Chapter 1 Introduction

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Introduction

Higher plants growing in nature constantly sense their environment and reflect to different stress situations by different signaling pathways that link a range of biochemical and molecular mechanisms (Jenks and Hasegawa 2014, Rejeb et al. 2014). Although there is no central system for adapting to simultaneous abiotic and biotic stress, several key nodes were reported as general regulators in the complex network. A number of such nodes in plant cells are formed by the family of 14-3-3 proteins, which play a role in various physiological processes at the molecular level.

What are 14-3-3 proteins?

14-3-3 proteins are a family of conserved proteins involved in many cellular processes (de Boer et al. 2013). They were first discovered in the mid 1960s, and found in all eukaryotic organisms tested so far (Moore and Perez 1967, Aitken 2006). A 14-3-3 database analysis showed that a total of 153 isoforms were identified in 48 different species (Rosenquist et al. 2000). In Arabidopsis, 14-3-3 proteins act as key components in signal transduction of a large number of cellular processes and were therefore also named General Regulatory Factors (GRF) (Rooney and Ferl 1995). Arabidopsis expresses thirteen different 14-3-3 genes, GRF1 through GRF13, and in analogy with their animal counterparts they are referred to with the Greek letters: Epsilon (ε), Iota (ι), Kappa (κ), Lambda (λ), Mu (μ), Nu (ν), Omicron (ο), Pi (π), Upsilon (υ), Phi (φ), Chi (χ), Psi (ψ) and Omega (ω) and we will use the Greek letter designation in this thesis (Rosenquist et al. 2000). The amino acid sequences of Arabidopsis 14-3-3s are 60 - 92% identical amongst the different isoforms (Wu et al. 1997). According to the gene structure and amino acid sequence, the thirteen 14-3-3 genes can be grouped into an epsilon and non-epsilon group (Fig. 1). In addition, the non-epsilon isoforms can be further divided into three subgroups: kappa-, psi- and omega-group (Piotrowski and Oecking 1998). The epsilon members are found in all organisms and are thought to be involved in basal eukaryotic 14-3-3 functions, while the non-epsilon group may be responsible for organism-specific regulatory aspects (Jaspert et al. 2011). 14-3-3 proteins are phospho-motif binding proteins that translate internal and external signals into a physiological response by means of protein-protein interactions that affect protein activity, stability, conformation, and localization. In the last decades the family of 14-3-3 proteins has become a class of key adapter proteins in fundamental cellular processes such as ion transport, assimilation, metabolism, signal transduction and cell growth (Bachmann et al. 1996a, Huber et al. 2002, Schoonheim et al. 2007a, de Boer et al. 2013).

14-3-3 Structure

14-3-3 Proteins are acidic proteins with a monomeric weight of 25-32 kDa. Each monomer is composed of nine to ten alpha helices and organized into a highly conserved
core region and a variable N- and C-terminus (Chung et al. 1999, DeLille et al. 2001). The C-terminal tail of some 14-3-3 proteins functions as an auto-inhibitor for several target proteins (Liu et al. 1996, Shen et al. 2003, Bornke 2005), whereas for the human 14-3-3π protein it has been reported that the last 10-15 residues of the C-terminus are required for binding to proteins like Cbl, Raf-1 and PI3-K (Liu et al. 1996). In *Arabidopsis*, the C-terminal tail of 14-3-3ω is necessary for the binding of phosphorylated nitrate reductase (pNR), and moreover, truncation of the C-terminal region of 14-3-3ω (T235 to Q259) causes the protein to be partially Mg\(^{2+}\)-independent in both pNR binding and activity inhibition (Shen et al. 2003). Crystal structures of plant 14-3-3 proteins have revealed that the proteins are homo- or heterodimers with an inner groove in each monomer which is the main docking site for phosphorylated target proteins (Fig. 2). This implies that a 14-3-3 heterodimer has the capability to bind one target protein at two different sites or bind two different targets simultaneously (Braselmann and McCormick 1995, Taoka et al. 2011). For example, 14-3-3 proteins act as an adapter linking Raf and Bcr into one complex. Nevertheless, Raf does not bind to Bcr directly in animal cells unless co-expressed with 14-3-3β (Braselmann and McCormick 1995).

**Fig. 1. Phylogenetic tree of the *Arabidopsis* 14-3-3 superfamily.** The phylogenetic tree was constructed using the full length amino acid sequences and Pi was used as outlier; the support branch values are in red. The division between the epsilon and non-epsilon group is indicated.

**Fig. 2. Ribbon representation of the structure of the human 14-3-3ζ dimer.** The
monomers are shown in green and red, where each consists of nine d, the s. Each monomer creates a groove that enables the binding of a phosphopeptide. From: (Reinhardt and Yaffe 2013).

14-3-3 binding motifs
In general, 14-3-3 proteins bind to target proteins at phospho-Serine/Threonine residues present in a conserved binding motif. Three canonical motifs, mode-I R/KXX(S/T)\(^9\)XP, mode-II R/KXXX(S/T)\(^9\)XP and mode-III at the C-terminal tip, (S/T)\(^9\)X\(_{1,2}\)-COOH (where X denotes any residue, and (S/T)\(^9\) denotes a phosphorylated serine or threonine ) have been well-defined so far. A recently developed computational method indicates that 14-3-3 isoforms show preference for specific amino acids at certain positions of the interaction motif (Li et al. 2016). In the last decade, over 40 targets in plants have been identified and well described (de Boer et al. 2013). Amongst the known phosphorylated 14-3-3-binding sites in the literature, the authors showed that LX(R/K)SX(pS/pT)XP was predominant in the plant 14-3-3-binding motifs (Aitken 2011). Additional sequences also allow 14-3-3 interaction; for example, the proline residue at the +2 position was not present in about half of the 14-3-3-binding sites identified so far. A binding motif may be suboptimal with a medium to low affinity for 14-3-3 proteins. Target proteins with two phospho-sites having suboptimal affinity for 14-3-3, may bind with high affinity through synergistic interaction of the two sites. Phosphorylation of the sites by different kinases places the complex formation at the cross-point of converging signaling pathways (Kleppe et al. 2014). In addition, 14-3-3 proteins can also bind some targets through non-canonical or unphosphorylated motifs such as Gly-His-Ser-Leu (GHSL) and Trp-Leu-Asp- Leu-Glu (WLDLE) (Andrews et al. 1998, Petosa et al. 1998, Šribar et al. 2003).

Post-translational modifications of 14-3-3 in plants
14-3-3 proteins contain a number of established and predicted modification domains, e.g. regions for divalent cation interaction, phosphorylation and acetylation. It is clear that the phosphorylation of different 14-3-3 isoforms has an essential role in protein interaction and dimerisation, suggesting that they themselves are regulated via phosphorylation. The first evidence of 14-3-3 phosphorylation in plants was the demonstration that Arabidopsis 14-3-3 can be phosphorylated in vitro (Lu et al. 1994). So far, multiple phosphorylation sites were confirmed in plant 14-3-3 proteins in several different species (Zeijl et al. 2000, Nakagami et al. 2010). In Arabidopsis, a total of 17 phosphorylation sites from eight different isoforms were identified by independent large scale phospho-proteomics studies (Benschop et al. 2007, Sugiyama et al. 2008, Jones et al. 2009, Reiland et al. 2009, Nakagami et al. 2010). The phosphorylation sites are not consistent among different isoforms. In Arabidopsis, the identified phospho-sites are mainly observed in the
non-epsilon group and between different isoforms several phosphorylation sites locate at different position of the proteins. Although the 14-3-3 phosphorylation sites were confirmed in vivo, the kinases responsible for these post-translational modifications have rarely been identified. A member of the sucrose non-fermenting-related kinase family SnRK2.8 was the first plant kinase known to phosphorylate 14-3-3 proteins at Ser-95 (Shin et al. 2007). Phosphorylation of S95 may be an important determinant of isoform specificity since this residue is absent in the 14-3-3 ψ/υ/ν clade and the epsilon group. The phosphorylation of a maize 14-3-3 protein (GF14-6) at Tyr-137 inhibits the interaction of a phospho-peptide derived from the H⁺-ATPase and represses the Fusicoccin induced activation of the H⁺-ATPase (Olivari et al. 2000). In mammalian cells, 14-3-3β has been reported to be phosphorylated by the sphingosine-dependent kinase SDK1 on Ser-58 and phosphorylation of this site promotes the conversion of the dimer to monomer form (Hamaguchi et al. 2003, Woodcock et al. 2010). In Arabidopsis, 14-3-3s are also phosphorylated at the equivalent position at Ser-62 by a calcium-dependent kinase (CPK3) (Lachaud et al. 2013, Denison et al. 2014). A recent study showed that phosphomimetic mutation of Ser-62 affected the Arabidopsis 14-3-3ω to form homodimers (Gökirmak et al. 2015). So far, analysis by means of mass spectrometry showed that Arabidopsis 14-3-3χ, ω, υ, λ and φ are acetylated on their N terminus in vivo (Fuller et al. 2006). Acetylation is the (reversible) transfer of an acetyl group (CH₃CO) to lysine and the conversion of the positively charged lysine to acetyl-lysine affects proteins structure and interaction with other proteins. It has been suggested that all 14-3-3 isoforms in Arabidopsis are acetylated according to the MS data and the previous mammalian 14-3-3 protein study (Martin et al. 1993). Although acetylation of 14-3-3 proteins is known to be affected by other post-translational modifications, thus far no specific role was assigned to acetylation regarding 14-3-3 protein function.

14-3-3 isoform specificity and redundancy

Previous studies have demonstrated that both gene specificity and functional redundancy exists amongst the 14-3-3 proteins. In barley (Hordeum vulgare L.) embryogenesis, 14-3-3 isoforms are expressed in a tissue-specific manner and differentially regulated during androgenesis induction and embryo pattern formation (Maraschin et al. 2003). The 14-3-3s are differentially expressed in various tissues and organs. The Arabidopsis 14-3-3 ψ, λ, μ and ε are expressed in leaves. 14-3-3χ is expressed in pollen grains and stigma papillar cells and 14-3-3τ appears to be specifically expressed in flowers (Rosenquist et al. 2001, Ferl et al. 2002). Recently, van Kleeff et al. conducted a series of growth experiments with higher order Arabidopsis 14-3-3 mutants and showed gene specificity and functional redundancy among non-epsilon group members in primary root elongation under control and under abiotic stress conditions (van Kleeff et al. 2014a). In addition, 14-3-3 proteins play an isoform-specific role in interaction with target proteins, what suggests that the
large number of 14-3-3 isoforms in plants may reflect functional specificity (Swatek et al. 2011). It has been proposed that the interaction specificity of certain 14-3-3 isoforms may involve the outer surface of 14-3-3, which shows variation between isoforms (Cardasis et al. 2007). For instance, 14-3-3 isoforms from tobacco (*Nicotiana tabacum* L.) present a difference in affinity towards Sucrose-6-phosphate synthase (SPS) in the yeast two-hybrid system (Bornke 2005). Furthermore, a large scale proteomics investigation showed that 14-3-3 target proteins bound differentially to 14-3-3 isoforms *chi* and *epsilon* during *Arabidopsis* seed development (Swatek et al. 2011).

Large scale 14-3-3 interactomics

In fungi, plant and animals systems, 14-3-3 acts as a key node in the interactome map constituted by various phosphoproteins (reviewed in (de Boer et al. 2013) and (Johnson et al. 2011)). The character of 14-3-3 proteins that they can bind to other proteins allows 14-3-3 proteins to participate in many cellular functions, e.g. (hormonal) signal transduction, metabolism, (a)biotic stress responses and apoptosis. Thus, it is valuable to identify more 14-3-3 targets and understand the mechanism of the regulation by 14-3-3 proteins. Compared to low-throughput studies, high-throughput interactome experiments have identified large pools of proteins that interact with 14-3-3s. Two main strategies were applied for 14-3-3 interactome studies: yeast two-hybrid (Y2H) screens and 14-3-3 affinity chromatography followed by identification using mass spectrometry. The first Y2H screen in plants yielded 130 putative interactors (Schoonheim et al. 2007b) and a number of these putative targets have been validated as direct interactors: neutral invertase (Gao et al. 2014a; this thesis), ABA-responsive element binding factors, ABRE’s (Schoonheim et al. 2007a, Hong et al. 2011), F-box proteins (Sepulveda-Garcia and Rocha-Sosa 2012) and the plasma membrane ATPase (Olsson et al. 1997). Y2H assays have been broadly applied for confirming the interactions between 14-3-3 and one specific binding partner (Yan et al. 2002, Bornke 2005). Identification of false positives occur in experiments using this method, since the high concentration of proteins expressed in the yeast cells may not be present in the plant cells. Recently, 14-3-3 affinity chromatography has been applied to identify 14-3-3 target proteins at a large scale. In short, cell extracts are applied to 14-3-3 affinity columns or magnetic beads in *vitro* and complexes that co-purify with the tagged 14-3-3 proteins are eluted and analyzed by mass spectrometry (Milne et al. 2002, Rubio et al. 2004, Alexander and Morris 2006, Gloeckner et al. 2007, Klychnikov et al. 2007, Schoonheim et al. 2007b, Woodcock et al. 2009, Swatek et al. 2011, Collins et al. 2013). To effectively eliminate proteins that bind non-specific (false positives), a peptide called R18 mimicking a phosphorylated mode I or mode II motif is frequently used to selectively compete off primary target proteins from the 14-3-3/targets complex. It is noteworthy that proteins found in the 14-3-3s affinity purification include not only primary or direct 14-3-3 targets but also secondary or
indirect targets as members of multiprotein complexes containing 14-3-3s. Proteins co-purified as part of stable 14-3-3 complexes can also provide valuable information about the in vivo regulatory significance of these proteins in the complexes. To date, over 300 putative 14-3-3 targets were identified in plants but only around 40 proteins have been successfully validated and characterized (reviewed in (de Boer et al. 2013)). Thus finding “gold standard” targets (proteins that directly interact with 14-3-3 proteins) and wherein the phospho-site has been identified allows us to distinguish the primary targets and secondary targets from hundreds of potential 14-3-3 targets (Johnson et al. 2011). De Boer et al. recently summarized three additional criteria for identifying an interaction site in a putative target: (1) has the protein been found in large scale 14-3-3 interaction studies, (2) is the putative site known to be phosphorylated in vivo? (see PhosPhat, http://phosphat.mpimp-golm.mpg.de), and (3) is the putative site in a disordered region of the protein (http:// www.disprot.org/pondr-fit.php) (de Boer et al. 2013). Based on these criteria, proteins from the large scale of 14-3-3 interactome studies can be selected for further detailed analysis.

**Functions of 14-3-3 proteins in plant physiology**

*14-3-3 proteins play a global role in plant metabolism*

To date, most of the 14-3-3 targets identified in plants mainly belong to metabolism related processes (Fig. 3). The interaction of 14-3-3 proteins with various metabolic enzymes suggests their involvement in a range of metabolic processes (reviewed in (Chung et al. 1999, Huber et al. 2002, Comparot et al. 2003)). The binding of the 14-3-3 protein is a highly regulated process involving both activation and inhibition of the targets. Many reports have shown the importance of 14-3-3 proteins in nitrogen, carbohydrate, amino acid metabolic pathways and the tricarboxylic acid cycle (TCA) (Swiedrych et al. 2002, Szopa 2002, Zuk et al. 2005, Diaz et al. 2011). Decreased levels of nitrogen containing metabolites, such as glycine, glutamine and asparagines were observed in metabolomics with 14-3-3 overexpression lines (Diaz et al. 2011). 14-3-3 proteins control nitrogen metabolism through interaction and alteration of nitrogen metabolic enzymes such as nitrate reductase and glutamine synthetase (GS). The interaction between nitrate reductase and 14-3-3 proteins has been well described in various plant species (Bachmann et al. 1996a, Athwal et al. 1998, Lambeck et al. 2012). In plant cells, NR is rapidly inactivated in the dark by phosphorylation followed by the binding to 14-3-3 proteins (Lambeck et al. 2012). Sugar metabolism is an indispensable process during life-sustaining processes like germination, flowering, senescence, etc. in green plants. Sugars act as energy compounds as well as signaling molecules that regulate the activity of e.g. NR (Koch 2004). Previous studies indicated that 14-3-3 proteins play an important role in sugar synthesis, hydrolysis and storage (Kulma et al. 2004, Bornke 2005, Harthill et al. 2006, Gao et al. 2014a). SPS plays a key role in sucrose biosynthesis in plants.
Several studies have shown that SPS interacts with 14-3-3 proteins both in vitro and in vivo (Toroser et al. 1998, Weiner and Kaiser 1999, Bornke 2005). However, so far there is no clear conclusion about the effect of 14-3-3 proteins on SPS activity. In potato, 14-3-3-repressed transgenic plants showed significantly higher levels of SPS activity (Szopa 2002), while an opposite effect of 14-3-3s on SPS was observed in Arabidopsis (Moorhead et al. 1999). In this thesis, CINV1, another critical enzyme in sucrose metabolism is extensively discussed. We showed the direct association between 14-3-3 and CINV1. Moreover, we showed the physiological relevance of invertase regulation by 14-3-3 binding by its light induced activation. A role for 14-3-3 proteins in starch synthesis has also been demonstrated through down-regulation of either 14-3-3\(\mu\) or 14-3-3\(\xi\) in Arabidopsis which resulted in increased starch accumulation (Sehnke et al. 2001). Many key enzymes of the TCA cycle were consistently identified in independent 14-3-3 interactome studies (Chang et al. 2009, Paul et al. 2009, Shin et al. 2011, Swatek et al. 2011). In yeast two-hybrid interaction assays, several malate dehydrogenases and isocitrate dehydrogenases were shown to interact with 14-3-3\(\kappa\), \(\chi\) and \(\psi\) and in addition 14-3-3 overexpression resulted in reduced activities of isocitrate dehydrogenase and malate dehydrogenase (Diaz et al. 2011).

14-3-3 and plant hormones

Hormonal signaling pathways are complex and are regulated by many factors. The 14-3-3 proteins clearly have an important function in hormonal signaling pathways by interacting with various intermediates or by regulating the transcription of key genes. The abscisic acid (ABA) signaling pathway is a major signal transduction pathways activated by drought and high salinity stress (Mehrotra et al. 2014, Yoshida et al. 2015). The expression level of 14-3-3 has been shown to be affected by ABA in various physiological processes including stomatal closure and seed germination (Leonhardt et al. 2004, Sirichandra et al. 2010a). Moreover, 14-3-3s interact with a range of transcription factors involved in the ABA signaling pathway, e.g. AREB/ABF/ABI5-like transcription factors that bind to ABA-response elements (Sirichandra et al. 2010a). In addition, three protein kinases identified as putative 14-3-3 targets, Sucrose non-fermenting1-related protein kinases 2 (SnRK2), Calcium dependent protein kinase (CDPK) and mitogen-activated protein kinase (MAPK), have been identified as ABA signaling factors (Mori et al. 2006, Danquah et al. 2015, Waadt et al. 2015). Gibberellins (GA) are essential regulators of plant growth processes and using 14-3-3 RNAi constructs in the barley aleurone transient expression system, it was demonstrated that silencing of five individual 14-3-3 isoforms suppressed GA induction of the alpha-amylase gene (Schoonheim et al. 2009). The calcium dependent protein kinase CDPK1 reduced the expression of enzymes essential for GA biosynthesis while it activated the expression of a 14-3-3 protein in rice seedlings (Ho et al. 2013). Increased concentrations of GA
activated CDPK1 of tobacco (NiCDPK1), what resulted in the phosphorylation of RSG (a tobacco transcriptional activator with a bZIP domain) creating a 14-3-3 binding site at Ser-114 (Ishida et al. 2008). The interaction between RSG with 14-3-3 proteins sequesters RSG in the cytoplasm, where it is unable to activate the transcription of GA biosynthetic enzymes (Igarashi et al. 2001a). The plant hormone ethylene plays an essential role in the response to biotic and abiotic stress conditions. 14-3-3s are linked to the ethylene biosynthesis pathway by interacting with a set of ethylene biosynthesis related proteins, including S-adenosylmethionine synthase (Phanchaisri et al.), ACC synthase and 1-aminocyclopropane-1-carboxylate synthase (ACS) (Chang et al. 2009, Shin et al. 2011, Yoon and Kieber 2013, Catalá et al. 2014). The role of 14-3-3 proteins in regulating ethylene levels has already been documented in potato (Solanum tuberosum), where the antisense expression of an endogenous 14-3-3 gene induced ethylene accumulation (Szopa 2002). Several studies revealed that a pool of S-adenosylmethionine (SAM) is a central factor in the response to abiotic stress and multiple 14-3-3 interactions can have significant impacts on ethylene signaling (Yao et al. 2007, Yoon and Kieber 2013, Catalá et al. 2014).

14-3-3 and Ion homeostasis

Ion homeostasis is essential for plants to adapt to salt stress. During salt stress the net K⁺ uptake is decreased and the Na⁺ influx increases in plant cells. Ample evidence has been provided that 14-3-3 proteins act as important regulators of ion homeostasis in plants (Shan'ko and Babakov 2002, Sottocornola et al. 2008, Latz et al. 2013a, Zhou H et al. 2014) (Fig. 3). Under high salinity condition, the transcription level of 14-3-3 genes was significantly up-regulated in cotton roots; in addition, the overexpression of Arabidopsis 14-3-3 Protein GF14λ in cotton significantly enhanced the salt tolerance (Yan et al. 2004, Wei et al. 2009). One mechanism by which 14-3-3s could act in the regulation of such environmental stress responses is through the regulation of ion channels. Several K⁺ channels have been identified as 14-3-3 downstream effector proteins by direct binding, including the inward rectifier K⁺ channel KAT1 and the vacuolar two-pore K⁺ channel 1 TPK1 (Sinnige et al. 2005b, Latz et al. 2008, Sottocornola et al. 2008, Sato et al. 2009). The H⁺-ATPase, the best characterized 14-3-3 target, plays a key role in Na⁺/H⁺ exchange under salt stress and is activated by binding of 14-3-3 protein to the phosphorylated C terminus (Shan'ko and Babakov 2002, Duby et al. 2009). In addition, under salt stress the level of cytosolic calcium increases and triggers changes in the activity of a number of Ca²⁺-binding proteins (Zhu 2002). In higher plants, three calcium sensor families have been identified: calmodulins (CaMs), calcineurin B-like proteins (CBLs) and calcium dependent kinases (CDPKs). Recently, Zhou et al reported that 14-3-3 proteins are involved in the Salt Overly Sensitive (Sepulveda-Garcia and Rocha-Sosa) pathway by interaction with the calcium-activated protein kinase SOS2 and repressing its kinase.
activity under control conditions, thus affecting the activity of ion transporters such as SOS1 (Zhou H et al. 2014). These studies indicate that 14-3-3 proteins act as important regulators of salt tolerance in plant cells.

Fig. 3. Summary of the plant 14-3-3 functional diversity. A selection of 14-3-3s target proteins which affect various physiological processes are shown, including transporters, hormone related proteins, Fe-deficiency responding proteins and metabolic enzymes which are mainly studied in this thesis. 14-3-3 proteins regulate the target proteins via phosphorylation-dependent binding which may mediated by protein kinases like CDPK and SnRK. In this thesis, we add the regulation of CDPKs, GORK and CINV1 to the 14-3-3 regulatory map. GORK, outward-rectifying K⁺ channel; KAT1, inward-rectifying K⁺ channel; TPK1, vacuolar Ca²⁺ activated outward rectifying K⁺ channel 1; AHA2, plasma membrane proton ATPase 2; SLAC1, guard cell anion channel; RSG, Repression of shoot growth; ABF, abscisic acid responsive element-binding factor; FIT, Fe-deficiency-induced transcription factor 1; FRO2, ferric chelate reductase 2; IRT1, iron-regulated transporter 1; NR, nitrate reductase; CINV1, alkaline/neutral invertase 1; SPS1, sucrose phosphate synthase 1; CDPK, calcium-dependent protein kinase; SnRK2, sucrose nonfermenting-1 related protein kinase. Symbol ‘P’ indicates phosphorylation.
**Chapter 1. Introduction**

**Scope and outline of the thesis**

The central theme of this thesis is the function of 14-3-3 proteins in sugar metabolism and ion homeostasis under abiotic stress conditions. Plants constantly sense and quickly adapt to environmental changes by utilizing cascades of molecular and biochemical mechanisms (Rejeb et al. 2014). In the last decades increasing evidence has shown that 14-3-3 proteins are important for physiological processes via direct protein-protein interactions. In the studies described in this thesis we incorporate various strategies including proteomic, metabolomic and molecular/genetic analyses to unravel the role of 14-3-3 in sugar metabolism and abiotic stress responses.

In **Chapter 2** we show the triangular relationship between the outward rectifying K⁺-channel GORK, calcium dependent phosphorylation kinase (CDPK) and 14-3-3s. We show that both PKA and CPK21 can phosphorylate the C-terminus of GORK and detected phosphorylation sites of GORK and CPK21 with mass spectrometry. Moreover, in a semi in vivo pull-down experiment, we showed that endogenous 14-3-3LAMبدا, PHI and NU were pulled down with the recombinant GORK C-terminus, suggesting a link between GORK and 14-3-3s. In addition, we showed binding between CPK and 14-3-3, whereby CPK21 and CPK23 showed enhanced activity upon 14-3-3 binding while the activity of two other CPKs, CPK3 and CPK6, was not affected. To study the physiological relationship between 14-3-3, GORK and CDPK, the vibrating probe technique was used to detect ion fluxes of both potassium (K⁺) and protons (H⁺) in gork, cpk21, aha2 and 14-3-3 single mutants at the onset of salt stimulation. The results indicate that gork-like characteristics (reduced K⁺-efflux at NaCl treatment) are observed in two 14-3-3 single mutants, phi and chi. In conclusion, our data show a potential link between GORK and 14-3-3’s either direct and/or indirect via CPK kinases.

In **Chapter 3** we show an interaction between a cytosolic invertase (CINV1, At1g35580) and 14-3-3s via a phosphorylation dependent manner. We demonstrate that Ser547 at the extreme C-terminus of the AtCINV1 protein is a substrate of calcium-dependent kinases (CPK3 and 21) and that phosphorylation creates a high-affinity binding site for 14-3-3 proteins. In addition, this binding results in enhancement of CINV1 activity. The analysis of a quadruple mutant kappa/lambda/phi/chi (klpc) shows strong reduction in the total cytosolic invertase activity and in hexose (glucose and fructose) levels in the roots. The physiological relevance of this novel mechanism of A/N invertase regulation is underscored by the light induced activation of 14-3-3 binding to CINV1 and invertase activity in roots. This is another example of the central role of 14-3-3 proteins in effectuating dark/light signaling.
In **Chapter 4** we sought to comprehensively investigate the molecular and physiological role of 14-3-3 in the response of *Arabidopsis* plants to Fe deficiency by utilizing interactomics and molecular strategies. Three 14-3-3 quadruple knockout mutants (*klpc, klun* and *unpc*) exhibited diverse physiological responses to Fe limitation: mutants that combined *kl* with *un* mutations or *pc* mutations (*klpc*) showed higher Fe uptake than Wt plants at low Fe in the medium, while this phenotype was not observed in mutants that combined *un* with *pc* (*unpc*) mutations. Moreover, the higher level of Fe in roots of *klun* was consistent with increasing transcript levels of Fe-deficiency-induced genes, including *14-3-3OMICRON*, *FIT* (FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTON FACTOR), FRO2 (IRON REGULATED FERRIC CHELATE REDUCTASE) and the FERROUS IRON TRANSPORTER, IRT1. By using quantitative affinity-purification mass spectrometry (qAP-MS) proteomics to characterize 14-3-3 interactomes with or without exposure to Fe deficiency, a total of 117 proteins were identified in the Wt roots (+ and -Fe), of which a subset of 27 proteins differentially interacted with 14-3-3 in roots grown without Fe for 24 h. Functionally, many of these -Fe responding proteins have a role in glycolysis and TCA cycle, the FoF1-synthase and in the cysteine/methionine synthesis. Also the 14-3-3 interactome of the *klun* roots showed significant differences with that of Wt roots under Fe sufficient conditions, where most of these differential binding proteins showed enhanced binding in the *klun* mutant. So, 14-3-3 proteins of the non-epsilon group clearly have an important role in adaptation to Fe stress and a clear explanation for the observed phenotypes awaits a more detailed analysis of the functional aspects of 14-3-3 binding to the target proteins identified in this study.

**Chapter 5** focuses on the 14-3-3 function in salt stress adaptation and signaling pathways. We demonstrated that the three 14-3-3 quadruple knockout mutants (*klpc, klun* and *unpc*) which are generated by crossing double mutants made of closely related gene pairs perform differently in Na\(^+\) uptake from soil. 14-3-3 mutants showed isoform specific salt dependent growth and Na\(^+\)/K\(^+\) accumulation and in particular the *klpc* mutant showed a clear Na\(^+\) and K\(^+\) phenotype. The lower levels of Na\(^+\) and K\(^+\) in *klpc* were not due to reduced transpiration rate under salt stress but correlated with an increase in the expression of the *HKT1* gene in this mutant. Further, we analyzed metabolites in roots of wild type and 14-3-3 mutants before and after salt stress. A total of 153 metabolites, including 80 named and 73 unknown compounds from the wild type and 14-3-3 qKOs were identified by using GC-MS. Notably, the reducing sugars glucose and fructose were lower in *klpc* under control and salt stress and their phosphorylated forms, Glucose-6P and Fructose-6P, were lower under salt stress as compared to Wt. Because *klpc* also accumulated significantly less Na\(^+\), it remains to be shown how the reduced levels of monosaccharides affects the growth performance of the *klpc* mutant under salt stress. The distinguished accumulation of metabolites between Wt and mutants may be responsible
for the different physiological responses of the 14-3-3qKO lines in response to high Na⁺ stress.

References
Chapter 1. Introduction


Chapter 1. Introduction

Chapter 1. Introduction

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Chapter 1. Introduction

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Chapter 1. Introduction


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