Roles of phosphatidic acid and its protein targets in mediating cellular responses of plants to salinity
McLoughlin, F.

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The answer to the ultimate question of life, the universe and everything is 42.

What do you get if you multiply six by nine.

Six by nine. Forty-two.

That’s it.

That’s all there is.

I always thought something was fundamentally wrong with the universe.

‘The hitchhiker’s guide to the galaxy’

Douglas Adams
Roles of phosphatidic acid and its protein targets in mediating cellular responses of plants to salinity

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in het openbaar te verdedigen in de Aula der Universiteit

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Chapter 1

Introduction

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Chapter 1

Introduction

During their lifecycle, plants encounter a wide range of unfavorable conditions. Low nutrient availability, pathogen infection, drought, heat, cold and water deprivation are all regular sources of stress for plants. In order to cope with these conditions, plants have to adapt adequately to ensure survival and reproduction. Since plants are sessile organisms they have evolved into masters of adaptation of both their development and metabolism. In order to accomplish growth and survival under adverse conditions, plants use a large array of signaling mediators, including hormones, protein kinases and phosphatases, Ca$^{2+}$, reactive oxygen species and low abundant phospholipids. Many of these signaling mediators are part of complex signaling cascades. In this thesis, I will focus on the contribution of phospholipids and protein kinases to these signaling events.

Environmental stress causes changes in phospholipid composition of cellular membranes. Several lipids, which are only present in small amounts under normal conditions, are synthesized rapidly and transiently in response to stress. They act as a lipid second messenger and can form docking sites to bind different proteins and thus provide spatial and transient signals needed to adequately respond to external stimuli (Meijer and Munnik, 2003; Wang, 2004; Munnik and Testerink, 2009; Xue et al., 2009; Munnik and Vermeer, 2010). The phospholipid phosphatidic acid (PA) is one of these signaling lipids, which accumulates rapidly in response to different environmental signals (Testerink and Munnik, 2005; Li et al., 2009; Testerink and Munnik, 2011).

The involvement and generation of PA in response to abiotic stress

Induced PA formation has been described in the response of plants to abiotic stress stimuli such as dehydration (Jacob et al., 1999), salt and osmotic stress (Munnik et al., 2000) and treatment with hormone ABA (Fan et al., 1997; Ritchie and Gilroy, 1998; Jacob et al., 1999), but PA also accumulates in response to biotic
stress stimuli (van der Luit et al., 2000; de Torres Zabela et al., 2002; de Jong et al., 2004). Different PA metabolizing pathways have been shown to contribute to the production of PA in response to abiotic stress (Munnik et al., 2000; Ruelland et al., 2002; Arisz et al., 2009; Bargmann et al., 2009; Li et al., 2009; Hong et al., 2010). The phospholipase D (PLD) enzyme hydrolyses primarily structural lipids such as phosphatidylcholine (PC), and phosphatidylethanolamine (PE), resulting in formation of PA and the remaining headgroup (Pappan et al., 1998). Phospholipase C (PLC) hydrolyses phosphatidylinositol lipids (PPIs) into water soluble inositol-bis or trisphosphate (IP₂, IP₃) and diacylglycerol (DAG), which remains in the membrane (Munnik and Vermeer, 2010). DAG can be subsequently phosphorylated to PA by DAG kinase (DGK) (Arisz et al., 2009).

The role of phospholipases in abiotic stress responses
Twelve PLDs have been identified in the model plant species Arabidopsis, which were initially classified in two groups based on their N-terminal lipid binding domain which consisted either of a Pleckstrin homology (PH) and PHOX (PX) or a calcium dependent lipid binding (C2) domain (Elias et al., 2002). In later studies the classes were further subdivided in six classes based on sequence homology and in vitro enzymatic activity: three α-, two β-, three γ-, one δ- and one ε- PLD with a C2 domain and two ζ-class PLDs that contain PH and PX domains (Qin and Wang, 2002; Bargmann and Munnik, 2006; Li et al., 2009). In the Arabidopsis genome, nine PLCs and seven DGK genes have been identified. PLC/DGK derived PA has been primarily implicated in responses to biotic stress (van der Luit et al., 2000; de Jong et al., 2004). Abiotic stress, in particular cold stress, also induces an accumulation of PLC/DGK-mediated PA formation, (Ruelland et al., 2002;
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Gomez-Merino et al., 2004). In addition to the formation of PA, PLC1 is thought to rise IP$_3$ levels necessary for maximal ABA-induced gene expression and full calcium release (Sanchez and Chua, 2001; Hunt et al., 2003; Mills et al., 2004).

Roots are the primary site of perception of salt stress, drought and low nutrient availability. To cope with these conditions, plants adapt growth and morphology of roots and several phospholipase D mutants are impaired in the adaption of their root system architecture to abiotic stress (Fig. 1). PLDζ1-inducible OE (overexpression) plants showed an increase in root hair initiation (Ohashi et al., 2003). Expression of PLDζ2 increased under low phosphate availability (Oropeza-Aburto et al., 2011) and in one study the pldζ2-KO (knock-out) mutant showed increased root hair growth when deprived of phosphate (Cruz-Ramirez et al., 2006). In accordance, less PA was formed in low phosphate conditions in the pldζ1/2 double mutant (Li et al., 2006a) and this mutant showed reduced lateral root and increased primary root growth in low phosphate conditions (Li et al., 2006b). In addition, pldζ2-KO also exhibited decreased sensitivity to the plant hormone auxin and a reduced root gravitropic response (Li and Xue, 2007). Another important nutrient for plants is nitrogen and Arabidopsis PLDε-OE lines displayed an increase in lateral root and root hair elongation and primary root growth in low nitrogen conditions. This effectively increased the dry weight of the plant under these conditions and indicated an important role for PLDε in growth and nitrogen signaling (Hong et al., 2009).

PLDα1 and PLDδ are involved in different responses to abiotic stress including reactive oxygen species (ROS) signaling in response to ABA
Introduction

(Sang et al., 2001; Zhang et al., 2003; Zhang et al., 2009; Uraji et al., 2012). The same phospholipases were also shown to play distinct roles in freezing tolerance (Welti et al., 2002; Li et al., 2004). Expression of PLDδ was elevated in response to dehydration and high salt stress (Katagiri et al., 2001). Salt stress induced formation of PA through PLDα1 and PLDδ and both single mutants showed a reduction in primary root growth in saline conditions and during dehydration. This was even clearer in the plda1/δ double mutant (Bargmann et al., 2009). A similar reduction in root growth was observed in plda3, which was more susceptible to salinity and water deficiency. In hyperosmotic stress conditions, the plda3 mutant displays a reduction in primary root growth and a reduction in lateral roots (Hong et al., 2008).

Figure 1. PA derived from different PLDs is involved in different molecular processes. PLDs regulate downstream targets through producing PA. Although all PLD isoforms hydrolyse structural lipids and generate PA in vitro, they have been identified to be involved in different processes and signaling cascades in vivo. The specificity of PLDs involved in different processes might be due to differences in substrate specificity, subcellular localization, tissue-specificity and activation mechanisms.
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Together, these studies show that PLDs are important for maintaining root growth in different conditions and imply an important signaling function for PA in roots. To determine how PA influences downstream components, it is important to identify which proteins interact with PA, and how these mediate the response that eventually leads to the acclimation to different stresses in roots.

PA protein targets
In addition to being an important component in membrane curvature and surface charge (Kooijman et al., 2003), PA exerts its function by recruiting target proteins to the membrane (Testerink and Munnik, 2005). In contrast to other signaling lipids such as phosphoinositides, no consensus PA-binding domain has been identified, which hampers the identification of new PA targets. PA targets identified in other parts of the plant will be discussed here.

A number of PA binding proteins have been identified involved in different cellular processes (Table 1). The Arabidopsis Phosphoinositide-Dependent Kinase 1 (PDK1) bound several phosphoinositides and PA through its Pleckstrin Homology (PH) domain (Deak et al., 1999). PA activated PDK1 and stimulated its downstream target, AGC2-1 (OXI1) (Anthony et al., 2004; Anthony et al., 2006). This signaling cascade induced a respiratory burst required for the full activation of MAP kinase 6 (MPK6) (Rentel et al., 2004), which was also shown to bind PA (Yu et al., 2010). The activation of MPK6 in response to salt was abolished in the \textit{pldca1} mutant background and might play a role in sodium homeostasis in roots in saline conditions since it was able to phosphorylate the Na$^+$/H$^+$ antiporter Salt overly sensitive (SOS1) \textit{in vitro} (Yu et al., 2010). Another phosphorylation target of PDK1, PINOID (PID), was shown to bind phosphoinositides and PA. PID is involved in the asymmetric distribution of Pin-formed (PIN) auxin transporters, which are key regulators of root development (Zegzouti et al., 2006).
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<tr>
<td>PDK1</td>
<td>Root hair development, defense to pathogens</td>
<td>(Deak et al., 1999) (Anthony et al., 2004) (Anthony et al., 2006)</td>
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<td>PTEN 2A</td>
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**Table 1. An overview of PA targets identified in plants.**

Abbreviations of all the PA targets are given (left column) with their putative function (middle column) and reference (right column). The abbreviations used: PDK1: Phosphoinositide-Dependent Kinase 1, ABI1: ABA Insensitive 1, SnRK2.10: Sucrose non-fermenting 1-Related protein Kinase 2.10, RCN1: Roots Curl in NPA 1, PID: PINOID, CP: heterodimeric actin Capping Protein, TGD2/4: Trigalactosyldiacylglycerol 2/4, AGD7: Arf Gap Domain 7, CTR1: Constitutive Triple Response 1, TaPEAMT1/2: PhosphoEthanolAmine N-MethylTransferase, RbohD/F: Respiratory burst oxidase homologue D/F, PEPC: PhosphoEnolPyruvate Carboxylase, MPK6: MAP kinase 6, MGD1: MonoGalactosyl Diacylglycerol synthase 1, PTEN: Phosphatase and TENsin homolog deleted on chromosome ten.

One of the best described PA targets in Arabidopsis is ABI1, which inhibits ABA-induced stomatal closure. PA formation inhibited the activity of ABI1 in a PLDα1 dependent manner, and effectively induced stomatal closure (Zhang et al., 2004). Furthermore, two NAPDH oxidases (RbohD and RbohF) have been shown to bind PA. In this case, lipid binding increased their activity and resulted in promotion of stomatal closure (Zhang et al., 2009).
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Constitutive Triple Response 1 (CTR1) is a plant homolog of one of the best characterized mammalian PA targets: the MAPKKK Raf-1 (Ghosh & Bell, 1997). CTR1 is a key negative regulator of ethylene responses. Kinase activity of CTR1 was shown to be inhibited in the presence of PA (Testerink et al., 2007) suggesting a role for PA in priming the plant for ethylene dependent signaling cascades, independently of ethylene production (Testerink et al., 2008).

Phosphoenolpyruvate carboxylase (PEPC) was identified as a PA binding protein and the enzymatic activity was inhibited by anionic lipids (including PA). PA binding affinity of PEPC increased under hypo-osmotic stress conditions, suggesting that a conformational change of the protein determines the interaction with PA (Testerink et al., 2004). A fractionation study was conducted, which revealed that PEPC present on the membrane has an altered mobility on SDS-PAGE, suggesting that PEPC bound to the membrane is largely modified (Monreal et al., 2010). This suggests that either post-translational modification plays a role in PEPC lipid binding affinity or that anionic phospholipids induce modification of the PEPC protein in the membrane.

Finally, the Sucrose non-fermenting 1-related protein kinase (SnRK) 2.10, an osmotic stress-activated protein kinase, was identified in a PA affinity screen (Testerink et al., 2004). Although, as described above, several PLDs play a role in root growth in saline conditions, very few PA targets involved in salt or osmotic stress have been identified so far. Therefore, SnRK2.10 and its close homologue SnRK2.4 were selected for further analysis in this thesis project.

SnRK2 kinases are key regulators in osmotic stress signaling
SnRK2 (Sucrose non-fermenting 1-Related protein Kinase 2) proteins belong to a plant-specific serine/threonine protein kinase family that is conserved in many plant species. These kinases have been identified to be key regulators in abiotic stress signaling. In Zea mays 11 members were isolated and their expression was found to be induced by several different abiotic stress stimuli (Huai et al., 2008), as
was found for PKABA1 identified in wheat (Anderberg and Walker-Simmons, 1992; Holappa and Walker-Simmons, 1995), SPK1 and 2 in soybean (Yoon et al., 1997; Monks et al., 2001) and NtOSAK in tobacco (Kelner et al., 2004). In Arabidopsis and rice, the SnRK2 family consists of 10 members, of which respectively 9 out of 10 and all 10 members can be activated upon osmotic stress (Boudsocq et al., 2004; Kobayashi et al., 2004).

Figure 2. A phylogenetic tree of the SnRK2 protein family in Arabidopsis.
A phylogenetic tree was constructed of the Arabidopsis SnRK2 family based on amino acid homology. Ten members of the SnRK2 family were classified and divided into three classes. These classes distinguish themselves in their responsiveness to the phytohormone ABA: class 1 is not responsive to ABA, class 2 to a lesser extent and class 3 SnRK2s strongly responsive to ABA (Boudsocq et al., 2004; Umezawa et al., 2004; Boudsocq and Lauriere, 2005).

Based on protein comparison, the SnRK2 family of Arabidopsis can be divided into three classes (Kobayashi et al., 2004) which differ in their activation in response to the phytohormone ABA (Fig. 2). In Arabidopsis three members, SnRK2.2 (SnRK2D), 2.3 (SnRK2I) and 2.6 (OST1; SnRK2E), make up class 3 and are strongly activated in the presence of ABA. The class 2 members SnRK2.7 (SnRK2F) and 2.8 (SnRK2C) are activated to a lesser extent in response to ABA, and the members of class 1, SnRK2.1 (SnRK2G), SnRK2.4 (SnRK2.4A), SnRK2.5 (SnRK2H), SnRK2.9 (SnRK2J) and SnRK2.10 (SnRK2B) are not activated at all by ABA.
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(Boudsocq et al., 2004; Umezawa et al., 2004; Boudsocq and Lauriere, 2005; Kulik et al., 2011). The PA binding SnRK2 members, SnRK2.4 and 2.10, belong to the ABA-independent class 1 SnRK2s and very little is known about their function.

In tobacco, a negative regulator of NtOSAK (Nicotiana tabacum osmotic stress-activated protein kinase, an orthologue of SnRK2.4) has been identified, the SnRK2-interacting Calcium sensor (SCS). SCS interacts with both ABA dependent and independent SnRK2s and might be a regulatory factor in their activity (Bucholc et al., 2011). In addition, nitric oxide (NO) activates NtOSAK, but no S-nitrosylation was observed after NO treatment. Glyceraldehyde-3-phosphate (GAPDH) was identified as a molecular partner of NtOSAK and was shown to be S-nitrosylated after NO treatment, suggesting that GAPDH might contribute to or regulate the activation of class 1 SnRK2s in response to NO (Wawer et al., 2010).

In addition, only very little is known about the function and downstream targets of class 1 SnRK2s. Overexpression of the SnRK2.4 wheat orthologue (TaSnRK2.4) in Arabidopsis showed an increase in primary root growth and resulted in more drought tolerant plants. This was explained by stronger water retention ability in these plants compared to wildtype (Mao et al., 2010). Overexpression of SAPK4 (a rice class 1 SnRK2 orthologue) increased tolerance to salinity and oxidative stress (Diedhiou et al., 2008).

Through a semi-degenerate peptide array screen, a preferential phosphorylation affinity peptide motive was identified for SnRK2.10, which is conserved in dehydrins (Vlad et al., 2008). Although the phosphorylation event still has to be confirmed in vivo, this might be a plausible phosphorylation target of the class 1 SnRK2s. Finally, a quadruple mutant of the class 1 SnRK2s (snrk2.1/2.4/2.5/2.10) contained elevated proline levels under hyperosmotic stress conditions (Fujii et al., 2011), indicating that this mutant is more susceptible to hyperosmotic stress. Overall, despite the evidence for a role for class 1 SnRK2s in osmotic stress signaling, the molecular mode of action and a possible role of PA therein is still unknown.
Outline of this thesis

This thesis focuses on the role of the lipid second messenger PA in the response of plant roots to salt stress. Although PA and several PLD isoforms are clearly involved in abiotic stress and nutrient sensing responses in roots, little is known about the PA target proteins that could mediate these responses. Therefore two kinases that were identified in a PA binding affinity screen, SnRK2.4 and 2.10, and were implicated in salt stress signaling, were further studied. Chapter 2 describes the role of SnRK2.4 and 2.10 in salt stress signaling, showing distinct roles for SnRK2.4 and 2.10 in maintaining root system architecture in saline conditions. Furthermore SnRK2.4-YFP was found to be targeted to the membrane and to accumulate in punctate structures in response to salt, supporting a role for PA in the localization of SnRK2.4. Chapter 3 describes the lipid binding properties of SnRK2.4 and 2.10. Both kinases specifically bound PA and snrk2.4-KO mutants showed a similar reduction in primary root growth as the plda1/δ in saline conditions, but no difference in activation of SnRK2.4/2.10 was observed in the plda1/δ mutant. Using a homology study between PA and non-PA binding SnRK2 members, several basic amino acid residues potentially responsible for PA binding were identified. A region with a high density of these candidate amino acids was shown to bind PA and this PA-binding region fused to YFP was shown to locate to punctate structures, similar to the full-length SnRK2.4. Mutating the candidate amino acids in this region to neutral amino acids abolished the PA binding capacity of this domain and reduced the PA binding capacity in the full-length protein.

To further increase our understanding of the role of PA in roots, a novel PA binding affinity screen was conducted. Although lipid-binding affinity has proven to be a good approach to identify PA targets, it is hard to determine which proteins are biologically relevant since many proteins have PA binding affinity in vitro. In Chapter 4 an approach is described where PA-binding proteins are purified from the fraction of peripheral membrane proteins in root tissue.
Arabidopsis roots were either control- or salt-treated and this allowed the identification of proteins that bind to PA and are recruited to the membrane in response to salt stress. Several PA binding proteins were identified that were targeted to the membrane after 7 minutes of salt stress, revealing new roles for PA in clathrin mediated endocytosis, potassium homeostasis and (lipid) metabolism amongst others.

Chapter 5 discusses the results presented in this thesis and summarizes the new insights gained during this study. Chapter 6 is a detailed protocol of the PA binding assay using PA beads and provides advice on what to take into account to successfully perform PA binding assays.

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Chapter 2

The Snf1-related protein kinases SnRK2.4 and SnRK2.10 are involved in maintenance of root system architecture during salt stress

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Chapter 2

Abstract

The Sucrose non-fermenting-1 related protein kinase 2 (SnRK2) family represents a unique family of plant specific-protein kinases implicated in cellular signalling in response to osmotic stress. In our studies, we observed that two class 1 SnRK2 kinases, SnRK2.4 and SnRK2.10, are rapidly and transiently activated in Arabidopsis roots after exposure to salt. Under saline conditions, snrk2.4 knockout mutants had a reduced primary root length, while snrk2.10 mutants exhibited a reduction in the number of lateral roots. The reduced lateral root density was found to be a combinatorial effect of a decrease in the amount of lateral root primordia and an increase in the amount of arrested lateral root primordia. The phenotypes were in agreement with the observed expression patterns of genomic YFP-fusions of SnRK2.10 and 2.4, under control of their native promoter sequences. SnRK2.10 was found to be expressed in the vascular tissue at the base of a developing lateral root, whereas SnRK2.4 was expressed throughout the root, with higher expression in the vascular system. Salt stress triggered a rapid re-localization of SnRK2.4-YFP from the cytosol to punctate structures in root epidermal cells. Differential centrifugation experiments of isolated Arabidopsis root proteins, confirmed recruitment of endogenous SnRK2.4/2.10 to membranes upon exposure to salt, supporting their observed binding affinity for the phospholipid phosphatidic acid. Together, our results reveal a role for SnRK2.4 and 2.10 in regulating root growth and architecture in saline conditions.
Introduction

Salinity stress is an increasing agricultural problem, limiting crop yield and plant productivity (Munns and Tester 2008). Salinity causes instant osmotic stress, similar to drought and cold stress, and additionally a gradual ion accumulation, which is toxic to the plant. Plants respond very quickly to salt, i.e. by regulation of ion channels, generation of lipid signals, including phosphatidic acid (PA) and phosphoinositides, and by activation of protein kinase pathways (Boudsocq and Lauriere 2005, Craig Plett and Moller 2010, Galvan-Ampudia and Testerink 2011, Hong et al. 2010, Kulik et al. 2011, Munnik and Vermeer 2010, Testerink and Munnik 2011, Zhu 2002). One family of protein kinases that is activated upon salt treatment is the plant specific Sucrose non-fermenting related kinase 2 family (SnRK2). Members of this protein kinase family have been identified in many different plant species, including Zea mays (maize) (Huai et al. 2008), Triticum aestivum L. (wheat) (Anderberg and Walker-Simmons 1992, Holappa and Walker-Simmons 1995), Glycine Max (soybean) (Monks et al. 2001, Yoon et al. 1997) and Nicotiana tabacum (tabacco) (Kelner et al. 2004) and were shown to be activated upon salt and osmotic stress (Mikolajczyk et al. 2000, Munnik et al. 1999). In Arabidopsis thaliana and Oryza sativa, the SnRK2 family consists of 10 members of which respectively 9 out of 10 or all 10 members are activated upon osmotic stress (Boudsocq et al. 2004, Kobayashi et al. 2004).

Based on phylogeny, the SnRK2 family is divided into three classes (Kobayashi, et al. 2004), which differ in their activation in response to the phytohormone ABA. Arabidopsis class 3, comprised by SnRK2.2 (SnRK2D), 2.3 (SnRK2I) and 2.6 (OST1; SnRK2E), are strongly activated in the presence of ABA, while class 2 members SnRK2.7 (SnRK2F) and 2.8 (SnRK2C) are activated to a lesser extent. In contrast, members of class 1, SnRK2.1 (SnRK2G), SnRK2.4 (SnRK2A), SnRK2.5 (SnRK2H), SnRK2.9 (SnRK2J) and SnRK2.10 (SnRK2B) are not

Class 2 and 3 SnRK2s were shown to phosphorylate Ser/Thr residues in the R-X-X-S/T motive of the Abscisic acid responsive element-Binding Factor 2 (ABF2) and ABF4 transcription factors (Fujii et al. 2007, Furihata et al. 2006, Yoshida et al. 2010). SnRK 2.7 and 2.8 are involved in drought signaling in an ABA dependent way (Mizoguchi et al. 2010). Using a phosphoproteomics approach, several targets of SnRK2.8 were identified that connect SnRK2.8 to metabolic processes (Shin et al. 2007). SnRK2.6 was shown to play an important role in the regulation of stomatal conductance (Mustilli et al. 2002, Yoshida et al. 2002), by targeting the KAT1 potassium channel (Sato et al. 2009), the slow-anion channel SLAC1 (Geiger et al. 2009, Lee et al. 2009) and AtrbohF NADPH oxidase (Sirichandra et al. 2009). SnRK 2.2 and 2.3 are also activated by ABA, but control responses of ABA in seed germination, dormancy and seedling growth (Fujii, et al. 2007). The snrk2.2/2.3/2.6 triple mutant is nearly insensitive to ABA, indicating redundancy between these genes (Fujii and Zhu 2009, Fujita et al. 2009, Nakashima et al. 2009). The activity of SnRK2.6 is directly inhibited by the PP2Cs ABI1, ABI2 and HAB1 and activation of SnRK2.6 occurs by de-repression of these phosphatases (Soon et al. 2012, Umezawa et al. 2009, Vlad et al. 2009, Yoshida et al. 2006), which in turn are contained by the soluble ABA receptors, PYR/PYL or RCAR in the presence of ABA (Ma et al. 2009, Park et al. 2009). The components PYR1, ABI1, SnRK2.6/2.2/2.3 and ABF2 were shown to be sufficient for ABA-induced-gene expression, showing that class 3 SnRK2s act in the core ABA signaling pathway (Fujii et al. 2009).

Compared to class 2 and 3 SnRK2 members, relatively little is known about the activation mechanism of class 1 members and their targets. Overexpression of the SnRK2.4 orthologue of wheat (TaSnRK2.4) in Arabidopsis has been shown to induce an increase in main root growth. Under drought conditions, overexpression lines had enhanced survival rates, which can be
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explained by their stronger water retention ability (Mao et al. 2010). Using a semi-degenerate peptide array screen, SnRK2.10 has been demonstrated to target a preferential phosphorylation affinity motive that is conserved in the S-segment of dehydrids (Vlad et al. 2008). Its orthologue in tobacco, NtOSAK, has been shown to directly interact with GAPDH (Wawer et al. 2010), linking its mode of action to metabolic processes, similar to the SnRK2.8 in Arabidopsis (Shin, et al. 2007). A quadruple mutant of the ABA-independent class I SnRK2 members (snrk2.1/2.4/2.5/2.10) revealed elevated proline levels in response to osmotic stress (Fujii et al. 2011).

To elucidate the function of the class 1 SnRK2s, we characterized the function of SnRK2.4 and 2.10, two previously described members (Boudsocq et al., 2004, Testerink et al., 2004) of this subgroup in Arabidopsis roots and found that both were activated within 1 min of salt treatment. Subcellular localization studies and mutant analyses revealed that SnRK2.4 and 2.10 exert their function at different locations within the root. SnRK2.4 was found to be targeted to the membrane structures upon lateral root emergence or in response to salinity in epidermal cells. Moreover, knocking out either gene alters the growth and the architecture of Arabidopsis roots in saline conditions. Together, our data reveal distinct roles of SnRK2.4 and 2.10 in maintaining both main and lateral root growth under salinity stress.

Results

SnRK2.4 and 2.10 are among the fastest activated protein kinases in Arabidopsis roots upon salt stress

To investigate their possible role in salt stress signalling, activation of SnRK2.4 and 2.10 was studied in A. thaliana roots. Plants grown hydroponically for 28 days were transferred to control or saline medium, and kinase activity was monitored using an in-gel kinase assay on root protein extracts. To identify
SnRK2.4 and 2.10 activation and to determine their roles in salt-related signalling, two independent T-DNA insertion lines were isolated for both kinases (snrk2.4-1: Salk_080588, snrk2.4-2: Salk_146522; snrk2.10-1: WiscDsLox233E9, snrk2.10-2: GABI_676G12) (Fig. S1a). Using an anti-SnRK2 (αSnRK2) antibody (Boudsocq, et al. 2004) these mutants were confirmed to be knock-outs at the protein level (Fig. S1b) activation of SnRK2.4 and 2.10 was studied in roots of hydroponically grown plants that had been stressed by transfer to different salt concentrations ranging between 100 and 150 mM NaCl or 200 mM mannitol (Fig. 1a, S2b). Crude protein extracts were separated by SDS-PAGE with the generic protein kinase substrate MBP immobilized in the gel. MBP can be phosphorylated by several protein kinase families, including MAPKs and SnRK2s (Boudsocq, et al. 2004, Droillard et al. 2002, Munnik, et al. 1999), allowing visualization of the activity of the endogenous kinases. Two kinases with a molecular weight of 45 and 48 kD (presumably MAPKs) are activated between 2 min-24 hr (48kD) and 5 min-6 hr (45 kD) in response to 150 mM NaCl (Fig. 1a, upper panel). At the expected molecular weight of SnRK2.4 and 2.10 (40 kD), a fast and transient activation of a protein kinase was observed (indicated by an arrow) between 30 s and 5 min and the activation increased again after 24 hr. To determine whether this protein kinase activity represented SnRK2.4, 2.10, or both, immunoprecipitations (IP) were performed with an antibody that recognizes both SnRK2.4 and 2.10 (Vlad et al. 2010) and the protein kinase activity was analyzed by an in-gel kinase assay (Fig. 1a, 2nd panel). In addition, kinase activation was investigated in the snrk2.4, snrk2.10 and snrk2.4/2.10 mutant backgrounds after two min salt stress (Fig. 1b). As shown in fig. 1a, the activation kinetics of the immunoprecipitated SnRK2s resembled the activation pattern observed at 40 kD in the crude extract. Activation was very fast (<0.5 min), peaking at 1 min and was rapidly repressed, but reappeared after 24 hr. The identity of SnRK2.4 and 2.10 as the 40 kD band in the crude extract was confirmed in the snrk2.4/2.10 mutant background, where the 40 kD band was completely absent after two min of salt stress. SnRK2.4 and 2.10 were activated to
a similar degree since the 40 kD kinase activity was similar in both single mutants (Fig. 1b). Western blot analysis with the same antibody on the crude extract, showed that there were no changes in protein abundance up to 6 hr of salt treatment (Fig. 1a, 3rd panel). No kinase activation was observed when plants were transferred to control medium (Fig. S2a). A similar activation pattern was observed when 200 mM mannitol was used (Fig. S2b), indicating that SnRK2.4 and 2.10 are activated in *Arabidopsis* roots in response to salt and osmotic stress.

**Figure 1.** SnRK 2.4 and 2.10 are among the fastest activated protein kinases in *A. thaliana* roots upon salt stress. a. In-gel kinase assay of protein extracts from hydroponically grown roots exposed to 150 mM NaCl. In the upper panel (crude extract, activity) the activity of a number of kinases is detected. At 40kD the activation of SnRK2.4 and 2.10 is observed (indicated with an arrow). In the second panel, in-gel kinase activity is shown after immunoprecipitation with an antibody against SnRK2.4/2.10, confirming the identity of these bands to be SnRK2.4/2.10. In the third panel, the Western blot analysis shows SnRK2.4 and 2.10 protein abundances at the different time-points and in the lower panel coomassie brilliant blue is shown as a loading control. b. SnRK2.4 and 2.10 are responsible for the kinase activity observed at 40kD. The same approach as shown in A was conducted in the single and double mutant background after 2 minutes of 150 mM salt stress. All the protein kinase assays were performed with MBP immobilized in the gel as substrate.
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SnRK2.4 and 2.10 play a role in maintaining root growth under saline conditions

In Arabidopsis, salinity has been shown to cause changes in the root system architecture (Zhao et al. 2011, Zolla et al. 2010), allowing plants to optimize their growth under this condition. To assess whether the SnRK2 protein kinases play a role in this response, primary root length and lateral root density was studied in the snrk2.4 and 2.10 mutants, in both control and saline conditions (85 or 115 mM NaCl) (Fig. 2). To avoid any problems with sucrose affecting root growth through direct uptake via the leaves (Macgregor et al. 2008), media were prepared without sucrose. Under control conditions, there were no differences in primary root length when Col-0 wild type and all mutants were compared (Fig. 2, left column). The addition of 85 mM NaCl to the growth medium did not change this, but when exposed to 115 mM NaCl, significant differences between wild type and the snrk2.4 mutant lines (snrk2.4-1, snrk2.4-2) appeared. A reduction of 35% in the primary root length was detected in snrk2.4-1, snrk2.4-2 and the snrk2.4-1/2.10-1 double mutant, whereas wild type and the snrk2.10 lines showed a reduction of only 20% when exposed to salt.

When studying the lateral roots (Fig. 2, right column), no differences in lateral root density (LRD) were observed between wild type and the mutants in control conditions. Col-0 showed a LRD reduction of 20% at 85 mM NaCl in comparison to control conditions. In the snrk2.10-1 and snrk2.10-2 single mutants, a significantly greater reduction was observed, showing a reduction of close to 50% at 85 mM NaCl in comparison to control conditions. The double mutant again phenocopied the single mutants, also showing a 50% reduction in LRD. Similar results were obtained when exposing Col-0 and the mutant lines to 115 mM NaCl. These data show that both protein kinases are involved in maintaining root system architecture under saline conditions, where SnRK2.4 exerts its function predominantly in the primary root and SnRK2.10 in the lateral roots.
Figure 2. SnRK2.4 and 2.10 are involved in maintaining primary and lateral root growth, respectively, in saline conditions. Arabidopsis thaliana seeds of Col-0, snrk2.4-1, snrk2.4-2, snrk2.10-1, snrk2.10-2 and snrk2.4/2.10 were sown on agar plates containing ½ MS. The plants were grown vertically under an angle of 70 degrees. After 4 days the seedlings were transferred either to control or plates supplemented with 85 or 115 mM salt. Plates were scanned and the primary root length (from the point of transfer to the root tip) and the lateral root density (LRD) were measured 8 days after the transfer (12-day old seedlings) using image analysis software and counting of visual LRs. The primary root length (left panel) and the lateral root density (number of lateral roots/cm primary root, right panel) were averaged. The number of replicates varied between 20 and 30 replicates.
per line and concentration and seedlings were randomized over different plates. The phenotypes were confirmed in 3 independent studies. The error bars represent the standard error and significant differences were determined using Tukey-b and are indicated by letters (p≤0.05).

**The reduced number of lateral roots in snrk2.10 and 2.4/2.10 mutants is primarily due to a reduction in the emergence of lateral roots**

The reduction in LRD in the single snrk2.10 and snrk2.4/2.10 double mutant could either be due to a reduction in the amount of lateral root primordia (LRP) or to a defect in their development. To investigate this in more detail, the developmental stages of all the primordia were studied.

As shown in fig. 3a, no difference in the primary root length was observed for the snrk2.10 mutant compared to Col-0 wildtype under any of the conditions tested. For Col-0, the LR density and the non-emerged LRP density were measured at control conditions, and media supplemented with 85 and 115 mM NaCl (Fig. 3b). In control conditions, 65% of the total lateral root primordia (LRP+LR) developed into a LR. When transferred to 85 mM NaCl, there was a 10% decrease in the total amount of primordia (LRP+LR), but only 30% of the LRP developed into a LR. At the higher salt concentration (115 mM NaCl), the total amount of primordia decreased by 25% compared to control conditions, but the percentage of LRP that developed into a LR was 30%, similar as observed at 85 mM NaCl (Fig. 3b).

In control conditions, no significant difference in either the LR or LRP density was detected between Col-0 and snrk2.10 (Fig. 3c). At both 85 and 115 mM NaCl, the density of total lateral root primordia (LRP+LR) was less in snrk2.10 compared to wild type. In addition to this reduction, a more pronounced effect was found in the percentage of LRP that developed into an LR (30% in Col-0, to 20% in the snrk2.10 mutant background), which effectively accounted for the overall reduction of over 30% in the emerged LR density (Fig. 3c). These results show that the reduction in LR density is partly due to a reduction in the total LRP density, but predominantly caused by a reduction in the percentage of LRP that developed into
a LR, showing that SnRK2.10 plays a role in the development of lateral root primordia in saline conditions.

**Figure 3. The snrk2.10 knockout line exhibits a reduction in the number of lateral root primordia in saline conditions.** Col-0, snrk2.10 were grown and treated similarly as in Fig. 2 a. Primary root length of Col-0 and snrk2.10 seedlings (n=19-22). b. Salt induces an increase in arrested lateral root primordia (LRP) and a reduction in the total amount of LRP. The LRD of Col-0 was measured similarly as in Fig. 2. The total number of LRP was counted using a binocular after fixing and clearing the roots. The non-emerged LRP (dark bars), LR (light bars) and the total amount of LRP (dark + light bars) were plotted per cm primary root. Asterisks in the dark and light bar represent significant differences in the density of non-emerged LRP and LRs, respectively. Asterisks above the bars indicate significant differences in the density of the total amount of LRP. c. snrk2.10 knock-out line shows a reduction in LRs and total LRP, but not in the non-emerged LRP. The number of replicates varied between 20 and 30 replicates per line and concentration and seedlings were randomized over different plates. The phenotypes were confirmed in 3 independent studies. The error bars represent the standard error and significant differences have been determined using a student’s T-test (* P<0.05).

**Tissue-specific localization of SnRK2.4 and 2.10 in Arabidopsis roots**

Their high homology at the amino acid sequence level indicated similar functions for the SnRK2.4 and 2.10 protein kinases. However, the knock-out mutants clearly
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showed different phenotypes in root system architecture in response to salt. To study their expression in roots, YFP-fusions of SnRK2.10 and SnRK2.4 under the control of their native promoter sequence were constructed and transformed into their respective mutant backgrounds.

In the primary root, SnRK2.10-YFP was not detectable in the root tip (Fig. 4a) and was predominantly expressed in the vascular tissue in distal root tissue (Fig. 4b-d). SnRK2.10-YFP was not detectable in the LRP during the initial stages of development in stages 3 and 6 (classification of LRP as described in (Malamy and Benfey 1997)) (Fig 4 b,c), but was highly expressed in the adjacent vascular tissue. Further from the root tip, SnRK2.10-YFP expression was higher in cortex cells (Fig. 4d). In addition, SnRK2.10-YFP specifically accumulated in the developing vascular tissue of a newly emerged LR (Fig. 4d).

SnRK2.4-YFP was expressed in almost all cells proximal to the root tip of the primary root, excluding the columella cells. SnRK2.4-YFP accumulated highest in pericycle cells (Fig. 5a, lower arrow). Further distal from the root tip, SnRK2.4-YFP also accumulated in the endodermis (upper arrow), while its abundance in the adjacent cortex cells was reduced. Even further distal from the root tip, the expression in the epidermal cells was low (Fig. 5b-d). Interestingly, at the site of a developing LRP (stage 3), SnRK2.4-YFP accumulated in punctate structures in the LRP (Fig. 5b). At a later stage of LRP development (stage 6), SnRK2.4-YFP was present in the cytosol of all the lateral root primordia cells (Fig 5c). Remarkably, enhanced expression was observed in the cortex cells at the side of the developing LRP, which was not observed in the cortex cells on the other side. In addition, accumulation of SnRK2.4-YFP was observed in punctate structures in these cells (Fig. 5c, indicated by the arrow). In the emerged LR, SnRK2.4-YFP was expressed at low levels in the developing vascular tissue, similar to the expression pattern of SnRK2.10-YFP (Fig 5d).
Figure 4. SnRK2.10 is expressed in the vascular tissue at the site of a lateral root. (a-d) pSnRK2.10::SnRK2.10-YFP was stably transformed into the snrk2.10-2 mutant background. The mid-section of the primary root (a), LRP in stage 3 (b), 6 (c) and emerged (d) were imaged. SnRK2.10-YFP is primarily expressed in the vascular tissue distal from the root tip. The arrow indicates the accumulation of SnRK2.10-YFP in the developing vascular tissue of an emerged lateral root. Prior to imaging, plants were exposed to propidium iodide in MQ for 2 minutes. Plants were grown vertically under an angle of 70 degrees on agar plates containing ½ MS and 1% sugar for 7 days. SnRK2.10-YFP is shown in green and propidium iodide is shown in red. Wide-field images are depicted with their corresponding confocal images and all the pictures were taken with the same confocal settings.
Figure 5. SnRK2.4 is expressed in cells surrounding an emerging LRP, and accumulates in punctate structures in these cells. (a-d) pSnRK2.4::SnRK2.4-YFP was stably transformed into the snrk2.4-1 mutant. The mid-section of the primary root (a), LRP in stage 3 (b), stage 6 (c) and emerged (d) were imaged. 

- **a.** SnRK2.4-YFP accumulated in pericycle (indicated by the lower arrow), and the endodermis (indicated by the upper arrow).
- **b.** At the site of a stage 3 LRP, SnRK2.4-YFP was highly expressed and accumulated in punctate structures. The expression in epidermal cells was lower compared to the root tip.
- **c.** Cortex cells surrounding the LRP contained higher levels SnRK2.4-YFP and similar punctate structures as observed in a stage 3 LRP (indicated by the arrow).
- **d.** Accumulation in the developing vascular tissue of an emerged LR. SnRK2.4-YFP is shown in green and propidium iodide is shown in red. The growth conditions were the same and all the pictures were taken with the same confocal settings as used in Fig. 4.

**Re-localization of SnRK2.4-YFP upon salt stress**

To investigate whether localization of SnRK2.4 or 2.10 would change in response to salt treatment, seedlings were treated with 115 mM NaCl. SnRK2.4-YFP relocalized from the cytosol to punctate structures within 5 min of salt application in epidermal cells (Fig. 6). Two min after the start of the treatment (first frame), SnRK2.4-YFP was still predominantly cytosolic, but after 15 minutes most of the SnRK2.4-YFP had moved from the cytosol and accumulated at unknown punctate structures. To confirm that the fusion protein was functional, Western analysis and
an in-gel kinase assay were performed on the SnRK2.4-YFP expressing line, which showed that the fusion protein was intact and could be activated by salt treatment, similar to the endogenous SnRK2.4 protein kinase (Fig. S4a,b). Moreover, also an N-terminal GFP fusion of the SnRK2.4 protein could be activated by salt (S4a,b), and this fusion also re-localized to punctate structures upon salt treatment (S4c), similar to the SnRK2.4-YFP, ruling out any positional effect of the FP tag on localization of the protein. SnRK2.10-YFP localization seemed not affected by salt treatment (up to 30 minutes; data not shown).

Figure 6. SnRK2.4-YFP accumulates in punctate structures in root epidermal cells upon salt stress. 7 day-old snrk2.4-1 pSnRK2.4::SnRK2.4-YFP seedlings were stained with propidium iodide in MQ for two minutes and subsequently placed either in control medium or medium containing 115 mM NaCl. The first image was taken 2 minutes after the saline medium was applied. Images were taken in epidermal cells approximately 150 µm above the root tip. In the lower right corner the same area is shown after 10 minutes of control treatment. SnRK2.4-YFP is shown in green and propidium iodide is shown in red. The growth conditions were the same and all the pictures were taken with the same confocal settings as used in Fig. 4.
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A co-localization study with FM4-64, a lipophilic dye to label membranes, was performed to further investigate the nature of the accumulation of SnRK2.4-YFP (FigS5). After treatment with the dye for 2 hr prior to the 15-min salt stress treatment, co-localization occurred in some of the punctate structures that were closer to the membrane (indicated by the arrows), indicating that SnRK2.4-YFP is targeted to cellular membranes.

Biochemical fractionation reveals membrane association of SnRK2.4 and/or SnRK2.10 upon salt stress

In order to further confirm the salt-induced recruitment to membranes, endogenous SnRK2.4/SnRK2.10 levels were analyzed after a sub-cellular fractionation in control and salt-stressed roots. This approach consisted of a number of different centrifugation steps, allowing isolating proteins that are associated to the membrane. A similar approach conducted on Sorghum bicolor has been described earlier (Monreal et al. 2010).

Using antibodies against proteins that reside in different cellular compartments, different fractions were analyzed (Fig 7a). Protein concentrations of all fractions were normalized in order to determine relative alterations between fractions. The total, 50,000 x g supernatant and the Brij-58 wash fraction mainly consisted of cytosolic proteins in both control and salt-stimulated samples. SnRK2.4/2.10 were highly abundant in these fractions, confirming their cytosolic localization. Both the 10,000 x g (debris, intact organelles) and 50,000 x g (microsomal membranes) pellet fractions contained cytosolic contamination, but contained mainly trans- and peripheral membrane proteins. In comparison to the cytosolic marker, SnRK2.4/2.10 was enriched in these fractions indicating they were partially membrane bound in both control and saline conditions. When the cytosolic contaminants were removed by Brij-58 washing (Hardin et al. 2004, Johansson et al. 1995), a striking increase of SnRK2.4/2.10 was observed in the remaining pellet fraction of the salt-treated samples, but not in the control
samples. The peripheral membrane marker, V-ATPase ε subunit, was equally present in this fraction in both samples, indicating that the same protein pools were isolated. The localization of SnRK2.4/2.10, in these fractionation studies indicated not only cytosolic, but also membrane associated localization in saline conditions, confirming the confocal microscopy observations and showing that SnRK2.4 is recruited to the membrane upon salt stress.

Figure 7. **SnRK2.4/2.10 are targeted to cellular membranes during salt stress and bind PA in vitro.** a) Proteins from control or 150 mM NaCl treated *Arabidopsis* roots were isolated and fractionated using sequential centrifugation steps. Pellet fractions are indicated with P and the supernatant fractions are indicated with S. Western blot analysis was performed on these fractions using antibodies against different compartment markers or SnRK2.4/2.10. Membranes (50,000 x g pellet) were isolated and washed with Brij-58 to remove any cytosolic contaminants. From the upper to lower panel: Plasma membrane (PM ATPase), peripheral vacuolar membrane (PMP V-ATPase ε subunit), SnRK2.4/2.10 and the cytosolic marker fructose-1,6-biphosphate (Cyt. cFBPase). In the lowest panel a silver stain is shown as a loading control. b) Both SnRK2.4 and 2.10 bind liposomes containing PA. Purified GST-tagged recombinant protein (1 μg per sample) was mixed with liposomes containing PC, PC/PS (1:1) or PC/PA (1:1). Lipid concentrations available for binding were 2 mM for PS, and 2 mM, 0.5 or 0.1 mM for PA. The start fraction represents the input, the pellet fraction represents the fraction that bound to the liposomes and the supernatant represents the remainder. Relative loading: start 50%, pellet 100%, supernatant 50%.
Recombinant SnRK2.10 and SnRK2.4 both specifically interact with PA in vitro

Salinity stress is known to induce changes in the phospholipid composition of membranes in *Arabidopsis*, including the transient formation of PA (Bargmann *et al.* 2009). SnRK2.10 was identified in a proteomic screen for PA targets and was shown to bind PA affinity beads (Testerink *et al.* 2004). To test whether SnRK2.10 and its most related family member, SnRK2.4, directly binds to PA and to assess the specificity of lipid binding, recombinant GST-SnRK2.10 and GST-SnRK2.4 proteins purified from *E. coli* were tested in a liposome binding assay (Fig. 7b). Since liposomes consist of a lipid bilayer, interaction with a more naturally organized PA can be shown using this approach (Julkowska *et al.* 2012, Testerink *et al.* 2007). Both SnRK2.4 and 2.10 exhibited binding affinity for PA-containing liposomes in a concentration dependent manner, while no binding could be detected for liposomes containing another anionic phospholipid, phosphatidylserine (PS) or control lipids consisting only of the structural phospholipid phosphatidylcholine (PC) (Fig. 7b). Thus, SnRK2.10 and SnRK2.4 are able to bind PA directly and selectively *in vitro*, providing a possible molecular basis for the observed interaction of SnRK2.4 to cellular membranes upon exposure of roots to salt.

Discussion

In plants, salinity stress activates several protein kinases that are implicated in salt acclimation signaling cascades (Galvan-Ampudia and Testerink 2011, Kulik, *et al.* 2011). Most SnRK2-family members are activated in response to salt, and some of them were revealed to be important for ABA signaling and drought tolerance (class 2 and 3). The role of the ABA-independent SnRK2s (class 1) is largely unknown (Kulik, *et al.* 2011). Here we took an approach to functionally characterize two of the class 1 SnRK2s. Although it has been suggested that all five class-1 SnRK2's
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(SnRK2.1, 2.4, 2.5, 2.9 and 2.10) act redundantly (Fujii, et al. 2011), we found that SnRK2.4 and 2.10 play distinct roles in maintaining root system architecture, affecting different parts of the root, largely corresponding to their respective expression patterns.

SnRK2.4 and 2.10 have been found to be activated by salt and hyperosmotic stress before, when transiently expressed in protoplasts (Boudsocq, et al. 2004). Here, we show their activation by salt and mannitol in Arabidopsis roots (Fig. 1, S2b). Since activation is fast and transient, they most likely play a signaling role in the early responses to osmotic stress. Involvement of SnRK2.4/2.10 in abiotic stress signaling is consistent with the effect of overexpressing the SnRK2.4 orthologue in wheat (TaSnRK2.4), which increased the plant's tolerance to drought, salt and cold stress (Mao, et al. 2010). Knocking-out either SnRK2.4 or 2.10 in Arabidopsis affected root growth and architecture in saline conditions, but not in control conditions (Fig. 2), showing that SnRK2.4 and 2.10 are involved in maintaining root growth during salt stress. As the SnRK2.4 orthologue in wheat has been described to be involved in the response to additional abiotic stress stimuli (Mao, et al. 2010) and SnRK2 class 1 kinases are generally activated in response to osmotic stress, it is likely that the function of these kinases is not restricted to salt stress signaling and might play a broader role in abiotic stress signaling, including mechanical stress occurring during LR development.

Although SnRK2.4 and 2.10 are highly homologous at the amino acid level (91%), different phenotypes were observed under saline conditions for each mutant; the absence of SnRK2.4 resulted in a reduction of primary root growth, while the absence of SnRK2.10 resulted primarily in a reduction of the lateral root. The expression patterns of both kinases are consistent with the observed phenotypes in the knockout mutants (Figs. 4, 5). This indicates that although their knockout phenotypes are different, cellular functions of both kinases could be similar.
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SnRK2.10 was shown to be important in the development from a LRP to a LR (Fig. 3). The LRP were classified in the developmental stages 1-7, emerged or LR (Fig. S3a) as described in (Malamy and Benfey 1997). In Col-0, there is an increase in non-emerged LRP in saline conditions (Fig. S3b). This was due to an increase in the amount of LRP arrested in stage 5 and 6 (Fig. S3b). The observed reduction in lateral root formation in Col-0 in salt stress in consistent with most published studies (Deak and Malamy 2005, Galvan-Ampudia and Testerink 2011). LRP stages 5 and 6 were not overrepresented in the snrk2.10 mutant (Fig. S3c), but LRs were rather arrested at the emergence stage, indicating that SnRK2.10 plays a role during or right after the emergence of a newly formed lateral root. This observation is consistent with the expression pattern of SnRK2.10 in the root, since it accumulated specifically in the developing vascular tissue of an emerged LR and was hardly present in the earlier LRP developmental stages (Fig. 4). These data all together indicate that SnRK2.10 plays a role in the emergence and further outgrowth of lateral roots during salt stress.

Although, the expression pattern of SnRK2.4 at the site of a developing LR also points to a function in LR development, no LR phenotype was observed in the snrk2.4 mutants, possibly due to redundancy with another SnRK2 class 1 member. In the cortex cells, where the LRP is applying mechanical pressure to its neighboring cells, high expression of SnRK2.4, but not 2.10 was observed (Fig. 5c). Here, SnRK2.4-YFP accumulated in punctate structures, similarly to the first stages of LRP development (Fig. 5b) or in response to salt stress (Fig. 6). Since all cells that contain these punctate structures are exposed to mechanical stress, this could be a plausible cause of the re-localization. In accordance, salt has been shown to induce swelling of cortex cells after 24 hrs, which would result in mechanical strain (Dinneny et al. 2008). Although the SnRK2.4 re-localization observed is much faster (i.e. within 5-10 minutes), it would be possible that initial changes in the cortex cells that lead to swelling would trigger the localization of SnRK2.4 to punctate structures.
Co-localization with the lipophilic dye FM4-64 suggested that SnRK2.4-YFP is associated to intracellular membranes (Fig S5). This was confirmed through a cellular fractionation of root extracts (Fig. 7a), where only after being exposed to salt stress, SnRK2.4/2.10 was found to be associated with membrane fractions. As SnRK2.10 was not expressed in the epidermal cells in which SnRK2.4 was found to re-localize, and the biochemical approach cannot distinguish the individual isoforms, it is unknown whether SnRK2.10 could also re-localize, similar to SnRK2.4.

Membrane association of both isoforms is consistent with the identification of SnRK2.10 in a proteomics screen for binding to the phospholipid, phosphatidic acid (PA) reported earlier (Testerink, et al. 2004) and the specific binding of both SnRK2.10 and SnRK2.4 to PA-containing liposomes observed here (Fig. 7b). PA rapidly accumulates in response to several stress conditions and is an important signalling lipid in all eukaryotes, affecting the localization and function of a diverse set of target proteins (Testerink and Munnik 2011) among which are several plant protein kinases. These include the Arabidopsis Constitutive Triple Response 1 (CTR1) (Testerink, et al. 2007), Phosphoinositide-Dependent Kinase 1 (PDK1) (Anthony et al. 2004), Mitogen-Activated Protein Kinase 6 (MPK6) (Yu et al. 2010) and a Zea mays Calcium-Dependent Protein Kinase (CDPK) (Klimecka et al. 2011). Re-localization of SnRK2.4 might thus be mediated through an interaction with PA, which could possibly affect interaction of class I SnRK2 isoforms with their direct phosphorylation targets. For SnRK2.4 and 2.10 no in vivo phosphorylation targets have been described. Yet, a conserved S-segment of stress-related dehydrin protein family members was found to be preferentially phosphorylated by SnRK2.10 in vitro (Close 1996, Vlad, et al. 2008). Dehydrins are implicated in drought, cold and salt stress and have binding affinity to anionic, negatively charged lipids through their conserved K-segment (Close 1996, Koag et al. 2003, Koag et al. 2009). One Arabidopsis dehydrin, Lti30, was further characterized and was reported to interact electrostatically with several anionic
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lipids, including PA. The interaction of Lti30 with the membrane depends on its phosphorylation status and alters the fluidity of the membrane (Eriksson et al. 2011). Since SnRK2.4 binds PA and is recruited to the membrane in response to salt, PA might act as a docking station, possibly spatially facilitating dehydrin phosphorylation and docking to the membrane.

Another candidate target of SnRK2.4 is the glycolytic enzyme GAPDH, which is an interaction partner of NtOSAK, a SnRK2.4 orthologue in Nicotiana tabacum (Wawer, et al. 2010). We have identified GAPDH to be a PA-binding protein using PA-beads (Chapter 4). Post-translational modification has been shown to influence the interaction of GAPDH with the surface of mitochondria and lipid-protein interactions are proposed to be necessary for its stabilization (Graham et al. 2007).

In this work, clearly novel physiological roles, localization and cellular dynamics of SnRK2.4 and 2.10 have been uncovered. Our findings suggest that class 1 SnRK2s play a role in linking salt stress perception to modulation of root growth and development. Since root system architecture in both control and saline conditions is highly controlled by phytohormones including auxin, cytokinin, ABA and ethylene (Fukaki and Tasaka 2009, Galvan-Ampudia and Testerink 2011), a possible role of SnRK2.4 and 2.10 in these signaling cascades should be investigated. In addition, identification and/or verification of phosphorylation targets of SnRK2.4 and 2.10 could reveal the molecular basis of the SnRK2.4 and 2.10 phenotypes in root growth and development.

Material and methods

Isolation of homozygous T-DNA insertion lines and generation of plants expressing YFP and GFP-fusions
Homozygous lines were selected by PCR using gene specific primers for two independent T-DNA insertion lines (Alonso et al. 2003) for both SnRK2.4
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(At1g10940) and SnRK2.10 (At1g60940) using; snrk2.4-1 (Salk_080588), snrk2.4-2 (Salk_146522), snrk2.10-1 (WiscDsLox233E9), and snrk2.10-2 (GABI_676G12). Primers are listed in Table S1. The snrk2.4-1 and 2.10-1 mutants were crossed to obtain a double mutant. Western blot analysis with an αSnRK2 antibody (Boudsocq, et al. 2004) of crude protein extracts of 8 days old seedlings was performed to confirm absence of the proteins. The SnRK2 antibody was used as primary antibody.

The genomic promoter and coding region of SnRK2.4 (chr.1: 3659208-3656052) were amplified using the attB1SnRK2.4 and attB2SnRK2.4 primers that contained Gateway adapters. The same was conducted for SnRK2.10 (chr.1: 22444519-22439397) using the attB1SnRK2.10 and attB2SnRK2.10 primers (Table S1). The fragments were recombined into pDONR207 using BP clonase (Invitrogen, Breda, the Netherlands). All the fragments were verified by sequencing. Subsequently the fragments were recombined into the expression vector pGreen0179 PL Gateway YFP HA using LR clonase (Invitrogen, Breda, the Netherlands). These constructs were transformed via the Agrobacterium tumefaciens strain GV3103 in their respective mutant backgrounds, snrk2.4-1 and snrk2.10-2, through floral dip transformation (Clough and Bent 1998). Several primary transformants were selected using 30 μg/ml hygromycin and the plants were allowed to self. Recombinant proteins of the correct size were confirmed by Western blot analysis using a αGFP polyclonal antibody (Molecular Probes, Bleiswijk, the Netherlands).

For the construction of the GST fusions and 35S::GFP-overexpression lines, SnRK2.10 and SnRK2.4 cDNAs were amplified using the specific primer sets R4F and R4R for SnRK2.10, and R5F and R5R for SnRK2.4. Subsequently, SnRK2.10 and SnRK2.4 PCR products were amplified with generic AttB1-F and AttB2-R primers to generate attB recombination sites, and were recombined into pDONR207. The resulting entry vectors were used in LR clonase recombination reactions with pDEST15, to generate GST-SnRK2.10 and GST-SnRK2.4 fusion
constructs for expression in *E. coli*, or with pK7WGF2, to generate 35S:GFP-SnRK2.10 and 35S:GFP-SnRK2.4 constructs for expression in plants.

**In-gel kinase assay**

*A. thaliana* plants were grown hydroponically (www.araponics.com) for four weeks under short day conditions (light/dark: 10/14, 21°C/70% humidity) with a weekly change of growth medium using the Flora series (GHE, Fleurance, France). 24 hours prior to stimulation, plants were transferred to smaller containers (3 plants per container). For each sample, 3 plants were treated by transferring the plants to containers containing control or medium supplemented with salt or mannitol.

Proteins were extracted from ground root tissue using 1 volume of extraction buffer (50 mM Tris/HCl pH 7.5, 5 mM EDTA, 5 mM EGTA, 2 mM DTT, 25 mM NaF, 1 mM Na₃VO₄, 50 mM β-glycerophosphate, 1x complete protease inhibitor cocktail (Boehringer Ingelheim, Alkmaar, the Netherlands)) and a 10 minutes 10,000 x g centrifugation step. Protein concentration was determined using Bradford (Bio-rad, Veenendaal, the Netherlands).

In case of immunoprecipitation, 500μg of proteins were combined with 25 μl αSnRK2.4/2.10 serum (Vlad, et al. 2010) and IP buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1x complete protease inhibitor cocktail (Boehringer Ingelheim, Alkmaar, the Netherlands), 0.2% [v/v] tergitol-type NP-40) was added to a total volume of 500 μl. Samples were gently rotated for 3 hours at 4°C. 50 μl of a 50% Protein G (GE Healthcare, Hoevelaken, the Netherlands) slurry in IP buffer was added and incubated overnight at 4°C while gently rotating. The samples were spun at 10,000 x g for 2 minutes and washed in IP buffer 3 times. The supernatant was completely removed after the last washing step and the proteins were eluted using 40μl 1x sample buffer (60 mM Tris/HCl, 2% SDS, 5% β-Mercaptoethanol, 10% glycerol, 0.02% Bromophenol blue).
Proteins were separated on a 12% polyacrylamide gel containing 0.2 mg/ml Myelin Basic Protein (Upstate, Lake Placid, USA). Gels were washed 3 times for 30 minutes in wash buffer (25 mM Tris/HCl pH 7.5, 500 μM DTT, 100 μM Na$_3$VO$_4$, 5 mM NaF, 500 μg/ml BSA, 0.1% [v/v] Triton X-100) at RT and additionally washed twice for 30 minutes and then overnight using regeneration buffer (25 mM Tris/HCl pH 7.5, 1 mM DTT, 100 μM Na$_3$VO$_4$, 5 mM NaF) at 4°C. Gels are washed one time at RT for 30 minutes in reaction buffer (25 mM Tris/HCl pH 7.5, 2 mM EGTA, 12mM MgCl$_2$, 1 mM DTT, 100 μM Na$_3$VO$_4$) and then incubated in reaction buffer supplemented with 25 μM of cold ATP and 50 μCi $^{32}$P γ-ATP for 1 hour. Gels are washed 6 times over a period of 5 hours in stop buffer (1% [w/v] Na$_2$H$_5$P$_2$O$_7$, 5% [v/v] trichloric acid). Gels were dried and the signal was visualized by exposing the gels to a phosphoimage screen (Amersham biosciences, Roosendaal, the Netherlands) and read by a Storm (Molecular dynamics, Sunnyville, USA).

**Root growth assays**

Seeds were surface sterilized in a dessicator in the presence of 100 ml household bleach supplemented with 3 ml HCl for 3 hours. Seeds were sown on square plates containing $\frac{1}{2}$ MS and 1% daishin agarose (Duchefa, Haarlem, the Netherlands), pH 5.8 (KOH) and vernalized at 4 degrees for 48 hours. Plants were grown under long day conditions (21°C, 70% hum., 16/8 light/dark) for 4 days until the plants were either transferred to control or plates supplemented with salt. At 8 days after transfer the plates were scanned and roots measured using Object Image software. To visualize the stages of primordia, roots were fixed and cleared as described in (Dubrovsky et al. 2009). Primordia were studied and classified using an Olympus BH-2 microscope.

**Confocal microscopy**

Plants were grown on square plates containing $\frac{1}{2}$ MS (Duchefa, Haarlem, the Netherlands), 1% Daishin agar, 1% sucrose (pH 5.8, KOH). Plants were either
grown on these plates for 7 days or transferred after 3 days to pre-fixed microscope slides and grown for an additional 4 days between the slides. For the salt treatment, control media were substituted with media containing the corresponding amount of salt. The fluorophores were either excited with: argon 514 nM, emission YFP: 525-555 nM, propidium iodide (600-650 nM). In the case of fig. S5: excitation: argon 488 nM, emission YFP: 525-555 nM, excited: argon 596 nM, emission FM4-64 570-620. Pictures were taken with a Nikon A1 with a 20x water lens. Pictures were processed using ImageJ.

**Fractionation**

Col-0 was grown similarly as for the in-gel kinase assay. 30 ml of root material was harvested of either control or salt stressed roots (approximately 160 plants per treatment). Fractionation was essentially performed as described in (Monreal, et al. 2010). Crude protein was extracted by grinding the tissue in liquid nitrogen and incubating it in protein extraction buffer (50 mM Tris pH 7.5, 300 mM sucrose, 5 mM EDTA, 5 mM EGTA, 2mM DTT, 1x Complete protease inhibitors (Boehringer Ingelheim, Alkmaar, the Netherlands)) for 10 minutes. Samples were filtered through miracloth and centrifuged at 1,500 x g, 2 min, 10,000 x g, 10 min for 6 times where the pellets were stored at -20°C for analysis. Membranes were isolated by spinning for 2 hours at 50,000 x g. The membranes were washed by homogenizing the pellet using protein extraction buffer with an additional 0.1% Brij-58 (Sigma-Aldrich, Zwijndrecht, the Netherlands) (Johansson, et al. 1995). Membranes were again spun down at 50,000 x g for one hour. The pellet fraction was washed twice again in protein extraction buffer as described above and the final pellet was dissolved in 1 ml of protein extraction buffer. The antibodies raised against specific protein markers were obtained from www.agrisera.com PM ATPase (At2g18960), PerM V-ATPase (At4g11150) Cyt. cFBPase (At1G43670). The SnRK2.4/2.10 specific antibody was described in (Vlad, et al. 2010). Silver staining was conducted as a loading control.
Lipid-protein binding assays

The constructs harboring GST-SnRK2.10 and GST-SnRK2.4 were transformed to *E. coli* strain BL21-A1 and expression of the fusion proteins was induced using 0.2% arabinose for 3 hours at 22 °C. GST fusion proteins were purified using affinity chromatography on glutathione agarose as described before (Testerink *et al.*, 2007). Bound protein was eluted from the glutathione agarose resin using elution buffer containing 20 mM reduced glutathione in 50 mM Tris-HCl pH 8.0. Liposome-binding assays were performed as described in (Julkowska, *et al.* 2012). Per sample 1000 ng protein was incubated with liposomes of varying lipid compositions as indicated for 1 hr, after which the liposomes were spun down and washed once. All phospholipids were obtained from Avanti Polar Lipids. Start, pellet and supernatant fractions were loaded on SDS-PAGE and proteins were detected using colloidal coomassie brilliant blue staining.

Acknowledgements

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Chapter 2

References


Chapter 2


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Figure S1. Isolation and confirmation of snrk2.4 and 2.10 knockout mutants and the generation of the SnRK2.4-YFP and SnRK2.10-YFP recombinant lines. a. A schematic overview of the coding region of SnRK2.4 and 2.10. The triangles indicate the locations of the T-DNA insertions with the according names and the insertion line identities. b. SnRK2.4 and 2.10 have a similar molecular weight. Proteins from seedlings were extracted and separated on a 12% polyacrylamide gel and subjected to Western blot analysis using the αSnRK2 antibody. SnRK2.4 was represented by the more intense upper band and SnRK2.10 by the less intense lower band. In the single mutants either of the proteins were gone corresponding to the mutant and in the double mutant both proteins were gone. c. The promoter and genomic sequence of SnRK2.4 and 2.10 fused to YFP were transformed to their corresponding mutant background. The proteins of 8-day-old seedlings were isolated and separated using SDS-PAGE. Recombinant proteins were detected through Western blot analysis using a polyclonal αGFP antibody. Both SnRK2.4-YFP and SnRK2.10 YFP show a recombinant protein at 70 kD, which corresponds to the calculated size. In the lower panel a loading control is displayed using CBB.
Figure S2. SnRK2.4 and 2.10 are activated when exposed to 100 mM NaCl as to 200 mM mannitol but not when transferred to control medium. Hydroponically grown plants were transferred to either control or medium supplemented with 100 mM NaCl, 200 mM mannitol or 150 mM NaCl. a. Transfer to control medium did not induce any visible kinase activation. Plants were transferred to control (C) or medium containing 150 mM NaCl (S) for 2 minutes or 24 hours and kinase activity was determined to exclude an effect of transferring the plants to fresh medium. The panels are constructed in the same way as described in Fig. 1. b. The activation in response to 100 and 150 mM and 200 mM mannitol is displayed in the upper three panels. The arrows indicate the activity of SnRK2.4 and 2.10. To confirm that the activity is due to SnRK2.4 and 2.10, the activity was also determined in the snrk2.4/2.10 double mutant two minutes after the transfer to saline medium. No activity
could be detected in the \textit{snrk2.4/2.10} mutants at 40 kD. 100 mM NaCl and 200 mM mannitol resulted in similar activation patterns. When treated with 150 mM NaCl there is also a transient activation, but the total activation is higher. In addition, the activation of SnRK2.4/2.10 after 24 hours only occurs when exposed to 150 mM NaCl. The lower three panels show a Western blot analysis using an antibody against SnRK2.4/2.10 to determine their abundances in each time-course. All kinase assays were performed with MBP as the substrate.

**Figure S3.** The distribution of LRP developmental stages is altered in \textit{snrk2.10}. \textbf{a}. A visual representation of the developmental stages of LRP with their corresponding nomenclature. \textbf{b}. In mild saline conditions less LRP develop into a LR and more LRP are arrested in stage 5 and 6. All the primordia on the primary root were classified and the distribution of the developmental stages over the total length of the primary root was determined. \textbf{c}. Less LRP developed into a LR in \textit{snrk2.10} compared to Col-0 in saline conditions. These data are obtained from the same experiment as shown in fig. 3 and the distribution of all the roots was determined and averaged. The error bars represent the standard error.
Figure S4. The SnRK2.4-YFP fusion is a functional protein kinase and re-localization of SnRK2.4 to punctate structures in response to saline conditions occurs independently of the location of the fluorophore. a. Both GFP-SnRK2.4 (N-terminally fused GFP) and SnRK2.4-YFP (C-terminally fused YFP) are detected by an αSnRK2 antibody. The GFP-SnRK2.4 was under the control of a 35S::promoter, therefore only 20% of the
amount of protein in comparison to the other samples was loaded on SDS-PAGE. CBB is shown as a loading control. b. GFP-SnRK2.4 and SnRK2.4-YFP are both activated in response to salt. Hydroponically grown *Arabidopsis* roots were treated with 150 mM NaCl. The crude protein extract was separated on SDS-PAGE and an in-gel kinase assay was conducted using MBP as substrate. c. 35s::GFP-SnRK2.4 (N-terminally fused GFP) was transformed to Col-0. The roots were exposed to 150mM NaCl for 2 and 5 minutes. GFP-SnRK2.4 also accumulates in punctate structures showing that the localization occurs independently of the localization of the fluorophore.

*Figure S5. The SnRK2.4-YFP fusion partially co-localizes with the endocytotic marker FM4-64*. 7 day-old seedlings were pre-treated with FM4-64 for 2 hours in advance before exposing them to saline conditions for 10 minutes. SnRK2.4-YFP is shown in the green and FM4-64 in the red channel. SnRK2.4-YFP in punctate structures close to the plasma membrane co-localized with the FM4-64 dye (indicated by the arrows). The co-localization was less striking further away from the plasma membrane.
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### Primers used

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Chapter 3

Identification of a phosphatidic acid-binding domain in the Snf1-related protein kinase SnRK2.4

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Abstract

Phosphatidic acid (PA) is an important signaling lipid in eukaryotes. The formation of PA is induced by a broad range of biotic and abiotic stress stimuli and is involved in various stress related signaling cascades. Two protein kinases involved in salt stress signaling, SnRK2.4 and 2.10, were identified as PA-binding proteins (Chapter 2). Using a differential centrifugation approach, SnRK2.4 and 2.10 were shown to be present in the peripheral membrane protein fraction after salt treatment \textit{in planta}. To determine the effect of PA on the activation of SnRK2.4 and 2.10, their activation in response to salt was observed in the \textit{phospholipase (pld) \alpha-1/\delta} mutant background, but no reproducible difference in activation was observed. A PA-binding domain of 42 amino acids was identified in subdomain 1 of SnRK2.4, which is required for osmotic stress responses. This PA-binding domain was fused to YFP and shown to locate in the cytosol and to accumulate in punctate structures in plants treated with salt. In order to disrupt the interaction between PA and SnRK2.4, seven basic amino acid residues, which were selected through a homology study with the non-PA-binding SnRK2.6, were mutated into Alanines. These changes completely abolished PA-binding affinity of the subdomain and reduced the PA-binding affinity of the full-length protein. In conclusion, this study describes a new PA-binding domain, which could be further developed as a biosensor for \textit{in vivo} PA production in plants. In addition, the identification of residues crucial for PA-binding is an important step towards elucidation of functional domains in SnRK2.4.
Introduction

Cellular membranes mainly consist of phospholipids that provide the structural basis of the membrane. Several low abundant phospholipids, including phosphoinositides (PPIs) and phosphatidic acid (PA) also act as lipid second messengers, involved in a wide array of cellular responses (Meijer and Munnik, 2003; Wang, 2004; Munnik and Testerink, 2009). PA is involved in stress responses as well as in development and metabolic processes (Testerink and Munnik, 2011). It is normally present in small amounts, but rapidly accumulates in the lipid bilayer in response to different biotic and abiotic stress stimuli. De novo PA is predominantly produced through two different pathways. Phospholipase D (PLD) hydrolyses structural phospholipids into PA and a remaining headgroup (Wang, 2004; Bargmann and Munnik, 2006) and phospholipase C (PLC) hydrolyses PPIs to produce diacylglycerol (DAG). DAG is subsequently phosphorylated to PA by DAG kinase (DGK) (Meijer and Munnik, 2003). Osmotic stress induces an increase in PA through both pathways (Arisz et al., 2003).

Several PLDs have been implicated in salt stress acclimation in Arabidopsis. A pldα3 knock-out (KO) mutant was shown to exhibit reduced primary root growth and a reduction in the number of lateral roots in hyperosmotic conditions (Hong et al., 2008). A similar observation was made in a pldα-1/δ double mutant. Less PA accumulated in response to salt and seedlings showed a reduction in primary root growth in saline conditions (Bargmann et al., 2009).

So far it has been proven to be a major challenge to identify in vivo protein targets of PA that are regulating the downstream signaling cascades. Nevertheless, several proteins involved in abiotic stress signaling have been shown to be PA targets. PLDα1 derived PA binds and inhibits the activity of the ABI1 protein phosphatase, and promotes abscisic acid (ABA)-induced stomatal closure (Zhang et al., 2004; Mishra et al., 2006). In addition, PA stimulates the activity of two
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NADPH oxidases, RbohD and RbohF, leading to an increase in reactive oxygen species needed for ABA-induced closure of stomata (Zhang et al., 2009). Another PA target involved in osmotic stress signaling is Map kinase 6 (MPK6), which is able to phosphorylate salt overly sensitive1 (SOS1) in vitro (Yu et al., 2010). Similar to the NADPH oxidases, MPK6 activity increased through PLDα1 derived PA.

Several additional proteins were identified in a proteomics screen for PA-binding proteins (Testerink et al., 2004). Using sepharose beads coated with PA, a number of proteins involved in metabolism and stress signaling were identified, including a SnRK2 protein kinase that is activated in response to salt and osmotic stress in Arabidopsis (Munnik et al., 1999; Mikolajczyk et al., 2000; Boudsocq et al., 2004). The protein kinases identified, SnRK2.10 (SnRK2B), and its close homolog SnRK2.4 (SnRK2.4A) belong to the plant specific Sucrose non-fermenting related kinase 2 family (SnRK2) (Kobayashi et al., 2004; Umezawa et al., 2004; Kulik et al., 2011). Based on phylogeny, the SnRK2 family has been divided into three classes (Kobayashi et al., 2004), which differ in their activation by the phytohormone ABA. In Arabidopsis, SnRK2.2 (SnRK2D), 2.3 (SnRK2I) and 2.6 (OST1; SnRK2E) are strongly activated in the presence of ABA (class 3). Together with the components PYR1, ABI1 and ABF2, the SnRK2.6/2.2/2.3 protein kinases were shown to be sufficient for ABA-induced-gene expression, showing that class-3 SnRK2s act in the core ABA signaling (Fujii et al., 2009). SnRK 2.7 and 2.8 are involved in drought signaling in an ABA dependent way (Umezawa et al., 2004; Mizoguchi et al., 2010). Using a phosphoproteomics approach, several targets of SnRK2.8 were identified that connect SnRK2.8 to metabolic processes (Shin et al., 2007). The members of SnRK2 class 1, SnRK2.1 (SnRK2G), SnRK2.4 (SnRK2.4A), SnRK2.5 (SnRK2H), SnRK2.9 (SnRK2J) and SnRK2.10 (SnRK2B) are activated by osmotic stress, but not by ABA (Boudsocq et al., 2004; Umezawa et al., 2004; Boudsocq and Lauriere, 2005). SnRK2.4 and 2.10 are amongst the most rapidly activated protein kinases in response to salt and are involved in the maintenance of the root system.
architecture under saline conditions (Chapter 2). SnRK2.4 binds PA in vitro and SnRK2.4-YFP accumulates in punctate structures in response to salt (Chapter 2), suggesting a role for membrane association in the response to salt stress.

Here, we show that SnRK2.4 targeted to punctate structures is transiently associated with cellular membranes in vivo. A PA-binding domain was identified within the SnRK2.4 protein. This domain fused to GFP localized in punctate structures in planta suggesting a role for PA in the recruitment to punctate structures in response to salt. Several basic amino acids in this PA-binding domain were replaced by Alanines, which resulted in loss of binding of the subdomain and in a reduction of PA-binding affinity of the full-length protein.

Results

**SnRK2.4 and 2.10 specifically bind to liposomes containing PA**
SnRK2.10 was identified to bind PA in vitro through a PA-binding affinity screen using PA-coated Sepharose beads followed by identification through mass spectrometry (Testerink et al., 2004). Both SnRK2.10 and 2.4 were shown to bind PA directly, using in vitro liposome assays (Chapter 2). To characterize their lipid binding affinity and specificity, liposome binding assays were performed with different phospholipid compositions (Fig. 1). The structural phospholipids PC and PE were used as the lipid backbone of the liposomes, in which different anionic and signaling lipids were mixed. Purified GST-fused SnRK2.4 and SnRK2.10 were tested for binding affinity to liposomes containing PA or the other phospholipids phosphatidylinerine (PS), phosphatidyl inositol phosphate (PIP) and phosphatidyl inositol (4,5) biphosphate (PIP_2) as controls. Both SnRK2.4 and 2.10 showed similar lipid binding properties and specifically bound to liposomes containing PA, but not to liposomes containing other anionic (PS) or anionic phosphorylated lipids (PPIs).
Figure 1. SnRK2.4 and 2.10 specifically bind liposomes containing phosphatidic acid.

GST-tagged SnRK2.4 and 2.10 were expressed in E. coli and purified. SnRK2.4 and 2.10 were incubated with 400 nmol (total lipid) liposomes containing (PC/PE/Pₓ) 1:1:2, where Pₓ represents the anionic lipids PS, PA, PIP or PIP₂. Both soluble and pellet fractions were loaded on SDS-PAGE and stained with colloidal Coomassie. The pellet fractions represent bound protein, and the supernatant fraction the unbound protein. In the left panels the input protein is shown as a loading control.

SnRK2.4/2.10 are identified in different subcellular fractions from Arabidopsis root tissue in saline conditions

SnRK2.4 is targeted to punctate structures in response to salt stress (Chapter 2, Fig 5) and is present in the microsomal membrane fraction in response to salt (Chapter 2, Fig. 6). To further investigate to which subcellular fraction SnRK2.4/2.10 relocalize upon salt treatment, Arabidopsis root extracts were subjected to differential centrifugation (Fig. 2). The distribution of SnRK2.4/2.10 over the different fractions is largely similar to the cytosolic marker (UGPase), showing that most of the SnRK2.4/2.10 is cytosolic. In contrast, SnRK2.4/2.10 are also present in the 10,000 x g (debris, intact organelles) and 50,000 x g pellet (microsomal membranes), showing that a sub-pool of SnRK2.4/2.10 is associated to the membrane or present in cellular compartments. The 50,000 x g pellet fraction was further divided in the Brij-58 wash fraction, peripheral membrane proteins and the remaining pellet. The pellet was first washed with Brij-58 to release any soluble proteins trapped in cellular compartments. Secretion-Associated and Ras-related protein1 (SAR1) is involved in intracellular protein transport between the endoplasmic reticulum (ER) and the Golgi, and is mainly present in the ER (Pimpl et al., 2000). SAR1 as well as SnRK2.4/2.10 are present in the Brij-58 wash fraction, indicating inclusion of SnRK2.4/2.10 in intracellular membrane structures. This observation is consistent with the localization in
punctate structures in response to salt stress (Chapter 2). In contrast to the ER marker, SnRK2.4/2.10 is also present in the peripheral membrane-protein fraction, showing that SnRK2.4/2.10 were not just trapped in vesicular or organellar structures but were also partially directly associated with the membrane, possibly in a PA dependent manner.

**Figure 2. SnRK2.4/2.10 is associated with the membrane after exposure to salt in Arabidopsis roots.** Proteins from *Arabidopsis* roots treated with 150 mM NaCl for 7 minutes were isolated and fractionated using sequential centrifugation steps. Western blot analysis was performed on these fractions using antibodies against different compartment markers and SnRK2.4/2.10. Membranes were isolated and washed with Brij-58 to release any soluble immobilized proteins. From the upper to lower panel: Plasma membrane (PM ATPase), peripheral vacuolar membrane (PerM V-ATPase), endoplasmic reticulum and involved in trafficking between the ER and the Golgi (ER/EndoM SAR1), Cytosol (UGP-ase) and SnRK2.4/2.10. In the lowest panel a silver stain is shown as a loading control.
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**PLDα1 and δ derived PA does not influence SnRK2.4/2.10 activation in response to salt.**

PLDα1 and δ have been shown to be important in salt stress responses (Bargmann et al., 2009). By knocking-out either or both, a reduction in the formation of PA was observed in response to a high concentration of salt (500-1500 mM NaCl). In addition, these mutants also showed a reduction in primary root growth in saline conditions (75/150 mM NaCl). A similar reduction in primary root growth in saline conditions was also observed in the *snrk2.4* mutant (Chapter 2). To determine a possible effect of PA formation on the activation of SnRK2.4 and 2.10 *in vivo*, SnRK2.4/2.10 activation was assessed in the *pldα1/δ* background.

![Figure 3. The activation of SnRK2.4 and 2.10 in response to salt is not affected in the PLD α1/δ double mutant.](image)

In-gel kinase assay of total protein extracts from hydroponically grown roots of Col-0 and a *pldα1/δ* mutant exposed for 2 and 10 minutes to 125 and 150 mM NaCl. In the upper panel (crude extract, activity) the activity of a number of kinases is detected. In the second panel, kinase activity is shown after immunoprecipitation with an antibody against SnRK2.4/2.10.

*Arabidopsis* roots were exposed to medium containing 125 and 150 mM NaCl for 2 and 10 minutes. Kinase activity of the crude extract (upper panel) and the immunoprecipitated SnRK2.4/2.10 (IP) is shown in Fig. 3. No reproducible differences in activation were observed in the SnRK2.4/2.10 IP samples at either concentration or time-point. To further examine the effect of PA-binding on the function of SnRK2.4, another strategy was followed: identification and disruption of the PA-binding site of SnRK2.4.
Multiple regions of SnRK2.4 bind PA-containing liposomes.

Liposome binding assays were conducted on purified *E. coli*-expressed SnRK2.4 recombinant protein at different concentrations of PA (Fig. 4a). SnRK2.4 was able to bind liposomes containing 50% PA at both 400 and 40 nm total lipid, while in contrast, the SnRK2.6 isoform did not bind to any of the liposomes tested. Since SnRK2.6 does not exhibit any PA-binding affinity, but has high homology to other SnRK2 members that do have PA-binding affinity (including SnRK2.1, data not shown), proteins were aligned to identify amino acids that are likely to be important for PA-binding. Lysine and Arginine residues are known to be preferred docking sites for PA (Kooijman et al., 2007). Therefore all of the basic residues conserved in the PA-binding SnRK2s and absent in SnRK2.6 are putatively involved in PA-binding (highlighted in red, Fig. 4b).

![Figure 4. SnRK2.6 does not bind liposomes containing PA.](image)

(a) GST-tagged SnRK2.4 and 2.6 were expressed in *E. coli* and purified. Both proteins were incubated with 400 and 40 nmol (total lipid) liposomes containing PC/PE/PA in the ratios 2:2:1 or 1:1:2. Pellet fractions, representing bound protein, were loaded on SDS-PAGE and protein was detected with anti-GST Western analysis.

(b) Amino acid alignment of three SnRK2s that have PA-binding affinity; SnRK2.1, 2.4 and 2.10 that binds and SnRK2.6 which does not bind liposomes containing PA. Basic amino acids conserved in the SnRK2 members that have PA-binding affinity and which are absent in SnRK2.6 are highlighted in red.
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A schematic overview of SnRK2.4 domains and location of candidate amino acids is displayed in Fig. 5a. Two candidate amino acids were found in the N-terminal kinase domain, five in domain 1, which is a 42 amino acid domain that is required for the osmotic stress response (Kulik et al., 2011) and three were found in the C-terminal acidic domain (domain 2) (Park et al., 1993). To further investigate which part of the protein contains the PA-binding domain, six fragments (A-F) of SnRK2.4 were produced, with an emphasis on the domain that is required for the osmotic stress response. Fragment A consisted of the kinase domain of SnRK2.4, fragment B is the regulatory domain. Fragment C covered the protein from the border of the kinase domain till the acidic domain including domain 1 and fragment D covered the acidic domain (domain 2). Since most candidate amino acids were identified in domain 1, two subfragments were produced for this domain; fragment E covers the N-terminal and F the C-terminal part including the region between domain 1 and 2.

Using liposomes containing 400 nmol lipids, all indicated fragments except fragment D (domain 2) were shown to bind PA. This indicates that multiple parts of the protein contribute to an interaction with PA (Fig. 5b). The SnRK2.4 kinase as well as the C-terminal regulatory domain exhibited PA-binding affinity. Although the kinase domain of SnRK2.4 and 2.6 are very similar, the SnRK2.6 kinase domain did not show any binding affinity to liposomes containing PA (Fig. 5c). The SnRK2.4 kinase domain has PA-binding affinity, but it only binds to the highest concentration of liposomes tested (400 nmol). On the other hand, fragment B was able to bind liposomes also at low concentrations of PA (40 nmol total lipids), indicating that the PA-binding affinity of the C-terminal regulatory domain is higher than the binding affinity of the kinase domain (Fig. 5d). Even at the lowest amount of total lipids tested (25 nmol), fragments B and C were able to bind PA, showing that the fragments containing the domain required for osmotic stress responses have the highest PA-binding affinity. The PA-binding strength of fragment E and fragment F were both lower than fragment C, which indicates that
the C-terminal PA-binding site is a combinatory domain of fragments E and F, and corresponds to the domain 1, which is necessary for the response to osmotic stress.

Figure 5. PA-binding occurs largely through the domain that is required for osmotic stress responses. a. Schematic overview of the coding region of SnRK2.4 with several domains and the locations of the candidate amino acids. Below, the locations of the SnRK2.4 fragments used are displayed. b. GST tagged SnRK2.4 and the fragments were expressed in E. coli and purified. SnRK2.4, the fragments and free GST were incubated with 400 or 40
nnmol (total lipid) liposomes containing either PC/PE in the ratio 1:1 or PC/PE/PA in the ratios 2:2:1 or 1:1:2. c. The kinase domain of SnRK2.6 does not bind PA. Liposome assays were conducted on full-length SnRK2.4 and the kinase domains of SnRK2.4 and 2.6 with 400 or 40 nmol (total lipid) liposomes containing either PC/PE in the ratio 1:1 or PC/PE/PA in the ratios 2:2:1 or 1:1:2. d. SnRK2.4 and a selection of truncated fragments were incubated with different amounts of liposomes ranging between 400 and 25 nm total lipid containing either PC/PE 1:1 or PC/PE/PA 1:1:2. Pellet fractions, representing bound protein, were loaded on SDS-PAGE and protein was detected with anti-GST Western analysis. In the left panels the input protein is shown as a loading control.

**The candidate amino acids present in domain 1 are necessary for PA-binding and a domain1::YFP fusion localizes to punctate structures in planta.**

A new fragment corresponding to domain 1, which encompasses the E fragment and the N-terminal part of fragment F (261-302) was cloned, and shown to exhibit affinity for PA (Fig. 6a). Within this PA-binding domain, the 5 candidate basic amino acids were mutated to Alanines. The resulting PA-binding domain PABD<sup>R266A, K278A, K279A, K294A, K300A</sup> did not exhibit any PA-binding (Fig. 6a), showing that these amino acids are indeed responsible for the PA-binding affinity of this domain.

![Figure 6. A 42 amino acid domain of SnRK2.4 binds PA and localizes to punctate structures in Arabidopsis roots.](image)

**Figure 6. A 42 amino acid domain of SnRK2.4 binds PA and localizes to punctate structures in Arabidopsis roots.** a. GST tagged PA-binding domain and the mutated equivalent were expressed in E. coli and purified. Both domains were incubated with different amounts of liposomes ranging between 400 and 25 nm total lipid containing either PC/PE 1:1 or PC/PE/PA 1:1:2. The loading control is shown in the left panels and the proteins that bound to the liposomes are shown in the right panels. A schematic representation is shown of the PA-binding domain with the selected Lysines and Arginines (red) and the same amino acids mutated to Alanine (light grey). b. pUbiquitin::PA-binding domain-YFP was stably transformed to Col-0. The fluorescence of the PA-binding-domain-YFP in roots is shown in green.
The PABD\textsuperscript{wt} was fused to YFP and expressed in \textit{Arabidopsis} to determine whether this domain would be important for the localization of SnRK2.4 in punctate structures (Chapter 2). The PA-binding domain was largely cytosolic in root cells, but also localized to punctate structures, similar to those observed for the full-length SnRK2.4-YFP. Although the accumulation in punctate structures was not as clear in comparison to the full-length protein, it shows that domain 1 contributes to the localization in punctate structures. Since this domain has the highest PA-binding affinity, it indicates that the localization to punctate structures could be dependent on an interaction with PA.

\textbf{PA-binding of full length SnRK2.4 does not solely depend on the candidate amino acid residues.}

Next, we studied the effect of mutating the 5 candidate amino acids present in the PABD on binding of the full-length SnRK2.4. A SnRK2.4\textsuperscript{R266A, K278A, K279A, K294A, K300A} mutant protein exhibited reduced PA-binding affinity at all lipid concentrations tested and no binding was observed at the lowest concentrations of lipids (25 nmol) (Fig. 7a). Since the kinase domain also exhibited PA-binding affinity, the candidate amino acids in the kinase domain where additionally mutated in the full-length protein. Surprisingly, the SnRK2.4\textsuperscript{K27A, K222A, R266A, K278A, K279A, K294A, K300A} still retained some PA-binding capacity, although the affinity was lower compared to SnRK2.4\textsuperscript{wt}. Thus, the candidate amino acids present in domain 1 and to lesser extent those in the kinase domain contribute to the PA-binding affinity of SnRK2.4. Although the PA-binding affinity was decreased in the mutated versions of the protein, our data indicate that there is an additional unknown interaction or domain contributing to the PA-binding affinity of SnRK2.4.
Figure 7. The identified PA-binding domain is not solely responsible for the PA-binding affinity of SnRK2.4. a. Five amino acids in the PA-binding domain were mutated to Alanine in the full length SnRK2.4 (SnRK2.4 K266A, K278A, K279A, K294A, K300A). Liposome binding assays of recombinant SnRK2.4 and SnRK2.4 K266A, K278A, K279A, K294A, K300A were performed using different amounts of liposomes ranging between 200 and 25 nm total lipid containing either PC/PE 1:1 or PC/PE/PA 1:1:2. On the right side a schematic overview of the location of the mutated amino acids is displayed. b. Mutating the two amino acids in the kinase domain reduced but did not abolish the PA-binding affinity. Five candidate amino acids in the PA-binding domain and two in the kinase domain were mutated to Alanine in SnRK2.4 (SnRK2.4 K277A, K222A, K266A, K278A, K279A, K294A, K300A). Liposome assays of the SnRK2.4 and SnRK2.4 K277A, K222A, K266A, K278A, K279A, K294A, K300A were conducted similarly as described above.

Discussion

The *Arabidopsis* SnRK2.4 protein kinase is activated in response to osmotic stress and binds the lipid second messenger PA (Chapter 2). The specificity of PA-binding of SnRK2.4 and 2.10 (Fig. 1) indicates the presence of a specific PA-binding site, rather than general, a-specific electrostatic interaction with anionic lipids. By testing different protein fragments of the SnRK2.4 protein for PA-binding, a domain of only 42 amino acids was identified that has high binding affinity for
liposomes containing PA. The PA-binding domain corresponds to the previously designated osmotic stress domain 1 (Kulik et al., 2011). *In vivo*, a fusion of this domain to YFP, localizes to punctate structures (Fig. 5) similar as observed before for the full-length SnRK2.4 (Chapter 2, Fig. 6).

Analysis of different cellular fractions and comparison with markers of known locations (V-ATPase and SAR1) revealed that a small but significant fraction of SnRK2.4/2.10, was truly associated with membranes (Fig. 3). On the other hand, the majority of the SnRK2.4/2.10 present in the microsomal membrane fraction was released during the Brij-58 wash (together with the SAR1 marker), suggesting that SnRK2.4/2.10 proteins in punctate structures are dissociated from the membrane, but remain within endomembrane structures. The PA-binding domain localizes to similar punctate structures supporting a role for PA-binding in this relocalization event. Since PA accumulation occurs more rapidly (Munnik et al., 2000) than the relocalization of SnRK2.4-YFP (Chapter 2, Fig. 6), PA-binding probably occurs prior the localization in punctate structures. This suggests an interaction with PA is necessary for the targeting of SnRK2.4 to punctate structures in response to salt stress. Whether re-localization would affect the salt-induced activation of SnRK2.4/2.10 (Chapter 2) remains unknown.

PA preferentially interacts with Lysines and Arginines (Testerink and Munnik, 2005; Kooijman et al., 2007). Altering basic amino acids was already shown to be effective in abolishing PA-binding of several PA-targets (Ghosh et al., 2003; Zhang et al., 2004; Zhang et al., 2009). Five positively charged amino acid residues (Lysines and Arginines), conserved in the PA-binding domain (domain 1) of the class 1 SnRK2 members, were identified here to be involved in PA-binding. Mutating the candidate amino acids in this domain completely abolished the PA-binding affinity of the PA-binding domain. Mutating the same amino acids in the full-length protein reduced the PA-binding affinity, but did not abolish it, indicating that the identified PA-binding domain is not the only part of the protein responsible for PA-binding affinity. Mutating additional amino acids in the kinase
domain did not result in complete disruption of the binding affinity either. For several PA targets it has been described that a hydrophobic region contributes to the lipid binding affinity (Frank et al., 1999; Koag et al., 2003; Koag et al., 2009). However, since SnRK2.4 is very hydrophilic and has no hydrophobic domain that is absent in SnRK2.6, the remaining PA-binding affinity present in SnRK2.4 K27A, K222A, R266A, K278A, K279A, K294A, K300A remains elusive. Although PA-binding of the mutated full-length SnRK2.4 is not completely abolished, studying the activation, localization and in planta function of the mutant protein will be instrumental to determine what the function of PA is in the regulation of SnRK2.4.

Here, we identified a novel PA-binding domain, that of SnRK2.4. Because the domain binds liposomes containing PA with high affinity, it could be exploited as a PA-biosensor to determine where PA is produced locally in a cell. Localization studies with the mutated PA-binding domain will have to be performed to validate its specificity in vivo. In addition, many pld, plc and dgk single and double mutants have been isolated and confirmed (Li et al., 2009; Munnik and Testerink, 2009; Testerink and Munnik, 2011). It will be interesting to investigate SnRK2.4 and 2.10 activation and localization in these mutant backgrounds.

Material and methods

Cloning and site directed mutagenesis
SnRK2.4, 2.10 and 2.6 FL CDS, fragments A-F, SnRK2.6 kinase domain, PABD, PABD R266A, K278A, K279A, K294A, K300A, SnRK2.4 R266A, K278A, K279A, K294A, K300A and SnRK2.4 K27A, K222A, R266A, K278A, K279A, K294A, K300A were amplified with primers containing the Gateway recombination attB1 and attB2 site sequences (Table S1), which are compatible with the recombination sites of pDONR207. The fragments were recombined in pDONR207 using BP2 Clonase according to the instructions of the manufacturer (Invitrogen, Breda, the Netherlands), resulting in pENTR(x) constructs, which were all verified by sequencing. Subsequently all constructs
were recombined into a pGEX-KG Gateway expression vector (Dhonukshe et al., 2010) using LR Clonase according to the instructions of the manufacturer (Invitrogen, Breda, the Netherlands). The constructs were transformed to *E. coli* strain BL21 DE3 for protein expression and purification. The pENTR-PABD was recombined into the expression clone pGII0125-R4R3 (Galinha et al., 2007) using 3-way Gateway, together with the ubiquitin 10 promoter, which was amplified by from genomic Col-0 DNA (1986bp upstream from the start codon) with primers containing appropriated attB recombination sites (table 1), (Galvan-Ampudia et al., unpublished) (Box1) and mVenus (Box3) (Nagai et al., 2002) using LR+ Clonase accordingly to the instructions of the manufacturer (Invitrogen, Breda, the Netherlands) (pGII0125-R4R3 Norf/pGEM, BOX1: promUBQ10/pDONR207, BOX 2: PCR product PABD/pGEM, BOX3: mVENUS FLAG t35). Constructs were transformed using the *Agrobacterium tumefaciens* strain GV3103 to Col-0 through floral dip transformation (Clough and Bent, 1998). Several primary transformants were selected using 0.3 μg/ml norflurazon and the plants were allowed to self-pollinate. Recombinant proteins of the correct size were confirmed by Western blot analysis using a αGFP polyclonal antibody (Molecular probes, Bleiswijk, the Netherlands) (Fig. S1).

Mutations were induced through site-directed mutagenesis with the indicated primers (Table 1). Mutations were sequentially applied in the pENTRY-SnRK2.4 and pENTRY-PABD clones. Mutations were introduced using *Pfu* polymerase (Promega, Leiden, the Netherlands) according to the manufacturers’ instructions. The PCR was conducted in a volume of 50 μl, using 10 ng plasmid as template, applying 21 cycles, annealing temperature: 52°C and an extension time of 16 minutes. The PCR product was digested with DPNI (Fermentas St. Leon-Rot, Germany) at 37°C for two hours and the digestion product was purified using the GeneJet PCR purification kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer’s instructions and eluted in 30μl MQ. The product was transformed
to *E. coli* strain DH5α. Plasmids were isolated and sequenced to determine if they contained the desired mutation.

**Induction and purification of GST-tagged SnRK2.4 protein fragments from *E. coli***

Transformed BL21 DE3 bacteria were grown over night at 37°C in 2xYT medium containing ampicillin. Four ml of o/n culture was diluted in 100 ml of pre-warmed 2xYT medium and was grown at 37°C until OD$_{600}$ reached 0.6. The production of recombinant protein was induced by adding IPTG up to 1mM final concentration. The cells were induced for 3 hours at 18°C. Subsequently cells were centrifuged at 5,000 x g for 15 minutes at 4°C. The pellet was snap-frozen in liquid nitrogen and stored at -80°C and subsequently dissolved in PBS containing 1x complete protease inhibitor cocktail (Boehringer Ingelheim, Alkmaar, the Netherlands). Cell contents were released by lysozyme treatment and sonication. Soluble proteins were isolated by spinning the cell suspension at 13,500 x g for 30 minutes at 4°C. The SnRK2.4 fragments were purified from soluble protein fraction using the GST-Sepharose beads. Proteins bound to the GST-Sepharose beads were eluted using elution buffer containing 20 mM reduced glutathione (50mM Tris pH 8.0). The protein concentration was determined by separating the proteins on SDS-Page, staining of the gel with colloidal Coomassie (Sigma-Aldrich, Zwijndrecht, the Netherlands) and compared to known BSA dilution series.

**Liposome binding assays**

Liposome assays were performed as described in (Levine and Munro, 2002; Loewen et al., 2004; Testerink et al., 2007) with some modifications. Per sample, 400 nmol of total lipids were used unless indicated otherwise. Synthetic 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), -phosphatidylethanolamine (DOPE), -phosphatidylserine (DOPS), -phosphate (DOPA) were used dissolved in chloroform and natural L-a-phosphatidylinositol-4,5—bisphosphate (brain,
porcine-triammonium salt) C:M:W (20:9:1) were used (all from Avanti Polar Lipids, Alabaster, AL, USA) (Julkowska et al. 2012). Lipids were mixed in the right molar ratios in chloroform, dried, and rehydrated in extrusion buffer (250 mM raffinose, 25 mM TRIS pH 7.5, 1 mM DTT) for 0.5 h. Unilamellar vesicles were produced using a lipid extruder with 0.2 µm membrane (Avanti Polar Lipids, Alabaster, USA) according to the manufacturer’s instructions. Liposomes were diluted in three volumes of binding buffer (125 mM KCl, 25 mM TRIS pH 7.5, 1 mM DTT, 0.5 mM EDTA) and pelleted by centrifugation at 50,000 x g for 15 min. Liposomes were resuspended in binding buffer, added to 500 ng purified GST-tagged protein, and incubated for 30–45 min in a total volume of 50 µl at room temperature. Liposomes were harvested by centrifugation at 16,000 x g for 30 min, washed once in binding buffer, and resuspended in sample buffer. Samples were incubated at 95°C for 5 minutes and run on 10% SDS–PAGE, blotted on Hybond and GST-tagged proteins were detected through Western blot analysis. IgG; αGST mouse monoclonal (Santa Cruz, Heidelberg, Germany) was used as the primary antibody and GAMPO (Sigma-Aldrich, Zwijndrecht, the Netherlands) as the secondary antibody according to the manufacturer’s instructions.

**Fractionation**

Col-0 plants were grown in hydroponics, similar to the in-gel kinase assay (Chapter 2). 40 ml of root material was harvested of salt stressed roots (150 mM NaCl, 7 minutes, approximately 200 plants per sample). Fractionation was essentially performed as described in (Monreal et al., 2010) and Chapter 2 with some modifications. The peripheral membranes were eluted by thoroughly homogenizing the pellet in protein extraction buffer supplemented with 100 mM Na₂CO₃. After homogenizing, samples were incubated for 15 minutes on ice and the sample was spun again at 50,000 x g for one hour. The supernatant is shown as the peripheral membrane proteins and the pellet is the remaining pellet. The antibodies raised against specific protein markers were obtained from Agrisera,
Vännäs, Sweden, unless stated otherwise, PM ATPase (Palmgren et al., 1991), PerM V-ATPase (At4g11150), ER/EndoM SAR1 (At3g62560), Cyt. UGPase (raised against barley), SnRK2.4/2.10 (Vlad et al., 2010). Silver staining was conducted as a loading control.

**Kinase assay**

In-gel kinase assays were performed on hydroponically grown roots of *Arabidopsis* Col-0 and *Pldα1/δ* (Bargmann et al., 2009) plants as described in Chapter 2.

**Confocal microscopy of PA-binding domain-YFP line.**

Plants were grown on square plates containing ½ MS (Duchefa, Haarlem, the Netherlands), 1% Daishin agar, 1% sucrose (pH 5.8, KOH) for 7 days. The fluorophore was excited with: argon 514 nM, emmision YFP: 525-555nM. Pictures were taken with a Nikon A1 with a 20x water lens and processed using ImageJ.

**Acknowledgements**

We would like to thank Lotte Caarls for technical assistance, Micheal Palmgren for supplying the anti- H+-ATPase antibody, Bea Horváth and Ben Scheres for supplying the Norf vector and the van Leeuwenhoek Centre for Advanced Microscopy (LCAM) at the University of Amsterdam for making their confocal microscopes available and technical support.

**References**


**PA-binding domain of SnRK2.4**


**Hong Y, Pan X, Welti R, Wang X** (2008) Phospholipase Dalpaha3 is involved in the hyperosmotic response in Arabidopsis. The Plant cell 20: 803-816


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Li M, Hong Y, Wang X (2009) Phospholipase D- and phosphatidic acid-mediated signaling in plants. Biochimica et biophysica acta 1791: 927-935


**PA-binding domain of SnRK2.4**


**Supporting information**

**Figure S1. The PABD fused to YFP has the expected size in transgenic Arabidopsis seedlings.** The PA-binding domain under control of the ubiquitin promoter, fused to YFP was transformed to Col-0. The proteins of 8-day-old seedlings were isolated and separated on a 10% polyacrylamide gel. Recombinant proteins were detected through Western blot analysis using a polyclonal αGFP antibody. In two independent transformants, a recombinant protein of 37 kD is detected, which corresponds to the expected size.
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#### Primers used

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Chapter 4

Identification of novel candidate phosphatidic acid binding proteins involved in the salt stress response of *Arabidopsis thaliana* roots

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Chapter 4

Abstract

Phosphatidic acid (PA) 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 stress and several PA-binding proteins have been identified that function in the response to salt, specifically in ABA (abscisic acid) and ROS (reactive oxygen species) signaling. Remarkably, very little is known about the role of PA in the initial response to salt stress in root tissue. In this study, we have combined an approach to isolate peripheral membrane proteins of Arabidopsis 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 mass spectrometry, eight proteins were found to be enriched in the membrane fraction after seven minutes of salt stress. Among these were two clathrin heavy chain (CHC) isoforms and two E/ANTH-domain clathrin assembly proteins. In addition, a protein involved in regulating potassium transport, two ribosomal proteins, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and a type II phosphatidylinositol (PI) 4-kinase γ bound PA and were recruited to the membrane upon salt stress. PA-binding and salt-induced membrane recruitment of GAPDH and CHC were confirmed by Western blot analysis in different fractions. In conclusion, the approach presented here was shown to be 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.
Isolation of PA targets involved in salt stress

Introduction

Cellular membranes primarily consist of phospholipids that separate the cytoplasm from organelles and the external environment. Besides this structural function, certain phospholipid species also provide spatial and transient information needed for adequate cell signaling (Meijer and Munnik, 2003; Munnik and Testerink, 2009; Xue et al., 2009; Munnik and Vermeer, 2010). One species that has been shown to be an important signaling lipid is phosphatidic acid (PA) (Testerink and Munnik, 2005; Li et al., 2009; Testerink and Munnik, 2011). PA is normally present in small amounts, but rapidly accumulates in lipid bilayers in response to different biotic and abiotic stresses (Munnik et al., 2000; Wang et al., 2006). Although involved in different signaling cascades, the molecular function of PA in cellular processes and plant physiological responses remains largely elusive (Testerink and Munnik, 2011).

Induced PA formation has been described in response to abiotic stress stimuli such as ABA (Fan et al., 1997; Ritchie and Gilroy, 1998; Jacob et al., 1999), dehydration (Jacob et al., 1999; Katagiri et al., 2001) and salt and osmotic stress (Munnik et al., 2000; Meijer et al., 2002; Hong et al., 2010). Different PA synthesis pathways have been shown to contribute to the production of PA in response to abiotic stresses (Munnik et al., 2000; Ruelland et al., 2002; Arisz et al., 2009; Bargmann et al., 2009; Li et al., 2009; Hong et al., 2010). Phospholipase D (PLD) hydrolyses structural phospholipids to form PA and a remaining headgroup (Pappan et al., 1998). Phospholipase C (PLC) produces diacylglycerol (DAG), which can subsequently be phosphorylated to PA by diacylglycerol kinase (DGK) (Meijer and Munnik, 2003; Arisz et al., 2009).

In Arabidopsis, PLD isoforms α-1, α3 and δ are required for maintenance of root growth in saline conditions (Katagiri et al., 2001; Hong et al., 2008; Bargmann et al., 2009). The increased salt sensitivity of these PLD mutants suggests an important role for PA in salt stress signaling. 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 phosphoinositide-dependent kinase 1 (PDK1). *Arabidopsis* PDK1 was shown to bind and to be activated by both PA and several PPIs (Deak et al., 1999). In later studies it has been shown that one of the phosphorylation targets of PDK1, PINOID (PID), also has affinity for PA (Anthony et al., 2004; Anthony et al., 2006; Zegzouti et al., 2006). Another target of PDK1 is oxidative signal-inducible (OXI1) which is required for the full activation of MAP kinase 6 (MPK6) (Rentel et al., 2004), which was also shown to bind PA (Yu et al., 2010) indicating that PA could play a role in spatially facilitating these signaling cascades. The *Arabidopsis* MAPKKK constitutive triple response 1 (CTR1) is homologue of the mammalian PA target Raf-1 (Ghosh and Bell, 1997) and was shown to bind PA. Moreover, its activity was inhibited in the presence of PA (Testerink et al., 2007). PTEN binds PPIs and hydrolyses the 3-phosphate from PI(3,4,5)P₃ in animal cells (Maehama and Dixon, 1998). Surprisingly, while the plant homologue, PTEN2A, did hydrolyze several polyphosphoinositides (PPIs), it did not show any binding affinity to PPIs but instead specifically bound to PA (Pribat et al., 2012). A PH domain, similar as found in PDK1, was found in Arf-Gap domain 7 (AGD7) and was identified to stimulate the Arf1 GTPase activity in a PA-dependent manner in vitro (Min et al., 2007). Dehydrins bind different lipids, including PA, through a lipid-binding domain that resembles a class 2 amphipathic alpha-helix domain (Koag et al., 2003; Koag et al., 2009) depending on the phosphorylation status of the protein (Eriksson et al., 2011). 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 lead to the discovery of several PA targets. Addition of PA induced ABA-dependent stomatal closure (Jacob et al., 1999), probably mediated by the protein phosphatase 2C (PP2C) ABA insensitive 1 (ABI1), because this protein was later identified as a direct PA target (Zhang et al., 2004). 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 (Zhang et al., 2004; Mishra et al., 2006). Adding exogenous PA induced ROS production in leaves (Sang et al., 2001) and activation of two NAPDH oxidases, RbohD and RbohF, was dependent on PA (Zhang et al., 2009). 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 (Zhang et al., 2009). Exogenous PA also induced an increase in the amount of filamentous actin in Arabidopsis cell suspension, which lead to the identification of the heterodimeric capping protein (CP). Binding of PA and PI(4,5)P₂ caused a reduction in the activity of this protein, effectively promoting actin polymerization (Huang et al., 2006).

Lipid-binding affinity assays have proven to be successful tools in identifying new PA-binding proteins. Using PA coupled to sepharose beads, phosphoenolpyruvate carboxylase (PEPC) was identified in an earlier PA-affinity screen (Testerink et al., 2004). The activity of C₄ 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 (Monreal et al., 2010). Other proteins identified in this screen include a member of the Snf1 related protein kinase 2 (SnRK2) family, SnRK2.10, which is activated and targeted to the membrane in response to salt stress (Chapter 2) and the PP2A regulatory subunit RCN1 that is involved in ethylene, ABA and auxin signaling (Testerink et al., 2004). Although lipid binding affinity screens have been 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, but biologically relevant PA-binding proteins, we realized that a pre-fractionation step had to be conducted to avoid overcrowding of the PA-affinity beads by abundant proteins. In previous studies this was realized by performing a pre-purification based on charge or size (Manifava et al., 2001; Krugmann et al., 2002; Testerink et al., 2004). In the present study, a pre-purification step was incorporated to specifically select for proteins that are peripherally bound to membranes. Moreover, by exposing plants to a PA inducing-stress stimulus and analyzing which PA-binding proteins are recruited to the membrane specifically in response to the stimulus, proteins can be identified that are recruited to the membrane in vivo, in a PA-dependent manner. Since PA accumulates in response to a wide array of stress stimuli, regulating different processes, stimuli-specific targets may exist. Here, we present this novel approach to isolate and identify candidate PA-binding proteins that are involved in the response of roots to salinity. A set of novel putative PA targets that are associated with membranes in salt-stressed Arabidopsis roots was isolated. Among the 42 PA targets identified, eight were found to be recruited to the membrane in response to salt, by using both iTRAQ labeling and a label-free MS² approach. Proteins identified are involved in the regulation of potassium homeostasis, metabolism and clathrin-mediated endocytosis, amongst other functions.
Isolation of PA targets involved in salt stress

Results

Identification of PA targets specifically involved in the response to salt stress.
To isolate PA-targets that are specifically involved in salt stress signaling, proteins that are not only recruited to the membrane upon salt treatment (Fig 1a), but also show binding affinity to PA-beads, were isolated (Fig 1b). 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 labeling of peptides and label-free MS analysis (Fig 1c).

Figure 1. Identification of PA-binding proteins that are targeted to the membrane in response to salt using two different mass spectrometry-based approaches. a. Peripheral membrane proteins were isolated from hydroponically grown Arabidopsis roots. Grey circles represent structural lipids and red circles 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. The proteins of the PA-binding fractions were identified and quantified using iTRAQ labeling and label-free MS. The flowscheme of the approaches is summarized. By concentrating only on the peripherally membrane-associated proteins, and by quantification of difference in PA-bound proteins of salt-treated vs control samples, candidate PA targets that are targeted to the membrane in response to salt in a PA-dependent manner can be identified.
A biochemical fractionation approach allows specific isolation of peripheral membrane proteins.

To isolate the peripheral membrane proteins (PMP) from root tissue of hydroponically grown plants, a differential centrifugation approach was applied. The flow-scheme of centrifugation steps to isolate peripheral membrane proteins is shown in fig. S1. The quality of the fractionation was determined by Western blot analysis of the different fractions using antibodies raised against different cellular compartment markers; plasma membrane (PM) (ATPase), mitochondrial inner membrane (MitM) (cytochrome oxidase subunit II (COXII)), peripheral membrane (PMP) (epsilon subunit of tonoplast ATPase (V-ATPase)) and a cytosolic marker (UDP-glucose pyrophosphorylase (UGPase)) (Fig. 2).

The total crude protein extract was first centrifuged at 10,000 x g for 10 min to remove cell debris and intact organelles, and the resulting pellet was indeed enriched in the MitM and to a lesser extent with PMP- and the PM-marker. The 50,000 x g crude pellet fraction contained the PM- and PMP-, and to a lesser extent the MitM-marker, while little cytosolic marker (UGPase) was detectable. The 50,000 x g crude pellet fraction was subsequently washed with a buffer supplemented with 0.1% Brij-58, which inverts liposome like structures, to release trapped hydrophilic contaminants. In this Brij-58 wash the remaining cytosolic proteins are released. This is necessary to further reduce the amount of cytosolic and to purify only genuine peripheral membrane proteins. These proteins were subsequently eluted from the membranes using protein isolation buffer supplemented with 100 mM Na₂CO₃, resulting in a PMP fraction that exclusively contained the PMP-marker in both the control (C) and the salt stressed (S) sample.
Isolation of PA targets involved in salt stress

**Figure 2. Isolation of a protein pool enriched in peripheral membrane proteins.** Arabidopsis plants were either control or salt treated (150 mM, 7 minutes). Proteins were isolated from the roots and fractionated using sequential centrifugation steps, after which several protein fractions were analyzed. Pellet fractions are indicated with P and the supernatant fractions are indicated with S. Intact organelles and cellular debris was removed at 10,000 x g (10,000 x g pellet). Sequentially, the microsomal membrane fraction (50,000 x g crude pellet) was isolated and washed with protein isolation buffer supplemented with 0.1% Brij-58 (Brij-58) to release any trapped soluble contaminants. After several additional washing steps using protein isolation buffer, the peripheral membrane proteins (PMP) 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 (Plasma membrane (PM ATPase), Mitochondrial membrane (MitM COXII), peripheral vacuolar membrane (PMP V-ATPase ε subunit), and the cytosolic marker UGP-ase (Cyt. UGPase)). Silver staining was performed as a loading control (lower panel).

**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 control and salt-treated samples using PA beads. The use of PA beads has already been shown to be
successful to isolate PA-targets (Manifava et al., 2001; Testerink et al., 2004). Protein profiles of the total extract, the 50,000 x g pellet (CP), PMP and the PA-binding fractions of control and salt-treated roots were visualized using silver staining (Fig. 3). Clear differences in protein compositions between different fractions were observed. The proportion of the total amount of proteins remaining in the sequential fractions was determined through protein quantification. The 50,000 x g crude pellet consisted of 5% of the total protein extract, the PMP consisted of 2.5% of the 50,000 x g crude pellet and of the PMP, 5% bound to the PA beads which results in a 1/15,000 fraction of total proteins in the final PA-binding protein pool.

![Figure 3. Detection of PA-binding proteins enriched at the membrane after 7 minutes of salt stress. A fractionation of control and salt treated Arabidopsis root protein extracts was conducted similar as described in figure 3. In addition, PA affinity purification was performed using the peripheral membrane protein fraction as input. By silver staining, differences in the protein composition of different fractions and between the different treatments were visualized. Open circles indicate proteins that were less abundant and the closed circles indicate proteins that were more abundant in the salt treated sample compared to the control in the PA-binding fraction.](image)

When comparing control and salt-treated samples of each fraction, no clear differences in individual protein abundances were detectable in either the total, 50,000 x g pellet or the PMP fraction. Only in the PA-binding protein fraction, differences in abundance were observed between the control and the salt treated sample (closed/open circles, fig. 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 mass spectrometry methods.
Protein identification and quantification of control-and salt-treated PA-binding-protein pools.  
Hydroponically grown, 28 days-old Arabidopsis Col-0 plants were transferred to salt (150 mM) or to control media for 7 min, after which root material was harvested, homogenized and proteins extracted. 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 labeled with iTRAQ, while one replicate was analyzed using label-free MS² quantitation. The total number of peptides, proteins and proteins enriched in the salt-treated samples for either the iTRAQ labeled or label-free approach are summarized in Fig. 4a. 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 (S/C) of the 42 proteins identified are shown in Fig. 4b. The proteins are arranged according to their S/C ratio, starting with the largest enrichment in the salt stressed sample. Most proteins are present in similar quantities in both the control and the salt treated sample. Eight of the 42 identified proteins (Table S1) were enriched in the salt-treated sample (S/C>\(^2\log 0.5\)), while no proteins were enriched in the control treated sample using the \(^2\log 0.5\) treshold. The S/C ratio of the eight proteins enriched in the salt-stimulated samples is shown in Fig. 4c and Table 1.
more than 0.5 on the $\log$-scale are considered as increased in their binding to PA in the peripheral membrane fraction. c. The ten proteins that were enriched in the PA-binding peripheral membrane fraction are plotted on a linear scale in a bar graph, error bars denote the standard error of the mean.

Table 1. Salt induced membrane targeted Pk-binding proteins. ^a

<table>
<thead>
<tr>
<th>AGI number</th>
<th>Protein</th>
<th>protein length (aa)</th>
<th>Exp.1 iTRAQ</th>
<th>Exp. 2 iTRAQ</th>
<th>Exp.3 Labelfree MS^b</th>
<th>mean ratio^c S.E.M.</th>
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</thead>
<tbody>
<tr>
<td>At1g04690</td>
<td>Potassium channel beta subunit (KAB1)</td>
<td>378</td>
<td>3.69</td>
<td>3.69</td>
<td>1.82</td>
<td>2.76, 0.93</td>
</tr>
<tr>
<td>At1g77940^d</td>
<td>60S ribosomal protein L30</td>
<td>152</td>
<td>2.46</td>
<td>n.d.</td>
<td>1.43</td>
<td>1.95, 0.52</td>
</tr>
<tr>
<td>At1g08530</td>
<td>Clathrin heavy chain</td>
<td>1703/1705</td>
<td>1.60</td>
<td>1.77</td>
<td>2.32</td>
<td>1.90, 0.17</td>
</tr>
<tr>
<td>At2g25430</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, 0.19</td>
</tr>
<tr>
<td>At3g35536</td>
<td>40S ribosomal protein S3</td>
<td>248</td>
<td>1.38</td>
<td>n.d.</td>
<td>1.92</td>
<td>1.65, 0.27</td>
</tr>
<tr>
<td>At2g01600^e</td>
<td>Putative clathrin assembly protein</td>
<td>178</td>
<td>1.29</td>
<td>1.59</td>
<td>1.84</td>
<td>1.57, 0.16</td>
</tr>
<tr>
<td>At1g13440/At1g04120</td>
<td>Glucosidase 3 phosphate dehydrogenase</td>
<td>278/212</td>
<td>1.66</td>
<td>1.20</td>
<td>1.63</td>
<td>1.49, 0.15</td>
</tr>
<tr>
<td>At1g64460/At2g46500^f</td>
<td>PI 4-kinase gamma</td>
<td>566/301</td>
<td>1.32</td>
<td>1.58</td>
<td>n.d.</td>
<td>1.45, 0.13</td>
</tr>
</tbody>
</table>

*Results are from independent experiments quantitatively analyzed by iTRAQ-TOF MS or labelfree by data-independent analysis (MSE).
^aThe average S/C ratios of the candidate proteins with the standard error of the mean.
^bPeptide fragment spectra sufficient for identification, but intensity too low for quantification.
^cProducts from both loci were represented by proteotypic peptides, but quantitation was based on shared peptides.
^dQuantitation includes semi-proteotypic peptide shared with L30e and L30-3 (encoded by At1g77940 and At3g18740, resp.).
^eA concurrent increase in the product of At1g14910 cannot be excluded; the latter was, in contrast to At2g01600, not represented by proteotypic peptides.
^fNo proteotypic peptides detected; quantitation was based on shared peptide.
Abbrev.: S.E.M., standard error of the mean; n.d. not detected.
The eight proteins identified are described to be involved in various cellular processes. The identification of potassium channel beta subunit 1 (KAB1) implicates a role for PA in the maintenance of potassium homeostasis in saline conditions. In addition, two clathrin heavy chain (CHC) isoforms and two clathrin assembly proteins 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 PI 4-kinase gamma type.

**PA dependent-membrane targeting is confirmed for clathrin heavy chain and GAPDH using Western blot analysis.**

The abundance of two prominent membrane-targeted candidate PA-binding proteins, clathrin heavy chain and GAPDH, was determined in the different fractions including the PA-binding fraction, using specific antibodies (Fig. 5). As previously shown (Fig. 2), the plasma-membrane marker was enriched in the 50,000 x g pellet fraction, while the PMP marker was strongly enriched in the 50,000 x g pellet and present in the peripheral membrane-protein pool. The PMP-marker could not be detected in the PA-binding protein pool. The latter fraction did contain both CHC and GAPDH (Fig. 5), confirming their identification by mass spectrometry as PA-binding proteins. Importantly, salt stress increased CHC and GAPDH abundance in the PMP fraction and the PA-binding fraction of root extracts. This confirmed salt-induced recruitment of both proteins to the membrane and illustrated the reliability of the purification procedure for PA-binding proteins.
Figure 5. Clathrin heavy chain and GAPDH are targeted to the membrane in a PA-dependent manner. Protein isolation and PA-binding was carried out in the same way as described in Fig. 4. Western blot analysis was performed using antibodies against two compartment markers (plasma membrane (PM ATPase) and peripheral vacuolar membrane (PMP V-ATPase ε subunit), and against two candidate PA targets in response to salt stress, (clathrin heavy chain (CHC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)). Both CHC and GAPDH were confirmed to be targeted to the membrane in response to salt and to bind PA. Silver staining was conducted as a loading control.

Discussion

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

Identifying proteins that are recruited to the membrane in a PA dependent manner allows identification of PA targets that are involved in a specific response, in this case salt, in an unbiased fashion. 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 x g P) with protein isolation buffer is not sufficient, since a large portion of the plasma membrane vesicles that are formed upon homogenization are oriented with the cytosolic side inwards, potentially trapping cytosolic proteins. Polyoxyethylene acyl ether Brij-58 is a non-ionic detergent that inverts these vesicles (Johansson et al., 1995), thereby releasing trapped cytosolic proteins. In addition, Brij-58 washing also reduces the amount of proteins present in organelles such as ER, plastids and mitochondria in the microsomal membrane fraction (Zhang and Peck,
2011). \( \text{Na}_2\text{CO}_3 \) was used to elute peripherally membrane bound proteins, because it disrupts the electrostatic interaction of these proteins with integral membrane proteins and the polar heads of lipids (Santoni et al., 1999) which was confirmed with the results obtained (Fig. 2).

**No previously described PA targets were identified in the present screen.**

Proteins with PA affinity were purified from the PMP fraction using PA-coated sepharose beads. Remarkably, none of the previously described PA targets were identified here. So far, few PA targets have been described from root tissue, although MPK6 and SnRK2.10 are known to bind PA and play a role in root growth in saline conditions (Yu et al., 2010) (Chapter 2,3). However, as shown in Chapter 3, although SnRK2.10 associates with cellular membranes, it is largely removed by Brij-58 washing, indicating that the interaction with PA is transient. The trace amount of SnRK2.10 remaining in the salt stimulated PMP fraction (Chapter 3), that could end up in the final PA-binding fraction, was apparently below the detection limit of our mass spectrometry approach.

**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 enriched on membranes after salt stress (Table I) are implicated in clathrin-mediated endocytosis (CME). These include two clathrin heavy chain proteins (At3g08530 and At3g11130) and two putative clathrin assembly proteins (At2g25430 and At2g01600). The latter belong to the family of E/ANTH-domain-containing accessory proteins, which in mammals and yeast are known to bind phospholipids, in particular PIP\(_2\), in order to support vesicle budding from the plasma membrane and trans-Golgi network. E/ANTH-domain containing proteins interact directly with proteins of the clathrin coat and are also speculated to be involved in cargo binding (Holstein and Oliviussson, 2005;
Amino acid motifs involved in clathrin binding are present in all eight *Arabidopsis* ANTH-domain containing proteins, but the sequence implicated in phospholipid binding is highly variable and different from the mammalian homologues.

In the initial phase of coated pit formation, clathrin triskelia, composed of three heavy chains and three light chain proteins, are recruited from the cytosol to adaptor proteins on the nascent budding site (Chen et al., 2011). Although clathrin is known to stabilize the interaction between adaptors and membrane lipids, there is no evidence for direct interactions of clathrin and lipids (McMahon and Boucrot, 2011). Nevertheless, both clathrin heavy chains encoded by the *Arabidopsis* genome were found in the PA-associated protein fractions (Table I). Although a direct interaction cannot be excluded, it is possible that the clathrin heavy chain was co-extracted in complex with the accessory (ANTH) proteins. Whether direct or not, in both cases the re-localization of CHC to the membrane in response to salt would be dependent on the production of PA.

In mammalian systems, PA and PA-generating enzymes such as PLD and DGK have been implicated in various aspects of vesicle transport (Manifava et al., 2001; Corda et al., 2002; Jang et al., 2012). PA is suggested to interact with a wide array of trafficking-related proteins, including Arf-GAP, coat protein 1 (COPI), N-ethylmaleimide sensitive factor (NSF), kinesin, endophilin and PI4P 5-kinase. 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 (Antonescu et al., 2010). 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 (Kooijman et al., 2003; Roth, 2008; Shin and Loewen, 2011). 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 (Dhonukshe et al., 2007) (Robert et al., 2010) and in environmental stress-induced
vesicle trafficking. Interestingly, the endocytosis and cycling of KAT1 potassium channels (Sutter et al., 2007) and several aquaporins (Luu et al., 2011), 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 plasma membrane 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 alpha subunits of inward-rectifying voltage gated-potassium channels. The *Arabidopsis* genome encodes a single homologue of the animal potassium channel β subunit, viz. KAB1 (At1g04690 (Tang et al., 1995)), which, as a tetramer, associates with the transmembrane α subunits of KAT1 channels (Tang et al., 1996). KAB1 is present in both membrane and soluble fractions (Tang et al., 1996). While *AtKAT1* expression is prevalent in leaves rather than roots, *AtKAB1* was immunolocalized in leaves, flowers, and distinctively in roots. Therefore, KAB1 is speculated to bind not exclusively to KAT1 but also to a different α subunit, viz. AKT1, which is selectively expressed in *Arabidopsis* roots (Tang et al., 1996).

KAB1 contains conserved amino acid motifs that, in animal Kvβ homologues confer pyridine nucleotide-dependent oxidoreductase activity. Whilst the functional relevance of this activity is unclear, the binding of NADP+/NADPH as co-factor in these proteins seems to be critical to the folding of Kvβ subunits and the intracellular trafficking of Kv1 channels (Campomanes et al., 2002).

Interestingly, in mammalian axons, the Kvβ2 subunit functions as an adaptor, linking Kv1-containing vesicles to motor proteins on microtubules (Gu and Gu, 2010) and targeting potassium channels to specific sites along the axonal
membrane. The interaction of Kvβ with such trafficking proteins is regulated by phosphorylation (Vacher et al., 2011). Mammalian Kvβ proteins contain 13 phosphorylation sites, three of which are conserved in Arabidopsis KAB1, which contains eight in total (Tang et al., 1995) suggestive of a similar regulatory mechanism.

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 K+ channels in stomatal guard cells. Interestingly, inactivation is faster than internalization, suggesting the involvement of another regulatory mechanism (Sutter et al., 2007). The consequences of ABA and salt stress on root K+ 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 (Aleman et al., 2011; Dreyer and Uozumi, 2011). Whether KAB1 plays a role in the modulation of AKT1 localization and/or activity in the salt stress response remains to be investigated.

**PI 4-kinase γ**

The two identified PI 4-kinases (At1g64460/At2g46500; Table I), which could not be discerned based on MS-generated amino acid sequences, belong to a family of eight type II PI 4-kinases γ in Arabidopsis (Galvao et al., 2008). In mammalian cells, type II PI 4-kinase has been implicated in clathrin-mediated transport from the Golgi (Wang et al., 2003), and together with a PIP kinase, in the generation of PIP2 required for clathrin coat assembly on lysosomes (Arneson et al., 1999). Similarly, in Arabidopsis, salt stress-induced PIP2 has been proposed to be associated with CME (Konig et al., 2008). However, whether Arabidopsis PI 4-kinases γ play a similar role is unknown, especially because two Arabidopsis PI 4-kinases, the isoforms γ4 and γ7 have been shown to display in vitro protein kinase rather than lipid kinase activity (Galvao et al., 2008).
Other potential novel PA targets

The finding of the cytosolic GAPDH proteins (At1g13440/At3g04120) as candidate PA targets in salt stress (Table I, Fig. 4) is in line with previous proteomics studies suggesting the salt-induced tonoplast association of glycolytic enzymes (Barkla et al., 2009), although the latter studies applied salt stress (75 mM) for at least 4 days, which is different from the fast recruitment (7 min) of GAPDH in our study. By binding to specific membranes/organelles, glycolytic complexes may concentrate in regions of high demand for ATP, fuelling ion transporters or proton pumps (Dhar-Chowdhury et al., 2007; Graham et al., 2007). 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 (Barkla et al., 2009). In addition, GAPDH has also been shown to bind PLDδ and is needed for full activation of PLDδ in response to ROS (Guo et al., 2012) and might therefore also function in the targeting of PLDδ 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 (Alvarez et al., 2011; Sengupta et al., 2011).

In conclusion, we set up and successfully carried out a PMP proteomics approach to isolate PA-binding proteins recruited to the PM in response to salt treatment of root tissue. The approach presented here 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, our results reveal putative novel targets of PA and suggest a function of PA in salt-induced regulation of CME which would likely influence a large array of cellular processes.
Chapter 4

Acknowledgements

We kindly thank M.C. Shih for providing the GAPDH antibody and Chris de Koster for making the mass spectrometres available. This work was supported by the Netherlands Organisation for Scientific Research (NWO), grants Vidi 700.56.429, ALW 820.02.017 and NGI Horizon project 93511011.

Material and Methods

Growth conditions

Arabidopsis plants were grown hydroponically (Araponics, Liège, Belgium) for four weeks under short day conditions (130 \( \mu \text{mol/m}^2/\text{s} \), light/dark: 10/14 h, 21°C/70% humidity) with a weekly change of growth medium using the Flora series (GHE, Fleurance, France). 24 hours prior to stimulation, plants were transferred to fresh medium. For each MS sample 160 plants were used.

Isolation of peripheral membrane proteins

Hydroponically grown Arabidopsis roots were treated by transfer to salt-containing medium or control medium and thereafter were drained of excess water, harvested and snap-frozen in liquid nitrogen. A simplified version of the fractionation has originally been described in (Monreal et al., 2010). The root tissue was ground in liquid nitrogen and proteins were isolated by incubating the tissue in one volume protein extraction buffer (50 mM Tris pH 7.5, 300 mM sucrose, 5 mM EDTA, 5 mM EGTA, 2mM DTT, 1x Complete protease inhibitors (Boehringer Ingelheim, Alkmaar, the Netherlands)) for 10 minutes on ice. Samples were filtered through Miracloth (EMD Millipore, MA, USA) and centrifuged twice at 1,500 x g for 5 min (debris) and subsequently up to six times at 10,000 x g to remove debris and intact organelles. Membranes were isolated by spinning for 2 hours at 50,000 x g (50,000 x g crude pellet). The membranes were washed by
homogenizing the pellet using protein extraction buffer with 0.1% Brij-58 (Sigma-Aldrich, Zwijndrecht, the Netherlands) (Johansson et al., 1995). Membranes were again spun down at 50,000 x g for one hour. 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 Na₂CO₃ by homogenizing the samples and keeping them on ice for 10 minutes. An overview of all the centrifugation steps and the origin of all fractions described are given in fig. S1. The antibodies raised against specific protein markers were obtained from Agrisera (Vännäs, Sweden): PM ATPase (At2g18960), MitM COXII (Atmg00160), PMP V-ATPase (At4g11150), Cyt. UGPase (raised against barley), CHC (At3g11130). The GAPDH antibody has been described in (Wang et al., 1997). Silver staining of the different fractions was performed as a loading control.

**PA-binding assay**

The PA-binding assay was conducted as described in chapter 6. As starting material 5μg protein from the PMP fraction was used (approximately 500 μl) per sample. The soluble fraction was transferred to a clean 2 ml eppendorf and was supplemented with lysis buffer to a total volume of 1,9 ml. 50 μl of PA beads (net volume) was 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 Tween-20. Synthesis of the phosphatidic acid beads has been described (Lim et al., 2002).

**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 minutes at 60°C before carbamidomethylation by 15 mM iodoacetamide for 30 minutes at room temperature 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 done at 37°C under continuous mixing. The supernatant containing tryptic peptides was collected after centrifugation at 10,000 x g for 5 minutes 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-MS by the HILIC cleaning method.

iTRAQ labeling (Applied Biosystems)
The collected supernatants of the samples were dried for 1 hour in a centrifugal vacuum concentrator at 35 °C. The dried samples were reconstituted with in dissolution buffer from the iTRAQ kit according to the manufacturer’s instructions (Applied Biosystems, Carlsbad, CA). The control samples were labeled 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 three hours 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 to a final concentration of 95% ACN. For the peptide enrichment TT2HIL TopTips (Glygen, Columbia, MD) were used in combination with the centrifuge adaptor, a micro-centrifuge and a 2 ml eppendorf vial. The samples were spun down for one minute at 1,500 x g. Before using the TopTips, they were washed three times with 0.1% TFA followed by three times with 95% ACN. The samples were loaded with consecutive 100 µl portions on the HILIC TopTip followed by a three times wash step with 95% ACN. The peptides were collected in 30 µl 0.1% TFA and stored at -20°C.
Mass spectrometry

Nanoscale LC separation of tryptic peptides was performed with a NanoAcquity system (Waters Corporation). Samples were loaded onto a Symmetry C18 5 μm, 2-cm x 180-μm trap column (Waters Corporation) at a flow rate of 5 μl/min prior to separation on a Bridged Ethyl Hybrid C18 1.7 μm, 25-cm x 75-μm analytical reversed phase column (Waters Corporation) by application of a 90 minute gradient from 1% acetonitrile, 0.1% formic acid to 40% acetonitrile, 0.1% formic acid at a column flow rate of 0.250 μl/min. Analysis of eluted tryptic peptides was performed using a Synapt G2 quadrupole time of flight mass spectrometer (Waters Corporation, Manchester, UK) equipped with a nanolockspray source (Waters Corporation) fitted with a pico-tip emitter (New Objective, Woburn, MA) operated around 3 kV capillary voltage, The collision gas used was argon, maintained at a constant pressure of 2.0x10^{-3} mbar in the collision cell. The lock mass, [Glu^1]-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 nanolockspray source. The data were post-acquisition lock-mass corrected using the monoisotopic mass of the doubly charged precursor of [Glu^1]-Fibrinopeptide B, delivered through the reference sprayer, which was sampled every 120 seconds. For Label-free experiments accurate mass precursor and fragment ion LC-MS data were collected in data independent LCMS^n mode of acquisition (Geromanos et al., 2009). For iTRAQ labelled samples LC-MS data was collected in tandem-MS data dependent mode of acquisition.

In addition, iTRAQ labelled samples were separated on a reversed phase capillary column (150 mm x 75-μm PepMap C18; LC Packings, Amsterdam, The Netherlands). Sample introduction and mobile phase delivery at 300 nl/min. were performed using an Ultimate nano-LC-system (Dionex, Sunnyvale, CA). For separation of peptides, a gradient from 0% acetonitrile, 0.1 % formic acid to 50% acetonitrile, 0.1 % formic acid over 60 minutes was used. Eluted peptides were electrosprayed into a Q-TOF quadrupole time of flight mass spectrometer (Waters,
Manchester, UK). 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, Manchester, UK). Fragmentation was conducted with argon as collision gas at a pressure of 4 x 10^{-5} bars measured on the quadrupole pressure gauge.

**Data analysis**

iTRAQ labelled samples: Raw data files of data dependent acquisitions 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 an in-house licensed version of Mascot. MASCOT search parameters were as follows: peptide and fragment error tolerances of 0.3 Da, ions-score cut-off 12, digest reagent: trypsin, allow 1 ‘missed cleavage’, fixed modifications: cysteine carbamidomethylation 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 quantitation iTRAQ 4plex activated for ratio include of 114/117.

Continuum LCMS® data were processed and searched using ProteinLynx GlobalSERVER version 2.5 (PLGS 2.5, Waters Corporation). Parameter settings: digest reagent trypsin, allow 1 ‘missed cleavage’, search tolerances automatic, typically 5 ppm for precursor and 15 ppm for product ions, fixed modification cysteine carbamidomethylation, and variable modification methionine oxidation. Protein identifications were obtained by searching an *Arabidopsis thaliana* database (UniProt release 2012_03) to which common protein contaminants were appended. Estimation of false positive identification rates was performed by searching a randomized version of the abovementioned *Arabidopsis thaliana*
protein database generated within PLGS 2.5. Peptide identification data were exported as csv-files and imported into Excel (Microsoft corporation, Redmond CA) with the ASAP-utilities add on installed (Zwolle, the Netherlands).

Peptide identification data were filtered, so only peptides identified in at least two out of three replicate injections of a sample were deemed as reliable identifications. Using this strict criterion no peptides from the decoy database were identified. Subsequently the signal intensities (area under the peak) reported by PGLS 2.5 per injection were normalized by the sum of the signal intensities of all identified peptides in that injection. Following normalization the average of the replicate injections were calculated and the ratio calculated for salt/control for each individual peptide identified. Using a pivot table, the average ratio, standard deviation, no. of peptides and no. of proteotypic peptides per protein were extracted. Proteins which did not have proteotypic peptides for which the peptides identified more than three different homologues were filtered out to create the final protein quantification table from the LCMSE data.

Proteins quantitation obtained by the iTRAQ labelled samples were combined with the label free LCMSE data, and only proteins quantified in at least two out of three samples are reported in figure 4, Table 1 and S1.

References


Chapter 4


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**Isolation of PA targets involved in salt stress**


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Li M, Hong Y, Wang X (2009) Phospholipase D- and phosphatidic acid-mediated signaling in plants. Biochimica et biophysica acta 1791: 927-935


Chapter 4


Supporting information

**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. Fractions analyzed by immunoblots or silver stain (Fig. 2,3,5) are boxed.
## Table S1. Identified PA-binding peripheral membrane proteins

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Chapter 5

General discussion

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Chapter 5

Biological function of SnRK2.4 and 2.10 in salt stress responses: interacting proteins and phosphorylation targets

In recent years, significant progress has been made in characterization of the molecular interactions and functions of SnRK2 protein kinases. Most of the progress has been made in the ABA-dependent (class 2 and 3) members, while the function of class 1 SnRK2 kinases remains largely unknown, although some (potential) cellular interactors have been identified (Fig. 1). Kinase activity of class 3 SnRK2s is directly inhibited by several PP2Cs and activation occurs by de-repression of these phosphatases (Yoshida et al., 2006; Umezawa et al., 2009; Vlad et al., 2009; Soon et al., 2012). Although the phosphorylation mechanisms of class 1 and class 3 SnRK2s are different (Burza et al., 2006; Vlad et al., 2010) class 1 SnRK2s could be activated through a similar mechanism. The SnRK2.4 orthologue in tobacco, NtOSAK (*Nicotiana tabacum* osmotic stress-activated protein kinase), was shown to interact with the SnRK2-interacting Calcium sensor (SCS). This sensor was shown to interact with SnRK2 members from all classes (Bucholc et al., 2011) and might mediate their inactivation that is observed after 10 minutes of salt stress (Chapter 2, Fig. 1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also identified as a cellular partner of NtOSAK. Nitric oxide (NO) treatment also induced activation of NtOSAK, but no S-nitrosylation was observed in this protein. This post-translational modification was observed for GAPDH, which suggests it might play a role in regulating the activity of NtOSAK in response to NO (Wawer et al., 2010). Nitric oxide (NO) treatment also induced activation of NtOSAK, but no S-nitrosylation was observed in this protein.

So far, no *in vivo* phosphorylation targets have been identified for the class 1 SnRK2 members yet, but SnRK2.10 was shown to exhibit a preference for a phosphorylation motif that is conserved in dehydrins (Vlad et al., 2008). Dehydrins are important for cold, salt and drought stress and function in protecting macromolecules besides several other potential protective roles (Allagulova Ch et al., 2003). Binding of dehydrins to the membrane is dependent on their
phosphorylation status (Erikkson et al., 2011) and they might therefore be targeted to the membrane in response to osmotic stress in a class 1 SnRK2 dependent manner, to function in membrane protection.

**Figure 1. SnRK2.4 is transiently activated by osmotic stress.** This model is based on data obtained on the class 1 SnRK2 members in different plant species. Abbreviations: PP: Protein Phosphatase, SCS: SnRK2-interacting calcium sensor, PA: Phosphatidic acid, NO: nitric oxide, SNO: S-Nitrosylated, GAPDH, glyceraldehyde-3-phosphate. Osmotic stress induces the production of PA and NO and the release of Ca^{2+}. These signals regulate the activity and the localization of SnRK2.4.

**Activation and subcellular localization of SnRK2.4**
SnRK2.4 is rapidly and transiently activated in saline conditions and is targeted to punctate structures in epidermal and cortex cells in Arabidopsis roots (Chapter 2). Using observations obtained in this study, combined with recent findings of other groups, a model was constructed describing the events happening within minutes after the application of salt stress (Fig. 2). In control conditions, SnRK2.4 is repressed, presumably by a protein phosphatase (PP), and interacts with its cellular partner GAPDH. After 1 minute of salt stress, the inhibition of the PP activates SnRK2.4. Meanwhile, PA is formed at the membrane to which SnRK2.4 is targeted. GAPDH was also shown to be targeted to the membrane in response to salt in a PA dependent manner (Chapter 4) suggesting these proteins might associate with the membrane as a complex. Calcium signaling events usually
within seconds after sensing salt stress (Kader and Lindberg, 2010) so after 1 minute SCS is likely to be already activated (Bucholc et al., 2011) but SnRK2.4 is still active at this point in time (Chapter 2, Fig. 1).

PA is likely to simply act as a docking site for SnRK2.4 and does not influence the activity since adding exogenous PA in vitro did not affect its activity (data not shown). Dehydrins bind different lipids including PA (Koag et al., 2003; Koag et al., 2009) depending on their phosphorylation status (Eriksson et al., 2011). When active SnRK2.4 would indeed phosphorylate dehydrins, it might increase lipid-binding affinity of the dehydrin to the membrane. Recruitment of SnRK2.4 to the membrane might be necessary to interact with its phosphorylation targets present on the membrane or to phosphorylate proteins that have a function near or on the membrane. After 5 minutes, SnRK2.4 activity is largely repressed, but it is unclear if this inactivation is due to an interaction with SCS or a protein phosphatase. S-nitrosylation of GAPDH in a NO dependent manner occurs after 5 minutes of NO treatment and coincides with the inactivation of SnRK2.4 (Wawer et al., 2010). A local increase in PA can induce membrane curvature and the negative charge facilitates vesicle formation (Kooijman et al., 2003), which might be important for the transport of SnRK2.4.

After 15 minutes of salt stress, SnRK2.4 (and presumably its cellular partners) are transported to punctate structures. SnRK2.4 present in punctate structures is largely dissociated from the membrane, indicating a reduced amount of PA in the membrane of these punctate structures or reduced PA-binding affinity of SnRK2.4 at this stage. Dehydrins are likely to stay bound to the membrane as long as they are phosphorylated since they also have binding affinity for other lipids like phosphatidylcholine, phosphatidylglycerol and phosphatidylserine. Since SnRK2.4 remains inactive, the interaction with the PP and/or SCS presumably remains. A similar regulatory mechanism can be predicted for SnRK2.10. However, no accumulation in punctate structures was observed for this protein, which is probably due to the cell-types SnRK2.10 is expressed in, because
no re-localization of SnRK2.4 was observed in these cell-types either (data not shown).

Figure 2. SnRK2.4 relocates to punctate structures in response to salt. This model describes the localization and activation events of SnRK2.4 and its potential downstream target, dehydrins (and other unknown targets). The curvature in the membrane after 5 minutes of salt stress represents PA-dependent membrane curvature. Abbreviations: PP: Protein Phosphatase, SCS: SnRK2-interacting calcium sensor, PA: Phosphatidic acid, NO: nitric oxide, SNO: S-Nitrosylated, GAPDH, glyceraldehyde-3-phosphate.

SnRK2.4-YFP expression was high and accumulated in punctate structures in cortex cells adjacent to an emerging LRP (Chapter 2, Fig. 5). This observation strengthens the idea that the localization in punctate structures of SnRK2.4-YFP was caused by mechanical stress. This could also be the cause of the re-localization of SnRK2.4 in epidermal cells in response to salt since salt stress induces radial
swelling of the outer tissue layers of roots (Dinneny et al., 2008). Since SnRK2.4/2.10 is targeted to the membrane in response to salt, and possibly also during mechanical strain, it might have a function in modulating the membrane in order to cope with the mechanical strain.

Although the identity of the punctate structures is unknown, they are likely to be Golgi bodies, based on their appearance. Another PA-binding protein kinase, MPK6 (Droillard et al., 2002; Yu et al., 2010), which is involved in salt stress signaling and root development, has been described to accumulate in Golgi bodies (Muller et al., 2010). Co-localization studies of SnRK2.4-YFP with different Golgi markers could establish the identity of the punctate structures.

A possible role of SnRK2.4 and 2.10 in auxin signaling

The question remains how SnRK2.4 and 2.10 contribute to the maintenance of the root system architecture in saline conditions. Snrk2.10-KO and to lesser extent snrk2.4-KO mutants displayed a reduced lateral root density in saline conditions. Lateral root development is regulated by different hormonal signaling pathways. Two plant hormones; auxin and cytokinin, act antagonistically in the regulation of lateral root initiation and development (Laplaze et al., 2007). Cytokinin alters the distribution of LRP s and inhibits root initiation by acting on lateral root founder cells (Laplaze et al., 2007), which is not the phenotype observed in snrk2.10 or the double mutant, which are arrested much later in their LRP development (Chapter 2, Fig. S3). The high expression of SnRK2.4-YFP in the pericycle indicates a possible relation with auxin since lateral root patterning occurs in an auxin dependent manner (Casimiro et al., 2001), but the reduction in lateral root density in saline conditions was primarily explained by a reduction in emergence. Lateral root emergence is also regulated by auxin, but auxin required for the emergence is derived from the shoot so accumulation of SnRK2.4 in the pericycle in roots would not explain a possible relation (Bhalerao et al., 2002). Remarkably, SnRK2.10 expression was found to be down-regulated in the
presence of the synthetic auxin 2,4D (Raghavan et al., 2006). Although no clear model can be constructed at this moment based on these data, a link between auxin and SnRK2.4/2.10 signaling deserves further investigation. Studying the distribution of auxin in the snrk2.4-KO and snrk2.10-KO and determining what the effect of auxin is on the root system architecture in these mutants in saline conditions could establish a possible relation between SnRK2 function and auxin signaling.

The challenges of studying lipid signaling

Significant progress has been made over the last decades in studying PA signaling in plants (Chapter 1,3,4). Using different approaches, several phospholipases and lipid targets have been identified involved in lipid signaling. The most widely applied approaches to study lipid signaling are investigation of phospholipase mutants, assessing the effect of exogenous PA and the identification of PA-binding proteins. Although all these approaches contributed to the understanding of the role of PA in the response to different external stimuli, some of these strategies potentially introduce false positive results. Studying phospholipase mutants certainly gives a good indication where these phospholipases are involved in, but since PA is also an important intermediate lipid it is hard to distinguish between the role of PA in lipid turnover and its role in signaling or protein recruitment (Testerink and Munnik, 2011). Adding exogenous PA disrupts membrane integrity, potentially leading to technical artifacts. Identification of PA targets through PA-binding affinity has proven to be a valuable tool to study the function of PA, but since there are a lot of proteins that exhibit PA-binding affinity in vitro, it is hard to determine which proteins are biologically relevant. Mutating residues responsible for PA-binding in PA binding proteins and studying their function in vivo is the most elegant approach to study the role of PA. However, mutations might also change the properties of the protein and therefore reduce or abolish the functionality of the protein independently of PA. To thoroughly investigate the role
of PA in any biological process, preferably evidence from multiple approaches should be obtained. This is a very difficult and time consuming process and will remain a challenge in the years to come.

**Biochemical fractionation to identify peripheral membrane proteins.**

In this thesis an approach is described to isolate peripheral membrane proteins. In addition to its use in identifying new PA targets involved in salt stress, it has also proven to be a valuable tool to study re-localization events of, in this case, SnRK2.4/2.10 in response to salt (Chapter 2, Fig. 7) and to confirm membrane docking (Chapter 3, Fig. 2). Even though SnRK2.4/2.10 was detected in the peripheral membrane protein pool, it was not identified in the mass spectrometry data following the same protocol (Chapter 4). Apparently the SnRK2.4/2.10 concentration was not high enough or the PA-binding affinity of SnRK2.4/2.10 was too low to be identified in this screen. In addition, the peripheral membrane marker used is specifically enriched in the PMP fraction, but while this protein is attached to the membrane through a protein-protein interaction, SnRK2.4/2.10 membrane binding is supposedly dependent on an interaction with PA. Dissociation from the membrane of SnRK2.4/2.10 during the Brij-58 wash could occur, because of the weaker electrostatic interaction with the membrane. Unfortunately, no standard can be suggested in this respect since protein-protein and protein-lipid interactions amongst themselves also differ. Although it would also be very interesting to identify PA targets that have a lower PA-binding affinity, it would entail reducing the severity of washing of the microsomal fraction. This would ultimately result in an increased amount of cytosolic contaminants, which increases the amount of false positives.
iTRAQ versus label free quantification

The label-free approach resulted in many additional peptides in comparison to the iTRAQ labeled approach. This resulted in an increase in both the amount of proteins identified and the amount of peptides identified per protein, resulting in more reliable protein quantifications. The reduction in peptide identification using iTRAQ is likely to be due to the absorption of a large amount of the collision energy by the iTRAQ labels. The proteins that were most prominently up-regulated in the salt-treated sample using either approach were reproduced. Still, some potentially interesting proteins were identified that were targeted to the membrane in response to salt and bind PA, but were only detected in one replicate. HSP-70 (At5g02500) was 1.8 times more abundant in the salt stimulated sample (data not shown) and modulates ABA-dependent physiological responses (Clement et al., 2011) and binds to dissociated clathrin heavy chain to prevent aggregation in the cytosol (Eisenberg and Greene, 2007). This protein is likely to be isolated in complex with the clathrin heavy chain, indicating that the additional clathrin heavy chain identified in the salt stimulated sample is recently recruited from the cytosol. Another interesting protein that was recruited to the membrane and bound to PA is Annexin 4 (At2g38750) (2.5 times more abundant in the salt stressed sample, data not shown). Annexins have previously been described to bind acidic phospholipids in a Calcium dependent manner (Gerke and Moss, 1997) and to be involved in ABA and osmotic stress signaling and are targeted to the membrane in response to salt (Lee et al., 2004). This is in line with a re-localization to the membrane in a phospholipid dependent fashion in response to salt. Finally, several protein kinases have been described to be modulated by phosphatidic acid and one kinase was identified that was close to twice as abundant in the salt stimulated sample; Calcium-dependent Protein Kinase 21 (CPK21, data not shown). CPK21 is activated in response to different abiotic stress stimuli and targets the slow anion channel SLAC1 (Geiger et al., 2010), which is also targeted by SnRK2.6 (Geiger et al., 2009; Lee et al., 2009).
Chapter 5

The timing and salt concentration were chosen arbitrarily and it would be interesting to determine which proteins are associating and dissociating from the membrane through time. PA also accumulates in response to drought, ABA, freezing and wounding stress and determining which proteins are targeted to the membrane in a PA-dependent manner in response to these stress stimuli could lead to new insights how PA can mediate diverse signaling cascades. In addition, this approach could be applied for studying lipid targets of other signaling lipids, like PPIs. Altogether, this tool offers the possibility to identify biologically relevant phospholipid targets specifically involved in certain responses.

In conclusion, significant process has been made in this study in understanding the role of PA in the response to salt in roots. In addition to revealing the role of SnRK2.4 and 2.10, newly found PA targets implicate involvement in additional cellular processes. Further studies will have to pinpoint how SnRK2.4 and 2.10 ultimately regulate the maintenance of the root system architecture by further unraveling their signaling cascades. In-depth characterization of the new PA-binding proteins involved in salt stress could establish the effect of PA in determining the localization and/or activity of these proteins.

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General discussion


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Chapter 6

Lipid affinity beads – From identifying new lipid binding proteins to assessing their binding properties

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Chapter 6

Abstract

Lipid affinity beads can be used to identify novel proteins with lipid binding capacity or to determine binding prerequisites of known lipid-binding proteins. Here we describe several applications for which this tool can be used and which considerations have to be taken into account. In addition to a precise protocol, several suggestions are made for experimental set-ups to facilitate identification of in vivo lipid binding targets.

Introduction

Cellular membranes primarily consist of phospholipids. Only few of these, including phosphatidic acid (PA) and polyphosphoinositols (PPIs), have a function as signaling phospholipids (Munnik and Testerink, 2009). Several proteins implicated in signaling cascades upon abiotic and biotic stress, have been described to bind PA in plants. In addition, PA also binds proteins that function in growth and general metabolism (as reviewed in (Testerink and Munnik, 2011)). Different lipid affinity beads can be used to isolate proteins with affinity for various signaling lipids. The protocol described here has been optimized for PA.

PA beads can be used to isolate and identify proteins from complex cellular extracts. Strategies to identify less abundant PA-binding proteins generally include a pre-purification step based on protein characteristics (e.g. size, charge) (Manifava et al., 2001; Krugmann et al., 2002; Testerink et al., 2004) to avoid saturation of the PA beads by very abundant PA binding proteins. Alternatively, pre-fractionation based on cellular localization (i.e. presence on the membrane, in organelles) can be performed (Fig. 2).

Not all the proteins that bind to PA beads will be bona fide in vivo PA targets since PA present on the beads is not in its natural form. To exclude false positives or to get a better idea which proteins are genuine PA targets, several
Lipid affinity beads

approaches are possible. One option is to determine if the protein of interest is competed off the beads by a more naturally occurring form of PA, through the addition of liposomes containing PA or soluble PA to the PA-binding assay. Tomato (Solanum lycopersicum) phosphoenolpyruvate carboxylase (PEPC) was identified in this way, as it was competed off the beads by the presence of soluble PA (Fig. 1a; (Testerink et al., 2004)). In addition, it was shown that PEPC has a higher PA binding affinity after a hypo-osmotic stress treatment, which is another indication that PEPC is an in vivo target of PA (Fig. 1b; (Testerink et al., 2004)). Later, it was shown that Sorghum C₄ PEPC activity was inhibited in the presence of anionic lipids and that the PEPC fraction targeted to the membrane of sorghum (Sorghum bicolor) leaf cells is largely modified (Monreal et al., 2010).

\[ \text{Figure 1. PA-binding characteristics of tomato PEPC.} \quad \text{modified from} \quad \text{Testerink et al., 2004 with permission.} \quad \text{a. PEPC is competed off the beads in the presence of soluble PA.} \quad \text{b. PEPC PA-binding affinity increases after hypo-osmotic stress treatment.} \]

Depending on the nature of the protein of interest, the nucleotide binding or phosphorylation state or several other posttranslational variations can be varied in vitro in order to determine if this has an effect on the PA binding affinity. It has been shown that the nucleotide binding state of NSF, a protein involved in intracellular trafficking in sheep brain, is important for PA binding. The binding to PA occurs only in its ADP-bound state (Manifava et al., 2001). Additionally, the PA binding specificity was determined using beads coupled to another anionic lipid, phosphatidyl inositol 4,5-bisphosphate (PI(4,5)P₂) as a negative control. The
specificity of a protein binding to a certain anionic lipid and not to others indicates specific binding capacities. Although beads can be used for this application, a liposome-binding assay is a more suitable technique to determine this, since it is easier to vary between different lipid compositions (as described in [Julkowska et al., 2012]).

Another way to determine if a protein is targeted to PA is to determine if proteins are relocated in vivo upon a PA inducing stimulus. An Arabidopsis thaliana cell suspension was exposed to control and PA-inducing conditions (250 mM NaCl, 10 min.). Subsequently the peripheral membrane protein pool was isolated from the control (C) and the salt induced (T) sample and a PA binding assay was conducted on these samples (Fig. 2). Proteins that were altered in their location upon a PA inducing stimulus, in particular proteins that are enriched on the membrane upon salt treatment, are very likely to be targeted to PA in the membrane in vivo.

Figure 2. Several proteins with PA binding affinity are either enriched or decreased in the peripheral membrane protein pool after salt stress treatment. A. thaliana cell culture was either control or salt (250 mM NaCl for 10 minutes) treated. The peripheral membrane protein pool (PMP) was isolated by isolating and washing cellular membranes and subsequently eluting the peripheral bound proteins with 100 mM Na2CO3. These protein pools were used as input for the PA binding assay. Solid arrows indicate proteins that are enriched in the PA-binding peripheral membrane protein pool when salt stimulated and the dashed arrows indicate proteins that are decreased in this sample compared to the control. This approach allows identification of proteins that are re-localized upon a PA-inducing stimulus. The proteins were visualized using silver staining.
2. Materials

2.1 Sepharose beads coupled to different kind of lipids
Synthesis of phosphatidic acid and phosphatidyl inositol beads have been described in (Lim et al., 2002). Beads can be stored at 4°C in distilled water containing 0.02% sodium azide.

2.2 Competition assay
In case of a competition assay, specific requirements and additional steps are performed. Essential steps, solutions and equipment are marked with #. Additional steps will be described in the notes.

2.3 Stock solutions for PA-binding assay
The Tris-HCl, KCl, EDTA, NaCl and azide stock solutions can be made beforehand and stored at room temperature.

1. 1 M Tris-HCl pH 6.8 and pH 7.5 and 8.0: Dissolve 121.14 g Tris in 500 ml of distilled water. Adjust the pH to the desired pH with concentrated HCl. Bring the total volume to 1L with distilled water. Store at room temperature.

2. 1 M KCl: Dissolve 74.55 g potassium chloride in 1L of distilled water. Store at room temperature.

3. 0.5 M EDTA: Dissolve 73 g ethylenediaminetetraacetic acid in 250 ml distilled water. Adjust the pH to 8.0 with concentrated NaOH otherwise EDTA will not dissolve. Store at room temperature.

4. 5 M NaCl: Dissolve 146.1 g NaCl in 500 ml of distilled water. Store at room temperature.

5. 1 M DTT: Dissolve 1.54 g dithiothreitol in 10 ml distilled water. Store in 200 µl aliquots at -20°C.
6. Phosphatidic acid (di C18:1) dissolved in chloroform according to the instructions (Avanti). Alternatively dissolve C8:0 lipids to a 1M concentration in 1 M Tris-HCl pH 7.5.

7. 2% Sodium azide: Dissolve 200 mg of sodium azide in 10 ml of distilled water.

2.4 Working solutions for PA binding assay

Lysis buffer (without the complete protease inhibitors) and IPP buffer can be stored at 4°C up to three months. Sample buffer (4x) and B-buffer can be prepared beforehand and stored at -20°C.

1. Lysis buffer 2x: 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 10 mM EDTA, and autoclave. Add 1% IGEPAL CA-630 (Sigma) after autoclaving. Add complete protease inhibitors (Boehringer) freshly.

2. IPP buffer 1x: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and autoclave. Add 0.1% Tween 20 after autoclaving.

3. Sample Buffer 4X: Dissolve 0.8 g of SDS in 4.6ml 87% glycerol, 2ml β-mercaptoethanol, 2.4 ml 1M Tris-HCl pH 6.8 and 1ml distilled water. Add 8 mg Brome Phenol Blue. Store in 1ml aliquots.

4. B-buffer 1x: 50mM Tris-HCl, pH 7.5, 2% SDS, 100 mM DTT

2.5 Materials for PA binding assay:

1. 0.5 and 1.5ml (preferably Safelock) Eppendorf tubes
2. Cooled table top Eppendorf centrifuge
3. Rotator at 4°C
4. 95°C heat block
5. Probe sonicator (see Note 3)
3. Methods

It is essential to keep the samples between 0-4°C throughout the whole procedure to prevent protein degradation.

1. Spin 225 µl soluble protein (ca. 1 to 2 mg/ml) in a tabletop centrifuge at 16,100 g for 10 min (see Note 1).
2. Add the supernatant (soluble fraction) to 225 µl 2x lysis buffer. (Lysis buffer is diluted 1:1 in protein isolation buffer). (see Note 2 and 3).
3. Spin at 16,100 g for 4 min to remove any insoluble matter, transfer the supernatant to a new tube and keep on ice (see note 4).
4. Spin an appropriate amount of PA beads (see notes 5-7), stored in water plus 0.02% sodium azide, at 400 g for 2 min. (see note 8).
5. Wash the beads twice with IPP buffer (by spinning at 400g for 2 min and replacing the IPP buffer) and make a 10% suspension in this buffer.
6. Pipet 60 µl 10% beads (=6 µl drained volume) in each 0.5ml tube (see note 9).
7. Add the soluble fraction of your protein sample to each tube containing the beads.
8. Rotate the samples at 4°C for 1.5 to 2 hours. (see note 10).
9. Spin at 400g for 2 min.
10. Wash the beads 4 times in 500 µl IPP (by spinning at 400g for 2 min and replacing the IPP buffer) and keep samples on ice during the process. (see note 11).
11. Elute the bound protein by adding 30 µl 2x sample buffer and incubate for 10 min at 4°C (see note 12).
12. Transfer sup to new tube and boil for 3 min. (see note 13).
13. Store samples at -20°C.
To re-use the beads:

1. Collect the used beads in an Eppendorf tube.
2. Add B-buffer to make a 25% bead suspension and leave for 10 min at room temperature (see note 14).
3. Wash the beads 6 times with IPP buffer (spin at 400g for 2 min) and store at 4 °C as a 10% suspension in IPP.
4. Add sodium azide to a final concentration of 0.02%.

4. Notes

1. For some applications (for example if the goal is to identify proteins using mass spectrometry) the volume of the protein sample could be bigger than 225 μl. In this case, transfer the sample to a 1.5 ml tube (or an even bigger volume) and keep the ratio of the lysis buffer and protein sample 1:1. Increasing the amount of beads accordingly to the increase in volume is advisable.
2. The pH of the mixture should be between 7.5 and 8.0. This is usually the case but if the pH of the protein extract is much higher or lower than the desired pH it is advisable to check the pH of your mixture by pipetting a small quantity on pH paper.
3. Dry down PA from the chloroform stock (e.g. di C18:1). Add lysis buffer and sonicate for 5 min or alternatively dilute soluble PA dissolved in 1M Tris-HCl pH 7.5 in lysis buffer (end concentration 100μM). Use either solution instead of regular lysis buffer at this step to add to the protein. Incubate for 20 minutes on ice before transferring the sample to the beads.
4. In some cases white precipitation occurs in later steps. The precipitation can be removed by using a tip with a very small diameter (for example Gilson p2 tips or tips for loading polyacrylamide gels).
5. Optimizing the ratio between the amount of protein and the amount of beads is best done in a trial experiment. The ratios given in the protocol are optimized for the PA beads we currently have. This can be used as a starting ratio for your experiment but it is advisable to test your protein sample with different amount of beads. This way the ratio at which the PA beads are the limiting factor can be determined.

6. Different protein samples require different bead-to-protein ratios. When using recombinant protein expressed in *E. coli* or purified protein, less beads are needed in comparison to a complex protein mix. In our experience, the ratio between beads and proteins can be reduced at least 4 times compared to the ratio described in the general protocol.

7. Calculate the total amount of drained beads you need and pipet double the volume out of the stock. This surplus is primarily needed to equalize the bead concentration between the first and the last sample. It is also possible that some beads are lost during the washing steps. The remaining beads can be stored again at 4°C.

8. The tip of standard yellow tips are not broad enough to homogenously pipet the beads. Tips with a broader diameter of the tip are available. Alternatively the last 5mm of the tip can be cut with a clean scalpel to broaden the diameter to facilitate the entering of the beads. This promotes equal distribution of the beads over the different samples.

9. For optimal distribution of the beads over the different samples, make sure the beads are homogenously distributed in the stock every time a sample is taken. This can be done by inversion or tapping the Eppendorf and quickly pipetting afterwards. Make sure to pipet from the mid-section of your stock. After the beads are distributed over the tubes always check if the amount of beads is the same in each tube.

10. Make sure the beads rotate head-over-head instead of rocking to increase the binding efficiency.
11. If the binding assay is performed in a 1.5 ml tube, the beads should be transferred to a 0.5 ml tube at this step. This decreases the chance of losing any beads during the washing steps. Mind that the diameter of the tip is large enough for the beads to pass.

12. For an optimal elution of your protein, gently tap the tubes after 2 and 4 minutes to optimize the exposure to the sample buffer.

13. It is not necessary to spin the samples before transferring the supernatant. A short spin is possible in case there is some sample stuck to the wall of the tube. Make sure you pipet the sample in the bottom of the new tube to avoid unnecessary loss of your sample.

14. Beads can be re-used up to five times. When the beads are reused for a couple of times it is possible that a white precipitate occurs between the beads. This can be removed before you start a new PA-binding assay using a pipet tip with a small diameter.

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Summary/
Samenvatting
Summary

Plants are sessile organisms and are unable to hide or walk away from different harmful external factors, therefore they have developed highly specialized mechanisms to deal with adverse conditions. In order to adequately respond to various environmental factors, it is of utmost importance that plants precisely determine the nature of the stress to maintain growth and survival. This is realized through complex signalling cascades, involving hormones, protein kinases and phosphatases, Ca^{2+}, reactive oxygen species and low abundant phospholipids. Salinity is one of the most severe problems in agriculture worldwide and is progressively becoming worse, affecting crop survival and yield. In this thesis I focussed on the contribution of certain phospholipids and protein kinases in early salt stress signalling events.

Phospholipids are the main component of cellular membranes and are essential to separate the content of the cell from the outside environment. Several low abundant phospholipids rapidly increase in response to salt stress and provide transient and spatial information from the outside environment. One these lipid second messengers is phosphatidic acid (PA). In addition to changing the physical characteristics of the membrane, lipid second messengers can bind proteins. The binding can result in activation, in-activation or relocalization of proteins. Certain phospholipids also provide docking-sites to spatially facilitate protein-protein interaction, effectively promoting signaling cascades. Two protein kinases involved in salt stress signaling, Sucrose non-fermenting-1 related protein kinase (SnRK) 2.4 and 2.10, were shown to have PA binding affinity. Protein kinases can transfer phosphate groups to specific substrates that alter the activity of the target protein.

Chapter 1 gives a general introduction to the areas of research described in this thesis. In chapter 2, we observed that SnRK2.4 and SnRK2.10 are rapidly and transiently activated in Arabidopsis roots after exposure to salt. In saline conditions, snrk2.4 knockout mutants had a reduced primary root length, while
snrk2.10 mutants exhibited a reduction in the number of lateral roots. The phenotypes were in agreement with the observed expression patterns of SnRK2.10 and 2.4. SnRK2.10 was found to be expressed in the vascular tissue at the base of a developing lateral root, whereas SnRK2.4 was expressed throughout the root, with higher expression in the vascular system. Salt stress triggered a rapid re-localization of SnRK2.4 from the cytosol to punctate structures in root epidermal cells. In addition, SnRK2.4/2.10 were shown to be recruited to the membrane after salt stress using cellular fractionation experiments, supporting their observed binding affinity for the phospholipid phosphatidic acid.

To further characterize the role of PA in the functionality of these protein kinases, a two-sided approach was applied in chapter 3. First, The activity of SnRK2.4 and 2.10 was determined in mutants which are disturbed in the production of PA, but no reproducible changes SnRK activation were observed. Second, the PA binding site of SnRK2.4 was determined to disrupt the interaction with PA. A PA-binding domain of 42 amino acids was identified in a subdomain, which is required for osmotic stress responses and this domain was shown to localize in the cytosol and punctate structures in roots treated with salt, supporting a role of PA in the relocalization event of SnRK2.4 in response to salt. The PA-binding affinity of this domain was completely disrupted by changing seven basic amino acid residues, which were selected based on homology study with the non-PA-binding SnRK2.6. This domain could be further developed as a biosensor for in vivo PA production in plants. In addition, the identification of residues crucial for PA-binding is an important step towards elucidation the role of PA in the functionality of SnRK2.4.

In chapter 4 we have combined an approach to isolate peripheral membrane proteins of Arabidopsis roots with lipid-affinity purification, to identify 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 mass spectrometry, eight proteins were found to be enriched in the membrane fraction after seven
minutes of salt stress. Among these were two clathrin heavy chain isoforms and two E/ANTH-domain clathrin assembly proteins, which are important for protein transport. In addition, a protein involved in regulating potassium transport, two ribosomal proteins, glyceraldehyde 3-phosphate dehydrogenase and a type II phosphatidylinositol 4-kinase γ bound PA and were recruited to the membrane upon salt stress. This approach was shown to be an effective way to isolate biologically relevant lipid-binding proteins and provides new leads in the study of PA-mediated responses of roots to salinity stress.

In chapter 5 the results obtained in this thesis are discussed and chapter 6 describes the applications of lipid affinity beads, together with a detailed protocol on how to use them.
Samenvatting

Planten kunnen zich niet verbergen of weglopen van verscheidene schadelijke invloeden van buitenaf en hebben daarom dan ook een groot aantal strategieën om zichzelf te beschermen tegen ongunstige omstandigheden. Om de schade van verschillende omstandigheden zo veel mogelijk te beperken is het van groot belang dat planten snel reageren en misschien nog wel belangrijker, de juiste mechanismen in werking stellen. Dit wordt gerealiseerd door middel van complexe signaaltransductiecascades, waarbij hormonen, eiwit kinases en fosfatafases, calcium, reactieve zuurstof intermediairen en laag abundante fosfolipiden een belangrijke rol spelen. De plant krijgt dus uit verschillende bronnen informatie over wat er in zijn omgeving gebeurd en gebruikt deze om vast te stellen welke mechanismen in werking gesteld moeten worden. Zoutstress is wereldwijd een van de meest ernstige problemen in de landbouw en wordt geleidelijk aan ernstiger door een gebrek aan zoet water en een toenemende verdamping van oppervlakte water. Dit proefschrift spits zich toe op de bijdrage van specifieke fosfolipiden en eiwit kinases die betrokken zijn in de vroege signalering van zout stress.

Fosfolipiden zijn de meest voorkomende componenten in de membranen die de cel scheiden van zijn omgeving. Verschillende fosfolipiden die normaal gesproken weinig voorkomen nemen heel snel toe als de plant wordt blootgesteld aan zout stress en geven tijdelijk en lokaal een signaal aan de cel. Een lipide die voldoet aan deze beschrijving is fosfatidylzuur (PA). Naast het wijzigen van de fysieke kenmerken van het membraan, wordt de toename van PA gebruikt als een sensor door de cel voor wat er zich buiten de cel afspeelt. Specifieke eiwitten binden aan PA en dit kan leiden tot activatie, in-activering of herlokalisatie van eiwitten. Phospholipiden zijn daarnaast ook een ontmoetingsplaats voor eiwitten, waardoor er complexen kunnen ontstaan of signalen kunnen worden doorgegeven aan andere eiwitten die een rol spelen in zout tolerantie. Twee eiwit kinases die
Samenvatting

betrokken zijn bij zoutstress signalering, Sucrose niet-fermenterende-1 gerelateerde eiwit kinase (SnRK) 2,4 en 2,10, hebben PA bindingsaffiniteit. Eiwit kinases kunnen fosfaatgroepen overbrengen aan specifieke substraten die de activiteit van het substraat wijzigen.

**Hoofdstuk 1** geeft een overzicht van wat er bekend is in de literatuur over verschillende nauw gerelateerde onderzoeksvelden en dient als introductie tot het beschreven onderzoek. In **hoofdstuk 2** is beschreven dat SnRK2.4 en SnRK2.10 snel en tijdelijk aangeschakeld worden in Arabidopsis wortels na blootstelling aan zout. In een zoute omgeving hebben planten waarbij snrk2.4 is uitgeschakeld is een verminderde hoofdwortel lengte en snrk2.10 is belangrijk voor het handhaven van de zijwortels. Deze defecten waren in overeenstemming met de waargenomen expressie patronen van SnRK2.10 en 2,4. SnRK2.10 bleek nadrukkelijk aanwezig te zijn in het vatweefsel aan de voet van een ontwikkelende zijwortel, terwijl SnRK2.4 nadrukkelijk in de hoofdwortel aanwezig was. Zoutstress leidde tot een snelle herlokalisatie van SnRK2.4 van het cytosol naar punt structuren. Daarnaast werd ook aangetoond door middel cellulaire fractionering experimenten dat SnRK2.4/2.10 aan de membraan bind, wat in overeenstemming is met een rol voor PA in de functionaliteit van deze eiwitten.

Om de exacte rol van PA in de functionaliteit van deze eiwit kinases te karakteriseren is een tweezijdige benadering toegepast in **hoofdstuk 3**. Ten eerste werd de activiteit van SnRK2.4 en 2.10 bepaald in verscheidene mutanten die aannemelijk verstoord zijn in de productie van PA, maar hier werden geen duidelijke verschillen gevonden. Ten tweede, door het PA-bindings domein van SnRK2.4 op te sporen en deze vervolgens te verstoren kan het exacte effect van PA op SnRK2.4 bepaald worden. Een PA-bindend domein van 42 aminozuren werd geïdentificeerd in een subdomein dat nodig is voor osmotische stress responsen en dit domein kwam voor in het cytosol en in punt structuren in wortels behandeld met zout, wederom ondersteunend voor een rol van PA in de herlocalizatie van SnRK2.4 in reactie op zout. De PA-bindingsaffiniteit van dit domein werd volledig
verstoor door het veranderen van zeven aminoxuren die werden geselecteerd door middel van een homologie studie met de niet-PA-bindende SnRK2.6. Dit domein kan mogelijk verder worden ontwikkeld als een biosensor om de productie van PA te volgen in cellen. Bovendien is de identificatie van dit PA bindend domein een belangrijke stap naar opheldering van de rol van PA in de functionaliteit van SnRK2.4.

In **hoofdstuk 4** staat een nieuwe methode beschreven om biologisch relevante PA-bindende eiwitten te identificeren die naar de membraan migreren tijdens zout stress. Hierbij is een techniek ontwikkeld waarbij eiwitten die perifeer aan de membraan zijn gekoppeld worden geïsoleerd, voor en na blootstelling aan zout. Hierbij ligt de nadruk op eiwitten die meer op de membraan voorkomen na zout stress. De eiwitten werden vervolgens geselecteerd op PA-bindings affiniteit. Van de 42 vermeende PA-bindende eiwitten die geïdentificeerd zijn door middel van massaspectrometrie zijn acht eiwitten verrijkt in de membraan fractie na zeven minuten zoutstress. Deze betroffen twee clathrine-zware-keten isovormen en twee clathrine assemblage eiwitten, welke een belangrijke functie hebben in eiwit transport. Daarnaast zijn een eiwit dat betrokken is bij het reguleren van kalium transport, twee ribosomale eiwitten, glyceraldehyde 3-fosfaat dehydrogenase en een type II fosfatidylinositol 4-kinase γ geïdentificeerd. Deze aanpak bleek een effectieve manier om biologisch relevante lipide-bindende eiwitten te isoleren en biedt nieuwe aanknopingspunten in het onderzoek naar PA-gemediaerde responsen tijdens zoutstress in wortels.

In **hoofdstuk 5** worden de resultaten uit deze thesis bediscussieerd en in een bredere context geplaatst en **hoofdstuk 6** beschrijft de verschillende toepassingen van lipide affiniteits bollen en bevat een gedetailleerd protocol hoe ze het beste gebruikt kunnen worden.
Publications


Manuscripts in preparation:


Contributions to books:


* these authors contributed equally
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