *47*) exhibited strongly altered transcript levels after Ca<sup>2+</sup> starvation but not after NaCl or K<sup>+</sup> starvation (Figure 6). This might indicate that general protein metabolism is particularly sensitive to Ca<sup>2+</sup> depletion. Analysis of the 10% most significantly up- and down-

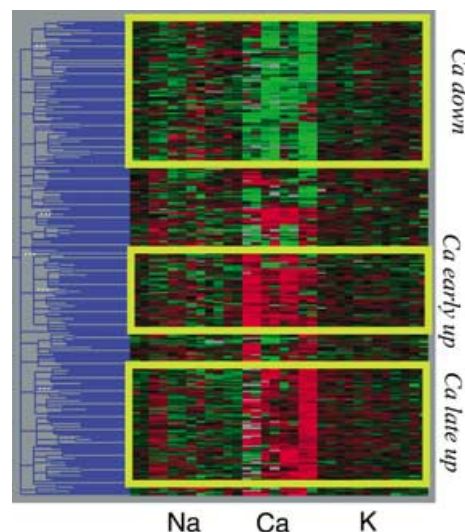


**Figure 7.** Expression profiles of AMT genes during cation stress. K-means clustering was used to identify common expression profiles within all genes that showed changes of transcript level in at least one treatment and time point (see Experimental procedures). Average ratios (average expression profiles) of all genes within a cluster are shown.

regulated transcripts (Figure 6) also identified novel genes that are candidates for a crucial role in ion stress responses, but whose function is unknown (indicated by MIPS protein accessions). Most of them were selected for this study on the basis of a high number of predicted transmembrane spanning domains. Future work such as reverse genetics approaches and heterologous expression will help establish the function in cation stress.

#### Co-regulated genes and promoter analysis

Figure 7 shows the results of cluster analysis of transcriptionally regulated AMT genes. In a first approach, K-means clustering was used to identify common expression profiles



**Figure 8.** Clusters of AMT genes that responded specifically to Ca<sup>2+</sup> starvation (compare Table 1). The gene tree was obtained using hierarchical clustering (see Experimental procedures). Clusters chosen for promoter motif search are shown in yellow boxes.

among all genes with respect to all treatments and time points. K-means clustering resulted in four distinct sets of genes with the following characteristics (Figure 7): transient downregulation by NaCl and transient upregulation by Ca<sup>2+</sup> deprivation (*Set 1*, 114 genes); up- or downregulation by Ca<sup>2+</sup> deprivation (*Set 2*, 147 genes and *Set 4*, 102 genes); transient upregulation by NaCl as well as downregulation during Ca<sup>2+</sup> and K<sup>+</sup> starvation (*Set 3*, 79 genes). In a second approach, genes that responded specifically to Ca<sup>2+</sup> starvation (see Table 1) were grouped using hierarchical clustering (Figure 8). From the Ca<sup>2+</sup>-specific gene tree, three clusters were selected for further analysis (yellow boxes in Figure 8), representing genes with the following expression profiles: downregulation (*Ca down*, 86 genes); early upregulation (*Ca early up*, 47 genes); late upregulation (*Ca late up*, 65 genes). Hierarchical clustering was also carried out for genes regulated by NaCl treatment only (data not shown). Clusters of down- and upregulated genes (32 genes and 13 genes, respectively) were identified, as well as clusters of genes that showed either initial downregulation followed by upregulation (35 genes) or initial upregulation followed by downregulation (21 genes).

The similarity of expression profiles within clusters might suggest that such co-regulated genes are under control of common transcription factors. To discover potential *cis*-acting regulatory elements in 5' upstream regions of co-regulated AMT genes, we used two different approaches based on different computer programs: namely SPEXS (Vilo, 1998) and BIOPROPECTOR (Liu *et al.*, 2001) (see Experimental procedures). The former approach identified sequence motifs of varying length over-represented in several subsets of fixed length (300, 600, 1000 and

2000 bp) of 5' upstream regions (SURs), allowing a relatively low degree of motif degeneracy. The BIOPROPECTOR approach identified sequence motifs of fixed length over-represented in 5' upstream regions of up to 1000 bp, allowing motifs to retain a certain level of degeneracy. The two approaches rely on different algorithms.

Table 2 presents statistically significant motifs identified with the SPEXS software, their binomial probabilities and coverage percentages for both the particular cluster and the corresponding background set. Only the motifs that passed the significance test outlined in the Experimental procedures section are shown here. Most motifs appear in only one group of maximal SUR length, indicating that they are localised in all genes within a certain distance from the start codon (close to the start codon if maximal SUR length is small; far away from the start codon if maximal SUR length is high). One motif (T..GACTC) was significantly over-represented in both SURs of up to 300 bp and SURs of up to 600 bp length, suggesting that this motif varies its position over a region of 600 bp upstream of the start codon.

Table 3 presents statistically significant motifs identified with BIOPROPECTOR software. The motifs given have a higher level of degeneracy than those identified with SPEXS, and consequently, the percentage coverage in both cluster and corresponding background sets are higher. In the *Ca down* and *Ca late up* clusters, motifs were identified that occurred in around 60% of genes belonging to those particular clusters, whereas motif scores in the background set were only 7 and 13%, respectively. Two of the motifs identified using BIOPROPECTOR reflect those found using SPEXS software, i.e. TCT[GT]A[TC][TC][TC]T[GT] in *Ca down*, which subsumes T..GACTC (Table 2), and the

**Table 2** Statistically significant motifs identified in the 5' upstream promoter regions of gene clusters using SPEXS software

Cluster <sup>a</sup>	No. of SURs <sup>b</sup>	Motif <sup>c</sup>	Binomial probability	% Hits in cluster (background set)
SUR length 300 bp				
<i>Ca down</i>	74	T..GACTC	9.14E-08	23.0 (4.9)
<i>Ca down</i>	74	ATGT.T.TGT	1.44E-06	13.5 (1.9)
SUR length 600 bp				
<i>Ca down</i>	65	T..GACTC	5.16E-09	35.4 (9.1)
<i>Ca down</i>	65	TGT.TC.AG	1.06E-06	18.5 (3.2)
<i>Set2</i>	111	AAA.AC.TTAT	1.78E-07	10.8 (1.5)
<i>Set2</i>	111	TAAATA.C.T	5.63E-07	14.4 (3.2)
SUR length 1000 bp				
<i>Ca late up</i>	33	ACACAT	4.45E-05	69.7 (34.7)
<i>Set3</i>	54	AACTACT	3.01E-06	29.6 (8.0)
SUR length 2000 bp				
<i>Set2</i>	56	CGTGCT	1.58E-05	28.6 (8.7)

<sup>a</sup>For clusters, see Figures 7 and 8.

<sup>b</sup>Size of a working cluster: number of genes in a given cluster with strictly upstream regions (SURs) of at least *L* bp length (*L* indicated in the header).

<sup>c</sup>Two wildcards (positions available to all four bases) per motif are allowed; these are marked by dots in a motif.

**Table 3** Statistically significant motifs identified in the 5' upstream promoter regions (up to 1 kb) of gene clusters using BIOPROSPECTOR software

Cluster <sup>a</sup>	Motif <sup>b</sup>	Significance Z score <sup>c</sup>	% Hits in cluster (background set)
<i>Ca late up</i>	[AT]CA[TC]A[GC]A[TC][AT][GC]	6.4	59.7 (12.8)
<i>Ca early up</i>	A[TC]TAATTA[AT][AC]	3.3	65.2 (18.1)
<i>Ca down</i>	TCT[GT]A[TC][TC][TC]T[GT]	4.3	59.8 (12.8)
<i>Ca down</i>	[TC]ACA[GT]AC[AC]C	4.4	57.3 (7.1)
<i>Set 2</i>	TCTCT[TC]TCTC	14.8	61.8 (19.7)
<i>Set 3</i>	CT[TC]TCTCTCT	6.5	69.3 (21.1)
<i>Set 4</i>	AGAG[AG][AG][AG]GAG	10.8	61.8 (17.5)
<i>Set 4</i>	TCTCT[GC]T[TC]TC	10.4	69.3 (21.1)

<sup>a</sup>For clusters see Figures 7 and 8.

<sup>b</sup>Searches were conducted for 8, 9, 10 and 11 nucleotide length motifs. Degenerate nucleotide positions are indicated between brackets.

<sup>c</sup>Difference between the motif score and the null distribution mean measured in the standard deviation units of the null distribution.

[AT]CA[TC]A[GC]A[TC][AT][GC] motif in *Ca late up* motif, which includes ACACAT (Table 2). BIOPROSPECTOR also identified various repetitive elements, e.g. (TC)<sub>n</sub>, with high scores, although such sequences were found in most clusters and therefore are unlikely to represent genuine *cis*-acting elements. No significantly over-represented motifs were found for Na<sup>+</sup>-specific clusters, most probably because of the small number of genes in these clusters (see above).

Although our promoter analysis suggests the presence of specific *cis*-acting sequences, particularly some involved in Ca<sup>2+</sup> deprivation, future experiments will have to establish whether the motifs in Tables 2 and 3 act as binding sites for transcription factors with a function in nutrient stress response.

The dehydration- and ABA-responsive elements DRE and ABRE have been shown to act as *cis*-acting elements with respect to a number of different stress conditions such as cold, salt and drought (Seki *et al.*, 2002). We assessed our co-regulated gene clusters for the presence of the YACGTGKC and TACCGACAT motifs, which, respectively, form the DRE and ABRE response elements. Our analysis revealed that the ABRE element occurred a total of 50 times across all clusters equivalent to a 7.9% hit score, whilst the background set showed 3425 hits (13.7%). The DRE element only occurred once (in *Set 1*), whereas 137 hits were found in the background data set. These results indicate that neither of these *cis*-acting elements is likely to play an important role in the cluster-specific expression profiles shown in Figures 7 and 8.

#### Cross-talk between different nutrient conditions

Interactions between K<sup>+</sup>, Na<sup>+</sup> and Ca<sup>2+</sup> are well documented (see Introduction). Although very few genes showed similar expression profiles during the time courses for the three treatments, some clear examples emerge where potential cross-talk between transport of different cations can take place. For example, the P-type Ca<sup>2+</sup> ATPase *ACA13*

transcript is upregulated by salt stress, and Ca<sup>2+</sup> starvation has a pronounced and negative effect on Na<sup>+</sup>:H<sup>+</sup> antiport expression. Furthermore, salt treatment leads to lower *CAX3* expression levels. These observations might point to genes involved in interactions between Ca<sup>2+</sup> deficiency and plant salt tolerance and the effects of excess Na<sup>+</sup> on Ca<sup>2+</sup> transport and Ca<sup>2+</sup>-based signalling pathways. NaCl also leads to transcriptional regulation of K<sup>+</sup> channel transcripts, i.e. reduced expression of *KCO5* and increased expression of *KAT3* (see Na<sup>+</sup>-specific list in Supplementary Material). Although simple transcriptome analysis does not enable a distinction to be made between direct effects on transporter expression (e.g. caused by high cytoplasmic Na<sup>+</sup> levels) and secondary effects, salt-induced changes in expression of K<sup>+</sup> transporters could be associated with prevention of the frequently observed K<sup>+</sup> deficiency that occurs during growth in saline conditions. For example, increased expression of K<sup>+</sup> uptake channels would contribute to maximising K<sup>+</sup> acquisition from the apoplast, whereas reduced K<sup>+</sup> efflux may function in the retention of K<sup>+</sup> in specific organs and compartments such as meristematic tissues and vacuoles.

All three treatments also affected the expression of genes associated with transport of other nutrients. This was particularly the case with NaCl stress and Ca<sup>2+</sup> starvation where nitrate (*NAR2-like*, *NRT2.1*), sulphate (*SULTR1*, *SULPH*) and phosphate (*PHT1.1*) transporters were among the most significantly changed transcripts (Figure 6; see also Kreps *et al.*, 2002). Wang *et al.* (2002) on tomato also showed that transporters specific for a given mineral nutrient were induced by the absence of other nutrients. For example, when the nutrients P, K<sup>+</sup> and Fe<sup>2+</sup> were withheld, all three treatments led to induction of the K<sup>+</sup> channel *LeKC1*, the Fe<sup>2+</sup> transporter *LeIRT1* and also of nitrate transporters.

The observations by Wang *et al.* (2002) and results reported here indicate that the sensing of nutrient status and responses to ion and nutrient stress are far more

complex than previously thought. The data suggest that, at the transport level, perturbations in the homeostasis of a particular nutrient leads to a relatively rapid modification in transport of other nutrients, possibly to maintain an overall balanced nutrient status.

### Concluding remarks

*Expression profiling provides important clues for functional annotation of unknown genes.* On the basis of sequence homology and transmembrane domain predictions, 1096 *Arabidopsis thaliana* membrane transporter genes were chosen for comprehensive gene expression profiling in response to cation stress. Only 15% of the genes represented on the AMT array were previously functionally characterised to some extent. Where available, annotation of the remaining 85% of AMT genes is currently based on sequence homology. Our study represents an important step forward in the functional annotation of transporter genes on the basis of their involvement in a particular nutrient stress response.

*Members of gene families are not redundant.* Over 60% of *A. thaliana* genes belong to gene families, several of which are very large and can contain over 100 members (as in the case of the ABC-transporter family; Sanchez-Fernandez *et al.*, 2001). This presumably frequently results in functional redundancy, a notion that is supported by the observation that many null mutations do not lead to an obvious phenotype (Bouché and Bouchez, 2001). By using an array that represents a full transporter complement, we were able to identify intrafamily responses of specific isoforms with respect to ion stress. For example, our data show specific downregulation of the H<sup>+</sup>-ATPase transcripts *AHA1* and *AHA7* by Ca<sup>2+</sup> starvation but not of other *AHA* isoforms, with no response to elevated NaCl or to K<sup>+</sup> starvation. Furthermore, NaCl stress strongly increased transcript levels of the nitrate transporters Nar2-like and NRT2.1 but not of other nitrate transporters. Ca<sup>2+</sup> starvation specifically affected *AMT1.2* transcript level, but none of the other ammonium transporters. Thus, although functional redundancy might occur frequently at a whole-plant level, our data show that specific isoforms respond transcriptionally to certain treatments but not to others, and expression studies, as presented here, can therefore help uncover functional nuances of highly homologous members within gene families.

*Responses to cation stress involve many unexpected players.* An important outcome of our study is the finding that nutrient stress caused by depletion or addition of specific ions in the growth medium did not affect specific gene families but rather resulted in a collective transcriptional response of genes across all transporter families. Although

the exact roles of individual genes remain to be revealed, the present study suggests functional interactions among very different types of transporters as plants orchestrate responses to nutritional stress. It is clear that a successful response to cation deficiency or abundance will involve not only uptake, efflux or sequestration of the cation in question, but also regulation of primary transport and fluxes of anions to ensure charge balancing and adjustment of fluxes for other nutrients. As expected, gene clusters based on common expression profiles included genes for cation and anion transporters as well as for primary pumps, but surprisingly clusters also contained many genes that were not anticipated to be linked to the cation in question, e.g. nitrate and ammonium transporters, amino acid and sugar transporters, ABC transporters and aquaporins. The strong response of aquaporins to cation stress even in absence of water stress is particularly interesting. Our findings suggest a central role of aquaporins in nutrient homeostasis, which is likely to comprise (i) support for ion fluxes through provision of accompanying water flow and (ii) active re-direction of apoplastic/symplastic water flow within tissues and the whole plant.

*Co-regulated transporters might share common promoters and signalling pathways.* Signalling networks in nutrient stress are underexplored at present. It can be anticipated that genes exhibiting parallel changes in expression patterns in response to the present treatments are co-regulated. Treatment-based clustering and statistical analysis of 5' upstream sequences revealed a number of novel sequence motifs as putative binding sites for transcription factors involved in the perception and signalling of cation stress in plants.

## Experimental procedures

### Plant growth and treatments

*Arabidopsis thaliana* (L) ecotype Columbia (0) seeds were surface-sterilised and placed on water/agar (0.8% w/v)-containing 1.5-ml tubes. After vernalisation at 4°C for 2 days, seeds were transferred to a growth cabinet and grown hydroponically in a standard medium containing 1.25 mM KNO<sub>3</sub>, 0.5 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.5 mM MgSO<sub>4</sub>, 0.625 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM NaCl and micronutrients, as described by Arteca and Arteca (2000). Growth medium was renewed every week. Plants were exposed to short-day conditions (10 h 270 μmol m<sup>-2</sup> sec<sup>-1</sup> light, 24°C, RH 70–80%) and harvested when they had developed a full rosette but not yet started flowering (growth stage 5.10, Boyes *et al.*, 2001). Prior to harvest, the following nutritional treatments were applied: (i) salt treatment: addition of 80 mM NaCl to standard medium; (ii) Ca<sup>2+</sup> treatment (starvation): replacement of 0.5 mM Ca(NO<sub>3</sub>)<sub>2</sub> by 1 mM NaNO<sub>3</sub>, reduction of NaCl from 2 to 1 mM NaCl and addition of 5 μM 1,2-bis(2-aminophenoxy)ethane-*N,N,N,N*-tetraacetic acid (BAPTA); (iii) K<sup>+</sup> treatment (starvation): replacement of 1.25 mM KNO<sub>3</sub> and 0.625 mM KH<sub>2</sub>PO<sub>4</sub> by 0.5 mM Ca(NO<sub>3</sub>)<sub>2</sub> and 0.625 mM

NaH<sub>2</sub>PO<sub>4</sub>, reduction of NaCl from 2 to 1.375 mM NaCl. For K<sup>+</sup> re-supply, plants were transferred back into standard medium. Plants were harvested at several time points after treatment (see below). Control plants were transferred to fresh standard medium at the onset of the respective treatments and were harvested at the same time as treated plants. Ten to fifteen plants were pooled for each experiment.

Sampling times were 5, 10, 24 and 96 h for all treatments plus additional times for specific treatments. A 2-h time point was added for NaCl stress, which was the only treatment that evoked some early responses of membrane transporters. Five-hour re-supply of K<sup>+</sup> after 24 and 96 h of K<sup>+</sup> deprivation was tested because of the generally weak response of transcripts to K<sup>+</sup> deprivation (labelled as 24 + 5 h or 96 + 5 h). The Supplementary Material online also contains results for 48 h of Ca<sup>2+</sup> deprivation.

#### Tissue water and ion analysis

Tissue water contents were determined by measuring FW and DW of root and shoot tissues (10–15 plants pooled). DW was obtained after drying of tissue at 80°C for 48 h. Ion analysis was carried out on dried root and shoot tissue after 12 h acid extraction with 0.2 M HCl using atomic absorption spectrometry.

#### Semiquantitative RT-PCR

Microarray results were evaluated by carrying out semiquantitative RT-PCR with the Ambion Quantum RNA kit (Ambion Huntingdon, UK). Gene-specific primers were designed spanning at least one intron sequence for the following genes: At4g30190 (*AHA2*), At3g46900 (*COPT3*), At5g14040 (Pi transporter), At2g24710 (*GLR2.3*), At4g01010 (*CNGC13*), At1g19910 (*VHA-c2*), At2g34390 (*NIP2.1*), At5g26340 (*STP13*), At4g23700 (*CHX17*), At1g10010 (*AAP8*), At1g59740 (*PTR35*), At4g30560 (*CNGC9*), At3g07520 (*GLR1.4*), At5g35735 (putative protein) and At4g03560 (amino acid transporter). PCR reactions were optimised for each pair of gene-specific and ribosomal 18S non-extending primers (competimers) to obtain products in the linear amplification range (typically 24–28 cycles). At least three reactions were carried out for each gene. PCR products were run on a 2% (w/v) agarose gel and quantified by densitometry using IS-1000 DIGITAL IMAGING SYSTEM (Alpha Innotech, Cannock, UK) software.

#### Microarray design

A list of mRNA sequences for known and putative AMTs was collated. Initially, AMT genes were identified by searching the *Arabidopsis* genome database at MIPS (MATDB) at <http://mips.gsf.de/proj/thal/> for homologues of all known plant transporters (e.g. ABC transporters, P-type pumps and K channels). The list of genes was extended to include genes with homology to transporters that had been described in other organisms. Finally, we added genes encoding proteins with greater than five predicted transmembrane spanning domains. The final list was checked against similar compilations, i.e. the Arabidopsis Membrane Protein Library by John Ward (<http://www.cbs.umn.edu/arabidopsis/>). In addition, 57 control genes were added including constitutive genes, transformation vectors, mammalian genes as spiking controls and previously described stress-induced genes.

Sequence specific oligonucleotides (50-mers) were designed at MWG (Ebersberg, Germany) on the basis of predicted mRNA sequences (<http://mips.gsf.de/proj/thal/>). In cases where confirmed mRNA sequences were available from NCBI, these were given

preference over the MIPS predictions. The oligonucleotides were synthesised and spotted onto glass slides by MWG. Every slide contains an ensemble of two identical arrays (upper and lower half), which are hybridised simultaneously.

#### Microarray hybridisation

Root RNA was isolated using RNeasy Plant Mini columns (Qiagen, UK) and pooled from three independent sets of plants for each treatment. Total RNA (50–100 µg) was polyA-primed with oligo(dT)<sub>20</sub> primer (MWG, Ebersberg, Germany) and reverse-transcribed with Superscript II reverse transcriptase (Life Technologies, UK). Fluorescent labelling was achieved by replacing dCTP in the dNTP mix (Sigma, UK) with Cy3-dCTP or Cy5-dCTP (Amersham, UK). Labelled target cDNA was cleaned on QIAquick spin columns (Qiagen, UK), vacuum-dried and re-dissolved in hybridisation buffer (MWG, Germany). Prior to hybridisation, microarrays were treated for 40 min at 42°C with blocking solution containing 5× SSC, 0.1% (w/v) SDS and 1% (w/v) BSA. For hybridisation, cDNA-buffer mix was applied to the microarray, covered with a Hybri-Slip (Sigma, UK) and incubated at 42°C for 16–20 h. After hybridisation, arrays were washed at room temperature in washing buffers containing 0.5–2× SSC.

#### Image analysis and gene expression analysis

Microarrays were scanned using AXON (Axon Instruments, Braintree, UK) or PACKARD LITE 4000 (Packard BioChip Technologies, Pangbourne, UK) and associated software. For image analysis, SCANALYZE software (B Eisen, Stanford, USA) or GSI LUMONICS QUANTARRAY 2.1 (Packard, see above) were used. Further analysis was carried out using GENECLUSTER and TREEVIEW software (B Eisen, Stanford, USA), Microsoft Excel and GENESPRING 4.2.1 (Silicon Genetics). Fluorescence intensities were measured and averaged over a defined spot area. Background signals were determined and averaged over a defined area in the immediate spot environment and subtracted from the spot signals. Spots were flagged P (present) or A (absent) according to signal/background ratios. The signal/background ratio cut-off for P/A classification was determined separately for every array and equalled the minimum ratio where >95% of negative controls (approximately 100 blank spots per array) appeared as flagged 'absent'. Typically, signal/background ratio cut-offs determined in this way were between 2 and 3. In addition to data exclusion on the basis of signal/background ratios, all images were visually inspected and flagged B (bad) in the event of aberrations in fluorescence or background signals.

Background-subtracted Cy3 and Cy5 signals were divided, obtaining treatment/control ratios independent of labelling. To correct for non-linearity of the Cy3/Cy5 plot, intensity-dependent normalisation was applied, where the ratio was reduced to the residual of the Lowess fit of the intensity versus ratio curve (GeneSpring, Silicon Genetics). Treatment/control ratios were normalised on each array half using the 50th percentile of all measurements as a synthetic positive control.

#### Cluster analysis

Cluster analysis was performed using both K-means and hierarchical clustering (GENESPRING 4.2.1 software, Silicon Genetics). As a first approach, K-means clustering was used to analyse expression profiles over the entire range of treatments and time points. Caution had to be taken to exclude low absolute signals

(A-labelled, see above), to avoid artificially high/low ratios. Therefore, K-means clustering was restricted to genes that exhibited significant (P-labelled) signals in at least 15 out of the 16 conditions tested (453 genes). A number of four was established as the minimum number of clusters needed to produce the maximum number of qualitatively distinct expression profiles. All treatments and time points were equally weighted. Similarity was measured using standard Pearson correlation. In a second approach, we concentrated on genes that had been identified previously as being specifically regulated by Ca<sup>2+</sup> starvation (see Table 1). Hierarchical clustering was used to arrange Ca<sup>2+</sup>-specific genes in a gene tree. Similarity was measured by Spearman correlation. Separation ratio was 0.5 and the minimum distance 0.001. Several clusters were selected for further analysis (yellow boxes in Figure 8).

#### Extraction of 5' upstream sequences

A library of 'strictly upstream regions' (SURs) for all *Arabidopsis* genes was generated from genomic data. The assembled sequences of five *Arabidopsis* chromosomes with the corresponding annotation files were downloaded from the FTP server at MIPS (<http://mips.gsf.de/proj/thal>). The annotation files contained coordinates (positions and strand) of different genomic features such as gene-protein, pseudogene, repeat region/unit, LTR, snRNA, tRNA, rRNA, transposon. We used in-house written software to find coordinates for maximum length SURs that do not overlap with annotated sequence. These coordinates were then used to extract the corresponding 5' sequences.

#### Identification of putative cis-acting elements

**Approach based on SPEXS.** SPEXS (Vilo, 1998) uses the suffix tree algorithm to find all motifs of unrestricted length that appear in at least  $t$  sequences in the cluster of size  $n$ . If a motif is found in  $k$  sequences, then SPEXS reports the probability that it is found by chance in at least  $k$  sequences of a cluster of size  $n$ . This probability is calculated from the binomial distribution, and the background probability is estimated from the frequency of occurrence of a given motif in a background set. For each of the clusters, we generated four subsets of gene SURs of lengths 300, 600, 1000 and 2000 bp. Using SPEXS software, we found all motifs containing up to two wild-cards (position available to any of four bases) and occurring in at least 10 SURs for each of the above 28 sets of SURs. As a background set, we used four complete sets of SURs of length 300, 600, 1000 and 2000 bp. These contained 23339, 19024, 14129 and 7317 SURs, respectively. The motifs were ranked according to the score equal to the inverse of the binomial probability. To determine which of the reported motifs have statistically significant scores, we repeated each of the above 28 calculations 40 times using randomly selected clusters of the same number of *Arabidopsis* SURs. Subsequently, we compared the score of the top three motifs of each original run with the distribution of the first, second and third hits in the 40 random runs, respectively. The motif was declared significant if its score was in the top 5% of the corresponding random distribution.

**Approach based on BIOPROPECTOR.** BIOPROPECTOR (Liu et al., 2001) is based on a Gibbs sampling algorithm to create a probability matrix for putative motifs. A motif score is produced that reflects the number of aligned segments for the motif and the probabilities of observing a particular nucleotide at a

particular position in the motif matrix, relative to the background set of sequences. BIOPROPECTOR uses zero- to third-order Markov background models. Searches for motifs of 8, 9 and 10 nucleotides were carried out using the data sets of each cluster and a background data set that contains SURs up to 1 kb of all *Arabidopsis* genes. To assess the statistical significance of putative motifs, for each cluster the BIOPROPECTOR software generated the null distribution of the score by fitting a normal distribution to the set of highest motif scores obtained for 200 randomised sequence sets of the same sequence number and length as the original cluster. The high-scoring motifs of a given cluster were declared significant if they scored at least three standard deviations above the null distribution mean (Z-score = 3).

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#### Supplementary Material

The following material is available from <http://www.blackwell-publishing.com/products/journals/suppmat/TPJ/TPJ1839/TPJ1839sm.htm>

Supplementary Material is available online in the following files. **AMT\_genes:** A list of all probes on the AMT chip with MATDB gene accession numbers, NCBI accessions for confirmed mRNA sequences, common names and preliminary annotation of transporter type.

**AMT\_Na\_specific (Ca\_specific, K\_specific, Na & Ca, Na & K, Ca & K, Na & Ca & K):** Lists of normalised treatment/control ratios for all genes that showed significant changes in the treatments indicated in the file name (compare Figure 4 and Table 1). As in Table 1, genes are sorted by transporter family. Significant changes in signal ratio are indicated in colours: red for up-, green for down-regulation.

**AMT\_clusters:** Accessions, names and annotation of genes belonging to the clusters shown in Figures 7 and 8.

**AMT\_all\_normratios:** This file contains the entire data set obtained in this project. Normalised treatment/control ratios of the Cy3, Cy5 fluorescent signals for all probes, all treatments and all time points.

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