RESEARCH ARTICLE

Identification of novel proteins and phosphorylation sites in a tonoplast enriched membrane fraction of *Arabidopsis thaliana*

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Plant vacuoles play essential roles in many physiological processes, particularly in mineral nutrition, turgor provision and cellular signalling. The vacuolar membrane, the tonoplast, contains many membrane transporters that are critical in the execution of these processes. However, although increasing knowledge is available about the identity of proteins involved in these processes very little is known about the regulation of tonoplast transporters. By studying the phosphoproteome of tonoplast-enriched membranes, we identified 66 phosphorylation sites on 58 membrane proteins. Amongst these, 31 sites were identified in 28 membrane transporters of various families including tonoplast anion transporters of the CLC family, potassium transporters of the KUP family, tonoplast sugar transporters and ABC transporters. In a number of cases, the detected sites were well conserved across isoforms of one family pointing to common mechanisms of regulation. In other cases, isoform-unique sites were present, suggesting regulatory mechanisms tailored to the function of individual proteins. These results provide the basis for future studies to elucidate the mechanistic regulation of tonoplast membrane transporters.

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1 Introduction

The large central vacuole of plant cells has important roles in nutrient storage, development and in responses to environmental change. A major function of the vacuole is as a repository for a range of anions and cations and sugars. Sequestration of these ions in the vacuole contributes to cell turgor

and/or stores the nutrients when in over supply. Release of ions from the vacuole occurs when nutrients become deficient in the cytoplasm, as part of signalling events or during cellular movement. Furthermore, the vacuolar lumen is typically acidic to facilitate secondary transport at the vacuolar membrane and to maintain a lytic compartment for the degradation of cellular compounds.

All these functions critically depend on the movement of ions across the vacuolar membrane, or tonoplast, a process mediated by membrane transporters. The general principles of transport processes at the tonoplast have been well documented [1] and several tonoplast transporters have been

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Abbreviations: TEF, low density membrane fraction; TMD, transmembrane domain

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functionally characterized and identified at the gene level, including pumps, carriers and ion channels [2, 3]. Nevertheless, for many hypothesized or observed transport activities at the tonoplast the genes and proteins that encode them remain unknown and even less is known with respect to how the activity of such proteins is regulated.

To improve our understanding of the composition and regulation of proteins of this important cellular compartment, proteomic analyses of vacuoles from a number of plant species have been performed, including *Arabidopsis* [4–7], barley [8] and rice [9], and cauliflower [10]. This work identified H^+ pumps involved in luminal acidification, carriers for inorganic nutrients, sugars, and peptides, aquaporins and occasionally ion channels that could contribute to the trans-tonoplast movement of cations and anions.

Although these studies provide an excellent basis for future research to elucidate the functions of transport proteins, they do not – *a priori* – provide any immediate insights into the potential mechanistic regulation of transport processes at the tonoplast. Reversible protein phosphorylation, mediated by the action of kinases, is one of the most prominent mechanisms for the post-translational modulation of protein activity (e.g. [11, 12]). Indeed, the activity of a number of pumps, channels, and transporters at other membranes has been found to be modulated by protein phosphorylation. Thus, identification of phosphorylation sites in tonoplast transporter proteins is likely to generate testable hypotheses for investigating the regulation of these important proteins. Recently, high-throughput phosphoproteomic techniques were applied to the plasma membrane fraction of suspension-cultured *Arabidopsis* cells [13–17]. These studies identified many new phosphorylation sites, some of which were demonstrated to have regulatory functions [15, 18].

To investigate posttranslational regulation of vacuolar proteins, we performed a phosphoproteomic analysis using a tonoplast-enriched membrane fraction isolated from intact *Arabidopsis* shoot tissue. In this work, we identified 66 phosphorylation sites on 58 membrane proteins, the majority of which consisted of novel sites. The potential physiological relevance of these sites in specific membrane transporters is discussed.

2 Materials and methods

2.1 Plant material

Arabidopsis thaliana ecotype Columbia was grown in Levington F2 soil in a Percival growth cabinet under short day conditions (10 h light at $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ intensity, 14 h dark, 23°C day, 17°C night and 70% RH).

2.2 Chemicals

All chemicals for membrane preparation, unless otherwise stated, were obtained from Sigma or Fluka.

2.3 Preparation of tonoplast-enriched fraction

Arabidopsis leaf material was harvested at fixed times during the light dark cycle from 5-week-old plants that had reached the mature rosette stage, and used directly for membrane preparation. The protocol is based on a discontinuous sucrose gradient centrifugation step to separate membrane fractions and typically leads to three-to-sixfold enrichment of tonoplast membrane [19]. To quantify the membrane composition, ATPase assays were carried out according to [20] using 50 mM KNO_3 , 0.1 mM vanadate and 3 mM azide as specific inhibitors of the tonoplast, plasma membrane and mitochondrial ATPase activity.

Leaf material (300 g/preparation) was homogenized in 400 mL of medium (1.1 M glycerol, 5 mM EGTA, 1.5 % w/v PVP, 1% w/v ascorbic acid, 50 mM Tris-Mes, pH 7.6). Homogenate was passed through Miracloth and centrifuged at $3600 \times g$ for 10 min. Supernatant was centrifuged at $100\,000 \times g$ for 30 min. The microsomal membrane pellet was resuspended in overlay medium (1.1 M glycerol, 5 mM Tris-Mes pH 7.6, 1 mM EGTA, 2 mM DTT) and applied to a 10/23% discontinuous sucrose gradient made up in homogenization medium. Gradients were centrifuged at $100\,000 \times g$ for 2 h, after which material at the 10/23% interface was removed, diluted with overlay medium and recentrifuged at $100\,000 \times g$ for 30 min. The low-density membrane pellet was carbonate washed [21]. Membrane preparations were stored at -80°C .

Prior to phosphoproteomic analysis, tonoplast-enriched fraction (TEF) samples were prepared by phenol back-extraction as described [22]. Briefly, the membrane samples were extracted with Tris-buffered phenol and then twice with Back Extraction Buffer (0.1 M Tris, pH 8.4, 0.02 M KCl, 10 mM EDTA, 0.4% v/v β -mercaptoethanol). Proteins were precipitated with 0.1 M NH_4OAc in methanol, washed once with 0.1 M NH_4OAc in methanol and twice with 80% acetone. Samples were maintained as acetone suspensions until phosphoproteomic analysis.

2.4 Phosphopeptide preparation

Proteins precipitated by acetone were resuspended in 50 mM ammonium bicarbonate with 0.05% Rapigest (Waters) and phosphatase inhibitors. Proteins were digested with trypsin (Promega) overnight, lyophilised, and dissolved in 5 mM ammonium formate, pH 2.7 in 30% ACN v/v, 0.1% formic acid v/v. Tryptic peptides were run on a cation exchange column (SCX; polysulfoethylaspartamide column size: $200 \times 3.2 \text{ mm}$, $3.5 \mu\text{m}$, 200 Å, PolyLC, Columbia, USA) for sample fractionation. SCX separation was performed using an elution gradient of eluent A (5 mM ammonium formate pH 2.7 in 30% ACN, v/v) and eluent B (500 mM ammonium formate pH 2.7 in 30% ACN, v/v) with increasing eluent B in 20-min steps from 0 to 8, 16, 24, 50 and 100%, and holding eluent B at 100% for 10 min. Flow rate was $400 \mu\text{L/min}$. Up to 18 1-mL fractions were collected and all fractions collected

from sample injection to 100% of eluent B were subsequently used for enrichment of phosphopeptides.

HPLC fractions were collected and lyophilized, dissolved in 10% v/v acetic acid and then run through a Ga³⁺- IMAC column (Pierce, Rockford, USA) to enrich for phosphopeptides. The manufacturer's protocol was used with following modifications: all wash volumes were increased to 500 μ L, wash one was performed with 0.2% acetic acid v/v and 100 mM NaCl, wash two was done with 0.2% acetic acid v/v and 20% ACN v/v. IMAC eluates were lyophilised and re-dissolved in 1% v/v formic acid for analysis by MS.

For each phosphoproteomics analysis, three TEF samples were pooled. In total, four phosphoproteomics analyses, as detailed in Supporting Information Table S5, were carried out.

2.5 MS

LC-MS/MS analysis was performed using a LTQ mass spectrometer (Thermo Electron, San Jose, CA) and a nanoflow-HPLC system (Surveyor, Thermo Electron). Peptides were applied to a precolumn (C18 pepmap100, LC Packings) connected to a self-packed C18 8-cm analytical column (BioBasic resin ThermoElectron; Picotip 75 μ m id, 15 μ m tip, New Objective). Peptides were eluted using a gradient of 2 to 60% ACN in 0.1% formic acid over 50 min at 50 nL/min. Data-dependent acquisition of MS/MS consisted of selection of the six most abundant ions in each cycle: MS m/z 300 to 2000, minimum signal 1000, collision energy 28, five repeat hits (*i.e.* a precursor ion was allowed to trigger MS2 five times before being added to an exclusion list), 300 s exclusion. MS3 were triggered if the neutral loss of phosphoric acid (49 m/z for 2+ parent ions) was detected in the three most abundant ions on the preceding MS2. Collision energy for MS3 was 35. In all cases, the mass spectrometer was operated in positive ion mode with a nano-spray source and a capillary temperature of 200°C; no sheath gas was employed and the source and focusing voltages were optimised for the transmission of angiotensin.

Raw data were processed using BioWorks 3.2 and TurboSEQUENT (Thermo Electron). MS/MS spectra as.dta files were collected by extract_msn (Thermo Electron Corp.) with dta generation parameters: MW range 300.00–3500.00, threshold absolute 1000, group scan 30, minimum 1, and minimum ion count 10. As the charge state cannot be reliably determined, the .dta files were generated with 2+ and 3+ charge states for each MS/MS spectrum and searched against the *Arabidopsis* genome supplemented with common contaminants (TIGR6 plus trypsin, keratins; sequences collated by Thermo Electron. The searched database contained 28 954 protein sequences in total). Fully tryptic peptides with one missed cleavage were specified allowing the following variable modifications; oxidized Met and phosphorylated Ser or Thr. Precursor mass tolerance was 2 Da, fragment tolerance 1 Da and mass values were averaged with one missed tryptic cleavage allowed. MS2 and

MS3 data were searched separately. Peptide hits were filtered by Xcorr and charge state [Xc (+1, 2, 3) 2.0, 2.2, 3.5] and peptide probabilities (Pp; calculated by BioWorks) of less than 0.01 gave false-positive rates of less than 1%. The false-positive rate was calculated using a reversed version of the database and is the percentage of twice the number of hits from the reversed database to the total number of hits. Each verified peptide was checked for redundant matches (to conserved peptides) by in-house blast (to *Arabidopsis* TAIR6) for short sequences. Peptide matches to more than one protein are indicated in Table 1 and Supporting Information Table 1.

2.6 Verification of spectra

All peptides passing the basic threshold criteria of Xcorr and Pp (see above) were entered into a custom built database for manual verification. The database served to combine the data from replicates and experiments, bring MS2 and MS3 spectra together (this is non-trivial using SEQUEST) and provide a rapid visualization assessment tool by colouring peaks blue (for the dominant loss of phosphoric acid in MS2), yellow if the peaks could be accounted for by b- or y-ion fragmentation, or red for unidentified peaks. Spectra were accepted if the S/N was visually accepted and if most of the main peaks could be accounted for. Peptides accepted by visual inspection were scored '3' when identified in two or more technical replicates (see Supporting Information Table S5 for details) in both MS2 and MS3 spectra. A '2' score denotes a peptide identified in two or more technical replicates by either MS2 or MS3, and a score of '1' denotes peptides that were identified once only. Finally, we implemented the scoring algorithm of Olsen and Mann [23, 24] to determine theoretical masses of b and y ions of the peptide. A match is called whenever a peak from the spectrum falls within a user-specified window of error around the theoretical ion mass. For our data, an error of ± 0.4 Da was selected. If the *p*-value for a specific position of phosphorylation was greater than three orders of magnitude less than the nearest alternative, we called that site 'determined' (marked with pS or pT in Table 1). If two or more potential sites were within three orders of magnitude then the sites were classified as ambiguous and marked (s) or (t) in Table 1. This annotation was only undertaken for phosphopeptides belonging to membrane proteins. Scoring information for all phosphopeptides identified in this study is presented in Supporting Information Table S4.

2.7 Annotated spectra

To generate annotated spectra for all final accepted MS/MS spectra, selected.dta files were extracted from our database (details of extraction and generation above) and searched against MASCOT (version 2.1, database *Arabidopsis* TAIR7

Table 1. Phosphopeptides from TEF membrane transporter proteins

Locus	TMD	Annotation	Peptide(s) ^{a)}	Precursor (m/z)	Xcorr	Charge (z)	SUBA localisation	Ref.
At2g26910	13	ABC transporter family	[s]t[il]sFKDEIEEELRS	954.16	3.43	2	ND	[1]
At4g15236	11	ABC transporter family	VIEYFM[ps]IPGVPKLK	901.42	3.00	2	ND	[1]
At1g51500	7	ABC transporter family	KVP[ps]JLSSLSR	572.11	2.31	2	ND	[1]
At3g62700	16	ABC transporter family	S[ps]JIESPR	636.57	4.00	2	TO (ms)	[1]
At4g13510 ^{b)}	9	ammonium transporter (AMT1)	ISSEDEMAGMDM[pT]IR	827.54	4.00	2	PM (ms/gfp)	[14, 18]
At2g39010 ^{c)}	6	Aquaporin putative	AYG[s]VR[s]QLHLSAS	824.67	2.22	2	PM (ms)	[14]
At2g37170/	6	Aquaporin PIP2B	SLG[ps]JFR[ps]AANV	635.14	2.78	2	PM (ms)	[14]
At2g37810/								
At3g53420 ^{b)}								
At2g18960 ^{b, c)}	10	ATPase 1 plasma membrane-type	GLDIDTAGHHY[pT]TV	740.31	3.00	2	TO/PM (ms)	[14, 17]
At4g30190 ^{c)}	8	ATPase 2 plasma membrane-type	EAVNIFPEKGI[ps]YR	746.88	3.00	2	TO/PM (ms/gfp)	[17]
At5g62670	9	ATPase plasma membrane-type	[pT]LHGLQAPDAK	567.08	2.32	2	PM (ms)	
At5g7110 ^{b, c)}	6	Calcium-transporting ATPase 8 plasma membrane-type	SEHAD[ps]JDSDTFYIPSK	939.95	5.00	2	PM (ms/gfp)	[14, 17]
At1g30450	9	Cation-chloride cotransporter	SMTGEQIQAPSL[ps]IPR	785.32	4.00	2	ND	
At5g40890/	9	Chloride channel protein (CLC-a/b)	TL[ps]SSTPLALVGAK	669.71	4.00	2	TO/unclear (gfp)	[25, 27]
At3g27170								
At5g49890	10	Chloride channel protein (CLC-c)	KI[ps]sGILDDG SVGFR	772.95	4.00	2	TO (ms)	[27]
			TTFG[ps]QILR	552.1	2.2	2		
At2g03620	2	Magnesium transporter (MRS2-5)	[ps]APV[ps]PVVGSTSGNFGK	826.87	3.57	2	PM (ms)	
At5g04930	10	Phospholipid-transporting ATPase 1	EYTFGLG[ps]IKR	596.12	2.2	2	PM (ms)	
At4g23640	14	Potassium transporter/tiny root hair 1 (KUP4/TRH1)	[s]l[s]EANIAAGSSRS	636.57	4.00	2	TO (ms)	[30, 31]
At4g33530	11	Potassium transporter family (KUP5)	ALE[ps]JGDDHND[pT]D[ps] EDDTLR	1267.51	5.00	2	TO (ms)	[30, 31]
At5g09400	12	Potassium transporter family (KUP7)	VD[ps]JFDEVALEVP GAPR	891.05	3.00	2	TO (ms)	[30, 31]
At5g14880	13	Potassium transporter putative (KUP8)	[s]DKPLNY[s]PDPENESGINERS	781.51	5.00	3	ND	[30, 31]
			LPNQQPADEQI[ps]S[ps]JFR	1122.19	3.28	2	PT (ms)	[30, 31]
At1g60160	13	Potassium transporter family (KUP12)	GFEKEDL[ps]PVVK	945.98	3.00	2	ND	
At1g52190	10	Proton-dependent oligopeptide transport (POT)	QTAEGSANPEPDQI[ps]IPR	714.81	3.00	2	PM (ms)	
At1g57990 ^{b)}	10	Purine permease-related low	GFVFPVPG[s]P[t]ERS	1046.37	4.00	2	PM (ms)	[14]
At3g05030/	12	Sodium proton exchanger putative (NHX2)		735.89	2.2	2	ND	[38]
At5g27150								
At1g71880	12	Sucrose transporter (SUC1)	DAAALE[il]sIPEDFDQPSPLRS	1134.51	3.00	2	TO/PM (ms/gfp)	[17, 29]
At5g17010	12	Sugar transporter family protein	SSGEI[ps]JPEREPLIK	811.72	3.00	2	TO (ms)	
At1g20840	10	Sugar transporter (TMT1)	PVPEQ[ss]LGLRS	689.36	3.00	2	TO (ms/gfp)	
			DNDYATDDGAGDDDD[ps]JDNILR	1235.41	4.00	2		
			DIESHYNKNDYATDDGAGDDDD[ps]JDNIL	1153.15	9.00	3		
At5g65687	11	Sugar transporter related	SNEV[s]EDDEVEEDKLESK	1081.3	4.55	2	ND	

a) Bold characters in square brackets indicate high confidence phosphorylation sites; lower case letters in square brackets indicate alternative phosphorylation sites. The \$ sign indicates one phosphorylation site, position uncertain.

b) indicates protein with known phosphopeptide(s) identified by Nühse *et al.* [14].

c) indicates protein with known phosphopeptide(s) identified by Niittyla *et al.* [17].

Full scoring information is presented in Supporting Information Table 1 (Sheet 4). Subcellular localisation in tonoplast (TO), plasma membrane (PM) or plastid (PT) is based on experimental data from MS (ms) or GFP-fusion proteins (gfp) as collated in the SUBA database (<http://www.plantenergy.uwa.edu.au/applications/suba/index.php>). ND denotes 'no data' available.

plus trypsin, keratins; sequences collated by Thermo Electron containing 32 721 protein sequences); precursor mass tolerance was 1.2 Da, fragment tolerance 0.6 Da, mass values were monoisotopic, fully tryptic peptides were specified with one missed cleavage allowed. Variable modifications of Oxidation (M), Phospho (ST), Phospho+PL (S), Phospho+PL (T), Phospho-NL (S), Phospho-NL (T) were included (PL and NL denote prompt and neutral loss of the phosphate group). Spectra for all peptides are available in the Supporting Information file 'PhosphoSpectra'.

2.8 Synthetic peptide

A phosphopeptide standard for CLC a or b (TL[pS]STPLALV-GAK) was synthesized by Thistle Research (Glasgow, UK). The lyophilized peptide was re-dissolved in PBS and desalted with a zip tip (Millipore) before MS analysis as described above.

2.9 Bioinformatics

Prediction of transmembrane domains (TMD) for phosphoproteomic datasets was carried out using the bulk data retrieval and analysis feature from the TAIR website (<http://www.arabidopsis.org/tools/bulk/protein/index.jsp>). Similarly, the functional categorisation of the verified phosphoproteins was carried out using the bulk download from TAIR (<http://www.arabidopsis.org/tools/bulk/go/index.jsp>). Analysis of membrane proteins was carried out using the Aramemnon database: <http://aramemnon.botanik.uni-koeln.de/>, including use of the consensus prediction of TMD for the purposes of creating models of the secondary structure of selected membrane proteins using the TOPO2 tool (<http://www.sacs.ucsf.edu/TOPO-run/wtopo.pl>).

The SUBA database (<http://www.plantenergy.uwa.edu.au/applications/suba/index.php>) was used to make comparisons with published proteomic datasets. Protein sequence alignment was carried out using Megalign/Clustal W within Lasergene v5.06.

3 Results and discussion

3.1 Membrane preparation

The vacuolar membrane, the tonoplast, plays important roles in cellular homeostasis [1]. Although ample data are now available regarding the tonoplast proteome composition [4–7], there has not yet been a concerted effort to analyze the phosphoproteome of this subcellular compartment. IMAC-based phosphoproteomics requires relatively large amounts of protein. We therefore used leaf material combined with a microsome-based membrane purification approach to obtain tonoplast-enriched membrane fractions. However, tonoplast proteomic analyses are complicated by

the difficulty in obtaining pure tonoplast membranes. Thus, cross-contamination with membranes from other compartments is a common problem. For example, recent work by Jaquinod *et al.* [7] who used vacuoles prepared from protoplasts showed the presence of many plasma membrane proteins such as P-type H⁺ ATPases. In the present study, well-established methods for isolating vacuolar membranes were used to obtain a low-density microsome fraction from *Arabidopsis* shoot tissue that was enriched in tonoplast [19]. Table 2 shows that NO₃-induced inhibition of ATPase activity increased from 17 to 65% after sucrose gradient centrifugation, indicating around fourfold enrichment in tonoplast. The small inhibitory effect of azide disappeared completely, showing that mitochondrial contamination is low after gradient separation. The vanadate-dependent inhibition showed only a small decrease from 36 to 26%. This remaining vanadate inhibition points to a considerable plasma membrane contamination and we therefore refer to our preparation as a "tonoplast-enriched fraction" (abbreviated to TEF).

Table 2. Residual ATPase activity in microsomal and low-density fraction (10/23% sucrose interface) in the presence of inhibitors

Inhibitor	Microsomal fraction	Low density fraction
KNO ₃	83 ± 10%	35 ± 14%
Azide	90 ± 7%	105 ± 6%
Vanadate	64 ± 15%	74 ± 11%

Inhibition assays were carried out using 50 mM KNO₃, 0.1 mM vanadate and 3 mM azide as specific inhibitors of the tonoplast, plasma membrane and mitochondrial ATPase activity. Specific activities in the control conditions were 124 and 281 nmol Pi min⁻¹ mg⁻¹ in microsomal and low-density fractions, respectively.

3.2 Analysis of the TEF proteome

Our phosphoproteomic analysis of the TEF revealed 130 proteins with one or more phosphopeptides (Supporting Information Table S1). Of the 130 proteins, 58 (45%) were classified as integral membrane proteins containing one or more TMD (Fig. 1A and Supporting Information Table S2). The latter group contains many transporters with clearly defined physiological functions in pH homeostasis, nutrient transport and signalling (Table 1 and Supporting Information Table S3).

As expected, the functional categories of the 130 identified phosphoproteins showed a high proportion of transporters (Fig. 1B). The second largest functional class contained kinases, which are often tethered to or associated with membranes. The latter is exemplified by the presence of membrane spanning regions in many of these proteins (data not shown). Apart from these membrane and membrane-

Figure 1. TMD distribution and functional classification of membrane proteins identified in TEF. (A) Predicted transmembrane domains (TMD) from all TEF proteins ($n = 58$) with verified phosphopeptides detected in this study. (B) Functional classification of TEF proteins with verified phosphopeptides ($n = 130$).

associated proteins, a large population of proteins showed diverse functional annotations and is likely to include both membrane-associated and soluble proteins.

Proteins with a well-defined annotation in membrane transport were further investigated. To analyse whether any were previously identified in vacuolar datasets we used the SUBA database, which integrates proteomics data from a large number of six studies ([25]; <http://www.plantenergy.uwa.edu.au/applications/suba/index.php>). The analysis shows that of 28 membrane transport proteins, 11 (39%) were previously identified as tonoplast proteins, the membrane location of six (21%) proteins is unknown and 11 (39%) are assigned to membranes other than the tonoplast (Table 1). Similar numbers of tonoplast proteins (33%) were recently reported using intact vacuoles from cauliflower [10].

3.3 Previously identified and new phosphorylation sites in the TEF phosphoproteome

Phosphosites were detected in a range of important transporter families (Table 1) and often for multiple members within families. The majority (22 out of 31) of membrane transporter phosphosites detected in this study has not been described previously. However, we identified nine phosphosites in nine proteins that were found in previous phosphoproteomic reports ([13, 14, 17] and Supporting Information Table S3). Existing and new sites were identified in H^+ and Ca^{2+} pumps (Table 1), the aquaporin PIP2;6 [17] and the

ammonium transporter AMT1;1 [13, 18], which contains a threonine residue at position 460 as the phosphorylation target and that was recently identified as playing a critical role in the allosteric regulation of the AMT1;1 oligomer activity [18].

3.3.1 Anion transporters

Previously unknown phosphopeptides were found in two CLC type proteins (Fig. 2A). One phosphosite near the N terminus was found in CLC-c and a second site in AtCLC-a or -b (the 100% sequence identity does not permit the identification of a specific isoform). A third phosphorylation site is located at the C terminus and present only in AtCLC-c. To confirm the phosphorylation site of serine 3 on CLC-a or -b and the general validity of our verification procedure, we synthesized a phosphopeptide to compare the MS fragmentation pattern of the *in vivo* and synthetic peptides. There are two possible phosphorylation sites in the identified peptide, at serine 3 and serine 4. Such close proximity would yield only the diagnostic fragment ions b3 and y10 to distinguish between the two residues. Phosphorylation at Ser 3 would give b3 = 382.33 and y10 = 957, ions that are clearly present in the fragmentation patterns from both experimental and synthetic peptides. The equivalent ions for phosphorylation at serine 4 (b3 = 302.35 and y10 = 1037.13) are not observed and the identical fragmentation patterns for both peptides in Fig. 2B therefore confirms the correct identification of this phosphorylation site at serine 3.

Figure 2. Phosphorylation sites in anion transporters. (A) Two N-terminal phosphopeptides were found in CLC-c (KI[pS]SILDDGSGVGR) and in CLC-a and/or b (TL[pS]STPLALVGAK; the 100% sequence identity does not allow unique isoform identification). A further C-terminal phosphosite was identified in CLC-c (TTFG[pS]QIL-R). (B) Comparison of MS/MS fragmentation patterns from experimental 'E' and the synthetic 'S' peptide [TL[pS]STPLALVGAK]. Loss of phosphate (H_3PO_4) from the y_{11} ion or the precursor ion is represented by Δ according to $y_n^\Delta = y_n - H_3PO_4$.

CLC-a [26] and CLC-c localize at the tonoplast whereas the location of CLC-b is unknown. In mammalian cells, CLC are typically involved in cellular volume control, and phosphorylation of specific CLC serine residues leads to channel deactivation [27]. In plants, the roles of CLC are largely unknown but clearly include nitrogen homeostasis. CLC-c was identified as a regulator of tissue nitrate level and mutations in *CLC-c* led to reduced nitrate levels [28]. More recent evidence showed that CLC-a also has an important role in nitrate homeostasis; however, CLC-a does not appear to be an anion channel but functions as an H⁺-coupled antiporter to drive vacuolar nitrate accumulation [26].

3.3.2 Sugar transporters

Another previously reported phosphopeptide was present in the sucrose symporter SUC1 (Table 1; [17]). SUC1 is believed to be exclusively expressed at the plasma membrane and is a main component of phloem sugar loading [17]. However, the vacuole is also important in sugar metabolism as a storage compartment for photosynthate. The latter requires coordinated deposition and release of sugars from this compartment via transporters and interestingly several (putative) sugar transporters were detected in the TEF with newly identified phosphosites. Two of these (At5g17010 and At1g20840 or TMT1) are annotated as tonoplast proteins whereas the membrane location of the third (At5g65687) and fourth (At5g65687) is not known. At5g17010 shows close alignment (80% similarity) to a protein annotated as a vacuolar glucose transporter (AtVGT1; [29]). The phosphorylated serine in At5g17010 aligns with a threonine in AtVGT1 (Fig. 3). TMT1 showed the presence of three phosphopeptides (Table 1).

Although several studies implicate phosphorylation as an important mechanism for plasma membrane sucrose transporters [17, 30] our data imply that similar mechanisms may also control trans-tonoplast sugar movement, for example during the circadian loading and unloading of photosynthate or in the regulation of osmotic balance.

3.3.2.1 Potassium (K⁺) transport

Several members of the KUP/HAK family were detected in the TEF, three of which were previously annotated as vacuolar proteins (KUP4, KUP5 and KUP7). KUP are highly

selective for K⁺ with substrate affinities ranging from low micromolar to millimolar levels. There are approximately 12 KUP in *Arabidopsis*, and these proteins are likely to be targeted to both the plasma membrane and the tonoplast [31]. The physiological roles of KUP include high affinity K⁺ uptake at the root:soil boundary, intracellular K⁺ distribution, turgor-driven growth [32, 32] and general K⁺ homeostasis [31, 34]. Expression of KUP is often modulated at the transcriptional level in response to K⁺ conditions, but little is known about modulation of their activity at the protein level.

Within the TEF, five KUP family members were shown to have phosphopeptides (Fig. 4A). Alignment of the KUP isoforms showed one phosphosite in the N terminus of KUP7 (Fig. 4B), which appears to be conserved only in KUP5 and KUP12. In contrast, a phosphosite between the second and third predicted TMD of KUP12 contained a well-conserved serine residue, which was present in most other KUP isoforms (Fig. 4C). KUP12 also has a phosphorylation site at position 188, which does not show such a high level of conservation. Three further sites were present in the C termini of KUP4, KUP5 and KUP8 (Fig. 4D) with two out of the three peptides overlapping. For the isoforms KUP5, KUP7 and KUP8 some topology prediction algorithms suggest an odd number of TMD, which implies the phosphopeptide would be located in the vacuolar lumen. Since the latter is very unlikely, topology predictions for the respective proteins may need to be amended.

Other members of the KUP family (KUP2 and KUP11) were previously shown to be phosphorylated [14]. The detected phosphorylation sites for these two additional family members occur in the N-terminal region (KUP11, Fig. 4B), and the C-terminal region (KUP2, Fig. 4D).

So far, no studies have been performed into modulation of plant KUP activity by phosphorylation. However, the yeast TRK1 protein, which is homologous to plant KUP transporters, is phosphorylated in response to K⁺ starvation [35]. Furthermore, K⁺ uptake in plants through other transporters such as AKT1 is also under control of phosphorylation [36] by specific kinases that are activated by low external K⁺. During such conditions, the phosphorylation dependent activation of plasma membrane KUP transport would augment cellular K⁺ uptake. K⁺ deficiency typically causes vacuolar K⁺ release to maintain cytoplasmic K⁺ levels [37]. Tonoplast KUP transporters have been suggested to mediate such K⁺ release since it may require energisation [37].

Figure 3. Phosphorylation sites in sugar transporters. The tonoplast localised sugar transporter At5g17010 contains a phosphopeptide (SSGEI[**pS**]PEREPLIK). The close homolog AtVGT1 (At3g39400), a vacuolar glucose transporter, shows a threonine residue at the same position.

Overall, there are now many data that indicate a primary role of protein phosphorylation in the maintenance of K^+ homeostasis.

3.3.2.2 ABC transporters

ABC transporters form a large family with over 150 members in *Arabidopsis*. These ATP fuelled pumps are involved in the transport of diverse substrates at various membranes and are particularly important in the deposition of toxic and xenobiotic compounds in the vacuole via glutathione conjugation [1]. Four ABC transporters with phosphosites were found. At3g62700, which encodes an MRP4 like ABC transporter, is localized to the tonoplast but membrane location for the other three ABC transporters is unknown. In addition, no data are available regarding substrate specificity of these proteins. In both bacteria and mammals, phosphorylation-

mediated regulation of ABC transporter activity has been described. Examples include the cystic fibrosis transmembrane conductance regulator (CFTR) in humans and the nitrate uptake ABC transporters in cyanobacteria [38]. Similar regulatory mechanisms for ABC transporters have not yet been described in plants.

3.3.2.3 Vacuolar H^+ cation antiport

The NHX family totals 6–8 isoforms in *Arabidopsis* [39] and is involved in the H^+ -coupled movement of K^+ and Na^+ across the membrane. A phosphopeptide was identified that is identical in both NHX1 and NHX2 and can therefore not be assigned to a specific isoform. From alignment of the protein sequences, it is apparent that the C-terminal phosphorylation site is not conserved in the other members of the NHX family (Fig. 5). Both NHX1 and NHX2 locate to the tonoplast.

Figure 4. Phosphorylation sites of KUP family transporters. (A) Schematic diagram of KUP secondary structure indicating position of all identified phosphorylation sites in KUP family. (B) Phosphosites at N terminus in KUP7 (VD[pS]FDVEALEVPGAPR) and KUP11 (DQKLDQ[pS]MDKEAG; [14]). (C) Phosphosite between second and third predicted TMD of KUP12 (LPNQPADEQI[pSpS]FR). (D) Phosphosites at C terminus for KUP2 (EDDNARsVQsNEsSSES; [14]), KUP4 ([s][s]EANIAGSSR), KUP5 (ALE[pS]DGDHND[pT]D[pS]EDDTLSR) and KUP8 ([s]DKPLNY[s]PDPENESGINER).

Figure 5. Alignment and phosphorylation sites in NHX family. The tonoplast located isoforms NHX1 and NHX2 show a phosphopeptide (GFVPFVPG[s]P[t]ER; the 100% sequence identity does not allow unique isoform identification) that does not appear to be conserved amongst any other NHX isoforms.

plast. However, although classified as $\text{Na}^+:\text{H}^+$ antiporters, increasing evidence indicates that vacuolar NHX are capable of transporting Na^+ and K^+ with equal affinity and that they may be important constituents of overall K^+ homeostasis

[39]. In addition, the role of NHX1 in salt tolerance has been well-documented [40] and some studies suggest NHX1 activity is modulated through phosphorylation by the serine/threonine kinase SOS2 [41].

3.3.2.4 Oligopeptide transport

One oligopeptide transporter was found belonging to the POT family, containing one phosphopeptide (Table 1). The identified phosphosite is at the end of the C terminus for At1g52190. These proteins typically transport di- and tripeptides with broad substrate specificity.

4 Concluding remarks

Several recent studies have provided examples that show the physiological relevance of transport modulation through phosphorylation of specific phosphopeptides [17, 18]. The data described in this study suggest that similarly essential phosphopeptides can be identified in vacuolar transporters. In several cases, the detected sites were well conserved across isoforms of one family, pointing to common mechanisms of regulation. In other cases, isoform-unique sites were present, suggesting modulatory mechanisms tailored to the function of individual proteins. A large task will be the identification of specific kinases that interact with the identified phosphoproteins. Generic techniques such as yeast two-hybrid screening will allow this to occur whereas subtractive methods such as those published recently by Shin *et al.* [42] may help identify targets of specific kinases.

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