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

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General discussion

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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 (Eriksson 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 phosphatidylyserine. 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
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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).
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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.

References


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