

This is a repository copy of *A proteomic and phosphoproteomic analysis of Oryza sativa plasma membrane and vacuolar membrane*.

White Rose Research Online URL for this paper:

<https://eprints.whiterose.ac.uk/4153/>

Version: Submitted Version

---

**Article:**

Whiteman, S.A., Nuhse, T.S., Ashford, D.A. et al. (2 more authors) (2008) A proteomic and phosphoproteomic analysis of *Oryza sativa* plasma membrane and vacuolar membrane. *Plant Journal*. pp. 146-156. ISSN 1365-313X

<https://doi.org/10.1111/j.1365-313X.2008.03578.x>

---

**Reuse**

Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

**Takedown**

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing [eprints@whiterose.ac.uk](mailto:eprints@whiterose.ac.uk) including the URL of the record and the reason for the withdrawal request.

*promoting access to White Rose research papers*



**Universities of Leeds, Sheffield and York**  
**<http://eprints.whiterose.ac.uk/>**

---

This is an author produced version of a paper, subsequently published in the **PLANT JOURNAL**. (This paper has been peer-reviewed but does not include final publisher proof-corrections or journal pagination.)

White Rose Research Online URL for this paper:  
**<http://eprints.whiterose.ac.uk/4153>**

---

**Published paper**

Whiteman, S, Nuhse, T, Ashford, D.A, Sanders, D, Maathuis, Frans J. M (2008)

*A proteomic and phosphoproteomic analysis of Oryza sativa plasma membrane and vacuolar membrane*

**Plant Journal** 56 1 (146-156)

---

**A proteomic and phosphoproteomic analysis of *Oryza sativa* plasma membrane and vacuolar membrane.**

Sally-Anne Whiteman<sup>1</sup>, Thomas S Nühse<sup>2</sup>, David A Ashford<sup>1</sup>, Dale Sanders<sup>1</sup> and Frans JM Maathuis<sup>1</sup>

<sup>1</sup>Department of Biology Area 9, University of York, York YO10 5DD, United Kingdom.

<sup>2</sup>Faculty of Life Sciences, Michael Smith Building, Oxford Road, Manchester M13 9PT, United Kingdom.

**Corresponding author:** F.J.M. Maathuis, Department of Biology, University of York Area 9, York YO10 5DD, United Kingdom.

E-mail: [fjm3@york.ac.uk](mailto:fjm3@york.ac.uk) , Tel: +44-1904-328652, Fax: +44-1904-328666.

**Running title:** Rice proteomics

**Abbreviations:** TFA: trifluoroacetic acid; MALDI: matrix-assisted laser desorption/ionization; MS: mass spectrometry

**Word count:** 6955

**Keywords:** Proteomics, Phosphoproteomics, Membrane transport, *Oryza sativa*, rice.

**Summary (word count 194):** Proteomic and phosphoproteomic analyses of rice shoot and root tonoplast-enriched and plasma membrane-enriched membrane fractions were carried out to look at tissue specific expression and to identify putative regulatory sites of membrane transport proteins. Around 90 unique membrane proteins were identified which include primary and secondary transporters, ion channels and aquaporins. Primary H<sup>+</sup> pumps from the AHA family showed little isoform specificity in their tissue expression pattern whereas specific isoforms of the Ca<sup>2+</sup> pump ECA/ACA family were expressed in root and shoot tissue. Several ABC transporters were detected, particularly from the MDR and PDR subfamilies, which often showed expression in either roots or shoots. Ammonium transporters were expressed in root but not shoot tissue. A large number of sugar transporters was expressed particularly in green tissue.

The occurrence of phosphorylation sites in rice transporters such as AMT1;1 and PIP2;6 agrees with those previously described in other species pointing to conserved regulatory mechanisms. New phosphosites were found in many transporters including H<sup>+</sup> pumps and H<sup>+</sup>:cation antiporters, often at residues which are well conserved across gene families. Comparison of root and shoot tissue showed that phosphorylation of AMT1;1 and several further transporters may be tissue dependent.

### **Introduction (word count 541)**

Homeostasis of cells and organisms critically depends on the function of membrane transporter proteins which mediate the movement of solutes between the environment and cytosol and between cell compartments. Genome sequencing has shown that a significant proportion of the genome (> 5%) encodes membrane transport proteins. Based primarily on phylogenetic relationships, most membrane transporters can be grouped into gene families and assigned a broad functional annotation. However, for most transporters detailed knowledge regarding physiological function is completely lacking and therefore a large task remains with regard to their functional annotation.

The latter will be facilitated by determining the subset of transporters that is relevant in plant development and growth. For *Arabidopsis*, membrane proteomics inventories are now available for the plasma membrane (e.g. Alexandersson *et al.*, 2004), vacuolar membrane or tonoplast (e.g. Carter *et al.*, 2004), mitochondrial membranes (Brugiere *et al.*, 2004) and plastidic membranes (Peltier *et al.*, 2004). Furthermore, large scale studies into protein phosphorylation are available

for both the *Arabidopsis* plasma membrane (Nühse et al., 2003; Nühse et al., 2004; Niittyla et al., 2007) and vacuole (Whiteman et al., 2008) identifying many phosphopeptides that are likely to be essential in the regulation of membrane transport.

These global analyses have greatly helped in establishing the roles of membrane proteins by localising them to specific tissues and organelles and have identified key aspects of membrane transport such as the presence of specific sugar transporters at the tonoplast (Endler et al., 2006), and regulation of the proton pumping activity of plasma membrane ATPases (Niittyla et al., 2007).

In contrast, the proteome and phosphoproteome of rice membranes remain little investigated in spite of the fact that rice is rapidly becoming a model system for plants. Transcriptomics studies in rice have helped to establish gene expression patterns for many membrane transporters but gene expression is not necessarily correlated with protein expression (Maathuis and Amtmann, 2005). In addition, transcriptomics surveys do not reveal information concerning membrane localisation and protein regulation. Previous studies in rice have mainly focussed on soluble proteins using gel-based methods that are less suited for the separation of hydrophobic proteins. Using this methodology, Komatsu and Tanaka (2005) identified around 5100 proteins from different tissues and organelles but only a small proportion consisted of membrane proteins. Other gel-based studies also showed a very low number of hydrophobic proteins (Tanaka et al., 2004).

We therefore undertook a study into the rice plasma membrane and tonoplast proteomes of both roots and shoots to provide a comprehensive inventory of membrane proteins in this plant and to assess whether proteins are differentially expressed between these tissues. Membrane fractions were analysed to catalogue the presence of membrane transporters involved in the movement of solutes and to map subcellular and tissue-specific localisation of isoforms. To discover putative regulatory mechanisms we also studied the phosphoproteomes of the membrane fractions. Our data show interesting similarities in expression patterns between root and shoot tissues for primary transporters and significant differences in the presence of secondary transport proteins that are involved in the movement of inorganic nutrients and photosynthates. In addition, we identified previously reported and many new phosphopeptides that may be involved in membrane transporter regulation. We show that for several transporters the phosphorylation state may be tissue dependent.

## **Results and discussion (word count 2490)**

*Membrane preparations:* Membrane proteomic analyses are complicated by the difficulty in obtaining pure fractions. We used well-established methods to obtain a low-density microsome fraction that was enriched in tonoplast (Lüttge et al., 2000; Whiteman et al., 2008) and a two phase partitioning fraction that was enriched in plasma membrane (Larsson et al., 1987). Nevertheless, cross contamination with membranes from other compartments is a common problem. Recent work by Jaquinod et al., (2007) who used vacuoles prepared from protoplasts showed the presence of many plasma membrane proteins such as P-type H<sup>+</sup> ATPases. Vice versa, proteomics on plasma membrane fractions typically detects vacuolar H<sup>+</sup> pumps, TIP-type aquaporins and other endomembrane proteins (e.g. Marmagne et al., 2004). Furthermore, membrane fractions often contain large numbers of non-membrane proteins. Some of these may be membrane associated but many are likely to be contaminating soluble proteins.

To maximise confidence in the obtained data from this study and to be able to improve their interpretation, we applied stringent criteria on the obtained peptides (see methods) but in addition only assigned proteins to a specific membrane and tissue if it was present in at least two out of the three biological replicates. To further provide a semi-quantitative measure of membrane and tissue expression, Table 1 denotes the total number of peptides that was identified for each protein in the four different fractions (root and shoot tonoplast and root and shoot plasma membrane). Although these numbers can not be equated with absolute expression levels they are indicative of relative abundance as is born out by the substantially higher scores for well known vacuolar proteins (e.g. pyrophosphatases and TIP aquaporins) in the tonoplast fractions and P-type ATPases in plasma membranes.

*Rice membrane proteome and regulation of transporters by phosphorylation:* Out of 231 unique proteins identified in the combined fractions (Supplementary Table), a total of 94 (40%) membrane proteins was detected. The 94 membrane proteins derived from various transport and non-transport functional categories (Fig. 1).

Within the membrane proteins 61 transporters with well defined functional annotations were present. Amongst these proteins, 30 phosphopeptides were identified by using immobilised metal affinity chromatography (Nühse et al. 2003; Nühse et al., 2004). The phosphorylation and dephosphorylation of proteins constitutes a well conserved generic regulatory mechanism of immense importance in living organisms. The phosphorylation state can affect protein activity in

many ways: by acting as an allosteric switch, by changing subcellular localisation or by targeting proteins for degradation. Thus, the identification of proteins that can be phosphorylated, and more specifically the residue that carries this modification, is crucial in understanding how particular proteins are regulated.

*Primary H<sup>+</sup> and Ca<sup>2+</sup> pumps:* A large number of primary pump proteins was found in all four membrane fractions. These mainly consisted of H<sup>+</sup>-ATPases from the plasma membrane AHA family, V-type ATPase subunits and vacuolar pyrophosphatases, which are all involved in the generation of trans-membrane H<sup>+</sup> electrochemical gradients. In addition, 3 isoforms of the Ca<sup>2+</sup> transporting ATPases from the ECA/ACA family were detected that are important in extrusion of Ca<sup>2+</sup> from the cytoplasm, either into the apoplast or into internal compartments such as the vacuole.

Although tissue specific transcript accumulation of AHA isoforms has been described in Arabidopsis (Morsomme and Boutry, 2000) very little isoform specificity was apparent between root and shoot rice tissues. Only AHA1, AHA4 AHA6 and AHA9 were detected exclusively in roots. Rice transcriptomics data (<http://signal.salk.edu> or <http://mpss.udel.edu/rice>) point to a fairly equal distribution of most AHA isoforms between root and shoot but also show expression is very sensitive to environmental conditions such as temperature. In Arabidopsis, AHA4 is abundantly expressed in the root endodermis and plays a role in plant ion homeostasis and nutrient transport (Vitart et al., 2001).

Some H<sup>+</sup> pumps were found to be differing in their phosphorylation state between roots and shoots: AHA7 was found non-phosphorylated in roots and phosphorylated in shoots whereas for AHA3 a shoot specific phosphosite was found. Although P-type ATPases undergo reversible phosphorylation at a conserved aspartate residue as part of the catalytic cycle, kinase dependent phosphorylation occurs at a C-terminal threonine (T<sup>956</sup> in OsAHA1) in the well conserved 14-3-3 protein binding domain (Gaxiola et al., 2007). This site was not detected in our study but another C-terminal peptide containing a serine at position 970 was found in AHA3 and in AHA7. This putative phosphosite is well conserved across all rice AHA isoforms except AHA10 (Fig. 2). The recently described 'new' phosphorylation site, (pT)LHGLQPK (Niittyala et al., 2007) in AtAHA1 and AtAHA2 was also found in the rice OsAHA3 (at T<sup>889</sup>) in both root and shoot samples.

Ca<sup>2+</sup> ATPases (ECA1, ACA9 and ACA11) were only detected in shoot PM. ECA1 and ACA9 transcript levels are similar in mature root and shoot tissues but ACA11 transcript is virtually

absent in mature roots. ECA1 is an ER localised protein that can transport both  $\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$ . It contributes to plant  $\text{Mn}^{2+}$  tolerance by removing this ion from the cytosol when plants are exposed to high concentrations of  $\text{Mn}^{2+}$  in the environment (Wu et al., 2002).

Rice contains a larger number of vacuolar  $\text{H}^+$  pumping pyrophosphatases (~12) than *Arabidopsis* (3 isoforms). Five isoforms were detected in the TO root and TO shoot fractions. Os01g23580, Os05g06480 and Os06g43660 were detected exclusively in shoot TO. Transcript levels for Os01g23580 and Os05g06480 in mature roots are considerably lower than those in found in hoots.

One vacuolar PPases (Os02g09150) was detected in phosphorylated and non-phosphorylated forms: Os02g09150 was present as a phosphoprotein in roots but not shoots. Little is known about regulation of pyrophosphatase activity and the physiological relevance of these sites is yet to be established.

*ABC-transporters:* Plant genomes encode many ABC-transporters and the rice genome contains around 100 genes that encode this type of ATP fuelled pump. ABC-transporters are expressed in both plasma membrane and tonoplast. In most cases their precise function is unknown but a general role includes detoxification and compartmentation of xenobiotics and heavy metals (Yazaki et al., 2006), and homeostatic and developmental functions such micronutrient homeostasis (Ducos et al., 2005) and lipid extrusion for the formation of leaf cuticles (Pighin et al., 2004). In rice roots and shoots three and two isoforms were expressed. Only MRP1 was found in both root and shoot tissue.

*Nitrogen transport:* Rice prefers  $\text{NH}_4^+$  over  $\text{NO}_3^-$  as a nitrogen source and its genome encodes a relatively large number (~12) of ammonium transporters from the AMT family. Two isoforms, AMT1;1 and 1;2, were detected exclusively in roots. This agrees with transcript profiles for these carriers and also with the notion that the large majority of  $\text{NH}_4^+$  is assimilated within the root. AMT1;1 has previously been described in *Arabidopsis* phosphoproteomics studies (Nühse et al. 2003; Nühse et al., 2004; Benschop et al., 2007; Whiteman et al., 2007) as a target for phosphorylation. The *Arabidopsis* AtAMT1;1 contains a threonine residue at position 460 as the phosphorylation target that was recently identified as playing a critical role in the substrate sensitive, allosteric regulation of this ammonium transporter (Loque et al., 2006). We identified a phosphosite in OsAMT1;1 with a threonine at exactly the same position indicating that this



mechanism of protein activity modulation in response to ammonium is conserved between different plant species.

In conjunction with AMT transporters, a putative  $\text{NO}_3^-$  transporter (Os10g42900) was also present in root membranes. Os10g42900 transcript level is primarily found in roots and its closest *Arabidopsis* ortholog is AtNRT1 a high affinity oligopeptide transporter (Rentsch et al., 1995). A second  $\text{NO}_3^-$  transporter (Os03g48180) was present in shoot tissue only. This protein is also alternatively annotated as a proton dependent oligopeptide transporter of the POT/PTR family and thus may be involved in intercellular movement of organic nitrogen. A large number of further peptide transporters and amino acid transporters is also likely to contribute to overall nitrogen homeostasis. Several other isoforms from the POT/PTR family and several amino acid transporters were found in roots and shoots indicating that such proteins are expressed at relatively high levels throughout the rice plant.

*Phosphate transport:* Like nitrogen, phosphorous is another essential mineral for plants which is frequently only available in very small amounts and thus requires high affinity mechanisms. Interestingly, no annotated phosphate carriers were detected in roots but our growth medium contains high levels of Pi (~0.6 mM) which may have led to suppression of P carriers in the root. Two isoforms (PT1 and PT8) of the extended PT family were expressed in the shoot PM fraction. OsPT1 transcript is relatively low in roots and reduced by mycorrhization (Pazskovski et al., 2002). OsPT1 transcript in shoot tissue is around three times higher but no functional data are available. Even less is known about PT8: its transcript levels are much higher in leaves than roots and are not affected by mycorrhization.

*Potassium transport:* The high affinity  $\text{K}^+$  carrier HAK1 was prominently present in root PM and not in any other fraction. A role for HAK1 in  $\text{K}^+$  uptake from the soil has been surmised previously on the basis of its expression pattern and induction by  $\text{K}^+$  deficiency (Banuelos et al., 2002). It also agrees with the notion that its closest *Arabidopsis* homolog is AtHAK5, the root expressed, high affinity  $\text{K}^+$  uptake system in this species (Gierth et al., 2004; Armengaud et al., 2004). In rice shoot tissue a second HAK-like transporter was detected which is homologous to *Arabidopsis* HAK8. AtHAK8 is mostly expressed in young leaves but also in other tissues (Ahn et al., 2004).

*Sugar transport:* A total of 4 sugar transporters was present in the various membrane fractions. The overall number found in green tissue was considerably larger than that found for roots with

respectively 4 and 1 isoforms. Several sugar transporters have been characterised to some extent such as MST3 which has H<sup>+</sup>-coupled transport activity for monosaccharides. MST3 has been detected previously in leaf blades, leaf sheaths, calli and roots, and is suggested to be involved in the accumulation of monosaccharides required for cell wall synthesis (Toyofuku et al., 2000).

One phosphopeptide was found in sugar transporters. Phosphorylation has been suggested as an important posttranslational mechanism to modify activity and affinity of sucrose transport (Niittyla et al., 2007) and we recently reported on several new phosphosites in *Arabidopsis* shoot tissue sugar transporters (Whiteman et al., 2008). In rice, phosphorylated sugar transporters were only observed in the shoot samples.

*Water transport:* Although water permeability of phospholipid bilayers is substantial, a large part of the water flux is mediated by aquaporins (Chrispeels et al., 2001) whose expression is especially prevalent at the tonoplast and plasma membrane. The rice genome encodes around 35 aquaporins of the PIP, TIP and NIP subfamilies and representatives of each family were found in both root and shoot tissue. Some PIP isoforms (PIP2;1, 2;2 and 2;6) were ubiquitously expressed and detected in three or more fractions but with a much higher peptide score in the PM. Conversely, TIP1;1 and 2;2 were detected in most fractions but with significantly higher scores in the TO. PIPs and TIPs are thought to be responsible for water movement across the PM and TO respectively and many are believed to express in a tissue specific manner. The latter was observed for PIP2;6 which showed a much higher peptide count in shoot tissue. PIP2;6 was previously found to have a C-terminal phosphosite in *Arabidopsis* (Niittyla et al., 2007; Whiteman et al., 2008). This site overlaps perfectly with a phosphosite in OsPIP2;6 (Fig. 3) containing a residue that is well conserved within the PIP2 subfamily. PIP2;6 was detected as a phosphorylated protein in shoots but non-phosphorylated in roots. Since phosphorylation gates aquaporins to the conducting state (Tornroth-Horsefield et al., 2006) this may suggest that PIP2;6 was actively mediating water flux in shoots only.

*Further transporters:* Root fractions also revealed the presence of transporters that may be involved in acquisition of other nutrients. Several transporters are present that may participate in uptake and distribution of the micronutrients Zn and Cu. Two copper transporters were detected at the root TO fraction. COPT5 is a member of the COPT family, believed to participate in Cu uptake and distribution, and particularly its deposition in vacuoles (Sancenon et al., 2003). The plasma membrane Zn transporter ZIP2 is likely involved in Zn and/or Fe uptake and homeostasis

but, interestingly, has also been implicated in Cu transport (Wintz et al., 2003). In yeast it shows a nanomolar  $K_m$  for Zn and Cu transport and its transcription is induced by deficiency of either metal (Wintz et al., 2003). The root PM fraction also shows the presence of an aquaglyceroporin, NIP2;1, which was recently characterised as the first plant silicon transporter. (Ma et al., 2006). Rice takes up relatively large amounts of silicon which is involved in cell wall integrity but also impacts on nutritional aspects such as the accumulation of tissue  $Na^+$  via a non-symplastic pathway.

Several exchangers were found in TO fractions with a putative Ca:Na exchanger in both root and shoot tissue. No functional data are available for this protein but it was present in phosphorylated form in shoots and non-phosphorylated in roots.

A H:Na antiporter was detected at the TO in shoots: NHX3 is part of the well characterised NHX family which has been shown to be important in the vacuolar deposition of monovalent cations such as  $Na^+$  and  $K^+$  and also for pH homeostasis. NHX3 showed phosphorylation at a C-terminal position at a residue, S<sup>471</sup>, that is conserved across rice NHX isoforms (Fig. 4) and also present as a threonine residue in *Arabidopsis* NHX isoforms. This suggests that this site may be important in regulation of vacuolar cation exchange. A putative phosphosite further downstream in *Arabidopsis* NHX1 and NHX2 has also been reported (Whiteman et al., 2008).

*Conclusions:* Rice is rapidly being established as a model species for cereal crops and is also a major target for genetic engineering. It is thus imperative to know the composition and function of the proteome that underlies rice growth and development. Many physiological features of this monocot crop may not be reflected in the dicot model *Arabidopsis*. We show that the expression of around 230 membrane and membrane associated proteins in plasma membrane and tonoplast fractions of rice root and shoot often occurs in a tissue specific manner and that around 20% of the detected proteins have clearly identifiable phosphosites that may be important in modulating protein activity. Many of these sites exactly match equivalent sites identified previously in *Arabidopsis* orthologues (e.g. Nühse et al., 2004) pointing to well conserved regulatory mechanisms. However, other sites are unique to rice thus confirming the need for experimental proteomic data in this species.

### **Experimental procedures (word count 1908)**

**Materials:** Chemicals were obtained from Sigma or Fluka unless otherwise stated.

**Plant growth conditions:** Rice plants (*Oryza sativa japonica* Nipponbare) were germinated on moistened filter paper and transferred to a hydroponic growth system. The growth medium contained 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> and micronutrients as described in (Maathuis et al., 2003). Plants were maintained in Percival growth cabinets under short day conditions (10 h light at 200  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  intensity, 14 h dark, 23°C day, 17°C night and 70% RH. Leaf and root tissue was harvested from 8 week old plants.

**Membrane preparation:** Plasma membrane and vacuolar membrane fractions from shoot and root tissue were prepared by employing two-phase partitioning (Larsson *et al.*, 1987) and sucrose gradient centrifugation (Lüttge et al., 2000) methods respectively.

To isolate plasma membranes, plant tissue was homogenised in buffer H (0.25 M sucrose, 2 mM EGTA, 10%(v/v) glycerol, 0.5% BSA, 50 mM BTP-MES, pH 7.8, 0.25 M KI, 2 mM DTT, 1 mM PMSF, 5 mM  $\beta$ -mercaptoethanol). The homogenate was centrifuged at 11,500g for 10 minutes and the supernatant re-centrifuged at 100,000g for 40 minutes. The pelleted material was resuspended in 5 ml of P-buffer (0.25 M sucrose, 3 mM KCl, 5 mM KH<sub>2</sub>PO<sub>4</sub>) and used as the microsomal preparation.

A batch preparation method was used to obtain a plasma-membrane enriched fraction, using a dextran-PEG two-phase system. The final composition of the phase system was 6.2% (w/w) dextran T500, 6.2% PEG 4000, 0.25 M sucrose, 5 mM K-phosphate, pH 7.8. The phase system was mixed by inversions of the tube, centrifuged at 720g for 10 minutes, and then 90% of the upper layer was removed and each layer was separately re-combined with fresh upper or lower phase as appropriate. Tubes were re-centrifuged, and the separation/extraction repeated once more. Upper phase fractions were recombined and centrifuged at 100,000g for 1 hour. Pellets were resuspended in 62.5 mM Tris-HCl, pH 6.8 and re-centrifuged at 100,000g for 1 hour.

For the isolation of tonoplast, leaf or root tissue was homogenized in Buffer A (50 mM MOPS-KOH, pH 7.6, 0.25 M sorbitol, 5 mM EGTA, 1% isoascorbic acid with Sigma plant protease inhibitor cocktail). The homogenate was centrifuged at 3,600g for 10 minutes and the supernatant from this was centrifuged at 150,000g for 20 minutes. The pellet was resuspended in Buffer B (10 mM potassium phosphate buffer, pH 7.8, 0.35 M sucrose, 1 mM EGTA, 1 mM DTT), overlaid with an equal volume of Buffer C (5 mM MOPS-KOH buffer, pH 7.3, 0.25 M sorbitol, 1 mM EGTA, 1 mM DTT) and centrifuged at 120,000g for 40 minutes. The protein present at the interface was collected, diluted 10-fold with Buffer A, and centrifuged at 150,000g for 20

minutes. The pellet was resuspended in Buffer A containing 20% (v/v) glycerol to yield a low density membrane fraction enriched in tonoplast.

All membrane fractions were carbonate washed according to Dunkley *et al.*, 2004 to remove soluble protein and stored at -80°C.

**Sample preparation for proteomic analyses:** Membrane fractions were separated by conventional 1D gel electrophoresis. Gels were stained with BioSafe Coomassie (BioRad) and the lanes cut into slices. Gel slices were macerated and destained in 25 mM ammonium bicarbonate in 50% (v/v) acetonitrile, dehydrated in acetonitrile, dried, and reduced in 10 mM dithioerythritol in 100 mM ammonium bicarbonate at 56°C for 1 hour. The samples were alkylated in 50 mM iodoacetamide in 100 mM ammonium bicarbonate. Samples were then washed, dehydrated and dried, and incubated with 0.2 µg sequencing grade modified porcine trypsin (Promega) overnight at 37°C. The liquid surrounding the gel pieces was removed and brought to 0.1% TFA.

For each of the four membrane fractions (root tonoplast, shoot tonoplast, root plasma membrane and shoot plasma membrane) three biological replicates were used resulting in 12 proteomics samples in total.

**Nano-LC-MALDI MS/MS:** Digests were brought to 0.1% TFA and then fractionated using a Dionex nano-LC with a monolithic PS-DVB capillary column (200 µm x 5 cm, Dionex). Fractions were spotted onto MALDI target plates after mixing with an equal volume of a freshly-prepared 5 mg/mL solution of 4-hydroxy- $\alpha$ -cyano-cinnamic acid (Sigma) in 50% aqueous (v/v) acetonitrile containing 0.1% (v/v) TFA. Positive-ion MALDI mass spectra were obtained using an Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems, Foster City, USA) in reflectron mode with an accelerating voltage of 20 kV. MS spectra were acquired with a total of 1000 laser pulses over a mass range of m/z 800-4000. Final mass spectra were the summation of 20 sub-spectra, each acquired with 50 laser pulses, and internally calibrated using the tryptic autoproteolysis products at m/z 842.509 and 2211.104. Monoisotopic masses were obtained from centroids of raw, unsmoothed data.

For collision-induced dissociation-MS/MS, a Source 1 accelerating voltage of 8 kV, a collision energy of 1 kV, and a Source 2 accelerating voltage of 15 kV were used. Air was used as the collision gas with a recharge threshold of  $9.9 \times 10^{-7}$  torr, producing a Source 2 pressure of about  $1 \times 10^{-6}$  torr. The precursor mass window was set to a relative resolution of 50, and the metastable

suppressor was enabled. Default calibration was used for MS/MS spectra, which were baseline-subtracted (peak width 50) and smoothed (Savitsky-Golay with three points across a peak and polynomial order 4); peak detection used a minimum S/N of 5, local noise window of 50 m/z, and minimum peak width of 2.9 bins. Filters of S/N 20 and 10 were used for generating peak lists from MS and MS/MS spectra, respectively.

**Nano-LC-ESI-MS/MS:** Aliquots of trypsin digests (1 - 3  $\mu$ L) were loaded onto an Ultimate nano-HPLC system (Dionex) equipped with a monolithic PS-DVB capillary column. The separation used a gradient elution with the following conditions: the initial eluant composition was 1% Solvent A; on injection, a linear gradient of Solvent B from 1% to 50% in 30 min was applied, followed by a 5 min wash at 95% Solvent B and finally re-equilibration in 1% Solvent B (Solvent A: 2% (v/v) acetonitrile, 0.1% (v/v) formic acid in H<sub>2</sub>O; Solvent B: acetonitrile, 0.1% (v/v) formic acid). The flow rate was 1.2  $\mu$ L/min and the column temperature was 70°C. Control and data acquisition were with Chromeleon v6.6 software (Dionex). The HPLC was interfaced with a QSTAR® API Pulsar *i* Hybrid LC/MS/MS System (Applied Biosystems) with a MicroIonSpray source (fitted with 20  $\mu$ m ID capillary). Positive ESI MS & MS/MS spectra were acquired over the range 300 – 2000 m/z using information dependent acquisition (IDA). ESI settings: ionspray voltage, 5200; nebulising gas, 5; curtain gas, 35; range, 300 – 2000 m/z; IDA settings: MS, 0.5 s; MS/MS, 0.5 s on 1st and 2nd most abundant ions that meet switch criteria; cycle time, 1.5 s; collision energy, 0 (calculated from IDA CE parameter table); Switch criteria: ions greater than 300, m/z; ions smaller than, 2000 m/z; charge state, 2 to 4; threshold, 20 counts; switch after, 1 spectrum; exclude former target ions for 60 s; ion tolerance, 100 ppm. Instrument control, data acquisition and analysis were carried out with Analyst QS v1.1 software.

**Nano-LC-MALDI MS/MS data analysis:** Mass spectral data obtained in batch mode were submitted to database searching using a locally-running copy of 'Mascot' (Matrix Science Ltd., version 2.1). Batch-acquired MS and MS/MS spectral data were submitted to a combined peptide mass fingerprint and MS/MS ion search through the Applied Biosystems GPS Explorer software interface (version 3.6) to Mascot. Search criteria included: Maximum missed cleavages, 1; Variable modifications, Oxidation (M), Carbamidomethyl; Peptide tolerance, 100 ppm; MS/MS tolerance, 0.1 Da. The most recent version of rice CDS protein database was used.

**Nano-LC-ESI-MS/MS data analysis:** Peptide MS and MS/MS data for database searching were submitted to Mascot (v2.2, Matrix Science). Query data were generated from IDA files using

either Mascot script (v1.6b21) or Mascot Daemon (v2.2) using the same database, modifications and tolerances as described above.

**Phosphoproteomic sample preparation:** Membrane preparations were washed sequentially with 100 mM Na<sub>2</sub>CO<sub>3</sub> and then 50 mM triethylammonium bicarbonate (TEAB). 400-700 µg membrane protein suspended in 50m TEAB at 2-3 mg/ml were digested over night at 37°C with sequencing grade trypsin (Promega) at a trypsin:protein ratio of 1:50. Digests were acidified with formic acid (2% final concentration), any precipitate removed by centrifugation (5 min at 20,000g), and the peptide supernatant lyophilised. For cation exchange chromatography, the peptides were redissolved in 0.5 ml 2.5% formic acid/ 30% acetonitrile and loaded onto a pre-equilibrated 30 mg/ 1 cm<sup>3</sup> Oasis MCX column. After washing with the same solution, peptides were step-eluted in twelve 0.7 ml fractions with 5-300 mM ammonium formate/formic acid (ph 2.7) in 30% acetonitrile. Eluates were vacuum-concentrated to remove acetonitrile and then freeze-dried. For IMAC, the fractions were redissolved in 300 µl 250 mM acetic acid/30% acetonitrile and incubated with 30 µl PhosSelect resin (Sigma). Phosphopeptides were eluted with 400 mM aqueous ammonia/30% AN, concentrated and acidified with 1% FA before mass spectrometric analysis.

**Phosphoproteomic mass spectrometry and analysis:** LC-MS/MS data were acquired using a NanoAcquity LC (Waters, Manchester, UK) coupled to a 4000 Q-TRAP (Applied Biosystems, Farmingham, USA). For each, 5 µL of sample was concentrated/desalted on a pre-column (20 mm x 180 µm i.d, Waters). Peptides were then separated using a gradient from 99% A (0.1% formic acid in water) and 1% B (0.1% formic acid in acetonitrile) to 30% B, in 40 min at 600 nL min<sup>-1</sup>, using a 100 mm x 100 µm i.d. 1.7 µM BEH C18, analytical column (Waters). The mass spectrometer was configured to acquire enhanced resolution and product ion scans for peptides with ion counts greater than 250000 cps, with a precursor ion selection window of *m/z* 400-1600. Tandem mass spectra were acquired from *m/z* 140-1400, upon the two most intense peaks, which after 2 occurrences were excluded for 12 s.

Raw data were submitted to a database search (TIGR rice genome release 5.0 January 2007) with Mascot version 2.1. The following settings were used: tryptic cleavage allowing max. 1 missed cleavage; Met oxidation and Ser/Thr phosphorylation as variable modifications; peptide tolerance +/- 1.2 Da, MS/MS tolerance +/- 0.6 Da; peptide charge 2<sup>+</sup> and 3<sup>+</sup>; monoisotopic; instrument ESI Quad-TOF. With these settings, peptide scores above 41 were significant (p<0.05) by Mascot

criteria. However, peptides with lower scores ( $\geq 20$ ) are reported if all of the following criteria were met: (1) most of the major peaks represent b or y ions, the precursor ion, or neutral loss of phosphoric acid or water from these ions; (2) a stretch of at least three sequential b or y ions matching the sequence is observed and (3) prominent ions are in agreement with well-known sequence specific fragmentation, e.g. N-terminal of Pro and typically Gly or C-terminal of Asp. Some peptides with scores above 41 that did not meet these requirements were also discarded. Where phosphorylation sites were ambiguous, i.e. there were more Ser/Thr residues than phosphate residues were present and the difference of the peptide scores for the isomers was less than 10, manual verification was carried out. Only if the expected fragment ions diagnostic for one isoform matched major ions substantially better than those of the other isoform was the former listed as a phosphorylation site “defined with high confidence”. Apart from the s/n ratio of potentially matched ions we also considered the fact that only fragments containing at least one unphosphorylated Ser or Thr gave rise to ion pairs spaced 18 Da (e.g. y and y<sup>0</sup>), while a genuine b-98 or y-98 ion never has a larger “twin peak” unless another unphosphorylated residue is present. Mascot results were processed with the Scaffold software (Scaffold-01\_07\_00) to obtain annotated spectra (see suppl. data).

**Acknowledgements:** This work was supported by the Biotechnology and Biological Sciences Research (grant C510032 to FJMM). We thank L Skiera and L Cheng for technical assistance and Dr D Knight and Ms S Warwood (Mass Spectrometry Core Facility, University of Manchester) for proteomic services and help with data evaluation.



## References (word count 1027)

- Ahn, S.J., Shin, R. and Schachtman, D.P.** (2004) Expression of KT/KUP genes in *Arabidopsis* and the role of root hairs in K<sup>+</sup> uptake. *Plant Physiol* **134**,1135-1145.
- Alexandersson, E., Saalbach, G., Larsson, C. and Kjellbom, K.** (2004) *Arabidopsis* plasma membrane proteomics identifies components of transport, signal transduction and membrane trafficking. *Plant Cell Physiol* **45**, 1543-1556.
- Armengaud, P., Breitling, R. and Amtmann, A.** (2004) The potassium-dependent transcriptome of *Arabidopsis* reveals a prominent role of jasmonic acid in nutrient signaling. *Plant Physiol.* **136**, 2556-2576.
- Banuelos, M.A., Garciasdeblas, B., Cubero, B. and Rodriguez-Navarro, A.** (2002) Inventory and functional characterization of the HAK potassium transporters of rice. *Plant Physiol.* **130**, 784-795.
- Benschop, J.J., Mohammed, S., O'Flaherty, M., Heck, A.J.R., Slijper, M. and Menke, F. L. H.** (2007) Quantitative phosphoproteomics of early elicitor signaling in *Arabidopsis*. *Mol. Cell. Prots.* **6**, 1198-1214.
- Brugiere, S., Kowalski, S., Ferro, M., Seigneurin-Berny, D., Miras, S., Salvi, D., Ravanel, S., d'Herin, P., Garin, J., Bourguignon, J., Joyard, J. and Rolland, N.** (2004) The hydrophobic proteome of mitochondrial membranes from *Arabidopsis* cell suspensions. *Phytochem* **65**, 1693-1707.
- Carter, C., Pan, S., Zouhar, J., Avila, E.L., Girke, T. and Raikhel, N.V.** (2004) The vegetative vacuole proteome of *Arabidopsis thaliana* reveals predicted and unexpected proteins. *Plant Cell.* **16**, 3285-3303.
- Chrispeels, M.J., Morillon, R., Maurel, C., Gerbeau, P., Kjellbom, P. and Johansson, I.** (2001) Aquaporins of plants, Structure, function, regulation, and role in plant water relations. *Aquaporins.* **51**, 277-334.
- Ducos, E., Fraysse, A.S. and Boutry, M.** (2005) NtPDR3, an iron-deficiency inducible ABC transporter in *Nicotiana tabacum*. *FEBS Letters.* **579**, 6791-6795.
- Endler, A., Meyer, S., Schelbert, S., Schneider, T., Weschke, W., Peters, S.W., Keller, F., Baginsky, S., Martinoia, E. and Schmidt, U.G.** (2006) Identification of a vacuolar sucrose transporter in barley and *Arabidopsis* mesophyll cells by a tonoplast proteomic approach. *Plant Physiol.* **41**, 196-207.

- Gaxiola, R.A., Palmgren, M.G. and Schumacher, K.** (2007) Plant proton pumps. *FEBS Letters* 581, 2204-2214.
- Gierth, M., Maser, P. and Schroeder, J.I.** (2005) The potassium transporter AtHAK5 functions in K<sup>+</sup> deprivation- induced high-affinity K<sup>+</sup> uptake and AKT1 K<sup>+</sup> channel contribution to K<sup>+</sup> uptake kinetics in *Arabidopsis* roots. *Plant Physiol.* **137**, 1105-1114.
- Jacquiod, M., Villiers, F., Kieffer-Jacquiod S., Hugouvieux, V., Bruley, C., Garin, J. and Bourguignon, J.** (2007) A proteomics dissection of *Arabidopsis thaliana* vacuoles isolated from cell culture. *Mol. Cell. Proteomics.* **6**, 394-412.
- Komatsu, S. and Tanaka N.** (2005) Rice proteome analysis: A step toward functional analysis of the rice genome. *Proteomics* **5**, 938-949.
- Larsson, C., Widell, S. and Kjellbom, P.** (1987) Preparation of high-purity plasma-membranes. *Meth. Enzymol.* **148**, 558-568.
- Loque, D., Lalonde, S., Looger, L.L., von Wiren, N. and Frommer, W.B.** (2007) A cytosolic trans-activating domain essential for ammonium uptake. *Nature.* **446**, 195-198.
- Lüttge, U., Pfeifer, T., Fischer-Schliebs, E. and Ratajczak, R.** (200) The role of vacuolar malate-transport capacity in Crassulacean Acid Metabolism and nitrate nutrition. *Plant Physiol.* **124**, 1335-1348.
- Ma, J.F., Tamai, K., Yamaji, N., Mitani, N., Konishi, S., Katsuhara, M., Ishiguro, M., Murata, Y. and Yano, M.** (2006) A silicon transporter in rice. *Nature.* **440**, 688-691.
- Maathuis, F.J.M., Filatov, V., Herzyk, P., Krijger, G.C., Axelsen, K.B., Chen, S., Green, B.J., Madagan, K.L., Sánchez-Fernández, R., Forde, B.G., Palmgren, M.G., Rea, P.A., Williams, L.E., Sanders, D. and Amtmann, A.** (2003) Transcriptome analysis of root transporters reveals participation of multiple gene families in response to cation stress. *Plant J.* **35**, 675-692.
- Maathuis, F.J.M. and Amtmann, A.** (2005) Transcriptional profiling of membrane transporters. In: *Plant Nutritional Genomics*, (Broadley, M.R. and White, P.J. eds), Blackwell, pp. 170-200.
- Marmagne, A., Rouet, M.A., Ferro, M., Rolland, N., Alcon, C., Joyard, J., Garin, J., Barbier-Brygoo, H. and Ephritikhine, G.** (2004) Identification of new intrinsic proteins in *Arabidopsis* plasma membrane proteome. *Mol. Cell. Proteomics.* **3**, 675-691.

**Niittyla, T., Fuglsang, A.T., Palmgren, M.G., Frommer, W.B. and Schulze, W.X. (2007)** Temporal analysis of sucrose-induced phosphorylation changes in plasma membrane proteins of *Arabidopsis*. *Mol. Cell. Proteomics*. **6**, 1711-1726.

**Nühse, T.S., Stensballe, A., Jensen, O.N. and Peck, S.C. (2003)** Large-scale analysis of in vivo phosphorylated membrane proteins by immobilized metal ion affinity chromatography and mass spectrometry. *Mol. Cel. Proteomics*. **2**, 1234-43.

**Nühse, T.S., Stensballe, A., Jensen, O.N. and Peck, S.C. (2004)** Phosphoproteomics of the *Arabidopsis* plasma membrane and a new phosphorylation site database. *Plant Cell*. **16**, 2394-405.

**Paszkowski, U., Kroken, S., Roux, C. and Briggs, S.P. (2002)** Rice phosphate transporters include an evolutionarily divergent gene specifically activated in arbuscular mycorrhizal symbiosis. *Proc Natl Acad Sci USA*. **99**, 13324-13329.

**Peltier, J., Ytterberg, A.J., Sun, Q. and van Wijk, K.J. (2004)** New functions of the thylakoid membrane proteome of *Arabidopsis thaliana* revealed by a simple, fast, and versatile fractionation strategy. *J. Biol. Chem.* **279**, 49367-49383.

**Pighin, J.A., Zheng, H., Balakshin, L.J., Goodman, I.P., Western, T.L., Jetter, R., Kunst, L. and Samuels, A.L. (2004)** Plant Cuticular Lipid Export Requires an ABC Transporter. *Science*. **306**, 702 – 704.

**Rentsch, D., Laloi, M., Rouhara, I., Schmelzer, E., Delrot, S. and Frommer, W.B. (1995)** NTR1 encodes a high affinity oligopeptide transporter in *Arabidopsis*. *FEBS Lett.* **21**, 264-268.

**Sancenon, V., Puig, S., Mira, H., Thiele, D.J. and Penarrubia, L. (2003)** Identification of a copper transporter family in *Arabidopsis thaliana*. *Plant Mol. Biol.* **51**, 577-587.

**Tanaka, N., Fujita, M., Handa, H., Murayama, S., Uemura, M., Kawamura, Y., Mitsu, T., Mikami, S., Tozawa, M., Yoshinaga, T. and Komatsu, S. (2004)** Proteomics of the rice cell, systematic identification of the protein populations in subcellular compartments. *Mol. Gen. Genomics*. **271**, 566-576.

**Tornroth-Horsefield, S., Hedfalk, K., Karlsson, M., Johanson, U., Wang, Y., Tajkhorshid, E., Horsefield, R., Nyblom, M., Backmark, A., Kjellbom, P. and Neutze, R. (2007)** Aquaporin gating. *J. Biomol. Struct. Dynam.* **24**, 719-721.

- Toyofuku, K., Kasahara, M. and Yamaguchi, J.** (2000) Characterization and expression of monosaccharide transporters (OsMSTs) in rice. *Plant Cell Physiol.* **41**, 940-947.
- Vitart, V., Baxter, I., Doerner, P., and Harper, J.F.** (2001) Evidence for a role in growth and salt resistance of a plasma membrane H<sup>+</sup>-ATPase in the root endodermis. *Plant J.* **27**, 191-201.
- Whiteman, S., Serazetdinova, L., Jones, A.M.E., Sanders, D., Rathjen, J., Peck, S.C. and Maathuis, F.J.M.** (2008) Identification of novel proteins and phosphorylation sites in the vacuolar membrane of *Arabidopsis thaliana*. *Proteomics* In press.
- Wintz, H., Fox, F., Wu, Y., Feng, V., Chen, W., Chang, H., Zhu, T. and Vulpe, C.** (2003) Expression profiles of *Arabidopsis thaliana* in mineral deficiencies reveal novel transporters involved in metal homeostasis. *J. Biol. Chem.* **278**, 47644-47653.
- Wu, Z.Y., Liang, F., Hong, B.M., Young, J.C., Sussman, M.R., Harper, J.F. and Sze, H.** (2002) An endoplasmic reticulum-bound Ca<sup>2+</sup>/Mn<sup>2+</sup> pump, ECA1, supports plant growth and confers tolerance to Mn<sup>2+</sup> stress. *Plant Physiol.* **130**, 128-137.
- Yazaki, K.** (2006) ABC transporters involved in the transport of plant secondary metabolites. *FEBS Letters*, **580**, 1183-1191.

**Suppl. material:** The supplementary file contains data summarising all proteins and all membrane proteins detected in the four respective membrane fractions. It also contains data on all identified phosphopeptides.

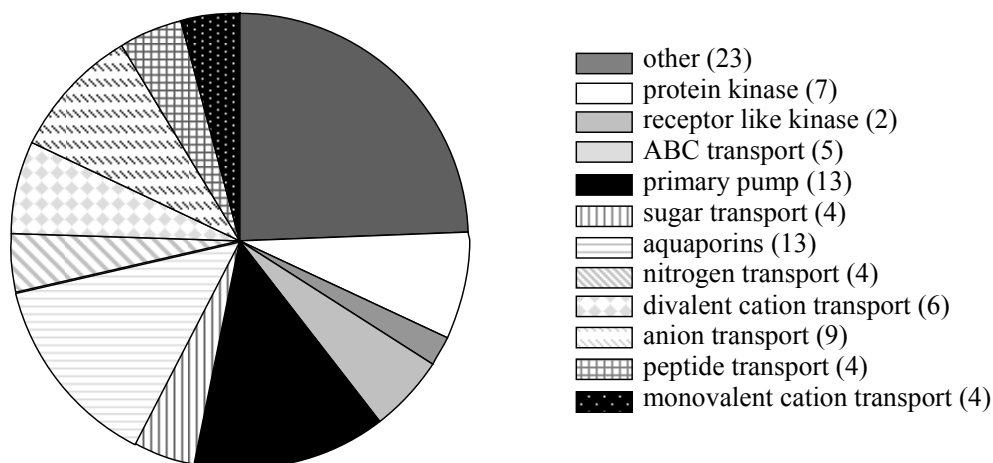
**Table 1:** Membrane transport proteins and phosphopeptides detected in rice membrane fractions enriched in root plasma membrane (PM), root tonoplast (TO), shoot PM and shoot TO. The third column denotes the number of transmembrane domains (TMDs) according to TMHMM predictions (<http://www.cbs.dtu.dk/services/TMHMM/>). Columns four to seven show the total number of peptides identified for each protein in each membrane fraction. Empty space signifies no peptide was identified. Columns eight and nine denote the identified phosphosites. Underlined characters indicate defined phosphorylation sites with high confidence (bold) or an alternative phosphorylation site where the exact position is uncertain (non-bold). Superscripts <sup>1</sup>, <sup>2</sup>, <sup>3</sup> or <sup>4</sup> denote identification of phosphopeptides in orthologous *Arabidopsis thaliana* proteins described in Nühse et al., 2004, Niittyla et al., 2007, Whiteman et al., 2008 or Benschop et al., 2007.

<b>Gene code</b>	<b>Root PM, annotation</b>	<b>TMD</b>	<b>RO PM</b>	RO TO	SH PM	SH TO	<b>peptide 1</b>	<b>peptide 2</b>
Os09g16330	ABC-transporter, PDR5-like	7	<b>4</b>					
Os08g03350	Amino-acid transporter HT1	10	<b>8</b>		5			
Os04g43070 <sup>1,2,3,4</sup>	Ammonium-transporter AMT1;1	11	<b>6</b>	4			ISAEDETS <span style="border: 1px solid black;">GMDL</span> <span style="border: 1px solid black;">[pT]</span> R	
Os02g40730	Ammonium-transporter AMT1;2	11	<b>8</b>					
Os02g44630	aquaporin PIP1;1	6	<b>6</b>		7			
Os04g47220	aquaporin PIP1;2	4	<b>11</b>	11	8			
Os02g57720	aquaporin PIP1;3	6	<b>6</b>					
Os07g26690 <sup>1</sup>	aquaporin PIP2;1	6	<b>17</b>		15	11	GKDEVME <span style="border: 1px solid black;">[pS]</span> GGAAGEFAAK	
Os02g41860	aquaporin PIP2;2	6	<b>14</b>		17	4		
Os04g44060	aquaporin PIP2;3	6	<b>14</b>					
Os04g16450	aquaporin PIP2;6	6	<b>3</b>	4	36	12		
Os03g48310 <sup>4</sup>	ATPase, plasma-membrane type, AHA1	8	<b>8</b>					
Os07g09340 <sup>4</sup>	ATPase, plasma-membrane type, AHA2	8	<b>10</b>		11			
Os12g44150 <sup>1</sup>	ATPase, plasma-membrane type, AHA3	6	<b>14</b>		19	13	<span style="border: 1px solid black;">[pT]</span> LHGLQPPDAK	
Os05g25550	ATPase, plasma-membrane type, AHA4	8	<b>2</b>					
Os02g55400	ATPase, plasma-membrane type, AHA6	8	<b>11</b>				QMEAVEEGR <span style="border: 1px solid black;">[pS]</span> IPASAK	
Os04g56160	ATPase, plasma-membrane type, AHA7	9	<b>33</b>	37	53	26		
Os03g08560	ATPase, plasma-membrane type, AHA9	7	<b>6</b>					
Os03g01700	Citrate transporter	9	<b>3</b>					
Os04g32920 <sup>1</sup>	Potassium-transporter, HAK1	12	<b>5</b>					
Os02g51110	Silicon influx-transporter OsNIP2.1	6	<b>6</b>	3				
Os05g39540	Zinc-transporter OsZIP2	8	<b>3</b>					
<b>Gene code</b>	<b>Root TO, annotation</b>	<b>TMD</b>	RO PM	<b>RO TO</b>	SH PM	SH TO	<b>peptide 1</b>	
Os04g52900	ABC-transporter, MRP1	14		<b>31</b>	8			
Os08g30740	ABC-transporter,	12		<b>9</b>				
Os12g42850	Amino-acid permease	10		<b>8</b>				
Os02g40710	Ammonium-transporter	9		<b>7</b>				
Os04g43070 <sup>1,2,3,4</sup>	Ammonium-transporter, AMT1;1	11	6	<b>4</b>				
Os02g51110	Silicon-influx-transporter OsNIP2.1	6		<b>5</b>			EG(SS)QKLSSFK	
Os04g47220	aquaporin, PIP1;2	6	11	<b>11</b>	8			
Os04g16450	aquaporin PIP2;6	6	3	<b>4</b>	36	12		
Os03g05290	aquaporin, TIP1;1	6		<b>8</b>	4	7		
Os02g44080	aquaporin, TIP2;1	6		<b>11</b>				

Os06g22960	aquaporin, TIP2;2	6		<b>18</b>	4	10		
Os04g56160	ATPase, plasma-membrane type, AHA7	9	33	<b>37</b>	53	26		
Os06g45120	ATPase, vacuolar, subunit A	0		<b>19</b>		29		
Os04g51270	ATPase, vacuolar, subunit G	0		<b>5</b>				
Os10g10500	V-ATPase glycoprotein subunit	6		<b>54</b>				
Os03g01700	Citrate-transporter, silicon efflux, OsSli2	9		<b>6</b>			ELGGGAEL	<b>[pS]</b> VVDGK
Os09g26900	COPT5	2		<b>2</b>				
Os01g56420	Ctr-copper-transporter family	2		<b>24</b>				
Os10g42900	Nitrate transporter, low affinity	10		<b>6</b>				
Os03g51050	Peptide-transporter	10		<b>9</b>				
Os01g65100	Peptide-transporter POT/PTR family	11		<b>8</b>				
Os02g51110	Silicon-influx-transporter OsNIP2.1	6	6	<b>3</b>			EG(SS)QKLSSFK	
Os01g11420	Sodium/calcium-exchanger protein	11		<b>4</b>		2		
Os10g39440	Sugar-transporter	10		<b>13</b>	6			
Os10g42960	Urea active-transport protein	15		<b>9</b>				
Os02g09150	V-type H <sup>+</sup> -translocating pyrophosphatase	14		<b>43</b>		16	I	<b>[pS]</b> TDASIK
Os02g55890	V-type H <sup>+</sup> -translocating pyrophosphatase	14		<b>106</b>		11		
<b>Gene code</b>	<b>Shoot PM, annotation</b>	<b>TMD</b>	<b>RO PM</b>	<b>RO TO</b>	<b>SH PM</b>	<b>SH TO</b>	<b>peptide 1</b>	<b>peptide 2</b>
Os04g52900	ABC-transporter, MRP1	14		31	<b>8</b>			
Os01g42380	ABC-transporter, PDR5-like	12			<b>6</b>		(SS)REEDDEEALR	
Os08g03350	Amino-acid transporter HT1	10	8		<b>5</b>			
Os09g19730	Anion channel, V dependent	11			<b>3</b>			
Os02g44630	aquaporin PIP1;1	6	6		<b>7</b>			
Os04g47220	aquaporin PIP1;2	4	11	11	<b>8</b>			
Os07g26690 <sup>1</sup>	aquaporin PIP2.1	6	17		<b>15</b>	11	ALGSFR	<b>[pS]</b> NA
Os02g41860 <sup>1,2</sup>	aquaporin PIP2.2	8	14		<b>17</b>	4		
Os04g16450 <sup>2,3</sup>	aquaporin PIP2.6	6	3		<b>36</b>	12	ALG	<b>[pS]</b> FRSNP <b>[pS]</b> N
Os09g36930	aquaporin PIP2.7	6			<b>4</b>		ALSSFR(ST)SVTA	
Os03g05290	aquaporin, TIP1;1	6		8	<b>4</b>	7		
Os06g22960	aquaporin TIP2.2	6		11	<b>4</b>	10		
Os03g17310	ATPase, calcium-translocating P-type ECA1	7			<b>4</b>			
Os04g51610	ATPase, calcium-translocating P-type ACA11	10			<b>4</b>			
Os02g08010	ATPase, calcium-translocating P-type ACA9	6			<b>3</b>		PVGVQAPEA	<b>[pS]</b> PGR
Os07g09340 <sup>4</sup>	ATPase, plasma-membrane-type, AHA2	6	10		<b>11</b>			







**Figure 1:** Functional classification of membrane proteins (any protein with 1 or more transmembrane domain) detected in rice membrane fractions enriched in root plasma membrane, root tonoplast, shoot plasma membrane and shoot tonoplast. The total number of unique membrane proteins detected in the combined samples was 94.

	960	970	980	989
OsAHA1	AEIARLRELH	TLKGHVE S	VV KLKGLDIDTI	H-QSYTV
OsAHA2	AEIARLRELT	TLKGRMES	VV KQKGLDLETI	Q-QSYTV
OsAHA3	AEIARLRELH	TLKGHVE S	VV KLKGLDIETI	Q-QSYTV
OsAHA4	AEIARLRELN	TLKGHVE S	VV KLKGLDIDTI	Q-QNYTV
OsAHA6	AEITRLRELH	TLKGKVE S	VA KLKGIDLEDV	NNQHYTV
OsAHA7	AEIARLRELH	TLKGHVE S	VV KLKGLDIDTI	Q-NHYTV
OsAHA8	AEIARLGDAH	MLRAHVQ S	VM RLKRVSDSVI	R-SAQTV
OsAHA9	AEVARLRELH	TLKGHVE S	VV KLKGLDIDTI	Q-QSYTV
OsAHA10	-----	-----	-----	-----

**Figure 2:** Phosphorylation sites in the plasma membrane AHA H<sup>+</sup>-ATPases. Two overlapping phosphosites were found in AHA3 and in AHA7 that include a well conserved serine at position 970 in the C-terminus of the proteins. Both AHA3 and AHA7 were observed as a phosphoprotein in shoot tissue and as non-phosphorylated proteins in root tissue.

	251	260	270	280	290		
OsPIP2;1	AWHNHWFVWV	GPFVGA	AIAA	FYHQYILRAG	AIKALG	SFRS NA	-----
OsPIP2;3	AWHDHWIFWV	GPLIGAAIAA	AYHQYVLRAS	AAKLG	SSSF	RG	-----
AtPIP2;6	AWDDQWIFWV	GPFVGA	AIAA	FYHQFVLRAG	AMKAYG	SFRS	QLHELHA
OsPIP2;6	AWDDHWIFWA	GPFIGALAAA	AYHQYILRAA	AIKALG	SFRS	NPSN	--
OsPIP2;7	AWKDHWFVWV	GPVIGAFLAA	AYHKLVL	RGE	AAKALS	SFRS	TSVTA

**Figure 3:** Phosphorylation sites in PIP aquaporins. Four overlapping C-terminal phosphopeptides were found in PIP2;1, PIP2;3, PIP2;6 and PIP2;7 that cover a highly conserved serine at position 286. *Arabidopsis* PIP2;6 and other isoforms show a phosphosite in the same position.

	431	441	451	461	471					
OsNHX1	FSTMVFGMMT	KPLIRLLLPA	S-----	GHPVT	SE-----	PS	S	PK SLHSPL		
OsNHX2	FSTMVFGF	F T KPLL	NLLIPP	R-----	P - - - - -	DIAADL	S	SQ S I IDPL		
OsNHX3	FSTMVFG	L T KPLIR	LLI PA	RHLNR	ESSAL	SD-----	PP	S	PKS FLDPL	
OsNHX4	FTTLV	FGF L T	RPLISAIL	PH QHRQ	STTPGT	GGGGR	STG	SN	S	PKDDFIMPF
AtNHX1	FSTMVFGML	T KPLI	S YLLPH	QNAT--	TSML	-----	S	DDN	T	PK S I HIPL

**Figure 4:** Phosphorylation sites in vacuolar NHX cation exchangers. A C-terminal motif was identified in NHX3 which includes a serine residue at position 490 that is conserved across two further rice NHX isoforms. *Arabidopsis* homologs such as AtNHX1 carry a threonine residue at the same position.