PA (phosphatidic acid) is a lipid second messenger involved in an array of processes occurring during a plant’s life cycle. These include development, metabolism, and both biotic and abiotic stress responses. PA levels increase in response to salt, but little is known about its function in the earliest responses to salt stress. In the present study we have combined an approach to isolate peripheral membrane proteins of Arabidopsis thaliana roots with lipid-affinity purification, to identify putative proteins that interact with PA and are recruited to the membrane in response to salt stress. Of the 42 putative PA-binding proteins identified by MS, a set of eight new candidate PA-binding proteins accumulated at the membrane fraction after 7 min of salt stress. Among these were CHC (clathrin heavy chain) isoforms, ANTH (AP180 N-terminal homology) domain clathrin-assembly proteins, a putative regulator of potassium transport, two ribosomal proteins, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and a PI (phosphatidylinositol) 4-kinase. PA binding and salt-induced membrane recruitment of GAPDH and CHC were confirmed by Western blot analysis of the cellular fractions. In conclusion, the approach of the present study is an effective way to isolate biologically relevant lipid-binding proteins and provides new leads in the study of PA-mediated salt-stress responses in roots.

Key words: cellular membrane, phospholipid signalling, phosphatidic acid, protein–lipid interaction, quantitative proteomics, salinity.

INTRODUCTION

Cellular membranes primarily consist of phospholipids that separate the cytoplasm from organelles and the external environment. Besides the structural functions of phospholipids, certain species provide spatial information for cell signalling in different organisms including plants [1,2]. One of these lipids is PA (phosphatidic acid) [3–5]. PA is normally present in small amounts, but rapidly and transiently accumulates in lipid bilayers in response to different biotic and abiotic stresses [6,7]. Although involved in different signalling cascades, the molecular function of PA in cellular processes and plant physiological responses remains largely elusive [4].

Induced PA formation in plants has been described in response to abiotic stress stimuli such as ABA (abscisic acid), dehydration, and salt and osmotic stress [8]. Different PA synthesis pathways have been shown to contribute to the production of PA in response to abiotic stresses [3,8,9]. PLD (phospholipase D) hydrolyses structural phospholipids to form PA and a remaining headgroup. PLC (phospholipase C) produces DAG (diacylglycerol), which can subsequently be phosphorylated to PA by DGK (diacylglycerol kinase) [1,9]. Previously NPC (non-specific PLC) 4, a phosphatidylinosine-hydrolysing PLC, has been implicated in various aspects of the response of Arabidopsis roots to salt stress [10,11]. Moreover, in Arabidopsis, PLD isoforms α1, α3 and δ are required for the maintenance of root growth in saline conditions [12–14]. The increased salt sensitivity of the corresponding PLD-knockout mutants suggested an important role for PA in salt-stress signalling. However, little is known about the function of PA and its binding partners in the response of roots to salt. The identification of proteins that bind PA is essential to determine the role of PA in this response.

Different approaches have been applied to identify the molecular targets of PA. Most plant PA-binding targets have been identified through studying orthologues of known phospholipid-binding proteins from other organisms, such as the mammalian PDK1 [PPI (phosphoinositide)-dependent kinase 1]. Arabidopsis PDK1 was shown to bind and to be activated by both PA and several PPIs, whereas in later studies it has been shown that one of the phosphorylation targets of PDK1, PINOID (PID), also has affinity for PA [15,16]. Another target of PDK1 is OXI1 (oxidative signal-inducible 1) which is required for the full activation of MKP6 (mitogen-activated protein kinase 6) [17], which was also shown to bind PA [18], indicating that PA could play a role in spatially facilitating these signalling cascades. The Arabidopsis MAPKKK (mitogen-activated protein kinase kinase kinase) CTR1 (constitutive triple response 1) is a homologue of the mammalian PA target Raf-1 and was shown to bind PA. Moreover, its activity was inhibited in the presence of PA [19]. PTEN (phosphatase and tensin homologue) binds PPIs and
hydrolyses the 3-phosphate from PtdIns(3,4,5)P$_3$ in animal cells. Surprisingly, whereas the plant homologue PTEN2A hydrolysed several PPIs, it did not show any binding affinity to PPIs, but instead specifically bound to PA [20]. A PH (plekstrin homology) domain, similar to that in PDK1, was found in AGD7 (Arf-GAP domain 7) and was identified to stimulate the Arf1 GTPase activity in a PA-dependent manner in vitro [21]. Dehydrins bind different lipids, including PA, through a lipid-binding domain that resembles a class 2 amphipathic $\alpha$-helix domain [22] depending on the phosphorylation status of the protein [23]. However, although several different PA-binding domains have been identified, a consensus PA-binding domain remains elusive, hampering the identification of additional PA-binding proteins using homology studies.

Adding exogenous PA is reported to induce several responses in plants, and identification of the effectors that cause these responses has led to the discovery of several PA targets. The addition of PA induced ABA-dependent stomatal closure, probably mediated by the PP2C (protein phosphatase 2C) ABI1 (ABA-insensitive 1), because this protein was identified as a direct PA target [24]. A PA-binding domain in ABI1, consisting of a region of basic amino acids, was identified and shown to be important for ABI1 localization and proper regulation of stomatal conductance [24,25]. Adding exogenous PA induced ROS (reactive oxygen species) production in leaves [26] and activation of two NADPH oxidases, Rboh (respiratory burst oxidase homologue protein) D and RbohF, was dependent on PA [27]. PA binding increases the activity of these enzymes, leading to an increase in ROS and ultimately in the closure of stomata in response to ABA [27]. Exogenous PA also induced an increase in the amount of filamentous actin in an Arabidopsis cell suspension. Binding of PA and PtdIns(4,5)P$_2$ caused a reduction in the activity of the actin capping protein, effectively promoting actin polymerization [28].

Lipid-binding affinity assays have proven to be successful tools in identifying new PA-binding proteins. Using PA coupled to sepharose beads, PEPC (phosphoenolpyruvate carboxylase) was identified by MS in an earlier PA-affinity functional proteomics screen [29]. The activity of C, PEPC was inhibited in the presence of anionic lipids and the PEPC fraction that is targeted to the membrane was found to be largely modified [30]. Other proteins identified in this screen include a member of the SnRK2 (Snf1-related protein kinase 2) family, SnRK2.10, which is activated upon salt stress in roots and was shown to be involved in the maintenance of lateral root density in saline conditions [31] and the PP2A (protein phosphatase 2A) regulatory subunit RCN1 that is involved in ethylene, ABA and auxin signalling [29]. Although lipid-binding affinity screens have proven to be a good approach to identify PA targets, it is hard to determine which of these interactions are biologically relevant since many proteins have PA-binding affinity in vitro.

To identify low abundant and biologically relevant PA-binding proteins, we realized that a pre-purification step had to be conducted in our lipid-affinity screen. In previous studies pre-purification based on charge or size was performed to avoid overcrowding of the PA-affinity beads by abundant proteins [29,32]. In the present study, a pre-purification step was incorporated to specifically select for proteins that are peripherally bound to membranes, on the basis of the rationale that genuine in vivo PA targets are recruited to cellular membranes. Moreover, by quantitatively comparing PA-binding proteins isolated from the membrane fractions of roots of control plants compared with plants exposed to a short (7 min) salt-stress stimulus, our goal was to selectively identify proteins that are recruited to the membrane in vivo, in a PA-dependent manner. In the present study we describe the methodology of the approach, and the resulting set of novel putative PA targets associated with membranes in salt-stressed Arabidopsis roots. Among the 42 PA targets identified, eight accumulated at the membrane fraction in response to salt, by using both iTRAQ (isobaric tags for relative and absolute quantification) labelling and a label-free MS$^e$ (elevated energy MS) approach. The proteins identified are involved in the regulation of potassium homeostasis, metabolism and CME (clathrin-mediated endocytosis), amongst other functions.

**EXPERIMENTAL**

**Growth conditions**

**Arabidopsis** plants were grown hydroponically (Araponics) for 4 weeks under short-day conditions (130 $\mu$mol/m$^{-2}$/s, light/dark: 10/14 h, 21°C/70% humidity) with a weekly change of growth medium using the Flora series (General Hydroponics Europe). At 24 h prior to stimulation the plants were transferred to fresh medium. For each MS sample 160 plants were used.

**Isolation of peripheral membrane proteins**

Hydroponically grown Arabidopsis plants were treated by transfer to salt-containing or control medium. The roots were subsequently excised and thereafter drained of excess water, harvested and snap-frozen in liquid nitrogen. A simplified version of the fractionation has originally been described previously [30]. The root tissue was ground in liquid nitrogen and proteins were isolated by incubating the tissue in 1 volume of protein extraction buffer [50 mM Tris (pH7.5), 300 mM sucrose, 5 mM EDTA, 5 mM EGTA, 2 mM DTT (dithiothreitol) and 1 x Complete™ protease inhibitors; Boehringer Ingelheim] for 10 min on ice. Samples were filtered through Miracloth (EMD Millipore) and centrifuged twice at 1500 g for 5 min (debris) and subsequently up to six times at 10000 g to remove debris and intact organelles. Membranes were isolated by spinning for 2 h at 50000 g (50000 g crude pellets). The membranes were washed by homogenizing the pellet using protein extraction buffer with 0.1 % Brij-58 (Sigma–Aldrich) [33]. Membranes were again spun down at 50000 g for 1 h. The pellet fraction was washed twice in protein extraction buffer (without Brij-58) and spun down. The peripheral membrane proteins were eluted using protein isolation buffer supplemented with 100 mM Na$_2$CO$_3$ by homogenizing the samples and keeping them on ice for 10 min. An overview of all the centrifugation steps and the origin of all fractions described is given in Supplementary Figure S1 (at http://www.biochemj.org/bj/450/bj4500573add.htm). The antibodies raised against specific protein markers were obtained from Agrisera: anti-PM (plasma membrane) ATPase] (TAIR ID At2g18960), anti-[MitM (mitochondrial membrane) COXII (cytochrome c oxidase subunit II)] (TAIR ID Atmg00160), anti-[PMP (peripheral membrane protein) V-ATPase (vacuolar ATPase)] (TAIR ID At4g11150), anti-[cytosolic UGPase (UDP-glucose pyrophosphorylase)] (raised against barley), anti-CHC (clathrin heavy chain; TAIR ID At3g11130). The anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) antibody has been described previously [34]. Silver staining of the different fractions was performed as a loading control.

**PA-binding assay**

The PA-binding assay was conducted as described [29] (F. McLoughlin and C. Testerink, unpublished work). As starting material 5 $\mu$g of protein from the PMP fraction was used.
(approximately 500 μl) per sample. The soluble fraction was transferred to a new 2 ml Eppendorf tube and was supplemented with lysis buffer [150 mM Tris/HCl (pH 8.0), 50 mM KCl, 10 mM EDTA and 1 % IGEPA (Sigma–Aldrich)] and complete protease inhibitors (Boehringer Ingelheim) to a total volume of 1.9 ml. A total of 50 μl of PA beads (net volume) were used per sample. Additionally, the beads were washed twice with Hepes (100 mM, pH 8.0) after the described washing steps to remove the majority of the Tween 20. For the specificity control experiments, Arabidopsis total protein extract was tested for binding to either the control or PA-coupled Affigel beads, that were treated identically except for the addition of PA to the coupling reaction. Synthesis of the PA beads has been described previously [36].

In-solution digestion including PA beads

The PA beads with the bound proteins from the control and stress-related conditions were reduced with 5 mM DTT for 30 min at 60°C before carboxymethylation by 15 mM iodoacetamide for 30 min at room temperature (21°C) in the dark, both reactions were carried out in 50 mM Hepes (pH 8.0). With a typical volume of 50 μl the beads could easily be resuspended in the buffer and the reaction chemicals. An overnight digestion with 2 μg of trypsin (Gold, Promega) was performed at 37°C with continuous mixing. The supernatant containing tryptic peptides was collected after centrifugation at 10 000 g for 5 min in a table-top centrifuge. The procedure was repeated once by washing the beads with 50 μl of Hepes buffer and both supernatants were mixed together. For label-free quantification the samples were prepared for LC (liquid chromatography)-MS by the HILIC (hydrophilic interaction LC) cleaning method.

iTRAQ labelling

The collected supernatants of the samples were dried for 1 h in a centrifugal vacuum concentrator at 35°C. The dried samples were reconstituted with dissolution buffer from the iTRAQ kit according to the manufacturer’s instructions (Applied Biosystems). The control samples were labelled with iTRAQ 114 and the salt-treated samples with iTRAQ 117 according to the manufacturer’s protocol. The samples were incubated at room temperature for 3 h and subsequently the control and salt-treated samples were mixed in a 1:1 ratio and prepared for LC-MS by the HILIC cleaning method.

HILIC purification

The samples were diluted with ACN (acetoniitrile) to a final concentration of 95 % ACN. For the peptide enrichment TT2HIL TopTips (Glygen) were used in combination with the centrifuge adaptor, a microcentrifuge and a 2 ml Eppendorf vial. The samples were spun down for 1 min at 1500 g. Before using the TopTips, they were washed three times with 0.1 % TFA (trifluoroacetic acid) followed by three times with 95 % ACN. The samples were loaded with consecutive 100 μl portions on the HILIC TopTip followed by a three washes with 95 % ACN. The peptides were collected in 30 μl of 0.1 % TFA and stored at −20°C.

MS

Nanoscale LC separation of the tryptic peptides was performed with a NanoAcquity system (Waters). Samples were loaded on to a Symmetry C18 5 μm, 2 cm×180 μm trap column (Waters) at a flow rate of 5 μl/min prior to separation on a Bridged Ethyl Hybrid C18 1.7 μm, 25 cm×75 μm analytical reversed-phase column (Waters) by application of a 90 min gradient from 1 % ACN and 0.1 % formic acid to 40 % ACN and 0.1 % formic acid at a column flow rate of 0.25 μl/min. Analysis of the eluted tryptic peptides was performed using a Synapt G2 Q-TOF (quadrupole time-of-flight) mass spectrometer (Waters) equipped with a nanolockspray source (Waters) fitted with a pico-tip emitter (New Objective) operated at a capillary voltage of approximately 3 kV. The collision gas used was argon, maintained at a constant pressure of 2.0×10⁻⁶ nbar (1 bar = 100 kPa) in the collision cell. The lock mass, [Glu1]fibrinopeptide B, was delivered from the auxiliary pump of the NanoAcquity system with a concentration of 100 fmol/μl at 0.5 μl/min to the reference sprayer of the nanolock-spray source. The data were post-acquisition lock-mass corrected using the monoisotopic mass of the doubly charged precursor of [Glu1]fibrinopeptide B, delivered through the reference sprayer, which was sampled every 120 s. For the label-free experiments accurate mass precursor and fragment ion LC-MS data were collected in the data independent LC-MS² mode of acquisition [37]. For the iTRAQ -labelled samples LC-MS data was collected in the tandem MS data-dependent mode of acquisition.

In addition, iTRAQ-labelled samples were separated on a reversed-phase capillary column (150 mm×75 μm PepMap C18; LC Packings). Sample introduction and mobile phase delivery at 300 nL/min were performed using an Ultimate nano-LC-system (Dionex). For separation of peptides, a gradient from 0 % ACN and 0.1 % formic acid to 50 % ACN and 0.1 % formic acid over 60 min was used. The eluted peptides were electrospayed into a Q-TOF mass spectrometer (Waters). The most abundant ions from the survey spectrum, ranging from m/z 350 to 1500, were automatically selected for collision-induced fragmentation in a data-dependent mode of acquisition using Masslynx (Waters). Fragmentation was conducted with argon as the collision gas at a pressure of 4×10⁻⁵ bars, measured on the quadrupole pressure gauge.

Three biological replicates were performed in total. Two biological replicates were analyses using iTRAQ labelling. These samples were twice injected on the Q-TOF and once on the Synapt G2 mass spectrometer and the average values were used for quantification. The third biological replicate was analysed label-free. Each sample was injected three times on the Synapt G2 mass spectrometer and the average values were used for quantification.

Data analysis

The raw data files of the data-dependent acquisitions of the iTRAQ-labelled samples were processed with Mascot distiller using different parameters for the Q-TOF- and Synapt G2-generated data, because of differences in resolution and peak width between the two mass spectrometers. The distiller output results were used to search the Arabidopsis database (UniProt release 2010-12-16) to which common protein contaminants were appended using in-house licensed Mascot software (version 2.3.02). The Mascot search parameters were as follows: peptide and fragment error tolerances = 0.3 Da, ions-score cut-off = 12, digest reagent was trypsin, allow 1 ‘missed cleavage’, and the fixed modifications were cysteine carboxamidomethylation and iTRAQ(K), iTRAQ(N-term). The individual peptide score for this database (P < 0.05) was 35. After evaluation of the output list the search was repeated with the same parameters, but now with quantification iTRAQ 4plex activated for a ratio include of 114/117. Continuum LC-MS² data were processed and searched using ProteinLynx GlobalSERVER version 2.5 (PLGS 2.5, Waters). The parameter settings were: the digest reagent was trypsin, allow 1 ‘missed cleavage’, search tolerances automatic, typically
Table 1  Salt-induced membrane-targeted PA-binding proteins

The results are from independent experiments quantitatively analysed by iTRAQ-TOF MS or label-free by data-independent analysis. n.d., not determined.

<table>
<thead>
<tr>
<th>TAIR ID</th>
<th>Protein</th>
<th>Protein length (aa)</th>
<th>Experiment 1 iTRAQ</th>
<th>Experiment 2 iTRAQ</th>
<th>Experiment 3 Label-free MS†</th>
<th>Overall</th>
<th>Mean ratio</th>
<th>S.E.M.</th>
</tr>
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<tbody>
<tr>
<td>At1g04690</td>
<td>KAB1</td>
<td>328</td>
<td>3.69</td>
<td>*</td>
<td>1.82</td>
<td>2.76</td>
<td>0.93</td>
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<tr>
<td>At1g77940†</td>
<td>6OS ribosomal protein L30</td>
<td>152</td>
<td>2.46</td>
<td>n.d.</td>
<td>1.43</td>
<td>1.95</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>At3g08530/At3g11130§</td>
<td>40S ribosomal protein L30</td>
<td>152</td>
<td>2.46</td>
<td>n.d.</td>
<td>1.43</td>
<td>1.95</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>ACG25430</td>
<td>Putative clathrin assembly protein</td>
<td>113</td>
<td>2.16</td>
<td>1.98</td>
<td>1.52</td>
<td>1.89</td>
<td>0.19</td>
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<td>At5g55350</td>
<td>Putative clathrin assembly protein</td>
<td>113</td>
<td>2.16</td>
<td>1.98</td>
<td>1.52</td>
<td>1.89</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>At2g01600‡</td>
<td>GAPDH</td>
<td>279</td>
<td>1.66</td>
<td>1.20</td>
<td>1.63</td>
<td>1.49</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>At1g19440/At3g04120‡</td>
<td>GAPDH</td>
<td>279</td>
<td>1.66</td>
<td>1.20</td>
<td>1.63</td>
<td>1.49</td>
<td>0.15</td>
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<tr>
<td>At2g16460/At2g46500¶</td>
<td>PKCy</td>
<td>566</td>
<td>1.32</td>
<td>1.58</td>
<td>n.d.</td>
<td>1.45</td>
<td>0.13</td>
<td></td>
</tr>
</tbody>
</table>

*Peptide fragment spectra were sufficient for identification, but too low for quantification.†Quantification includes a semi-proteotypic peptide shared with L30e and L30-3 (coded by AT1G77940 and AT3G18740 respectively).‡Products from both loci were represented by proteotypic peptides, but quantification was on the basis of shared peptides.¶A concurrent increase in the product of At1g14910 cannot be excluded; the latter was, in contrast with At2g01600, not represented by proteotypic peptides.

RESULTS

Identification of PA targets involved in the response to salt stress

To isolate the PA targets that are involved in salt-stress signalling, proteins that are not only recruited to the membrane upon salt treatment (Figure 1A), but also show binding affinity for PA beads, were isolated (Figure 1B). Salt-induced increases in PA formation are fast and transient, typically occurring within 5–15 min [6,13]. By applying salt stress for only 7 min, we focused on those proteins that are relocalized to the membrane, rather than proteins that were induced at the level of expression. Isolated proteins were digested with trypsin while still bound to the PA beads and subsequently peptides were identified and quantified using two different proteomic approaches: iTRAQ labelling of peptides and label-free MS analysis (Figure 1C).

A biochemical fractionation approach allows specific isolation of PMPs

Hydroponically grown 28 day-old Arabidopsis Col-0 plants were transferred to salt (150 mM) or to control media for 7 min, after which the root material was harvested, homogenized and the proteins extracted. To isolate the PMPs from the root tissue, a differential centrifugation approach was applied. The flowchart of the centrifugation steps used to isolate the PMPs is shown in Supplementary Figure S1. The quality of the fractionation was determined by Western blot analysis of the different fractions using antibodies raised against different cellular compartment markers: PM (ATPase), MitM (COXII), PMP (V-ATPase) and a cytosolic marker (UGPase) (Figure 2).

First, the total crude protein extracts of control and salt-treated roots were centrifuged at 10000 g for 10 min to remove the cell debris and intact organelles, and the resulting pellets were enriched with the MitM, and to a lesser extent with the PMP and PM marker. The 50,000 g crude pellet fractions contained the PM and PMP, and to a lesser extent the MitM marker, whereas little of the cytosolic marker (UGPase) was detectable. The 50,000 g crude pellet fractions were subsequently washed with a buffer supplemented with 0.1% Brij-58, which inverts liposome-like structures, to release trapped hydrophilic contaminants [33]. In this Brij-58 wash the remaining cytosolic proteins were released, yielding a fraction containing only peripheral and transmembrane proteins. Peripherally associated proteins were subsequently eluted from the membranes using protein isolation buffer supplemented with 100 mM Na2CO3, resulting in PMP fractions that exclusively contained the PMP marker.

Isolation of the membrane-associated PA-binding proteome

PA-binding proteins or proteins that interact with the membrane in a PA-dependent manner were purified from the PMP fraction of both the control and salt-treated samples using PA beads. The PA beads have already been shown to be effective in isolation of PA targets [29,32] and validated in the present study to purify proteins in a PA-dependent manner.
Isolation of PA targets involved in salt stress

Figure 1 Identification of PA-binding proteins that are targeted to the membrane in response to salt using two different MS-based approaches

(A) Peripheral membrane proteins were isolated from hydroponically grown Arabidopsis roots. Light ovals represent structural lipids and dark ovals represent PA. (B) Proteins that associated to the membrane in vivo in control or salt-treated root extracts were subsequently used as input material for an in vitro PA-binding assay. (C) Flow schemes summarizing the MS approaches, using either iTRAQ or label-free quantification. By concentrating only on the peripherally membrane-associated proteins, and by quantification of difference in PA-bound proteins of salt-treated compared with control samples, candidate PA targets that are targeted to the membrane in response to salt in a PA-dependent manner can be identified.

Protein profiles of the total extract, the 50000 g pellet, the PMP fraction and the PA-binding fractions of the control and salt-treated roots were visualized using silver staining (Figure 3). Clear differences in the protein compositions between the different fractions were observed. The proportion of the total amount of proteins remaining in the sequential fractions was determined through protein quantification. The 50000 g crude pellet consisted of 5% of the total protein extract and the PMP fraction consisted of 2.5% of the 50000 g crude pellet. The PA-binding protein pool made up 5% of the PMP fraction, equivalent to 1/15000 of the total proteins.

When comparing the control and salt-treated samples of each fraction, no clear differences in individual protein abundances were detectable in either the total, the 50000 g pellet or the PMP fraction. Only in the PA-binding protein fraction were differences in abundance observed between the control and the salt-treated sample (Figure 3). To identify and quantify the PA-binding proteins, they were tryptically digested directly on the PA beads and identified by two different quantitative gel-free MS methods.

Identification and quantification of the control and salt-treated PA-binding proteins

In total three independent biological replicates were performed on the roots of 160 plants per sample per experiment. For two experiments, the resulting PA-binding peptides obtained by tryptic digestion were labelled with iTRAQ, whereas one replicate was analysed using label-free MS quantitation. The total number of peptides, proteins and proteins enriched in the salt-treated samples for either the iTRAQ-labelled or label-free approach are summarized in Figure 4(A). More peptides and proteins were identified in the label-free approach, but this did not result in the identification of more proteins that were differentially abundant in the salt-stimulated sample. The combined data represent proteins identified in at least two of the independent replicate studies.

The ratios between the average protein abundances in the salt- and the control-treated sample of the 42 proteins identified are shown in Figure 4(B). The proteins are arranged according to their S/C (salt-treated/control) ratio, starting with the largest enrichment in the salt-stressed sample. Most proteins were present in similar quantities in both the control and the
Arabidopsis plants were either control or salt treated (150 mM, 7 min). Proteins were isolated from the roots and fractionated using sequential centrifugation steps, after which several protein fractions were analysed. Pellet fractions are indicated with P and the supernatant fractions are indicated with S. Intact organelles and cellular debris was removed at 10,000 g (10,000 g pellet). Sequentially, the microsomal membrane fraction [50,000 g crude pellet (CP)] was isolated and washed with protein isolation buffer supplemented with 0.1% Brij-58 to release any trapped soluble contaminants. After several additional washing steps using protein isolation buffer, the PMPs were eluted using protein isolation buffer supplemented with 100 mM Na2CO3. Western blot analysis was performed on these fractions using antibodies against different compartment markers: PM (PM ATPase), MitM (MitM COXII), peripheral vacuolar membrane (PMP V-ATPase ε subunit) and the cytosolic marker UGPase (Cyt. UGPase). Silver staining was performed as a loading control (lower panel). Molecular mass is shown on the right-hand side in kDa.

Salt-treated samples. A total of eight of the 42 identified proteins (Supplementary Table S1) were enriched in the salt-treated sample (S/C > 2Log 0.5), whereas no proteins were enriched in the control-treated sample using a 2Log 0.5 threshold. The S/C ratio of the eight proteins enriched in the salt-stimulated samples is shown in Figure 4(C) and Table 1.

The eight proteins identified have been described as involved in various cellular processes. The identification of KAB1 (potassium channel β subunit 1) implicates a role for PA in the maintenance of potassium homoeostasis in saline conditions. In addition, CHC isoforms and two clathrin assembly proteins were increased in the salt-stressed sample. Furthermore, two isoforms of GAPDH and two ribosomal proteins were targeted to the membrane in response to salt, as was a PI4Kγ (phosphoinositide 4-kinase γ).

Confirmation of PA-dependent membrane targeting for CHC and GAPDH using Western blot analysis

The abundance of two prominent membrane-targeted candidate PA-binding proteins, CHC and GAPDH, was determined in the different fractions including the PA-binding fraction using specific antibodies (Figure 5). As shown above (Figure 2), the PM marker was enriched in the 50,000 g pellet fraction, whereas the PMP marker was strongly enriched in the 50,000 g pellet and present in the PMP pool. The PMP marker could not be detected in the PA-binding protein pool. The latter fraction did contain both CHC and GAPDH (Figure 5), confirming their identification by MS as PA-binding proteins. Importantly, salt stress increased CHC
Control. Molecular mass is shown on the right-hand side in kDa. C, control; CP, crude pellet; P, response to salt stress (CHC and GAPDH). Both CHC and GAPDH were confirmed to be targeted (50 000 × g centrifuged supernatant). Thus, ether Brij-58 is a non-ionic detergent that inverts these vesicles and potentially trapping cytosolic proteins. The polyoxyethylene acyl ether Brij-58 is a non-ionic detergent that inverts these vesicles [33], thereby releasing the trapped cytosolic proteins. In addition, Brij-58 washing also reduces the amount of proteins present in organelles such as the ER (endoplasmic reticulum), plastids and mitochondria in the microsomal membrane fraction [38].

The basis of the PA-binding proteins identified and Western blot analysis, the PMP fractions used in the present study as an input for the PA-binding assay at least contained the PM, tonoplast, ER and mitochondrial membranes, but the presence of other membranes cannot be excluded. Na2CO3 was used to elute the peripheral membrane-bound proteins, because it disrupts the electrostatic interaction of these proteins with integral membrane proteins and the polar heads of lipids [39], which was confirmed with the results obtained (Figure 2).

**DISCUSSION**

Recruitment of proteins to specific locations within the cell is an important aspect of cellular signalling, and it is known that phospholipid-mediated recruitment plays a role in many plant stress-signalling responses [1,2]. In the present study, we set out to identify proteins that are involved in a specific response, in this case to salt stress, and are recruited to the membrane in a PA-dependent manner, using an unbiased lipid-affinity and differential proteomics-based approach.

**Isolating peripherally membrane-bound PA-binding proteins**

To determine which proteins are bound to the membrane it is essential to remove any cytosolic contaminants from the PMP fraction. Just washing the microsomal membrane fraction (50 000 g pellet) with protein isolation buffer is not sufficient, since a large portion of the PM vesicles that are formed upon homogenization are orientated with the cytosolic side inwards, potentially trapping cytosolic proteins. The polyoxyethylene acyl ether Brij-58 is a non-ionic detergent that inverts these vesicles [33], thereby releasing the trapped cytosolic proteins. In addition, Brij-58 washing also reduces the amount of proteins present in organelles such as the ER (endoplasmic reticulum), plastids and mitochondria in the microsomal membrane fraction [38]. On the basis of the PA-binding proteins identified and Western blot analysis, the PMP fractions used in the present study as an input for the PA-binding assay at least contained the PM, tonoplast, ER and mitochondrial membranes, but the presence of other membranes cannot be excluded. Na2CO3 was used to elute the peripheral membrane-bound proteins, because it disrupts the electrostatic interaction of these proteins with integral membrane proteins and the polar heads of lipids [39], which was confirmed with the results obtained (Figure 2).

Proteins that are recruited to the membrane in response to salt and bind to PA beads

A role for PA in clathrin-coated pit formation?

Several PA-binding proteins that associate with membranes after salt stress (Table 1) are implicated in CME. These include two CHC proteins (At3g08530 and At3g11130) and two putative clathrin assembly proteins (At2g25430 and At2g01600). The latter contain ANTH (AP180 N-terminal homology) domains and constitute, together with ENTH (epsin N-terminal homology) domain-containing proteins, a family of accessory proteins which in mammals and yeast are known to bind phospholipids, in particular PtdIns(4,5)P2, in order to support vesicle budding from the PM and trans-Golgi network. ENTH/ANTH domain-containing proteins interact directly with proteins of the clathrin coat and are also speculated to be involved in cargo binding [40]. Several Arabidopsis ANTH domain-containing proteins were shown to localize to cellular membranes [41] and the At2g01600 (AtECA1) isoform was demonstrated to bind several phosphoinositides, although PA binding was not tested [42].

In the initial phase of coated pit formation, clathrin triskelia, composed of three heavy chains and three light chains, is recruited from the cytosol to adaptor proteins on the nascent budding site. Although clathrin is known to stabilize the interaction between adaptors and membrane lipids, there is no evidence for the direct interaction of clathrin and lipids [40]. Nevertheless, both CHCs encoded by the Arabidopsis genome were found in the PA-associated protein fractions (Table 1). Their increased abundance in the salt-stressed membrane sample is consistent with earlier work in which clathrin light chains were found to be recruited to cellular membranes in response to osmotic stress [43].

In mammalian systems, PA generated via PLD and DGK activities has been proposed to function differentially in cargo-selective subsets of clathrin-coated pits, driving the internalization of e.g. epidermal growth factor receptor [44]. In addition, the induced formation of PA in a lipid bilayer may outline the shape of a pit and could play a structural role in vesicle budding by promoting the formation of membrane domains of positive curvature [45–47]. CME is emerging as a major route of vesicle-mediated transport in plants, both in constitutive endocytic cycling, e.g. of PIN auxin efflux carriers [48,49], and in environmental stress-induced vesicle trafficking. Interestingly, the endocytosis and cycling of KAT1 potassium channels [50] and several aquaporins [51], has been shown to be regulated in the early response to salt and osmotic stress conditions. This type of regulation allows cells to rapidly adjust the PM composition by exchange of components with closely apposed early endosomes. Certainly, future research should not only address the questions of the nature and specificity of the interactions of CME components with PA, but also investigate PA’s broader role in salt-induced vesicular trafficking.
Potassium channel β subunit

Potassium channel β subunits are hydrophilic polypeptides that interact with the cytoplasmic part of tetrameric pore-forming α subunits of inward-rectifying voltage-gated potassium channels. The Arabidopsis genome encodes a single homologue of the animal potassium channel β subunit, KAB1 (At1g04690), which, as a tetramer, associates with the transmembrane α subunits of KAT1 channels [52]. KAB1 is present in both membrane and soluble fractions [52] and contains conserved amino acid motifs that, in animal potassium channel β subunit homologues, confer pyridine nucleotide-dependent oxidoreductase activity. Interestingly, in mammalian axons, the potassium channel β2 subunit functions as an adaptor, linking potassium channel β1 subunit-containing vesicles to motor proteins on microtubules [53] and targeting potassium channels to specific sites along the axonal membrane.

The plant hormone ABA, which is known to control ion fluxes under water stress, has been shown to induce rapid inactivation and internalization of KAT1 potassium channels in stomatal guard cells. Interestingly, inactivation is faster than internalization, suggesting the involvement of another regulatory mechanism [50]. The consequences of ABA and salt stress on root potassium channel activities are less well understood. The root epidermal AKT1 channel plays an important role in potassium absorption from the soil and this system is challenged at high levels of sodium, which induces potassium deficiency. Potassium absorption from the soil and this system is challenged at high levels of sodium, which induces potassium deficiency [54,55]. Whether KAB1 plays a role in the modulation of AKT1 localization and/or activity in the salt-stress response remains to be investigated.

PI4K

The two identified PI4Ks (At1g64460 and At2g46500; Table 1), which could not be discerned based on MS-generated amino acid sequences, belong to a family of eight type II PI4Ks in Arabidopsis [56]. In mammalian cells, type II PI4K has been implicated in clathrin-mediated transport from the Golgi [57]. Similarly, in Arabidopsis salt stress-induced PtdIns(4,5)P2 has been proposed to be associated with CME [43]. However, whether Arabidopsis PI4Ks play a similar role is unknown, especially because two Arabidopsis PI4Ks, the isoforms γ4 and γ7, have been shown to display in vitro protein kinase rather than lipid kinase activity [56].

Other potential novel PA targets

The finding of the cytosolic GAPDH proteins (At1g13440/At3g04120) as candidate PA targets in salt stress (Figure 4 and Table 1) is in line with previous proteomics studies suggesting the salt-induced tonoplast association of glycolytic enzymes [58]. By binding to specific membranes/organelles, glycolytic complexes may concentrate in regions of high demand for ATP, fuelling ion transporters or proton pumps [59]. For example, the association of aldolase and enolase in the tonoplast of Mesembryanthemum crystallinum plants under salt stress has been speculated to contribute to the supply of ATP to the V-ATPase whose activity is required for pumping sodium ions into the vacuole [58]. In addition, GAPDH has also been shown to bind Ptdδ and is needed for full activation of Ptdδ in response to ROS [60], and might therefore also function in the targeting of Ptdδ to the membrane or promote its activity, further facilitating the formation of PA. The finding of ribosomal subunits, like glycolytic enzymes, is a common feature in differential proteomics studies of abiotic stresses in plants, and their implications are not well understood at present [61,62].

Conclusion

In conclusion, we set up and successfully carried out a directed proteomics approach to isolate PA-binding proteins recruited to membranes in response to the salt treatment of root tissue. The approach of the present study to investigate peripherally membrane-bound proteins, opens up the possibility to study the function of PA in the context of other stress stimuli, such as ABA treatment, wounding, temperature or biotic stress. With regard to salt stress, the results of the present study reveal putative novel targets of PA and suggest a function of PA in salt-induced regulation of CME, which would probably influence a large array of cellular processes.

AUTHOR CONTRIBUTION

Fionn McLoughlin and Christa Testerink conceived the study and designed the research. Fionn McLoughlin, Steven Arisz, Henk Dekker, Gertjan Kramer and Christa Testerink performed experiments. Fionn McLoughlin, Steven Arisz, Henk Dekker, Gertjan Kramer, Chris de Koster, Michel Haring, Teun Munnik and Christa Testerink analysed data. Fionn McLoughlin, Steven Arisz and Christa Testerink wrote the paper with input of all co-authors.

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Isolation of PA targets involved in salt stress


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SUPPLEMENTARY ONLINE DATA
Identification of novel candidate phosphatidic acid-binding proteins involved in the salt-stress response of Arabidopsis thaliana roots

Fionn MCLOUGHLIN*, Steven A. ARISZ*, Henk L. DEKKER†, Gertjan KRAMER‡, Chris G. DE KOSTER†, Michel A. HARING*, Teun MUNNIK* and Christa TESTERINK*2

*University of Amsterdam, Swammerdam Institute for Life Sciences, Section Plant Physiology, Postbus 94215, 1090 GE Amsterdam, The Netherlands, †University of Amsterdam, Swammerdam Institute for Life Sciences, Mass Spectrometry of Biomacromolecules, Postbus 94215, 1090 GE Amsterdam, The Netherlands, and ‡Department of Medical Biochemistry, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands

Figure S1 Flowchart of centrifugation, washing steps and the PA-binding assay that led to isolation of PA-binding proteins

Vertical arrows are used to indicate the isolation of the supernatant and horizontal arrows indicate the isolation of the pellet fraction. The fractions analysed by immunoblots or silver stain (Figures 2, 3 and 5 of the main text) are boxed.

1 Present address: University of Massachusetts, 1003 Lederle Graduate Research Center, 710 North Pleasant Street, Amherst, MA 01003, U.S.A.
2 To whom correspondence should be addressed (email c.s.testerink@uva.nl).
Figure S2  No binding of proteins to control uncoupled Affigel-10 beads

Crude protein extract was isolated from 4-week-old Arabidopsis plants (roots and leaves) and used as the input for a PA-binding assay with PA or control beads. The fractions were separated using SDS/PAGE and silver stained. Molecular mass is given on the right-hand side in kDa.
Table S1  Identified PA-binding PMPs

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