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DISSERTATION

Ca^{2+} Dependent Protein Kinases in *Arabidopsis thaliana*

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Verfasser:	Mag. Norbert Mehlmer
Matrikel-Nummer:	9820609
Dissertationsgebiet:	Genetik/Mikrobiologie
Betreuer:	Univ.-Prof. Dr. Markus Teige
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Zusammenfassung

Pflanzen sind nicht in der Lage, den Standort zu wechseln und müssen daher auf Änderungen der Umwelt adäquat reagieren um zu überleben und sich fortzupflanzen. Viele extrazelluläre Signale wie Licht, biotische und abiotische Stressfaktoren lösen in pflanzlichen - und auch in tierischen Zellen - eine kurzfristige Erhöhung der zellulären Ca^{2+} Konzentration aus, die als Signal wirkt (Harper et al., 2004; Cheng et al., 2002; Sanders et al., 2002). Ca^{2+} abhängige Protein Kinasen werden in der Gegenwart von erhöhtem Ca^{2+} aktiviert und leiten das ursprüngliche (Stress) Signal durch Protein Phosphorylierung weiter. Somit spielen diese Proteinkinasen eine zentrale Rolle in der Regulation der zellulären Stressantwort. In dieser Arbeit beschreibe ich die subzelluläre Lokalisierung von mehreren Ca^{2+} abhängigen Protein Kinasen (CDPKs) im Allgemeinem und CPK3 im Detail, und analysiere die Rolle von CPK3 in der Anpassung an abiotische Stressbedingungen. Durch Verwendung von CDPK-YFP Fusionsproteinen und biochemischer Zellfraktionierung war ich in der Lage zu zeigen, dass viele CDPKs Membranassoziiert sind. Ich konnte weiterhin zeigen, dass N-terminale Acylierungen (Myristoylierung und Palmitoylierung) für diese Lokalisierung verantwortlich sind. Diese Ergebnisse bestätigen die Beobachtung, dass N-Terminale Myristoylierung und Palmitoylierung eine wesentliche Rolle für die subzelluläre Lokalisation dieser Proteinkinasen spielen. CPK3 ist allerdings nicht palmitoyliert und infolgedessen auch im Zellkern lokalisiert. Dies erhöht die Anzahl von möglichen Zielproteinen, die durch CPK3 phosphoryliert werden können. Um molekulare Zielproteine von CPK3 zu isolieren, wurden mikrosomale Membranen isoliert und mit rekombinanter CPK3 phosphoryliert. Durch einen kombinierten Einsatz pho-spezifischer Antikörper und massenspektroskopischer Analyse konnten Phosphoproteine als CPK3 Targets identifiziert werden. Interessanterweise war ein Großteil dieser Proteine in den Transport von gelösten Substanzen und Ionen involviert, wie z.B. Ionen Pumpen und Kanal-Proteine, was für den salz-sensitiven Phänotyp der cpk3 Knock-out Linie verantwortlich sein dürfte.

Abstract

Plants as sessile organisms have to respond to various external stimuli such as different forms of stress (i.e. pathogens, abiotic stress) or different light intensities. Many extra cellular signals such as light, biotic and abiotic stress factors elicit changes in the cellular Ca^{2+} concentrations in plant and animal cells (Harper et al., 2004; Cheng et al., 2002; Sanders et al., 2002). In plants, decoding of these calcium signals is performed by protein kinases such as calcium dependent protein kinases (CDPKs), which mediate cellular responses by either directly changing enzymatic activities via protein phosphorylation, or indirectly by changing gene expression patterns. In this work I describe the subcellular localization of several CDPKs, in particular that of CPK3 in detail and analyse the role of CPK3 in adaptation to abiotic stress conditions. Using expression constructs, in which the CDPKs were fused to yellow fluorescent protein (YFP) and in biochemical cell fractionation experiments, I was able to demonstrate the attachment of several CDPKs to cellular membranes. These results are consistent with the observed N-terminal myristoylation and palmitoylation of many CDPKs. However, CPK3 is not palmitoylated and localizes also to the nucleus, thus enabling targeting a great number of proteins with different subcellular localizations. To identify molecular targets of CPK3 isolated microsomal membranes were phosphorylated by recombinant CPK3 and identified phosphoproteins were analyzed by MS. Interestingly a major part of the identified proteins is involved in transport of solutes and ions including porins and ion pumps/channel proteins, which could explain the salt sensitive phenotype of the *cpk3* knock-out line.

Table of contents

1 Introduction

1.1 Protein kinases in plant signaling

Plants as sessile organisms have to cope repeatedly with changes in their environment such as altered growth conditions or different forms of biotic or abiotic stress during their life-cycle. Therefore they have evolved a great number of adaptation mechanisms. Their survival depends on the ability to adapt to different environmental changes by recognizing those extracellular signals and to process them into a cellular response. This could either be an adaptation of their cellular metabolism or a switch in their developmental program. The recognition of external signals occurs at the molecular level by intracellular or cell-surface receptors, which can detect environmental changes or signaling molecules. Usually, the recognition of signals by the receptor causes its activation and could also change its biochemical features resulting for example in different enzymatic properties, conformational changes, interaction with other proteins or release of second messenger molecules. In this fashion the generation of chemical signals and their further release in the signaling process can activate numerous downstream receptors and thereby forward and amplify the signal within signal transduction pathways (McCarty and Chory 2000; Colcombet and Hirt 2008).

Mitogen Activated Protein Kinase (MAPK) cascades present a prototype of such a signaling pathway, which is evolutionary highly conserved in animals (Avruch, Nemenoff et al. 1982; Ray and Sturgill 1988), yeast (Errede, Cade et al. 1995; Herskowitz 1995), and plants (Colcombet and Hirt 2008). The signal transduction cascade starts with the activation of the MAP kinase kinase kinase by activation of a cellular receptor. In animal-cells, these receptors are for example G-protein-coupled receptors reacting to hormones. Accordingly these pathways were initially named mitogen-activated protein kinase pathways (Rossomando, Payne et al. 1989). The MAP kinase kinase kinase subsequently phosphorylates the MAP kinase kinase, which finally phosphorylates and activates the MAP kinase. This cascade thereby transmits the signal towards the final targets, i.e. transcriptional regulators in the nucleus, and furthermore amplifies the signal from recognition of only a few signals at the cellular surface to phosphorylation of a great number of target molecules. Transcription factors present bona fide targets for MAP kinases, which could be modified in many ways including control of their sub-cellular localization, expression, stability, or their ability to bind to

other components of transcriptional complexes and their ability to remodel chromatin structure. (Yang, Sharrocks et al. 2003; Whitmarsh 2007).

Another completely different strategy to transduce extracellular signals to cellular targets for adaptation processes is the generation of so-called secondary messengers. These are small molecules, which are generated in response to an extracellular signal. The second messenger Ca^{2+} is stored in the apoplast or organelles (i.e. vacuole, the endoplasmatic reticulum, and chloroplast) and its release is mediated by specific ion channels (Sanders, Pelloux et al. 2002). In contrast to the surrounding compartments, the cytosolic Ca^{2+} concentration is maintained at low levels (10-100 nM) under resting condition. Ca^{2+} reaches the cytoplasm through a large number of channels in organelles and can increase rapidly in concentration in response to either external or internal signals. The removal of Ca^{2+} from the cytoplasm is done by membrane transporters. But Ca^{2+} is not distributed randomly in the cytoplasm and it is known that the influx of Ca^{2+} generates localized domains of high calcium concentration next to membranes (Etter, Minta et al. 1996).

The detection of Ca^{2+} is mediated by receptors which can act as sensor responders or as sensor relays. Ca^{2+} binding proteins like the calmodulins represent “sensor relays”. Calmodulins are well known Ca^{2+} receptors, binding Ca^{2+} with their EF hands. Other Ca^{2+} binding proteins like the CBLs are able to bind Ca^{2+}. Sensor relays have different conformations in the Ca^{2+} bound state and therefore different binding properties to target proteins. Protein kinases like the CaMKs, CCaMKs, SRKs and SnRK3s are regulated by these calcium binding proteins. But also the regulation of membrane proteins like Ca^{2+}-ATPase ACA is regulated by an Ca^{2+} binding protein (Sanders, Pelloux et al. 2002). A sensor responder directly changes the enzymatic activity if Ca^{2+} is bound to it. CDPKs are sensor responders because the calmodulin domain is part of the CDPK and is activated by the binding of Ca^{2+} to the calmodulin. Also the Ca^{2+} sensing receptor (CAS), a membrane bound protein, is directly activated by Ca^{2+} and it is supposed to be responsible for the detection of the influx of Ca^{2+} from the apoplast of stomata (Han, Tang et al. 2003).

Interplay of Protein Kinases and plant hormones

Many different aspects of plant growth and development are regulated by signaling pathways, which involve plant hormones and include protein phosphorylation by

protein kinases at one or the other step. I will summarize recent evidence for interaction of protein kinase signaling and different plant hormones.

Auxins (Aux) are well-known signal molecules, which are involved in many developmental processes, including shoot and root growth, leaf formation, lateral root formation, apical dominance (Fleming 2006). Although Auxin can be synthesised in all tissue to some extent, the biosynthesis of Auxins occurs mainly in rapidly growing tissues, especially in shoots (Ljung, Bhalerao et al. 2001). Auxins are redistributed within the plant through the vasculature system by specific transporters, and polar auxin transport, mediated by PINs, is a major driving force in plant development. For example phyllotaxis, a developmental program resulting in the asymmetric architecture of plants, is established by Auxin gradients (Reinhardt, Pesce et al. 2003). Recently PIN7 has been shown to be phosphorylated *in planta* in a phospho-proteomics study (Nuhse, Stensballe et al. 2004).

Ethylene (ETH**)** is an example of a small gaseous signal molecule regulating many aspects of plant growth and development. It is synthesized from ACC, a side product from the methionine cycle. However, the rate-limiting step in the synthesis of ethylene is the synthesis of ACC by the ACC synthase, controlled by the stability of ACC synthase. The degradation of ACC synthase is regulated by the phosphorylation state on the N-terminus of ACC synthase. Un-phosphorylated ACS6 is fast turned over by the 26S proteasome pathway and it is known that truncated versions of ACC synthase lacking the phosphorylation sites are not degraded. It was recently shown that MAP kinases are responsible for the phosphorylation of the N-terminal domains stabilizing ACC synthase (Joo, Liu et al. 2008).

Gibberellins (GA) are plant hormones that regulate especially developmental processes, for example germination or flowering. It was shown that DELLA proteins, which act as transcription factors, play an important role in the negative control of GA signaling (Fleet and Sun 2005). These DELLA proteins are degraded via the ubiquitin proteasome pathway upon GA treatment (Itoh, Matsuoka et al. 2003). On the other hand light increases the amount of DELLA proteins by reducing the GA levels (Achard, Liao et al. 2007; Feng, Martinez et al. 2008). Achard et al. showed the complex interplay between abiotic stress signals and plant hormones during germination in a “quadruple-DELLA” mutant lacking four DELLA proteins (GAI, RGA, RGL1 and RGL2). Germination of this mutant was less sensitive to salt stress (Achard, Cheng et al. 2006).

Brassinosteroids (BR) are derivatives of cholestane that are considered to be the plant analogues of steroid hormones in the animal kingdom. Depending on the plant species, the distribution of the various BRs differs significantly. In general the application of BRs to plants shows a strong growth promoting effect. Consequently, mutations in the receptor for BR (bri1) show a dwarfish phenotype. BRI1 is a receptor like kinase localized on the membrane. If BRI1 binds BR, BRI1 interacts with BAK1 and both will be phosphorylated (Eckardt 2005).

Abscisic acid (ABA) is an important plant hormone that is involved in many phases of the plant life cycle, including seed development and dormancy, and in plant responses to various environmental stresses (Seo and Koshiba 2002). ABA is produced in root tissue under drought stress condition and then transported to the leaves, where it mediates the closure of stomata guard cells (Trejo, Davies et al. 1993; Trejo, Clephan et al. 1995). In these cells ABA mediates the release of Ca^{2+} from the vacuole into the cytoplasm blocking the uptake of K^{+} (McAinsh, Brownlee et al. 1992; MacRobbie 2000). For a long time it was not clear how the plant-cell recognizes ABA but finally receptors were found. The nuclear RNA-binding protein FCA which is involved in the control of flowering and a chloroplast H subunit of Mg-chelatase (CHLH) was identified (Sheen 1996; Razem, El-Kereamy et al. 2006). Also GPA1 a membrane bound G protein-coupled ABA receptor was identified (Liu, Yue et al. 2007). ABFs are basic leucine zipper-type (bZIP) transcription factors including ABF1, ABF2 (AREB1), ABF3, ABF4 (AREB2), and ABI5, and they can bind to the ABRE elements in the promoters of many ABA-induced genes (Choi, Hong et al. 2000; Uno, Furihata et al. 2000). But it was already known that the active forms of two Arabidopsis CDPKs CPK10 and CPK30 activate an ABA stress-inducible promoter in leaf protoplasts (Sheen 1996). Supporting evidences for the involvement of CDPKs in transcriptional induction came from a Yeast-Two-Hybrid screen, performed with ABF4 as bait. In this screen CPK32 was identified and confirmed as interacting partner by *in-vitro* binding and phosphorylation assays (Choi, Park et al. 2005). Also the protein-expression and kinase activity of CPK4 and CPK11 were induced by ABA and it was shown that ABA induced the transcription factors ABF1 and ABF4 were phosphorylated by both kinases. On the other hand, CPK4 and CPK11 knock-out lines showed an ABA insensitive phenotype, which could be complemented by the overexpression of CPK4 or CPK11 (Zhu, Yu et al. 2007). Similar results were obtained with loss-of-function mutants of

CPK3 and CPK6, which showed an impaired closure of stomata (Mori, Murata et al. 2006).

Light is quite obviously a key-issue for plant's life, not only for driving photosynthesis and thereby enabling the autotrophic life-style of plants, but also as important regulator of plant-growth and development. Therefore plants have developed different light-sensing systems. The detection of red light and far red light is done by phytochromes. These are localized in the cytoplasm or the nucleus, depending on the light conditions and the type of phytochromes (Sakamoto and Nagatani 1996). Phytochromes are synthesized in a red light–absorbing form Pr in the dark. The absorption of red light converts Pr to the far-red light-absorbing and biologically active form Pfr. Exposure to far-red light can revert Pfr back to its inactive form Pr. In *Arabidopsis* there are five known phytochromes (phyA-phyE), each of them has both unique and redundant functions (Devlin, Patel et al. 1998). Analysis of mutants has shown that the C-terminus is important for phytochrome function in vivo, but the biochemical mechanism of phytochrome signaling is still unclear (Quail 1997). There are evidences that phytA has a serine-threonine kinase activity induced by light (Yeh and Lagarias 1998). PKS1 (phytochrome kinase substrate 1) was identified in a Yeast-Two-Hybrid screen and is light dependently phosphorylated by phyA (Fankhauser, Yeh et al. 1999).

Calcium signaling pathways

Calcium mediated cell signaling is involved in a vast number of processes including stress response, growth and development. Many biotic and abiotic signals can cause the influx of Ca^{2+} elevating the cytosolic free calcium ($[Ca^{2+}]_c$) concentration (Knight, Trewavas et al. 1997). Also a large set of kinases is regulated directly or indirectly by Ca^{2+} including CDPKs, CaMKs, CCaMKs, SRKs and SnRK3s (Sanders, Pelloux et al. 2002).

Ca^{2+} activated protein kinases have different activation-mechanisms (figure 1.2). CDPKs are the only kinases which are directly fused to the Ca^{2+} binding and activation domain.

Calcium in the salt stress response of plants

High salinity is one of the most severe environmental stresses for plants and for crop production (Tuteja 2007). Therefore it is fundamental for productive plant growth to

keep the cellular concentration of toxic ions below a threshold level and to accumulate certain ions to correct ion imbalance. At the cellular level it is necessary for plants to keep the homeostasis between K^+ and Na^+ because increased concentrations Na^+ ions are toxic for cellular metabolism. Furthermore, plants need to establish high cytoplasmic K^+ concentrations to keep their turgor pressure, the driving force for plant growth (Hastings and Gutknecht 1978; Walker, Leigh et al. 1996). Accordingly a large set of genes is transcriptionally up regulated under salt stress conditions to counteract the negative effects of Na^+ toxicity and to maintain cellular ion-homeostasis. These genes include different Na^+ and K^+ ion channels (like SOS1, NHX1, NHX8, TPK1 and HKT1), receptors, metabolic enzymes for syntheses of compatible solutes acting as osmolytes, and signaling factors. One characteristic feature of Na^+ stress is the elevated cytosolic Ca^{2+} concentration, which regulates a wide range of Ca^{2+} dependent pathways. It is possible that a Ca^{2+} release also results from the activation of phospholipase C (PLC), leading to hydrolysis of PIP to IP3 and the subsequent release of Ca^{2+} from intracellular Ca^{2+} stores (Mahajan, Pandey et al. 2008).

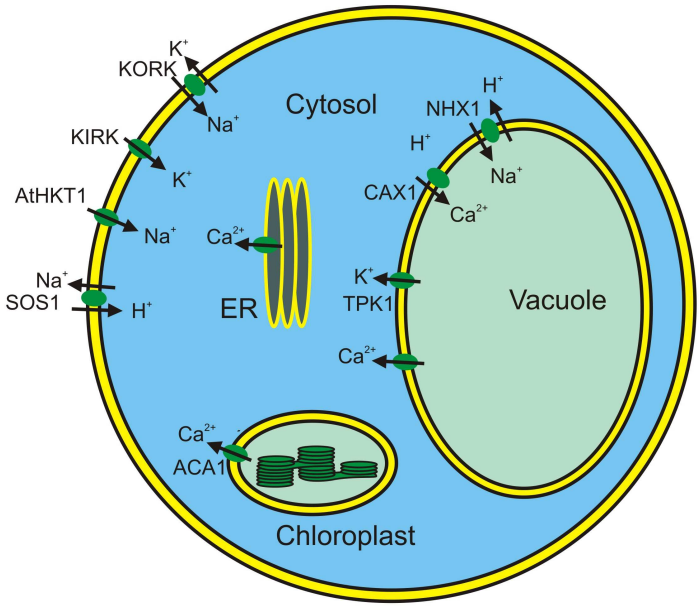

Figure 1.1: Overview on the most important ion channels in plants according to Mahajan et al., 2008

The uptake and removal of ions is regulated by a wide range of ion pumps/channels maintaining ion homeostasis (Figure 1.1). Some channels including the K^+ inward-rectifying channel have more selectivity to K^+ than for Na^+. These channels mediate the influx of K^+ upon the plasma membrane and selectively accumulate K^+ ions. The K^+ outward-rectifying channel opens during depolarization of the plasma membrane and

mediates the efflux of K^+ and the influx of Na^+ ions. The low-affinity Na^+ ion-transporter histidine kinase transporter (HKT1) blocks the entry of Na^+ into the cytosol. The efflux of Na^+ from the vacuole is done by the vacuolar Na^+/H^+ exchanger (NHX1) and the H^+/Ca^{2+} antiporter (CAX1) is responsible for the uptake of Ca^{2+} into the vacuole. The plasma membrane localized H^+/Na^+ antiporter SOS1 is responsible for the removal of Na^+ from the cytoplasm and was identified in a mutagenesis screen (Wu, Ding et al. 1996). The mechanism for regulation of SOS1 consists of a protein kinase complex, which is regulated by the presence of Ca^{2+}. The calcineurin B-like protein (CBL) SOS3 acts as sensor relay molecule by binding Ca^{2+} and subsequently activating protein kinase SOS2, which phosphorylates and activates SOS1 (Wu, Ding et al. 1996; Mahajan, Pandey et al. 2008).

SOS1 is not the only known target for the protein kinase complex SOS2/SOS3. Under salt stress condition the low-affinity Na^+ transporter AtHKT1 is repressed by the activated SOS2/SOS3 kinase complex. In addition to SOS1, the activated SOS2/SOS3 kinase complex also activates the vacuolar localized NHX1, which mediates the removal of Na^+ into the vacuole.

Calcium dependent Protein Kinases (CDPKs)

CDPKs are broadly distributed in the plant kingdom and in alveolate protists, including Eimeria (Bumstead, Dunn et al. 1995; Dunn, Bumstead et al. 1996), *Plasmodium falciparum* (Zhao, Kappes et al. 1993; Farber, Graeser et al. 1997; Gardner, Tettelin et al. 1998) and *Paramecium tetraurelia* (Kim, Messinger et al. 1998). Based on genomic sequence analysis 34 CDPKs have been annotated from the fully sequenced genome of *Arabidopsis thaliana*, and 29 CDPKs were found in rice (Asano, Tanaka et al. 2005). Sequence analysis of all 34 known *Arabidopsis* CDPKs showed that the overall identities are 39% to 95% and the similarities are 56% to 96%. According to their homologies the Arabidopsis CDPKs can be sub-grouped into 4 branches (figure 1.2) and phylogenetic analysis of the intron structure showed that CDPKs are evolved by the fusion of ancestral CaMK with ancestral calmodulin (Zhang and Choi 2001).

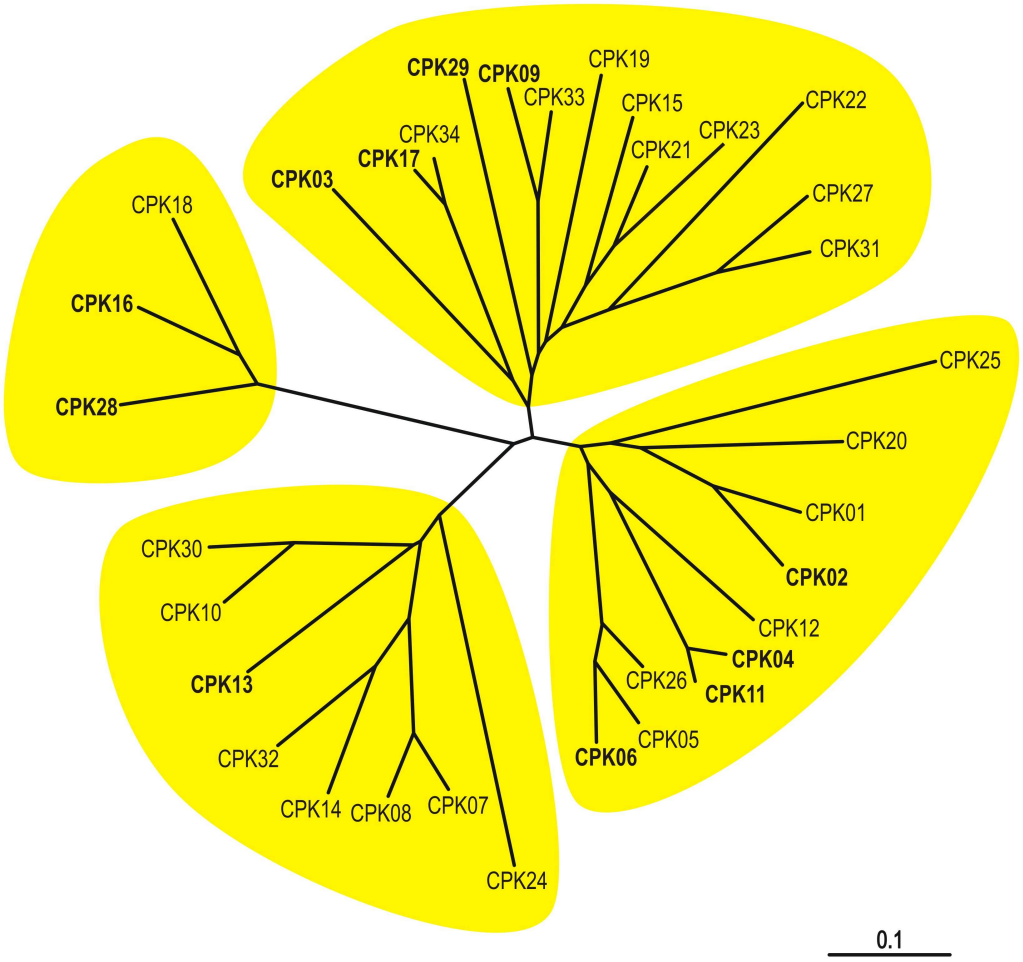

Figure 1.2: Phylogenetic tree of all 34 known CDPKs from Arabidopsis thaliana using ClustalW and PHYLIP.

The nearest CDPK homologues of Ca^{2+} dependent or activated protein kinases are the calmodulin-dependent protein kinases (CaMKs), calcium and calmodulin-dependent protein kinases (CCaMKs), the CDPK-related protein kinases (CRKs) and the SnRK3s.

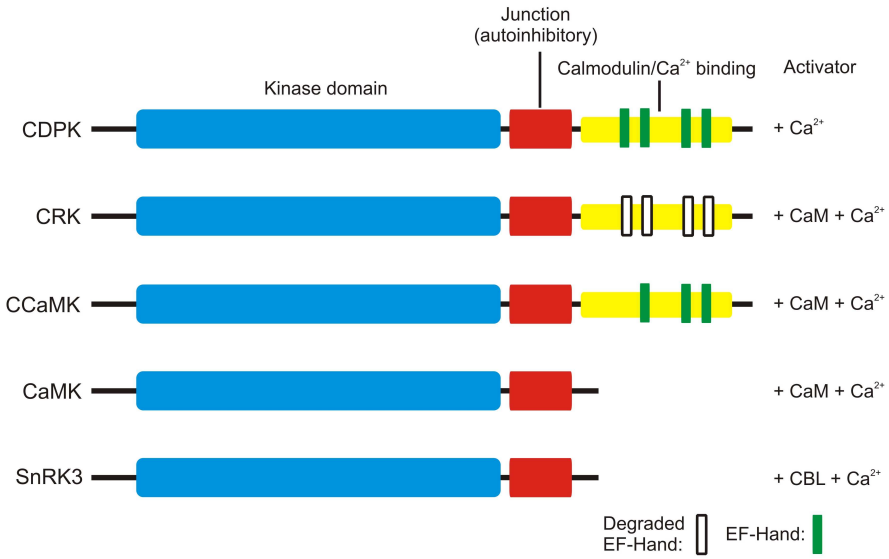

Figure 1.3: Ca^{2+}-activated protein kinases in plants (Harper, Breton et al. 2004). CDPK: calcium dependent protein kinase. CRK: CDPK-related portein kinase. CCaMKs calcium and calmodulin-dependent protein kinases. CaMK: calmodulin-dependent protein kinases. SnRK3: SNF1-related protein kinase 3.

The CaMKs are well characterized in animals and yeast, but there is only one putative representative known in plants (Watillon, Kettmann et al. 1995). CCaMKs contain calcium-binding domains but these are more similar to visinin than to calmodulins (Poovaiah, Xia et al. 1999). CRKs have kinase catalytic domains closely related to those of CPKs, and the C-terminal domains share sequence similarity to calmodulin, but the EF-hands are poorly conserved. CRKs are not directly activated by Ca^{2+}(Furumoto, Ogawa et al. 1996). It is assumed that they contain binding sites which activate the kinase. In contrast to the other Ca^{2+} dependent protein kinases SnRK3 kinases do not act on calmodulin, they recognize the Calcineurin B-Like Ca^{2+}-binding protein (CBL) in its Ca^{2+} bound form.

The protein structure of a CDPK consists of well-defined domains including N- and C-terminal variable region, a kinase domain, an auto-inhibitory domain and a calmodulin domain with EF hands as Ca^{2+} binding sites (Harmon, Gribskov et al. 2000)(figure 1.3 and 1.4). The amino acid sequences of the N-terminal and C-terminal domains have the highest variations and it is known that these domains affect the subcellular localisation (Dammann, Ichida et al. 2003). Most of the CDPK N-terminal sequences contain consensus motifs for myristoylation and palmitoylation, which are important for subcellular localisation.

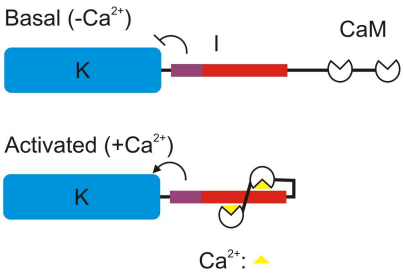

Figure 1.4: The activation mechanism of CDPKs (Harmon, Gribskov et al. 2000). Under low calcium condition the kinase catalytic domain is repressed by the binding to the auto-inhibitory domain. In the presence of Ca^{2+} the calmodulin domain changes the conformation by the binding of Ca^{2+} to the EF hands. The conformational change of the calmodulin removes the auto-inhibitory domain from the kinase catalytic domain and activates the kinase.

Known targets of calcium dependent protein kinases

Protein phosphorylation requires the physical contact between the protein kinase and the target protein substrate for the transfer of the phosphate group. The specificity for this interaction and phosphorylation depends on binding and phosphorylation motifs on the substrate protein and is in general different for each protein kinase family. The fact that a certain protein kinase can strongly interact with its substrate was used in Yeast-Two-Hybrid assays to identify substrates of kinases (Mizoguchi, Ichimura et al. 1998; Rodriguez Milla, Uno et al. 2006). But it is not said that a protein which interacts with a protein kinase is also phosphorylated by them, because this depends also on the appropriate consensus sequence in the protein substrate. For that reason interaction between a protein kinase and a prospective substrate has to be confirmed by phosphorylation assays. Besides these facts, the interaction and the phosphorylation is not a final evidence for a relevant connection between the protein kinase and the substrate. It is also necessary to show that both are co-expressed in the same cell, localized in the same cellular compartment and have biological relevance.

All these experimental methods were used to identify substrates for CDPKs in the past but for most of them at least one line evidence was missing. First experiments with Ca^{2+} dependent protein kinases were done with soluble proteins and with in-vitro phosphorylation assays. It was shown that the phosphorylation of the leaf nitrate reductase (NR) from spinach by a CDPK down-regulates the enzymatic activity of NR. The phosphorylation of the spinach NR on serine-543 is Ca^{2+} dependent and leads to the binding of a 14-3-3 protein to the phosphorylated loop in the NR protein, which shuts down its enzymatic activity (Bachmann, Shiraishi et al. 1996; Moorhead, Douglas et al. 1996; Douglas, Moorhead et al. 1998; MacKintosh 1998). Sucrose phosphate synthase

(SPS) is a soluble enzyme, which catalyzes sucrose synthesis in source leaves and responds rapidly to extracellular changes like altered light intensities or osmotic stress. A fast regulatory mechanism is necessary to adopt the enzymatic activity to environmental conditions. One important switching mechanism is protein phosphorylation (Huber and Huber 1996). Two phosphorylation sites at serine-158 and serine-424 were identified in spinach SPS. The phosphorylation at serine-158 occurs in response to changing light conditions and is catalyzed by a calcium independent stress induced kinase (McMichael, Klein et al. 1993). On the other hand, when SPS is phosphorylated at serine-424 in spinach by a CDPK under osmotic stress conditions, serine-424 phosphorylation leads to increased SPS activity (Toroser and Huber 1997). Similar to NR, SPS binds to 14-3-3 proteins after phosphorylation (Moorhead, Douglas et al. 1999). Sucrose synthase (SuSy), which is only found in plants and cyanobacteria, has a dual role in producing both UDP-glucose and ADP- glucose. Because of that it is necessary for cell wall, glycoprotein and starch biosynthesis. SySy was detected in the cytosolic and plasma membrane fractions (Amor, Haigler et al. 1995; Carlson and Chourey 1996; Sturm, Lienhard et al. 1999). A partially purified CDPK from maize leaves and soybean nodules was identified as kinase which phosphorylates SuSy (Huber, Huber et al. 1996; Zhang and Chollet 1997). This phosphorylation leads to a higher enzymatic activity of SuSy (Huber, Huber et al. 1996). The phosphorylation of the amino terminal part of SuSy affects the membrane association (Hardin, Winter et al. 2004). The actin-depolymerising factor ADF3 is phosphorylated by CDPK (Smertenko, Jiang et al. 1998; Allwood, Smertenko et al. 2001).

There is also increasing evidence for transcription factors as targets of CDPKs: In a Yeast-Two-Hybrid screen, using *Arabidopsis* CPK11 as bait, the nuclear zinc finger protein AtDi19 was identified and further confirmed as CPK11 interacting protein by a pull-down assay. It was also shown that CPK11 phosphorylates AtDi19 and both proteins co-localize *in vivo* (Rodriguez Milla, Uno et al. 2006). However, functional evidence for this interaction could not be provided by the authors. The dimerization and the DNA-binding affinity of the wheat basic/leucine zipper (bZIP) histone DNA binding protein (HBP-1a) is modulated by the phosphorylation of a CDPK (Tabata, Takase et al. 1989; Meshi, Moda et al. 1998). The ABA induced bZIP transcription factor ABRE Binding Factor (ABF4) from *Arabidopsis* interacts with CPK32 in the yeast two hybrid system and is dependent on the phosphorylation of serine-110 of ABF4 (Choi, Park et al. 2005).

Most of the CDPKs are believed to be associated with membranes because of their N-terminal myristoylation and palmitoylation, which is increasing the hydrophobicity of their N-terminal domains. This localisation also explains the great number of identified targets or substrates of CDPKs, which are associated with membranes. Phosphorylation of the potato NADPH oxidase Respiratory Burst Oxidase Homolog (RBOH) by St CPK5 is associated with the production of reactive oxygen species (ROS) (Kobayashi, Ohura et al. 2007). ACA2 is an ER localized calmodulin-dependent Ca^{2+} pump which was shown to be phosphorylated by a CDPK, resulting an inhibition of its Ca^{2+} pump, activity (Hwang, Sze et al. 2000). Furthermore, the nodulation specific trans-membrane channel protein nodulin 26 from soybean which is a member of the major intrinsic proteins (MIPs) is phosphorylated by a CDPK. The phosphorylation of nodulin 26 increases the permeability for water and is responsible for the voltage-sensitivity of this channel protein. (Weaver, Crombie et al. 1991; Weaver, Shomer et al. 1994; Lee, Zhang et al. 1995). Also the voltage dependent potassium channel 1 (KAT1) from *vicia faba*, which is localized to the plasma membrane is phosphorylated by a CDPK (Li, Lee et al. 1998). This is true as well for the seed specific protein vacuolar membrane protein α-TIP, a member of the Major Intrinsic Protein family (Johnson and Chrispeels 1992; Maurel, Kado et al. 1995).

1.2 Myristoylation and palmitoylation

Post-translational and co-translation modifications of proteins can alter their biochemical properties, such as localisation, stability, enzymatic activity and interaction with other proteins or molecular targets.

Myristoylation is an irreversible co-translational modification which occurs during translation on the nascent chain. It starts with the removal of the first amino acid methionine from the nascent chain by the methionine amino peptidase. This is followed by the attachment of myristic acid on the second amino acid by the N-myristoyl-CoA:protein myristoyltransferase (NMT). Myristoylation requires a glycine on the second amino acid and a consensus motif, which is recognized by the NMT. The attachment of the fatty acid myristic acid increases the hydrophobicity of the protein affecting the localisation (Resh 1999).

Palmitoylation is a similar protein modification, but the difference is that it takes place after translation and it is therefore a post-translational modification. Palmitic acid is also a fatty acid and it is attached to cysteine by palmitoyl acyl transferases. In contrast to

myristoylation, palmitoylation is reversible. Much less is known about the process of palmitoylation but it must be assumed that it occurs on membranes because proteins with increased hydrophobicity are more frequently palmitoylated (Resh 1999). For that reason it is believed that the increased hydrophobicity caused by myristoylation favours palmitoylation and therefore a strong membrane attachment.

Sequence comparison of the 34 known CDPKs from *Arabidopsis* reveals that 29 of them contain a glycine on the second amino acid and 28 out of the 29 CDPKs have also one or more cysteines in their N-terminus. There is only one CDPK (CPK3), possessing only a glycine on position two without cysteines in its neighbourhood. Interestingly, CDPKs without a glycine on position two do also lack cysteines and it is assumed that these CDPKs are not associated with membranes (Resh 1999; Dammann, Ichida et al. 2003). Thus the co-appearance of N-myristoylation and palmitoylation motifs could be taken as evidence for N-myristoylation and palmitoylation of these CDPKs because CDPKs without myristoylation do not contain a palmitoylation motif. It seems that both modifications are important for the appropriate cellular localisation of the CDPK, which will also be shown in this work.

1.3 Aim of this work

The aim of this work was to gain insights into the role of CDPKs in the context of cellular function and salt stress response by using knock-out and over-expression mutants of CPK3. It was found that *cpk3* mutants are salt sensitive and CPK3 overexpressor plants showed increased salt tolerance. This effect could not be explained by changes in transcription of typical stress-induced genes, and showed furthermore no interference with MAP kinase signaling. To investigate if protein phosphorylation is affected in response to salt stress in an unbiased approach, a 2D-gel separation of protein extracts from wild type and *cpk3* mutants was performed. Moreover, the endogenous localisation of CPK3 was determined to understand the molecular mechanism how CPK3 regulated the salt stress response in *Arabidopsis*. To test the effect of N-terminal myristoylation of CPK3, it was transiently expressed as YFP-fusion protein in tobacco leaves. Finally, recombinantly expressed, CPK3 was used for phosphorylation of isolated microsomal membranes proteins to identify phosphorylated targets by MS.

2 Material and methods

2.1 Buffer and Media

TAE: 40 mM Tris acetate, 1 mM Na_2EDTA, pH 8.0

Resuspension buffer P1: 50 mM TrisCl pH 8.0, 10 mM Na_2EDTA (including 100 µg/ml RNase)

Lysis buffer P2: 200 mM NaOH, 1% SDS

Neutralisation buffer P3: 3 M potassium acetate pH 5.5

TE: 10 mM TrisCl pH 8.0, 1 mM Na_2EDTA

CTAB buffer: 2% CTAB; 100 mM TrisCl (pH 8.0); 20 mM EDTA; 1.4M NaCl; 1% polyvinylpyrrolidone

REX buffer: 1% SDS; 10 mM Na_2EDTA

Phenol extraction buffer 1: 1% SDS; 1x TE

Phenol extraction buffer 2: 0.7 M sucrose; 0.1 M KCl; 0.5 M TrisCl, pH 7.5; 50 mM EDTA

Coomassie staining solution: 2.5 g Coomasie R250/G250 (4:1); 10% isopropanol; 10% acidic acid

Coomassie de-staining solution: 10% isopropanol; 10% acidic acid

SDS-PAGE equilibration buffer: 75 mM TrisCl at pH 8.8; 6 M urea; 2% SDS; 0.002% bromophenol blue

Anode buffer 1: 300 mM TrisCl pH 10.4; 10% methanol

Anode buffer 2: 25 mM TrisCl 10.4; 10% methanol

Cathode buffer: 25 mM Tris base pH 9.4; 40 mM glycine; 10% methanol

TBS-T: 50 mM TrisCl pH 7.4; 150 mM NaCl; 0,1% Tween

IP buffer: 25 mM TrisCl pH 7.5; 15 mM $MgCl_2$; 15 mM EGTA; 75 mM NaCl; 1 mM DTT; 0.1% Nonidet P-40; 15 mM beta-glycerophosphate; 0.5 mM Na_3VO_3; 1 mM NaF; 1 x Complete-EDTA free from Roche (per 50 ml)

IP wash buffer: 50 mM TrisCl pH 7.5; 5 mM EGTA; 5 mM EDTA; 0.1% Tween 20; 0.1% Nonidet P-40; 250 mM NaCl; 5 mM NaF; 1 x Complete-EDTA free from Roche (per 50 ml)

IP kinase buffer: 20 mM HEPES pH 7.5; 15 mM $MgCl_2$; 5 mM EGTA; 1 mM DTT

SDS-PAGE sample buffer: 125 mM TrisCl pH 6,8; 2% SDS; 50% glycerine; 5% β-mercaptoethanol; 0,01% bromphenole blue

Kinase buffer (without and with Ca^{2+}): 20 mM HEPES pH 7.5; 15 mM $MgCl_2$; 5 mM EGTA or 100 µM Ca^{2+}; 1 mM DTT

Homogenization buffer: 400 mM sorbitol; 40 mM HEPES KOH pH 7.0; 10 mM $Na_4P_2O_7$; 2.5 mM $MgCl_2$; 2 mM ascorbate

pTWIN buffer B1: 20 mM Na-HEPES pH 8.5; 1000 mM NaCl; 1 mM EDTA; 0.2 mM β-mercaptoethanol

pTWIN buffer B2: 20 mM Na-HEPES pH 7.0; 500 mM NaCl; 1 mM EDTA; 0.2 mM β-mercaptoethanol

Hoagland medium: 4.03 mM $Ca(NO_3)_2$ $4H_2O$; 0.522 mM $NH_4H_2PO_4$; 6.04 mM KNO_3; 1.99 mM $MgSO_4$ $7H_2O$; 0.125 mM NaOH; 89.6 µM EDTA; 89.6 µM $FeSO_4$ $7H_2O$; 9.68 µM H_3BO_3; 2.03 µM $MnCl_2$ $4H_2O$; 0.314 µM $ZnSO_4.7H_2O$; 0.21 µM $CuSO_4$ $5H_2O$; 13.9 nM MoO_3; 8.59 nM $Co(NO_3)_2$ $6H_2O$

LB: 1% peptone; 0,5% yeast-extract; 1% NaCl

YEB: 0.5% tryptone; 0.1% yeast extract; 0.5% peptone; 0,5 % Sucrose; 2 mM $MgSO_4$

Induction media: 1.38 % K_2HPO_4 $3H_2O$; 0.45% KH_2PO_4; 0.213% MES H_2O pH 5.6; 0.1% $(NH_4)_2SO_4$; 0,0246% $MgSO_4$ $7H_2O$; 0.2% Glucose; 150 µM Acetosyringone

***Arabidopsis thaliana* suspension media**: 0.5 x MS; 3 % sucrose; 1 µg/ml 2,4-D

TB Buffer: 10 mM $CaCl_2$; 10 mM PIPES-NaOH pH6.7; 15 mM KCl; 55 mM $MnCl_2$

S.C. Trafo solution (filter sterilized): 40% PEG (4.000); 200 mM LiAcetate; 100 mM DTT

2.2 Bacteria and yeast strains

***Escherichia coli* strain used for cloning:**

DH5α F-, ø80dlacZDM15, D(lacZYA-argF)U169, deoR, recA1, endA1, sdR17(rk,mk+), phoA, supE44, l-, thi-1, gyrA96, relA1

***Escherichia coli* strain used for protein expression:**

ER2566 fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10--Tet^S)2 [dcm] R(zgb-210::Tn10--Tet^S) endA1 Δ(mcrC-mrr)114::IS10

***Saccharomyces cerevisae* strain used for complementation:**

AXT3K Δena1::HIS3::ena4, Δnha1::LEU2, Δnhx1::KanMX4

***Agrobacterium* strain used for transient expression in tobacco epidermal cells and transformation of Arabidopsis thaliana:**

AGL1 AGL0 (C58 pTiBo542) recA::bla, T-region deleted Mop(+) Cb(R)

2.3 Plant lines

Col-0 Wild type ecotype Col-0

cpk3 T-DNA insertion line cpk3-2 KanR, SALK_02286246 (from NASC)

35S:CPK3-1 35S:CPK3-YFP KanR over expressing line in Col-0 background

35S:CPK3-2 35S:CPK3-YFP KanR over expressing line in Col-0 background

2.4 Antibodies

αCPK3 Affinity purified antibody from rabbit against the last 15 amino acids of CPK3 (mkkgnpelvpnrrrm)

αpThr Commercial antibody (Cell signaling: #9381) from rabbit against phosphorylated threonines

αGFP Commercial antibody (Roche: 11814460001) from mouse against phosphorylated threonines

αMPK4 Published antibody from rabbit against MPK4 (Teige, Scheikl et al. 2004)

αMPK6 Published antibody from rabbit against MPK6 (Teige, Scheikl et al. 2004)

2.5 Isotopes

L-[^{35}S] methionine: 1175 Ci/mmol^{-1} (Perkin Elmer)

[9, 10-^{3}H]-myristic acid: 60 Ci/mmol^{-1}(American Radiolabeled Chemicals)

[γ^{32}P]ATP: 6000 Ci/mmol^{-1} (Perkin Elmer)

2.6 Oliogonucleotides

Cloning and point-mutagenesis oligonucleotides		
Name	Sequence (from 5’ to 3’)	ID (TAIR)
CPK2nt	AGGGCCCATGGGTAATGCTTGCGTTGG	At3g10660
CPK2ct	AGCGGCCGCAATGTTTCAGAGAAATGCTAA	
CPK2G2Ant	TTGGGCCCATGGCTAATGCTTGCGTTGGA	
CPK3nt	AAAAGGATCCGGGCCCATGGGCCACAGACACAGCAAGTCCAAA TCCTCCG	At4g23650
CPK3ct	TTTTGTCGACCTAGCGGCCGCACATTCTGCGTCGGTTTGGCAC CAATTCTGGATTTCCC	
CPK3G2A-1	GCTGTGTCTGTGGGCGTAGGGCCCGGATCC	
CPK3G2A-2	GGGCCCATGGCCCACAGACACAGCAAGTCC	
CPK3K107R-1	ACAGGTCGCATGCCGGTCAATCCCTACGCG	
CPK3K107R-2	GGATTGACCGGCATGCGACCTGTTGTTTCG	
CPK3 F358A-1	CAGGATGAAACAAGCCCGGGCGATGAAC	
CPK3 F358A-2	CATCGCCCGGGCTTGTTTCATCCTGG	
CPK3 S242A-1	GGATCTTGTTGGCGCCGCATACTATGTTGCCCC	
CPK3 S242A-2	GTATGCGGCGCCAACAAGATCCTTAAACTTATCACC	
CPK3-S242D-1	TCTTGTTGGAGATGCATACTATGTTGCCCCAGA	
CPK3-S242D-2	GGGGCAACATAGTATGCATCTCCAACAAGATCC	
CPK4nt	TTGGATCCATGGAGAAACCAAACCCTAGAAGACCC	At4g09570
CPK4ct	TTGCGGCCGCGTGAATCATCAGATTTAGCAGTGCTGC	
CPK5nt	GGGCCCATGGGCAATTCTTGCCGTGGATCT	At4g35310
CPK5ct	GCGGCCGCACGCGTCTCTCATGCTAATGT	
CPK6nt	TTGGGCCCATGGGCAATTCATGTCGTGGTTCT	At2g17290
CPK6ct	AGCGGCCGCACACATCTCTCATGCTGATGT	
CPK6G2Ant	TTGGGCCCATGGCCAATTCATGTCGTGGTTCT	
CPK9nt	AAAGGATCCGGGCCCATGGGAAATTGTTTTGCCAAGAATCATG GATTG	At3g20410
CPK9ct	TTTGCGGCCGCCGAACAGCCGAGGTTGTTGTTGTTGTGG	
CPK9G2Ant	CCGGGCCCATGGCCAATTGTTTTGCCAAGAAT	
CPK11nt	GGGCCCATGGAGACGAAGCCAAACCCTAGA	At1g35670
CPK11ct	GCGGCCGCAGTCATCAGATTTTTCACCAT	
CPK12nt	GGGCCCATGGCGAACAAACCAAGAAC	At5g23580
CPK12ct	GCGGCCGCAGACATTCATAGACTCATCAG	
CPK13nt	TTGGGCCCATGGGAAACTGTTGCAGATC	At3g51850
CPK13ct	AGCGGCCGCATTCGTTGCCTAGGTTCAAAG	
CPK13G2Ant	TTGGGCCCATGGCCAACTGTTGCAGATC	
CPK16nt	TTGGATCCGGGCCCATGGGTCTCTGTTTCTCCTCCGCCGCC	At2g17890
CPK16ct	TTGCGGCCGCCCTTGCGAGAAATAAGAT	
CPK16G2Ant	CCGGGCCCATGGCCCTCTGTTTCTCCTCCGCC	
CPK16C4F-1	GGTCTCTTCTTCTCCTCGGCCGCCAAATCCTCCGGCCACAAC	
CPK16C4F-2	GTGGCCGGAGGATTTGGCGGCCGAGGAGAAGAAGAGACCCAT	
CPK17nt	AAGGGCCCATGGGAAATTGTTGCTCTCACGG	At5g12180
CPK17ct	TTGCGGCCGCGGAATGAAAGTTCACGCCGCTTCTTTGGG	
CPK17G2Ant	AAGGGCCCATGGCCAATTGTTGCTCTCACGG	
CPK24nt	TTTGGGCCCATGGGAAGTTGTGTTTCGTCGCCATTGAAAGGC	At2g31500
CPK24ct	AAGCGGCCGCTTAGACCTGAGGGTTTATAGGTTTTGGAGATCT GAAGC	
CPK24G2Ant	AGGGCCCATGGCCAGTTGTGTTTCGTCGCCATT	
CPK28nt	AAGGGCCCATGGGTGTCTGTTTCTCCGCC	At5g66210
CPK28ct	TTGCGGCCGCGAAGATTCCTGTGACCTGCAGGGC	
CPK28G2Ant	AAGGGCCCATGGCCGTCTGTTTCTCCGCC	

CPK29nt	TAGGGCCCATGCTTCAAAACCAACATAA	At1g76040
CPK29ct	TGGCGGCCGCATCTGATCAGCTTTGGATCTG	
KCO1nt	GGGCCCATGTCGAGTGATGCAGCTCG	At5g55630
KCO1ct	GCGGCCGCTCCTTTGAATCTGAGACGTGG	
KCO1Ntermct	GCGGCCGCCGATCACTCGCCTGAGATTCG	

RT PCR oligonucleotides		
Name	Sequence (from 5' to 3')	ID (TAIR)
ERF6-1	CCGTTGCCTACTACTGCCACC	At4g17490
ERF6-2	GCACTTTCTCAACCACCGTC	
ACS6-1	GAGCGGCGGCGCAACCGGAG	At4g11280
ACS6-2	CCACCCTGTCATTGTAAGAG	
GolS2-1	AAGGCTGTGTCGTGCGTGAG	At1g60470
GolS2-2	GGCTTGGATCCAGCTGCACAG	
STZ1-1	ATGGCGCTCGAGGCTCTTAC	At1g27730
STZ1-2	TCCTTCGTAGTGGCACCGC	
P5CS2-1	CGTCGTCAAGGTTGGGACTGC	At2g39800
P5CS2-2	TCTAGCGACAGAAGAGCGGC	
ERD10-1	TCTTCCTCTTCGAGTGATGAAG	At1g20450
ERD10-2	TCTCTTCCTCTCCAGTGG	
RD20-1	CCAAAACCATACATGGCAAGAGC	At2g33380
RD20-2	TGAAAGCCATCCAAAAGGATCG	
RD29a-1	AGCACCCAGAAGAAGTTGAACATC	At5g52310
RD29a-2	CGTTACATCCTCTGTTCCAG	
ACT3-1	ATGGTTAAGGCTGGTTTTGC	At2g37620
ACT3-2	AGCACAATACCGGTAGTACG	
CPK3i-1	AGATGTTCGCCGTGAAGTCC	At4g23650
CPK3i-2	ACGGATGATTTAGCACTTCCG	

2.7 DNA Methods

Agarose gel electrophoresis

0.7% - 2.0% agarose gels were prepared according to the size of the DNA molecules to be separated. TAE was used as running buffer and for preparation of agarose gels including 0.25 µg/ml ethidium bromide. The DNA was applied in DNA loading buffer to estimate fragment sizes. The GeneRuler™ 1kb DNA Ladder Plus from Fermentas was applied as well. Gels were run at 100-130 V. The DNA fragments were visualized under UV light.

Plasmid DNA mini preparation from *E. coli*

2 ml over-night-culture was centrifuged for 2 min at 16100 g and the supernatant was removed. The pellet was resuspended in 200 µl of resuspension buffer P1 followed by addition of lysis buffer P2 to lyse the cells. To mix the suspension, the tubes were inverted three times and incubated for 5 min at room temperature. The *E. coli* lysate was neutralized by the addition of 200 µl of neutralisation buffer P3 and mixed by inverting three times. After 20 min incubation at 4°C the suspension was centrifuged for 10 min at 16100 g. To precipitate the DNA the supernatant was mixed with 0.7 times isopropanol and incubated at -20°C for 20 min followed by an centrifugation step at 16100 g at 4°C. The supernatant was removed and the pellet was washed with 500 µl 70% EtOH. The pellet was dried in the speedvac and resolved in 50µl 0,5x TE.

Plasmid DNA midi preparation from *E. coli*

The midi preparation of plasmid DNA from *E. coli* was done with the Jetstar Midiprep II kit (GENOMED GmbH) in accordance with to the manufacturer´s instructions.

Isolation of genomic DNA from Arabidopsis thaliana

50-150 mg plant material was grinded in 200 µl CTAB buffer with a spatula tip of polyvinylpyrrolidone and sand until a fine suspension was generated. This suspension

was mixed with additional 200 µl of CTAB buffer and heated up to 65°C for 60 min. One volume of chloroform was added to the suspension, the mixture was vortexed and then centrifuged for 10 min. The supernatant was precipitated with 0.7 volume of isopropanol for 20 min at -20°C and centrifuged at 16100 g for 10 min. The pellet was washed with 500 µl 70% EtOH and dried in the speedvac. The pellet was resolved in 150 µl P1 with RNase A and incubated at 37 °C for 30 min. After the RNase A treatment the solution was well mixed with one volume of chloroform and centrifuged for 10 min. The supernatant was precipitated with 0.7 volume of isopropanol for 20 min at -20°C and centrifuged at 16100 g for 10 min. The pellet was washed with 500 µl 70% EtOH and dried in the speedvac. The pellet was resolved in 50 µl ddH_20.

Polymerase chain reaction

PCR reactions were carried out according to the manufacturer´s instructions for the used polymerase. The following polymerases were used: GoTaq from Promega, Vent from NEB and Turbo Pfu Stratagene.

Ligation of DNA

DNA fragments were cut out from an agraose and purified with the Wizard® SV Gel and PCR Clean-Up System from Promega, according to the manufacturer´s instructions. A total volume of 10 µl including ligase buffer 100 U ligase from NEB, 100-200 ng insert and 25-50 ng vector DNA fragments was ligated at room temperature for 20 min. The reaction was directly used for transformation into *E. coli*.

Analytical digestion of plasmid DNA

1.5 µg plasmid DNA was digested with the appropriate restriction endonuclease from NEB in a total volume of 20 µl, according to the manufacturer´s instructions. After digestion the DNA was separated by Agraose gel electrophoresis.

Preparative digestion of plasmid DNA

3 µg plasmid DNA was digested with the appropriate restriction endonuclease from NEB in a total volume of 40 µl, according to the manufacturer´s instructions. After

digestion the DNA was separated by Agraose gel electrophoresis. The appropriate bands were cut out and purified with the Wizard® SV Gel and PCR Clean-Up System from Promega.

Plasmid DNA vectors and cloning

pBAT: in-vitro transcription and translation vector with rabbit 3-globin leader (Annweiler, Hipskind et al. 1991).

pGEX4T1: IPTG-inducible *E. coli* vector for the expression of N-terminal GST-fusion proteins from Amersham-Pharmacia.

pTwin1: IPTG-inducible *E. coli* vector for the expression of N- and/or C-terminally intein-chitin tagged proteins from NEB.

pBIN19: binary plant transformation vector (Bevan 1984).

pSKII+: blue white selectable *E. coli* vector from Stratagene.

pTLT: CMV 35S promoter based plant expression vector for protoplast transformation and sub cloning into pBIN19. This vector was created in this work.

YEPlac195: yeast cloning vector with the yeast ura selection marker, blue white selectable *E. coli* multi cloning site and a 2µ origin of replication.

RNA isolation from Arabidopsis thaliana

10-14 day old Arabidopsis thaliana seedlings were frozen in liquid nitrogen and grinded to a fine powder. In a 1.5 ml Eppendorf-tube 100-200 mg of the plant material were mixed with 130 µl phenol pH 4.0 and 130 µl REX buffer. The mixture was grinded with a pre-cooled glass-rod Eppendorf homogenizer in the presence of 50 µl sea sand until a fine suspension was generated. 400 µl REX buffer and 400 µl phenol pH 4.0 were added, vortexed and centrifuged for 10 min at 16100 g. The aqueous upper phase was extracted twice with one volume of PCI and once with one volume of chloroform. The supernatant was mixed with 1/3 volume of 10 M LiCl and incubated over night at 4°C. To precipitate the RNA the mixture was centrifuged at 16100 g at 4°C for 20 min. The pellet was washed once with 2.5 M LiCl and two times with 80% EtOH. The RNA pellet was resolved in 25 µl H_2O and the concentration was determined by measuring at 260 nm and 280 nm.

Reverse transcription RT-PCR

Reverse transcription was carried out using the Promega M-MLV reverse Transcriptase, RNase H Minus, Point Mutant, according to the manufacturer's instructions. In a final volume of 14 µl 1 µg total RNA and 0.5 µg oligo(dT)$_{15}$ were mixed and heated to 70°C for 5 min, then cooled quickly on ice for 5 min followed by the addition of 5 µl M-MLV RT 5X Reaction Buffer, 1.25 µl 10 mM dNTP mix and 1µl M-MLV RT (H-). The reaction was mixed and incubated at 40°C for 60 min. 1-2 µl reverse transcripted cDNA was used for PCR amplification using the GoTaq from Promega.

2.8 Protein methods

Phenol protein extraction from Arabidopsis

2 g of plant material were grinded in liquid nitrogen. A second grinding in phenol extraction buffer 1 followed. The suspension was centrifuged at 12000 g and the supernatant was mixed with the equal amount of phenol pH 7.4. The mixture was vortexed for 1 min and centrifuged at 3500 g for 10 min at 4°C. The supernatant consisting of aqueous phase was replaced with the same volume of phenol extraction buffer 2, vortexed for 1 min and centrifuged at 3500 g for 10 min at 4°C. The supernatant consisting of the organic phenol phase was mixed with the same volume of phenol extraction buffer 2 and vortexed for 1 min. The mixture was centrifuged at 3500 g for 10 min at 4°C. The supernatant consisting of the organic phenol phase was mixed with 5 times methanol including 100 mM ammonium acetate. To precipitate the proteins the suspension was incubated over night at -20°C and centrifuged at 16100 g for 10 min. The supernatant was removed and the pellet was washed one time with 100% MetOH and 2 times with 100% acetone. The pellet was dried in the Speedvac and resolved in the appropriate buffer (Isaacson, Damasceno et al. 2006).

SDS-PAGE

Protein molecular weight determination was done by electrophoresis using 12% and 8-15% gradient polyacrylamide gels using Mini Protean 3 from BIORAD and Multigel-Long from Whatman Biometra. As running buffer a solution of 25 mM Tris, 250 mM

glycine and 0,1% SDS was used (Fling and Gregerson 1986). The electrophoresis was performed, according to the manufacturer's instructions. For determination of the molecular weight the PageRulerTM Prestained Protein Ladder Plus from Fermentas was used.

Coomassie staining

After electrophoresis, the SDS-PAGE gel was placed in Coomassie staining solution and was heated up to ~ 60°C and incubated under shaking for 15 min. When the gel turned blue the Coomassie staining solution was replaced by the Coomassie de-staining solution heated up to ~60°C and incubated under shaking for 15 min. The last step was repeated until the gel was completely de-stained.

Silver stain

All incubation steps were done with light shaking. The SDS-PAGE was incubated in 50% methanol and 5% acidic acid for 20 min. After 10 min washing in 50% methanol the gel was rinsed for 2 h in H_20. The gel was sensitized for one min in 0.03% $Na_2S_2O_3$, rinsed two times in water and incubated in 0.1% AgNO3 for 20 min at 4°C. After two washing steps in H_2O for one min, the developing was started by application of 2% Na_2CO_3 and 0.00014% Formaldehyde until the protein bands turned to dark. The reaction was stopped by replacing the staining solution with 5% acetic acid. (Shevchenko, Wilm et al. 1996)

2D-Gel electrophoresis

The protein was purified as described in phenol protein extraction from Arabidopsis. 200 µl 2d-gel sample containing 100 µg, 7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer (pH 3-11) NL and 0.002% bromophenol blue was applied on a 11 cm Immobiline DryStrip (pH 3-11) NL from GE Healthcare. The proteins were focused using an IPGphor from GE Healthcare according to the manufacturer's instructions. After isoelectric focusing, the IPG strip was equilibrated for 15 min in SDS-PAGE equilibration buffer with 100 mg DTT. Then the strip was incubated for additional 15

min in SDS-PAGE equilibration buffer with 250 mg iodoacetamide. The separation in the second dimension was carried out using a 8-15% SDS-PAGE.

Western blot

Transfer of proteins on PVDF membranes was done with the Tran-Blot® Semi-Dry blotting chamber from Biorad. Protein samples were separated by SDS-PAGE. After electrophoresis, the gel was incubated in cathode buffer under light shaking for 15 min. The PVDF membrane was a short time equilibrated in methanol for 15 sec, rinsed with H_2O for 2 min and incubated in anode buffer 1 under light shaking for additional 5 min. Two layers of Whatman papers soaked in anode buffer 1 were placed on the metal anode of the blot-chamber followed by one layer of Whatman paper soaked in anode buffer 2. On top of the Whatman paper the membrane, the gel and three layers of Whatman papers soaked in cathode buffer were placed. The blot-chamber was closed by pressing the metal cathode on top of the last Whatman layers. The transfer was performed for 90 min at 1.8 mA/cm^2. The blotting efficiency was verified by staining the proteins on the membrane with 0.1% Ponceau S in 5% acetic acid and washing them with H_2O. The Ponceau S was removed by washing the membrane with TBS-T. After blotting, the membrane was blocked in TBS-T with 5% fat free dry-milk and incubated on a horizontal shaker for 30 min. The blocking solution was replaced with the primary antibody diluted in TBS-T with 5% fat free dry-milk and incubated for 2 h at room temperature or over night at 4°C under light shaking. To eliminate unspecific bound antibodies the membrane was washed 3 times for 10 min with TBS-T. The membrane was incubated with the secondary antibody coupled to HRP diluted in TBS-T with 5% fat free dry-milk for 45 min. To get rid of unspecific bound secondary antibodies the membrane was washed 3 times for 10 min with TBS-T. For detection of the secondary antibodies, the SuperSignal® WestPico Chemiluminescent Substrate from PIERCE was used, according to the manufacturer´s instructions.

Kinase assay of immunoprecipitated proteins

About 150-250 mg Arabidopsis seedlings were grinded in IP buffer and clarified by centrifugation at 16100 g for 10 min at 4°C. After determination of the protein content 100 µg were incubated with 10 µl protein A-Sepharose. The clarified supernatant was

immunoprecipitated for 1 hour with 20 µl of protein A-Sepharose and 3 µl antibody at 4°C. The beads were washed three times with IP buffer, once with IP wash buffer and finally once with IP kinase buffer. Kinase reaction was started by adding to the beads 20 µl IP kinase buffer including 2 µg MBP and 2 µCi γ-32ATP. The reaction was terminated after 30 min at room temperature by adding the SDS-PAGE sample buffer. The sample was analyzed by SDS-PAGE. The incorporation of radioactivity was measured by exposing the dry gel over night on a storage phosphor screen which was scanned in a GE Healthcare Typhoon 8600 Variable Mode Imager. (Bogre, Calderini et al. 1999)

Kinase assay of recombinant proteins

Kinase assay was performed by incubating about 1 µg recombinant protein kinase, 3-10 µg substrate (histone S3, N-Terminus of Kco1 and microsomal membranes) and 2 µCi γ-^{32}ATP in kinase buffer without and with Ca^{2+}. The amount of kinase and substrate was 10 times higher for 2-D gel electrophoresis. After incubation at room temperature for 30 min the reaction was terminated by adding SDS-PAGE loading buffer. The sample was analyzed by SDS-PAGE. The incorporation of radioactivity was measured by exposing the dry gel over night on a storage phosphor screen that was scanned in a GE Healthcare Typhoon 8600 Variable Mode Imager.

Isolation of microsomal membranes form Arabidopsis thaliana

10 g of *Arabidopsis thaliana* suspension culture were first grinded in liquid nitrogen and then homogenised in 30 ml homogenisation buffer including Complete-EDTA. The suspension was filtered through 2 layers of Miracloth and centrifuged at 500 g for 5 min at 4°C. To remove large insoluble cell compartments the supernatant was centrifuged at 10000 g for 15 min. The microsomal membranes were precipitated by ultra centrifugation at 100000 g for 90 min at 4°C. The pellet was washed once with homogenisation buffer without Complete-EDTA free and stored at -80°C until it was used.

Protein purification using the pTWIN protein purification system

The isolation of recombinant proteins was done using the IMPACT™-pTWIN protein purification system from NEB, according to the manufacturer's instructions. All CDPKs were expressed as N-terminal fusion. An over night culture of *E. coli* (ER2566) carrying the pTWIN expression plasmid was inoculated in 200 ml LB-ampicillin and adjusted to OD_{600} of 0.2. The culture was incubated at 37°C under vigorous shaking until OD_{600} of 0.6 was reached. Then the expression was induced by the application of 100 µl of 1 M IPTG. The best expression was achieved by incubating the culture at 16°C over night under vigorous shaking. *E. coli* were harvested by centrifugation for 10 min at 2900 g at 4°C and resuspended in 10 ml pre-cooled pTWIN buffer B1. To break the cells the suspension was sonificated and clarified by centrifugation at 16000 g at 4°C for 20 min. All the following purification steps were performed at 4°C. The supernatant was applied to a column packed with 2 ml (bed volume) of chitin beads which were washed in pre-cooled pTWIN buffer B1. The column was washed with 20 ml of pre-cooled pTWIN buffer B1 and fast flushed with 4 ml pre-cooled pTWIN buffer B2. To induce protein cleavage the column was incubated on room temperature over night. After the elution with pTWIN buffer B2, the buffer was exchanged to kinase buffer (without Ca^{2+} and EGTA) and the protein was concentrated by ultrafiltration in an Amicon® Ultra-4 filter device from MILLIPORE.

Protein determination by the Bradford method

50 µl protein sample was mixed with 950 diluted Bradford (BIO-RAD Protein assay) reagent and measured at 595 nm. For determination of protein concentration a BSA calibration curve was made with the following protein concentrations: 1.0, 0.5, 0.25, 0.125, 0.0625 mg/ml.

In-vitro Myristoylation assay

Myristoylation assay was done by coupled in-vitro transcription/translation in a cell free system using the TNT Coupled Wheat Germ Extract System from Promega. 2 µg of pBAT-CPK plasmid DNA was linearized with NotI and in-vitro translated. The reactions were either carried out in the presence of 10 µCi of L-[^{35}S] methionine for total protein labelling, or 50 µCi of [9,10-^{3}H]-labelled myristic acid. Immediately before

starting the 90 min reaction at 30°C, the [3^{H}] myristic acid was dried in the speed-vac and resuspended in the reaction-mix by pipetting. The samples were separated by SDS-PAGE. After Coomassie staining the gel containing the ^{3}H labelled proteins was soaked in Amplify™ Fluorographic Reagent from Amersham for 30 min and dried in the gel-drier. Light emission was measured by incubating the dried gel with an x-ray-film for 5-14 days at -80°C. The uptake of L-[^{35}S] methionine was measured by exposing the dry gel over night on a storage phosphor screen. This was scanned in a GE Healthcare Typhoon 8600 Variable Mode Imager.

2.10 Plant methods

Vapor-phase sterilization of *Arabidopsis* seeds

Seeds were surface sterilized using the vapour-phase method published by (Clough and Bent 1998). Open Eppendorf tubes containing about 200 µl seeds were placed in a 10 l plastic box. Next to the Eppendorf tubes a 400 ml beaker was placed with 150 ml bleach (2.8 % NaClO). After the plastic box was placed in fume hood the generation of chlorine gas was started adding about 50 ml of 5 M HCl to the bleach and the lid was closed immediately. Seeds were sterilized at least for 2 h or over night.

Cultivation of *Arabidopsis thaliana*

Sterile cultivation of *Arabidopsis* plants (Col-0) was done on ½ MS agar plates or ½ MS agar plates, containing 50 µg/ml kanamycin under 16 h light with 80 µmol m^{-2} sec^{-1} light intensity at 25°C. Stratification was carried out prior putting the seeds into light for 2 days in the dark at 4°C. Non sterile cultivation of Arabidopsis was done by placing them on soil (10 parts Spezialblumenerde, Diwoky; 3 sand: Rasenquarz, Körnung 0.2-2.0 mm, Quarzwerke Österreich GmbH, Melk, Austria; one part perlite: Granuperl S3-6, #50140050, KNAUF Perlite GmbH, Vienna, Austria) or by sawing them directly.
Hydroponical cultivation of Arabidopsis plants was done according to Tocquin et al., 2003. Seeds were placed directly into 0.5 ml PCR tubes filled with ½ Hoagland medium with agar and grown for one week. The bottom of the PCR tube was cut off and the tube was placed into a 1 ml Gilson (Middleton, WI, USA) tip box filled with ½ Hoagland medium which was covered with aluminium foil. To maintain a high humidity the box

was covered with transparent foil for about 3 weeks. Then the cover was opened partly during one week and then entirely removed (Tocquin, Corbesier et al. 2003).
Soil and hydroponical cultured Arabidopsis were grown with an light intensity of 200 µmol m^{-2} sec^{-1} under short (8 h light / 16 h dark) or long day (16 h light / 8 h dark) condition.

Agrobacterium mediated expression in *Nicotiana tabacum* leafs

Transient expression of proteins in *Nicotiana tabacum* was done, according to the method described in (Bucher, Sijen et al. 2003). pBin19 35S: C-terminal YFP constructs were transformed via electroporation in *Agrobacterium* (AGL1) plated on LB media containing 50 µg/ml kanamycin. One clone was inoculated in liquid YEB containing 50 µg/ml kanamycin and incubated over night at 30°C under shaking. On the next day 50 ml of YEB containing 50 µg/ml kanamycin was inoculated with the pre-culture and adjusted to an OD_{600} of 0.05. Then the culture was incubated at 30°C under vigorous shaking until an OD_{600} of 0.2 was reached. The bacteria suspension was pelleted by centrifugation at 2900 g at room temperature for 10 min and resuspended in 25 ml induction media. After 2 h incubation at 30°C the cells were again centrifuged at 2900 g at room temperature for 10 min and resuspended in 10 ml of 5 % sucrose containing 300 µM acetosyringone. This suspension was used to infiltrate young *Nicotiana tabacum* leafs with a 1 ml syringe grown under Arabidopsis short day condition. Directly after infiltration plants were incubated in the dark over night and were placed back in the growth chamber on the following day. Two days after infiltration the expression of proteins could be detected.

Transformation of *Arabidopsis thaliana* (floral dip)

Stable transformation of *Arabidopsis thaliana* was performed by the floral dip method (Clough and Bent 1998) and (Zhang, Henriques et al. 2006) by using the *Agrobacterium* strain GV3101 (Bevan 1984) carrying pBIN19 plasmid. 250 ml *Agrobacterium* suspension was grown in LB under selective condition until an OD_{600} of 0.8 was reached. Then the cells were precipitated by centrifugation and resuspended in 5% sucrose containing 0.05% Silvet L-77. Flowers of 5-8 week-old Arabidopsis thaliana (Col-0) plants grown under long day condition were dipped into the bacteria suspension.

Then the Arabidopsis thaliana plants were covered with plastic foil and placed in the dark over night. On the next day the plastic foil was removed and the plants were put back into the grow chambers until the seeds were matured. The selection of positive Arabidopsis thaliana plants was carried out on ½ MS plants containing 50 µg/ml kanamycin.

Arabidopsis suspension culture

Arabidopsis thaliana suspension culture (provided by Andrij Belokurow, group Hirt) was grown in adequate media. 45 ml of suspension culture was grown in a 250 ml flask at 22°C under shaking at 140 rpm. Every 7 days the suspension culture was diluted 1:3 with Arabidopsis thaliana suspension media.

2.10 Yeast and bacteria methods

Preparation of chemical competent *E. coli*

A pre-culture of LB supplemented with 20 mM $MgSO_4$ was inoculated with 3-5 independent *E. coli* clones and incubated at room temperature under shaking over night. 600 ml of LB supplemented with 20 mM $MgSO_4$ was adjusted with the pre-culture to OD_{600} of 0.2 and incubated under shaking at room temperature until an OD_{600} of 0.5 was achieved. Cells were harvested by centrifugation at 700 g for 10 min at 4°C and resuspended in 50 ml ice cooled TB buffer. After incubation on ice for 30 min the cells were again harvested by centrifugation at 400 g for 10 min at 4°C and resuspended in ice cooled TB buffer supplemented with 7% DMSO. The suspension was incubated on ice for additional 30 min, spitted into 450 µl aliquots and frozen away at -80 °C. (Inoue, Nojima et al. 1990)

Transformation of chemical competent *E. coli*

In a total volume of 10 µl 2-50 µg plasmid DNA was transferred into a 1.5 ml reaction tube and placed on ice. Competent *E. coli* suspension was thawed on ice. 50 µl of the E. coli suspension was mixed with the 10 µl plasmid DNA and incubated on ice for 15 min. The reaction mix was heat-shocked at 37°C for 45 sec and placed back on ice. To

recover the cells 800 µl LB media was added and the suspension was incubated for 60 min at 37°C under shaking. The cells were harvested by centrifugation at 16100 g for 1 min at room temperature, resuspended in about 80 µl LB and plated on appropriate selective LB media.

Transformation of *S. cerevisae*

150 µl of S.C. Trafo solution (including 20 µg carrier RNA) were mixed with approximately 50 µl of fresh *S. cerevisae* cells grown on YPD over night. 2 µg of plasmid DNA were added and mixed by vortexing. The suspension was incubated at 30°C for 20 min and heat-shocked for additional 20 min on 44°C. 1 ml sterile H_2O was added and mixed by inverting the tube. The cells were precipitated by centrifugation at room temperature at 2000 g for 2 min. Most of the supernatant was removed and the cells were resuspended in 50-100 µl sterile H_2O and plated on selective media.

Preparation of electro competent Agrobacteria

Over night culture of Agrobacteria was inoculated in 600 ml LB and incubated at 30°C under vigorous shaking for 1.5 days until an OD_{600} of 1.5-2 was reached. The suspension was cooled down on ice for 10 min. The cells were precipitated by centrifugation for 15 min at 6000 g at 4°C and resuspended in 50 ml of 1 mM HEPES pH 7.0. Cells were again precipitated by centrifugation at 4000 g at 4°C for 15 min and resuspended in 1 mM HEPES pH 7.0. This washing step was additionally repeated twice and then the 1 mM HEPES pH 7.0 was replaced by 10 % glycerol. The cells were again precipitated by centrifugation at 4000 g at 4°C for 15 min and resuspended in 4-6 ml 10 % glycerol. Aliquots of 400 µl per 1.5 ml Eppendorf tube were frozen in liquid nitrogen and stored at -80°C.

Transformation of electro competent Agrobacteria

1-2 µl (1 µg/µl) of plasmid DNA were placed into a sterile pre-cooled electroporation cuvete. 60 µl of electro competent Agrobacteria suspension were added and mixed by pipetting. Electroporation was carried out at 200 Ohm and 1,4 kV and 25 µF with a Bio-Rad (Hercules, CA, USA) Pulse Controller electroporation device. After electroporation

800 µl LB were added the suspension was transferred to a 1.5 ml Eppendorf tube and incubated at 30°C for 30-60 min. Cells were precipitated by centrifugation at 14000 g for 2 min, resuspended in 50-10 µl LB and plated on LB media containing the appropriate antibiotics.

2 Results

2.1 Characterisation of CPK3

Phenotype of *cpk3* mutants

To elucidate the functional role of CPK3 *in planta*, a *cpk3* knock-out line was obtained from the Salk Arabidopsis Insertion Library (N522862; SALK_022862, Salk Institute Genomic Analysis Laboratory, La Jolla, CA, USA, http://signal.salk.edu/). This line was further analyzed by mapping the T-DNA insertion via PCR and subsequent sequencing of the PCR products. This mutant could be confirmed as null mutant at the protein level by Western blot analysis using a specific antibody against the C-terminal 15 amino acids of CPK3 (figure 2.1).

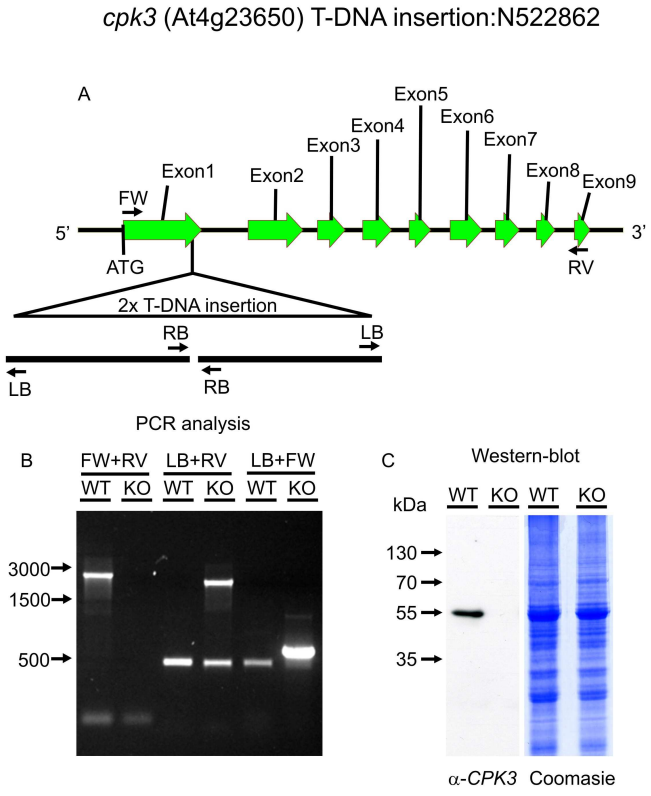

Figure 2.1: CPK3 T-DNA insertion mapping. A: The T-DNA insertion was mapped by PCR, using primers specific to the CPK3 gene and the T-DNA. B: PCR products were produced with the primer combination LB+RV and LB-FW which indicates a double T-DNA insertion in the first exon of CPK3 locus. C: Western blot analysis using a specific antibody for CPK3 shows that the CPK3 knock-out line lacks the endogenous CPK3 protein.

Since activation of CPK3 kinase activity by salt stress had previously been shown in the laboratory (M. Teige, unpublished), germination assays on ½ MS plates were carried out in order to test for a salt sensitive phenotype of *cpk3* mutants. *CPK3* knock-out

(*cpk3*) and two independent CPK3 overexpression lines (*CPK3-1* and -*2*) were germinated on ½ MS plates, either without salt, or in the presence of 150 mM NaCl. The germination rate on media without salt was 100% for all lines, thus indicating that observed changes in germination must be due to salt stress. On the plates containing 150 mM NaCl, the germination rate of all lines was impaired. However, a very clear differentiation emerged: Wild type plants had a germination rate of 22.3% ± 7.82, and the *cpk3* line was severely impaired showing a strongly reduced germination rate of only 8.4% ± 7.08 (figure 2.2). In contrast, the CPK3 overexpression lines displayed an increased germination rate which moreover correlated to the level of CPK3 expression as revealed by RT-PCR. The weak overexpressor line *CPK3-1* had a germination rate of about 29.2% ± 2.48, and the strong overexpression line *CPK3-2* had a germination rate of 55.1% ± 16.95 (figure 2.2). In summary, these experiments clearly showed the essential role of CPK3 for the salt stress adaptation in *Arabidopsis*.

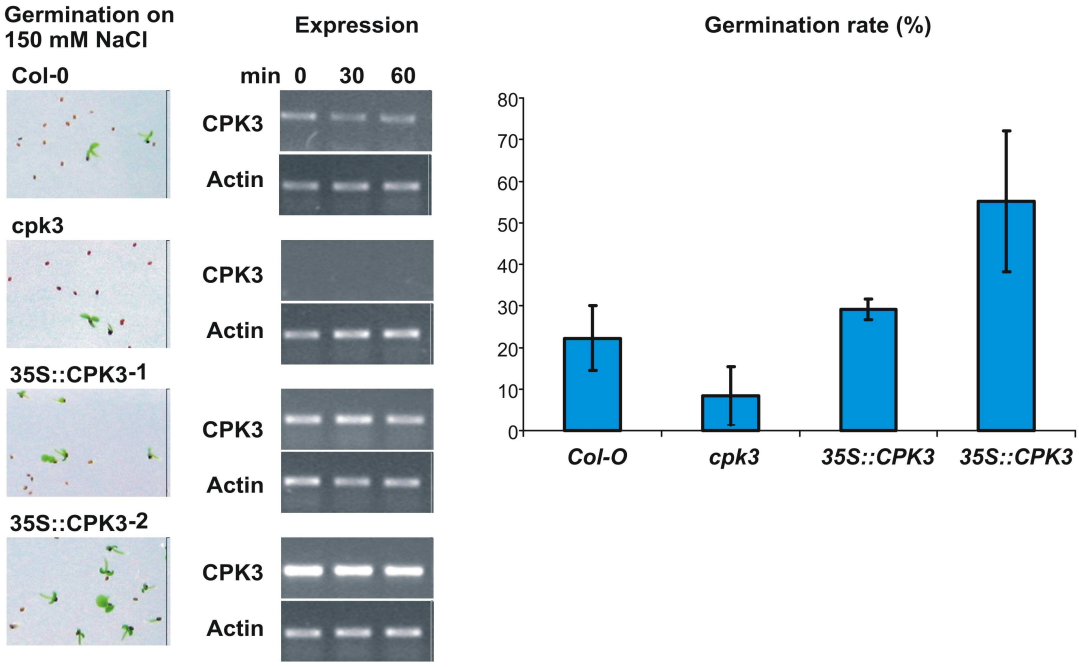

Figure 2.2: Salt sensitive phenotype and analysis of CPK3 expression-levels in the CPK3 knock-out mutant (*cpk3*) and two independent over-expressor lines (CPK3-1 and CPK3-2). Germination was scored for seven days on ½ MS plates supplemented with 150mM NaCl and survival was scored for seven days. Statistical analysis of survival rates from three independent plates, n>300 per plate and plant line.

Crosstalk with salt stress triggered MAP kinase pathways?

To understand how CPK3 could mediate adaptation to salt stress at the molecular level, and which downstream targets could be regulated the further analysis was extended also

to related signaling pathways which are known to be involved in salt stress signaling. Furthermore, the question of cross-talk between different signaling pathways in salt stress response should be addressed.

MAP kinase pathways are not affected by CPK3

MAP kinase pathways represent well characterized examples in this respect, and the MAP kinases MPK4 and MPK6 are activated by various abiotic and biotic stresses, including salt stress (Ichimura, Mizoguchi et al. 2000; Petersen, Brodersen et al. 2000; Asai, Tena et al. 2002; Teige, Scheikl et al. 2004). A MAP kinase pathway which regulates MPK4 and MPK6 includes the upstream MAP kinase kinase MKK2 and is also required for the cold- and salt stress response in *Arabidopsis* (Teige, Scheikl et al. 2004). The fact that *mkk2* mutants showed a similar phenotype on salt media suggests a related regulation mechanism. Therefore *CPK3* overexpressor and *cpk3* knock-out lines were used to determine the activation of MPK4 and MPK6 in response to salt stress by immuno-complex kinase assay using MPK4 and MPK6 antibodies (figure 2.3).

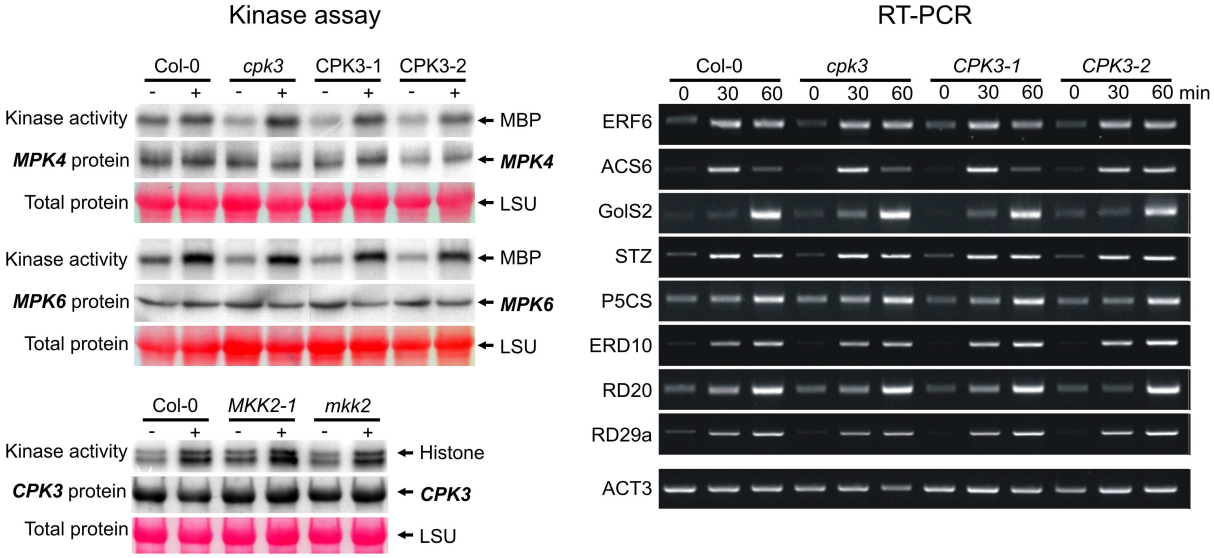

Figure 2.3: Salt triggered activation of MPK6 in wild type (Col-0), cpk3 knock-out, and two independent CPK3 overexpressor lines (see Figure 2.2) towards myeline basic protein (MBP) as generic substrate. Kinase assay: Kinase activities were measured in immunocomplex kinase assays upon salt treatment of 14-days-old seedlings for 15 min. RT-PCR: Salt triggered induction of known salt stress responsive marker genes was compared between wild type (Col-0), cpk3, and the two CPK3 overexpressor lines by RT-PCR and compared to Actin (ACT3) as internal control. 14-day-old seedlings were treated with 150 mM NaCl.

However, no difference in the activation of either MPK4, or MPK6 could be detected. *Vice versa*, the salt stress induced activation of CPK3 was determined in *mkk2*

knock-out and MKK2 overexpressing lines. But again, no difference in activation of CPK3 could be detected, clearly demonstrating, that these pathways function completely independently at the level of the involved protein kinases

CPK3 does not affect the transcriptional induction of known salt stress marker genes

To look further downstream in the stress response pathway, the expression levels of known marker genes for salt stress adaptation were analyzed. MAP kinase pathways are known to target predominantly transcriptional responses in animal cells (Whitmarsh 2007). For plant cells the same seems to hold true as may be seen for studies on gene expression in MAP kinase mutant plants (Teige, Scheikl et al. 2004; Qiu, Zhou et al. 2008) and from identified targets involved in transcriptional regulation (Qiu, Fiil et al. 2008). The MKK2 MAP kinase pathway plays an essential role in signaling and adaptation to cold- and salt-stress in *Arabidopsis* by regulating a set of 127 genes which are required for adaptation to these stresses (Teige, Scheikl et al. 2004). To compare transcriptional regulation, downstream targets of the MAP kinase- and the Ca^{2+} dependent protein pathways were analyzed. The expression of marker genes was analyzed by RT-PCR specifically to address the question if these MAP kinase pathways and the Ca^{2+} dependent CPK3 signaling pathway cross-talk at the level of target gene expression. For that reason 22 known target genes which are known to be regulated in response to salt stress based on microarray analysis were selected and analyzed (Kreps, Wu et al. 2002; Seki, Ishida et al. 2002; Taji, Seki et al. 2004).

The result of these experiments is exemplified in figure 2.3 for 8 genes involved in ethylene signaling and biosynthesis (*ERF6* and *ACS6*), synthesis of the compatible solutes galactinol (*GolS2*) and proline (*P5CS*), transcriptional regulation (*STZ*), and general stress response factors (*ERD10, RD20,* and *RD29a*). The transcriptional induction was analyzed by RT-PCR 30 and 60 min after salt stress treatment in wild type plants (*Col-0*), a *cpk3* knock-out mutant, and two different CPK3 overexpressor lines which had all been characterized before. It turned out that all salt stress-responsive genes were equally induced in all lines. The complete set of genes which was analyzed in these studied included furthermore the Na^+/H^+ antiporters *NHX1* and *SOS1,* the Na^+-induced K^+ channel *KC1,* the trehalose synthesis genes *TPS1, TPS11,* and *T6PP*; proline catabolism (*PDH*), ABA- and salt stress-responsive protein phosphatases (*AHG3,*

PP2C); and the general stress-response factors *ERD15*, and *RD29b*. But also in these cases no difference of the induction patterns of salt-responsive genes emerged between the different lines studied. In line with previous microarrays (Kreps, Wu et al. 2002) *CPK3* expression itself was also not found to be regulated at the mRNA level (figure 2.3).

Localisation of CPK3

These findings indicate that CPK3 is not involved in transcriptional induction of salt stress response genes and acts independently of MAP kinase pathways. This suggests that CPK3 uses a different mechanism to mediate salt stress response. Therefore I decided to study the localization of the kinase in different tissues and at the subcellular level to get a further idea on the function of CPK3 in salt stress adaptation.

The amount and tissue-specific distribution of endogenous CPK3 protein from different plant tissues was determined using a specific antibody (Figure 2.4 C) against the C-terminal variable region (Figure 2.4 B). The endogenous level of CPK3 protein in Arabidopsis was nearly the same in all compartments with slightly increased CPK3 protein levels in old leaves and roots (figure 2.4 A). A biochemical fractionation of cell extracts from *Arabidopsis* leaves and root suspension culture revealed that CPK3 enriches in un-soluble sub-cellular fractions and co-purified with microsomal membranes. No CPK3 could be detected in the cytosolic fraction (figure 2.4 A).

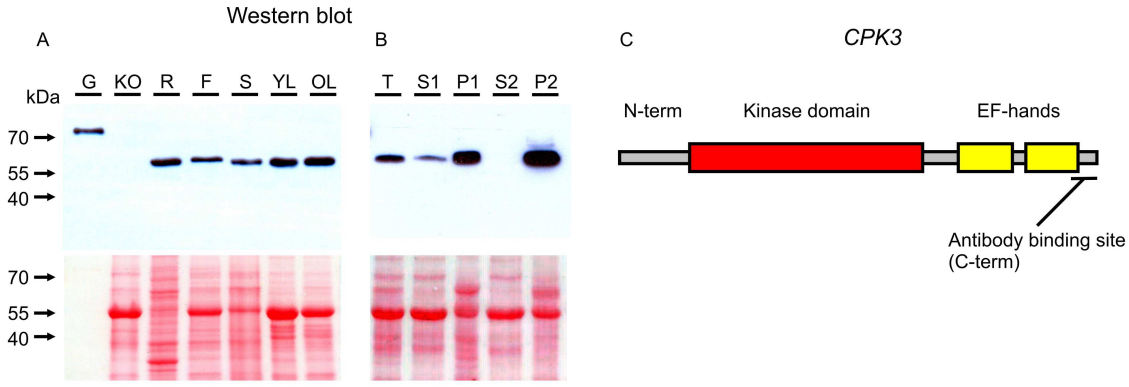

Figure 2.4: Localization (A and B) and structure (C) of the CPK3 protein. (A) Tissue specific expression of CPK3 and specificity of the antibody. In the first lane 20 ng of recombinant GST-CPK3 protein was loaded and in the following lanes total protein extracts from the *cpk3* mutant, and from different tissues of wild type plants: root; stem; flower; young (20 dpg), and old leaves (40 dpg. (B) Detection of endogenous CPK3 in subcellular fractionation from wild type plants. In lane 1, total cell extract was loaded (T), and in the following lanes protein from the 13.000 x g (13k) supernatant (S1) and pellet (P1), as well as the 100.000 x g (100k) k supernatant (S2) and pellet (P2).

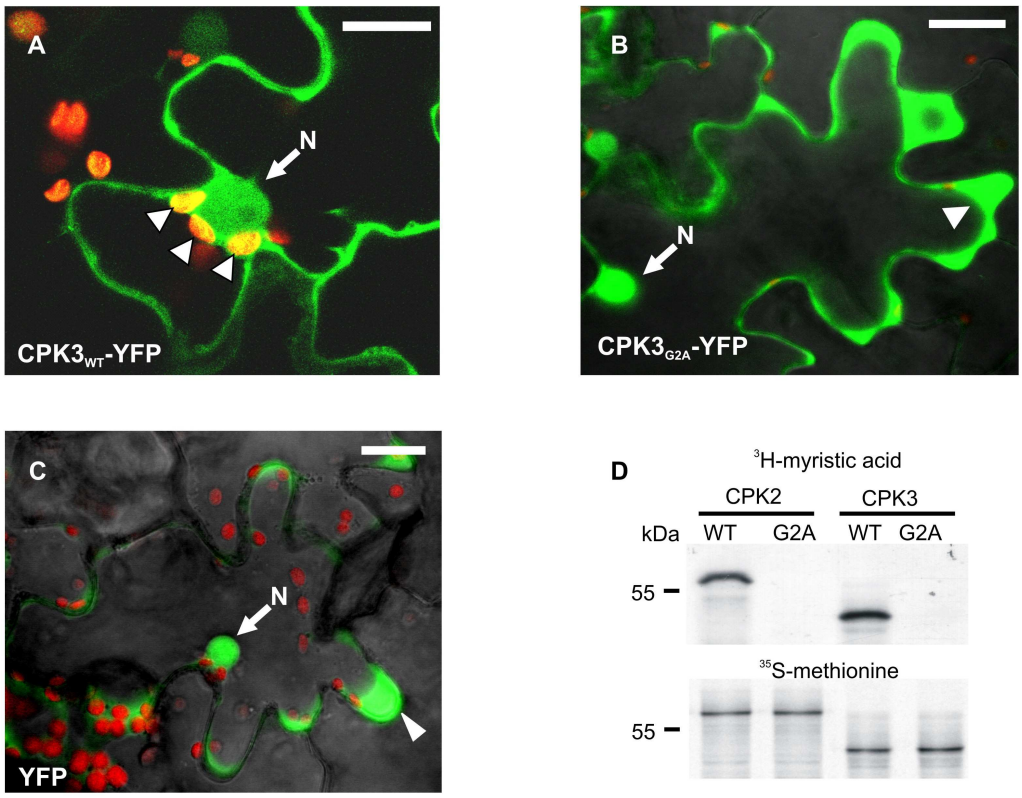

Figure 2.5: Localization of $CPK3_{WT}$-YFP fusion proteins (A), CPK3G2A-YFP fusion proteins (B) and YFP protein alone (C) in leaf epidermal cells, 2 days after infiltration. Infiltration of tobacco leaves was done as described in experimental procedures. The nucleus (N) is marked by an arrow, and three chloroplasts adjacent to the nucleus are marked by a triangle in A. Cytoplasmic lobes of the epidermal cells are marked by the triangle in B and C. The scale bar represents 20 µm. Chlorophyll auto-fluorescence is shown in red. (D) *In vitro* myristoylation of CPK3 and CPK2 as positive control. The wild type (WT) and non-myristoylable G2A mutants of CPK2 and CPK3 were translated *in vitro* in wheat germ extracts. This happend in the presence of either ^{3}H-labelled myristic acids or 35S-labelled methionine as described in experimental procedures and subsequently separated by SDS-PAGE. Incorporation of the label was scored by autoradiography.

To extend this study into the sub-cellular level, I studied the *in vivo* localisation of CPK3-YFP fusion proteins by confocal laser scanning microscopy in *Nicotiana tabacum* (SR1) leaves. Transient transformation was done by infiltration of *Agro-bacteria*, containing the constructs for expression under control of the cauliflower mosaic virus *CaMV 35S* promoter. To investigate the N-terminal mediated subcellular localisation of CPK3, the YFP portion was fused to the C-terminus of the full-length CPK3 coding sequence (figure 2.5). A potential effect of N-terminal myristoylation based on localisation was studied by using a mutated form of CPK3, lacking the myristoylation site on glycine (G2A). This mutant cannot be myristoylated per se, and its localization was compared to either YFP, expressed under the 35S promoter, or the wild type version of CPK3-YFP. Expressed CPK3-YFP showed nuclear and membrane associated localisation. In contrast, the mutated CPK3-YFP G2A appeared more nuclear

and cytosolically localized, which was similar to non-fused YFP. That the observed difference in localization could indeed be explained by N-myristoylation of CPK3 was confirmed by *in vitro* myristoylation using wheat germ extract. CPK3, and CPK2 as positive control, were *in vitro* translated either in the presence of ^{35}S-methionine, or ^{3}H-labelled myristic acid in a coupled transcription/translation system as previously described for CPK2 (Lu and Hrabak 2002). The incorporation of the label was detected by autoradiography after SDS-page (figure 2.5 D). Both, CPK2 and CPK3 were clearly found to be effectively N-myristoylated in these assays.

Salt stress mediated alteration in protein phosphorylation pattern of *cpk3* knock-out plants

The previous data indicating that CPK3 activity has no influence on salt stress dependent gene expression gave rise to the question how the salt sensitive phenotype of the *cpk3* knock-out mutants could be explained. To investigate salt stress triggered changes in protein phosphorylation patterns in wild type and cpk3 knock-out mutants an unbiased approach was done. Using different phosphoamino acid-specific antibodies, phosphorylated serine and threonine residues were analyzed by 2D-gel Western blotting of total proteins from salt-stressed and untreated plant extracts. Considering the important role of roots for salt stress adaptation (Munns and Tester 2008) and also the strong expression of CPK3 in this tissue, the focus of this studies was based on root tissue, isolated from hydroponically grown plants. Phosphorylation patterns of proteins before and 30 min after application of salt stress (150 mM) were compared. 100 µg of total proteins were separated by 2D-gel electrophoresis. In figure 2.6 the results are shown for detection with the anti phospho-Thr antibody.

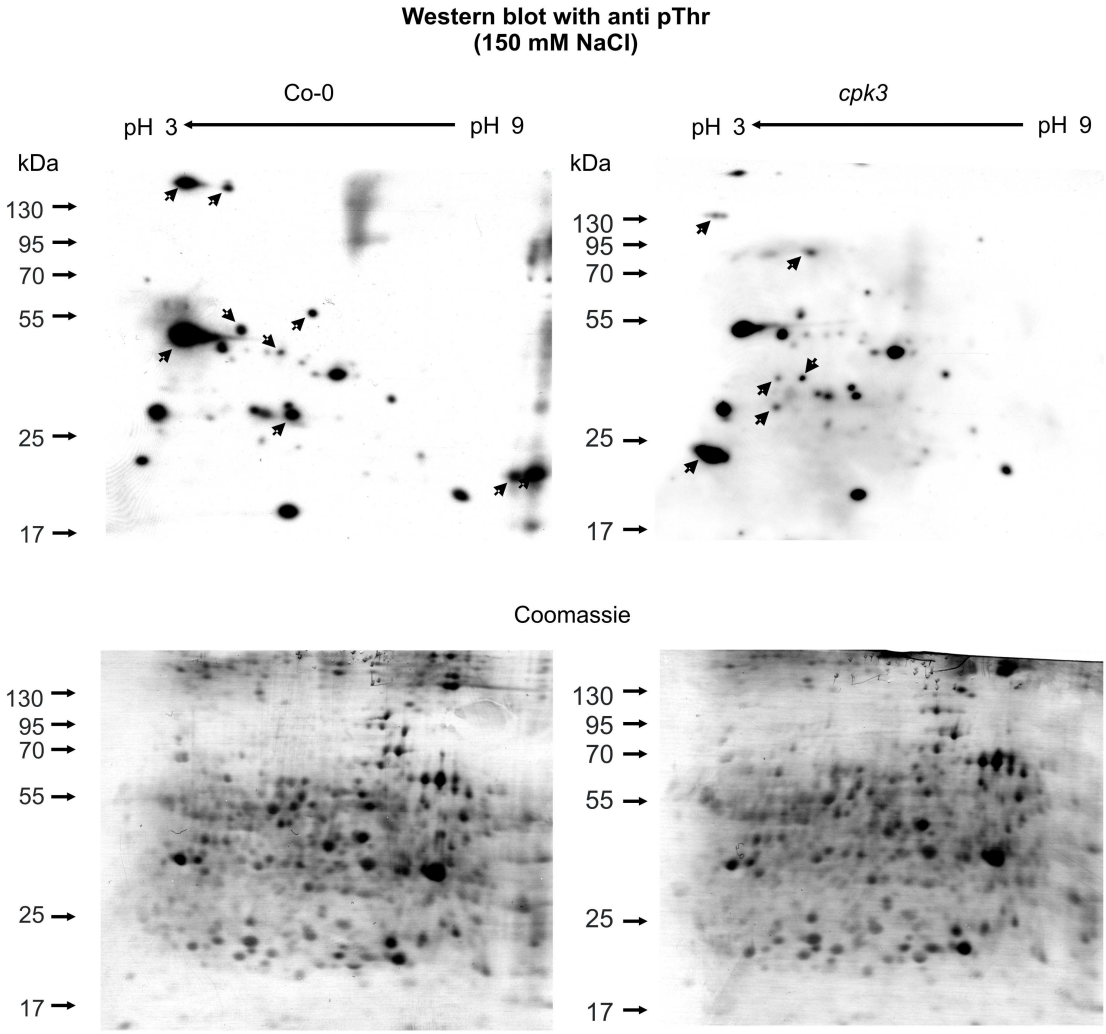

Figure 2.6: CPK3 mediated protein phosphorylation patterns in response to salt stress. Arabidopsis wild type (Col-0) and cpk3 plants were grown in hydroponic culture for four weeks before salt stress (150 mM NaCl) was applied for 30 min and protein extracts were prepared from roots as described in materials and methods. Briefly, 100 µg of total protein was separated on pH 3-11 (NL) immobiline dry strips in the first dimension, followed by 8-15% gradient SDS-PAGE in the second dimension. Protein phosphorylation on threonine residues was detected after Western blotting using an anti-phospho-Thr antibody (upper panels), and total protein was scored by Coomassie-brilliant blue staining of the membranes (lower panels). The arrows in the upper panels mark significant changes in the phosphorylation level.

These separations typically resulted in more than 300 spots, which could clearly and reproducibly be detected by Coomassie-staining after blotting on PVDF membranes. Clear differences in phosphorylation were visible between wild type (Col-0) and *cpk3* mutants in response to salt stress (upper panels). Most importantly, there were no detectable differences in total protein spots by Coomassie-staining (lower panels). This indicates that the observed differences are indeed due to phosphorylation and not to differences in expression.

This result was further confirmed by a comparison of untreated wild type and *cpk3* samples (figure 2.7). Also here no differences in phosphorylation could be observed pointing out that the discovered differences in Thr-phosphorylation between wild type and *cpk3* are dependent on CPK3 kinase activity. The arrows in the upper panel of figure 2.6 indicate the major changes.

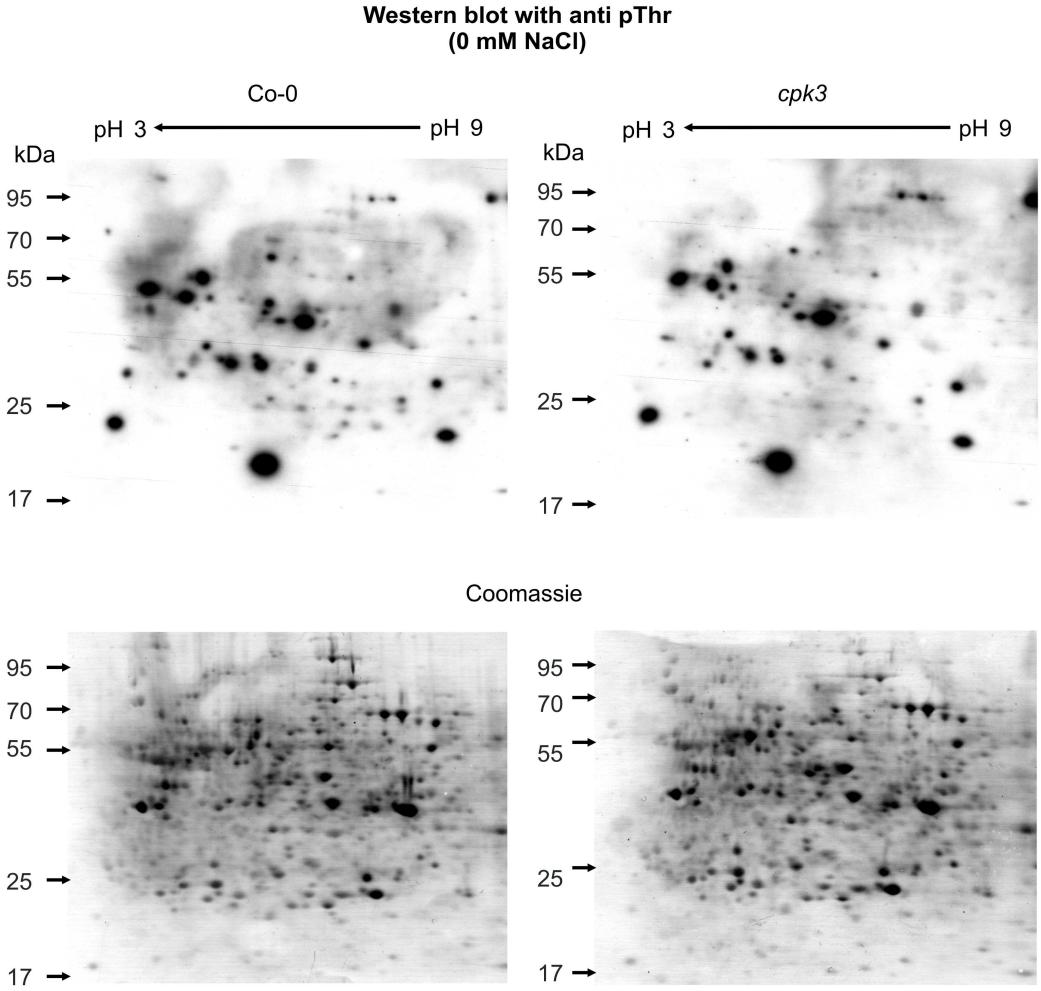

Figure 2.7: Phosphorylation patterns without salt stress from Col-0 and *cpk3*. Arabidopsis wild type (Col-0) and *cpk3* plants were grown under the same condition but not treated with NaCl (see figure 2.6).

In summary, 9 spots appeared either new or in strongly enhanced intensity in wild type as compared to the *cpk3* mutant; whereas 6 spots appeared new or enhanced in the *cpk3* mutant as compared to the wild type. Taken together, a clear difference in the Thr-phosphorylation of 15 proteins between *cpk3* mutants and wild type roots 30 min after salt stress was detectable.

Regulation of ion homeostasis

The ability to maintain ion homeostasis under stress conditions is of fundamental importance for plants to survive salt stress. Accordingly, plants apply a plethora of different transport systems in order to either exclude Na^+ from the cell or to sequester it into the vacuole via Na^+/H^+ antiporters (Tuteja 2007). These channels are tightly controlled, which becomes particularly important when the plant has to counter act environmental changes. For that reason plants are able to adopt to changes in soil ion concentrations by removing toxic ions like sodium from the cytoplasm. Different ion channels achieve this by pumping out these ions from the cytoplasm in order to maintain the critical K^+/Na^+ ratio. Salt stress results in a transient rise in free cytosolic Ca^{2+} concentration which is perceived by calcium sensor molecules.

The SOS pathway is not activated by CPK3 in yeast

SOS1 is a Na^+/H^+ antiporter in the plasma membrane which is responsible for salt tolerance in *Arabidopsis* (figure 2.8). It is known that salt stress leads to elevated cytosolic Ca^{2+} concentration resulting in the activation of the SOS-pathway (Shi, Ishitani et al. 2000; Quintero, Ohta et al. 2002; Zhu 2002). In this pathway, the activation of SOS1 antiporter activity is regulated by the Ca^{2+}-sensing SOS2/SOS3 complex. SOS3 (also known as CBL4) is a calcineurin B-like protein (CBL), which can bind Ca^{2+} and therefore recognizes elevated Ca^{2+} concentration. The Ca^{2+} bound form of SOS3 binds to SOS2, which phosphorylates SOS1 and activates the Na^+/H^+ antiporter (Qiu, Guo et al. 2002). Therefore the possibility that also CPK3, containing both a protein kinase domain and a Ca^{2+}-sensing domain, could be able to activate SOS1 should be tested in a different approach.

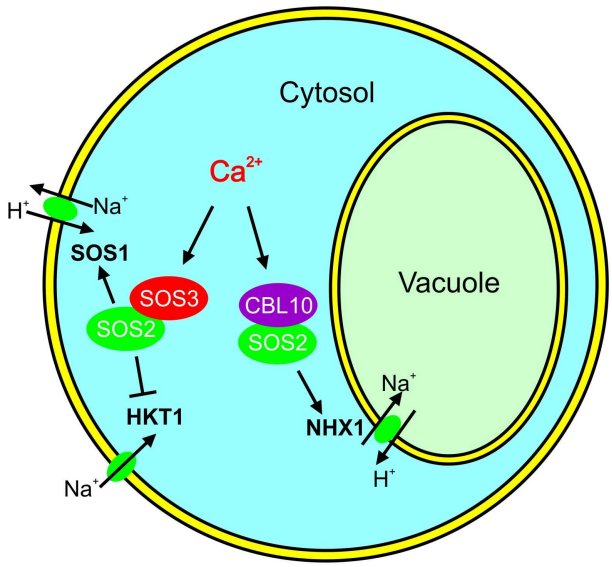

Figure 2.8 Schematic representation of the SOS pathway. Increased Ca^{2+} levels are recognized by the SOS2-SOS3 and by the CBL10-SOS2 complexes. The calcium bound SOS2-SOS3 complex activates Na^+/H^+ antiporter SOS1 and inhibits low-affinity potassium transporter, which transport Na^+ ion under increased salt condition. CBL10-SOS2 is also activated by increased Ca^{2+} levels and activates the vacuolar Na^+/H^+ pump NHX1 (Mahajan, Pandey et al. 2008)

The activation of SOS1 was tested by a yeast functional complementation assay in the yeast strain AXT3K (MATalfa, 7ena1::HIS3::ena4, nha1::LEU2, nhx1::KanMX). This strain lacks four different sodium transporters (Quintero, Ohta et al. 2002) rendering this strain extremely salt sensitive towards sodium. CPK3 was overexpressed under the control of the ADH promoter from the YEPlac112 plasmid together with SOS1 which was overexpressed when using the original plasmid (pSOS1) from Quintero et al., 2002. The transformed yeast cells were plated on media containing 100 and 150 mM NaCl, and growth on salt was compared to transformants, complemented with the complete SOS-pathway. It was found that the combination of SOS1-SOS2-SOS3 was able to restore survival on salt media, but none of the CPK3 constructs was able to compensate the function of the SOS2/SOS3 complex (figure 2.9). Altogether, these experiments showed that CPK3 is not able to activate the SOS pathway.

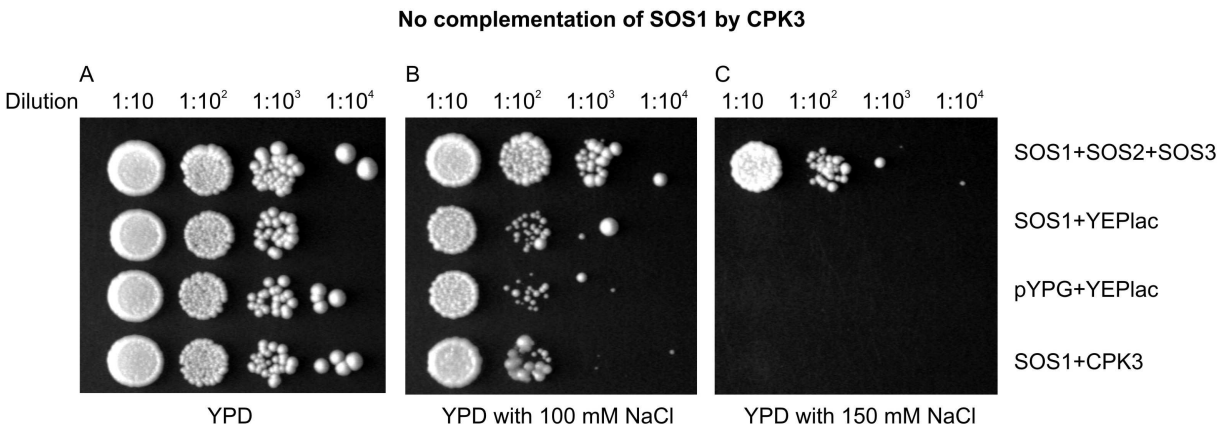

Figure 2.9: The yeast strain AXT3K lacking sodium transporters was used to test the activation of the *Arabidopsis* Na^+ transporter SOS1 by CPK3. The positive control (SOS1+SOS2+SOS3) was clearly able to counteract both high NaCl concentrations of 100 mM and 150 mM NaCl (B and C). But the negative control (SOS1 with the empty vector YEPlac and both empty vectors) and SOS1 with CPK3 showed no increased growth on media containing 100 mM and 150 mM NaCl.

Phosphorylation of microsomal membrane proteins by CDPKs

The finding that CPK3 does not regulate the SOS pathway does of course not rule out other membrane proteins as cellular targets, particularly since CPK3 revealed a localization at cellular membranes (shown in figure 2.4 B). Therefore membrane associated or membrane localized proteins had to be analyzed in particular as targets of CPK3. An important feature of those proteins is that they function at the interface between two cellular compartments.

Exchange of solutes between different cellular compartments is mediated by membrane integrated channel proteins which are often highly specific for particular soluble molecules. The regulation of these membrane proteins is an essential mechanism to maintain normal cellular function, and phosphorylation is a known regulation mechanism. It can mediate the adaptation to different environmental conditions.

To identify membrane associated molecular targets of CDPKs, I performed kinase assays using isolated microsomal membranes that had been isolated from an *Arabidopsis* root suspension culture. The phosphorylation of microsomal membranes was done by the incubation of recombinant CDPKs (CPK3, CPK4, CPK5, CPK11 and CPK6) with microsomal membranes in the presence of γ^{32}-ATP. The phosphorylation assays were carried out in kinase buffer, either in the presence of Ca^{2+} or without Ca^{2+} (EGTA). As negative control microsomal membranes alone were incubated with γ^{32}-ATP (without CDPK). Interestingly, each CDPK showed different phosphorylation

patterns, indicating that each CDPK has different phosphorylation targets and furthermore illustrating the specificity of this assay (figure 2.10).

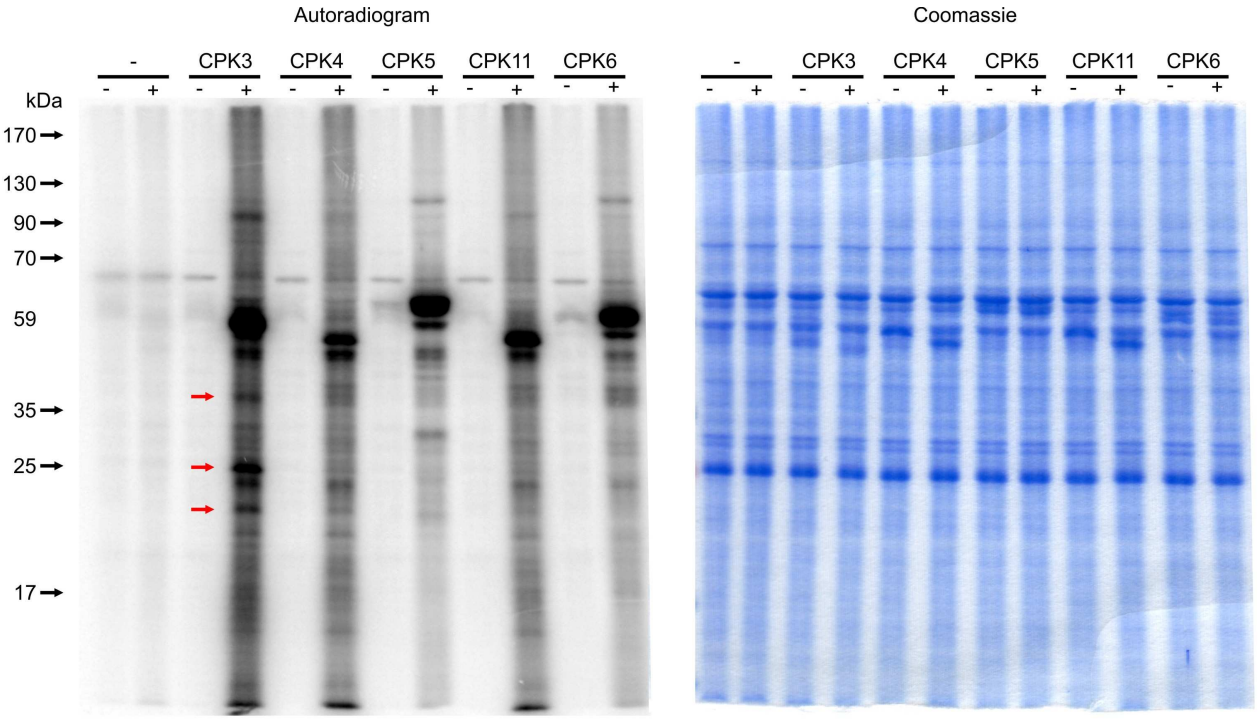

Figure 2.10: Microsomal membranes isolated from Arabidopsis root suspension culture were incubated with CPK3, CPK4, CPK5, CPK11 and CPK6 in the absence or presence of Ca^{2+}. The uptake of radioactivity was measured by autoradiogram (left) and the total amount of protein was detected by Commassie staining (right). Red arrows idicate potential phosphorylation of potential targets.

Phosphorylation of TPK1 by CPK3

TPK1, the dimeric-outward-rectifying-membrane-K^+-channel localized to the tonoplast, is known to be Ca^{2+}- and voltage-dependent (Bihler, Eing et al. 2005). Recently, it was also shown that 14-3-3 proteins can interact with TPK1. The 14-3-3 binding domain of TPK1 contains a consensus sequence for serine/threonine protein kinases and it can be demonstrated that the phosphorylation of this motif triggers the binding of the 14-3-3 proteins, resulting in the activation of the K^+ channel (Latz, Becker et al. 2007).

The phosphorylation by CDPKs was tested with the N-terminal soluble part of TPK1 (first 80 amino acids including the 14-3-3 binding motif) fused to GST which was lacking the trans-membrane domain and the EF hands for Ca^{2+} binding. In *in-vitro* kinase assays the phosphorylation of the 14-3-3 binding motif was done with recombinantly expressed CPK3, CPK4, CPK5 and CPK11 in the absence or presence of Ca^{2+} (figure 2.11 B). CDPKs are able to phosphorylated TPK1 in the presence of Ca^{2+}

and CPK3 showed the highest kinase activity in comparison to CPK4, CPK5 and CPK11 which are indicated by rel. activity in figure 2.11 B. The *in vivo* localisation of TPK1 was confirmed by *Agrobacteria* mediated transient expression in tobacco epidermal cells of TPK1-YFP under the control of the CMV 35S promoter (figure 2.11 C).

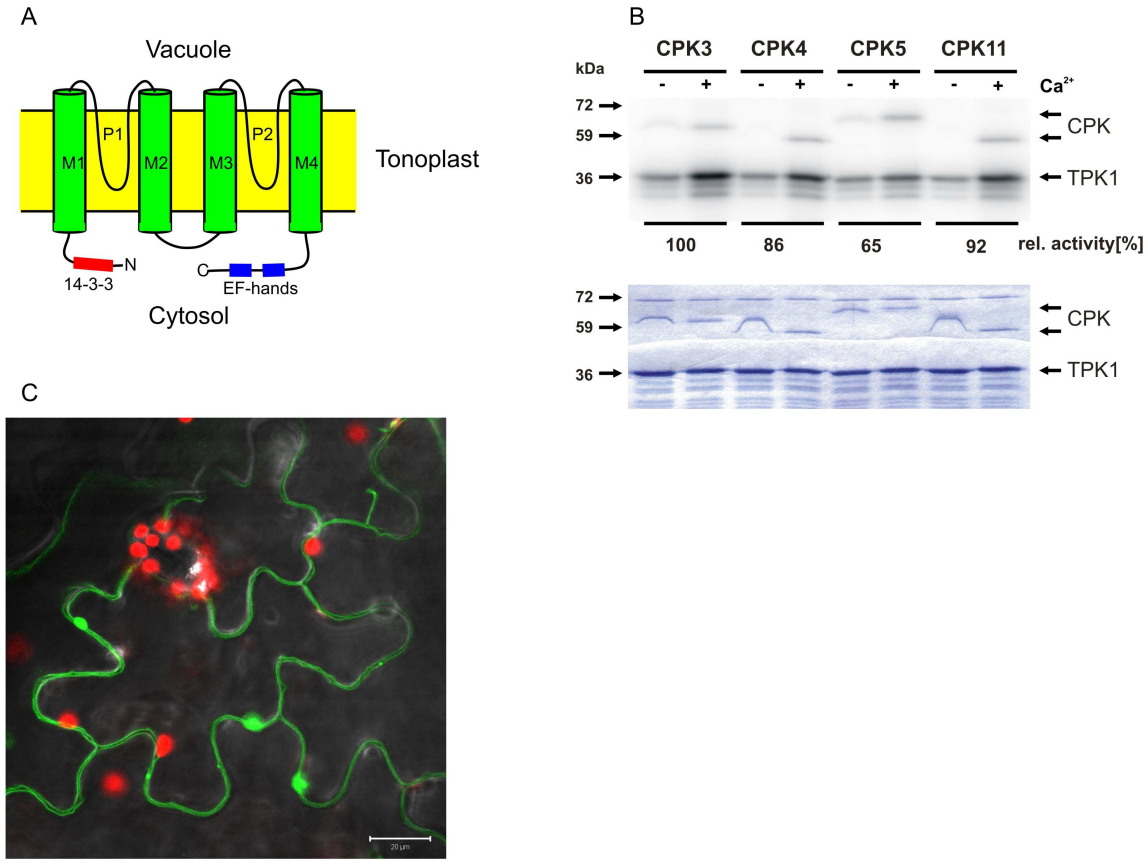

Figure 2.11: (A) Topology of TPK1 with four transmembrane domains (M1-M4) and two pore regions (P1-P2). (B) Kinase assay of TPK1 with CPK3, CPK4, CPK5 and CPK11. (C) *Agrobacterium* mediated transient overexpression of WT TPK1-YFP under the control of the CMV 35s promoter in epidermal leaf cells.

The previous kinase assays (figure 2.10) showed that recombinant CPK3 is able to phosphorylate different membrane bound proteins in a Ca^{2+}-dependent manner. Notably, a protein phosphorylated band with a size comparable to TPK1 appeared in these assays when microsomal membranes, isolated from *Arabidopsis* root suspension culture, were phosphorylated with recombinantly expressed CPK3 (figure 2.12 C). Therefore the kinase assay was repeated with non-radioactive ATP and the phosphorylation site was tested using a phosphorylation site specific antibody against

the 14-3-3 binding motif. Also in this case the antibody was able to recognize a protein of the appropriate size of TPK1 (figure 2.12 B).

To show that the binding of the 14-3-3-phosphorylation-site-specific antibody is specific for phosphorylated TPK1, the non-radioactive kinase assay was repeated while using the recombinant N-terminal part of TPK1 fused to GST as substrate and CPK3 as protein kinase (figure 2.12 A). These assays did clearly show that CPK3 does phosphorylate the vacuolar K^+ channel TPK1 specifically at its N-terminal 14-3-3 binding motif *in vitro*.

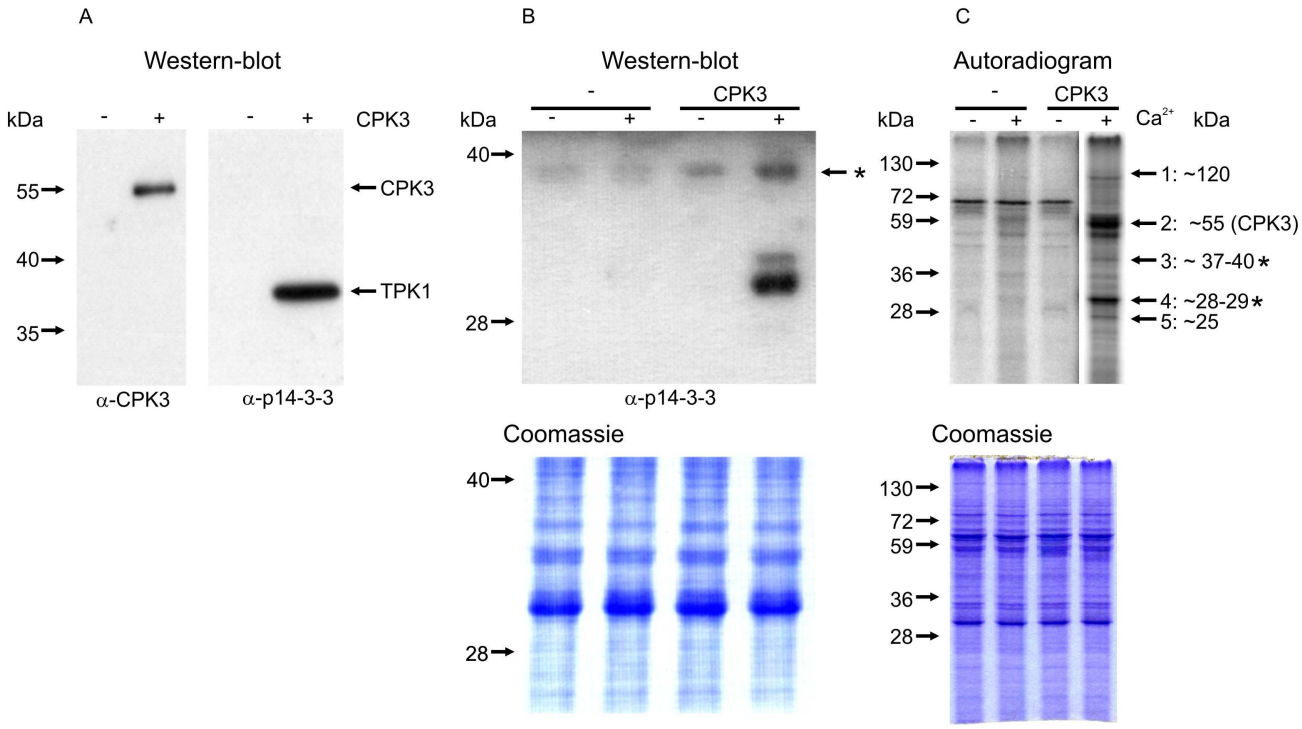

Figure 2.12: Detection of the CPK3 phosphorylated 14-3-3 binding motif on the N-terminal part of TPK1. CPK3 was detected with the CPK3 antibody (A). Microsomal membranes were phosphorylated by recombinant CPK3 in the absence or presence of Ca^{2+}. Phosphorylation of the 14-3-3 binding motif was detected by Western blot analysis (A). Total phosphorylation was detected by incubation with γ^{32}-ATP (C).

Characterisation of the CPK3 - TPK1 interaction

To uncover the molecular properties of CPK3, a structural model of CPK3 was generated together with the collaborators Thomas Müller and Dirk Becker at the University of Würzburg (Latz and Mehlmer et al., submitted for publication). The model was based on the coordinates of two different kinases, the calmodulin-dependent kinase I, which shares the highest amino acid similarity with CPK3, and the phosphorylase b kinase gamma. The model is restricted to the kinase domain with the

N-lobe in the open conformation and has no ATP analogue bound in the ATP binding cleft. On the substrate site, the residues of the PKI5-24 peptide were replaced by residues Asn26 to Arg45 of TPK1. Side-chain-rotamer-searches and several steps of energy refinement ensured that the substrate interacted tightly with the kinase domain and no bad van der Waals-contacts are present in the final model. Residues Asn26 to Arg33 of TPK1 were modelled to form a short α-helix similar to the PKI peptide, whereas residues Arg38 to Arg45 form an extended strand-like structure. The C-terminal end of the α-helix of the substrate contains multiple positively charged amino acids, mainly arginines, which interact possibly via ionic and polar bonds with the highly conserved Glutamate residues Glu157, Glu160, Glu163, and Glu206 of the β5α2-loop, the helix α2 and the substrate binding loop in the kinase domain of CPK3 (figure 2.13 A and B).

To determine the molecular basis for the observed kinase specificity of the tested *Arabidopsis* CPKs towards TPK1 as substrate, a multiple sequence alignment of the region responsible for substrate binding in the tested CPKs was performed (figure 2.13 C). Highly conserved glutamate positions (Glu160) are crucial for interaction with arginine residues in the TPK1 N-terminus according to the model. This alignment showed that exactly at one of them an alanine is present in CPK5 and CPK1 (figure 2.13 marked by the red arrow). Thus the reduced ionic interaction between these CPKs and the TPK1 N-terminus could explain the difference in target phosphorylation.

The next from the structural model deducable insight was the role of one autophosphorylation site in CPK3, exactly residing in the substrate binding loop of the kinase domain (indicated by the arrow in Figure 2.13 C). Upon activation by calcium, autophosphorylation at different serine and threonine residues has been observed for many CDPKs (Hegeman, Rodriguez et al. 2006), but so far a functional consequence of this autophosphorylation is not known.

By using the recombinant protein, an autophosphorylation of CPK3 at multiple sites could be detected. 6 different protein spots became visible after 2D separation by IEF and subsequent SDS PAGE (Figure 2.13 D). In cooperation with Edina Csaszar from the mass spectrometry facility at the MFPL the excised spots from the gel were further analysed by tandem mass spectrometry. It was found that they correspond to differentially phosphorylated forms of CPK3 by its neutral loss of phosphoric acid from the serine residue in the indicated peptide fragmentation pattern (Figure 2.13 E).

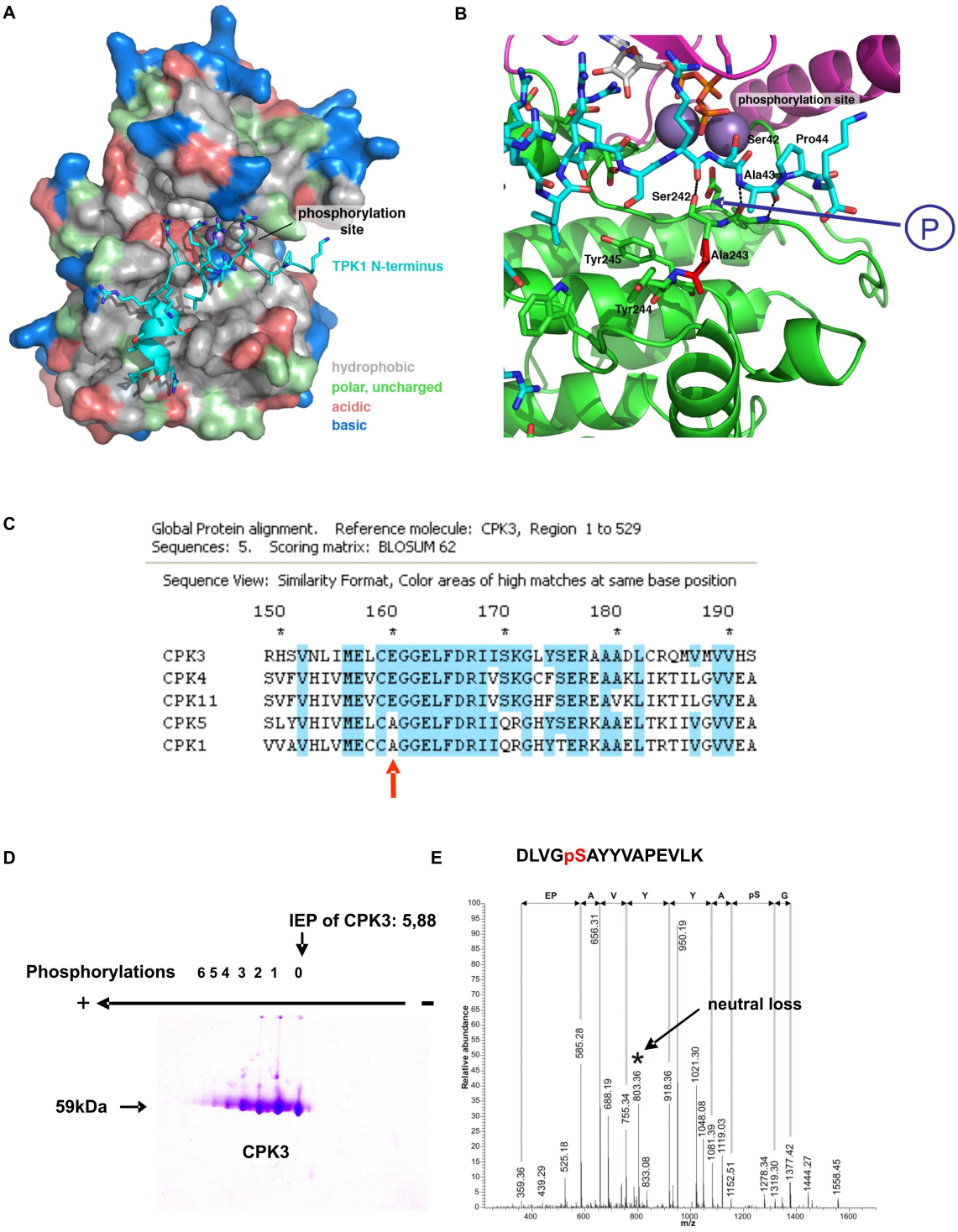

Figure 2.13: (A & B) Molecular modelling of the CDPK TPK binding. (C) Multiple sequence alignment of the substrate binding domain of the CPKs. The sequence alignment was done using BLOSUM 62 for the substrate binding regions identified in the molecular modelling. The difference at the position of Glu160 (for CPK3) is indicated by an arrow. (D) 2D gel separation of recombinant CPK3 after auto-phosphorylation. 30 µg recombinant protein were separated on a 2D gel as described in material and methods and visualized by Coomassie brilliant blue staining. The direction of the non-linear IEF-gradient (pH 3-11 from right to left) and the phosphorylation status of CPK3 are indicated at the top of the figure. (E) Positive ionization MS/MS spectrum of the phosphorylated CPK3 peptide DLVGSAYYVAPEVLK. The doubly charged ion was chosen for CID fragmentation and the phosphorylated residue is labelled with "p". The peak generated by the characteristic loss of phosphoric acid is indicated by the arrow.

In this analysis, Ser242 was unambiguously identified as phosphorylated residue by both data base search programs (Mascot and Bioworks). The doubly charged peptide DLVGSAYYVAPEVLK was fragmented several times both with phosphorylated and with unphosphorylated Ser242 residues. The spectra of the phosphorylated peptide (figure 2.13 E) show the characteristic loss of phosphoric acid from the parent ion, a series of y fragment ions and several fragment ions indicating the loss of phosphoric acid. This serine residue is localized at the very beginning of kinase domain VIII and strongly conserved in all CDPKs. Autophosphorylation of the equivalent serine residue has been reported for CPK4, CPK11, CPK16, and CPK28 (Hegeman, Rodriguez et al. 2006).

The identified Ser242 of CPK3 seems to be important for the recognition between kinase and TPK1, but in Yeast-Two-Hybrid experiments an interaction between CPK3 with the N-terminal part of TPK1 could not be detected. For that reason a mutational analysis of the Ser242 in CPK3 was performed. Ser242 was replaced by an alanine to mimic the non-phosphorylated state and aspartate to mimic the phosphorylated state. The Lys107Arg mutant results in an inactive kinase domain and was used because this might stabilize the transient interaction between the kinase and its substrate. However, also with these mutants it was not possible to obtain data on interaction between CPK3 and the N-terminal part of TPK1 in a Yeast-Two-Hybrid system. Subsequently, I tried to adopt the split-ubiquitin system (Dualsystems) for analysis of this interaction. Unfortunately this approach did also fail due to the high auto-activation of TPK1 in this system.

In subsequent kinase assays, I wanted to test a potential functional consequence of this auto-phosphorylation of CPK3. These experiments, using the Ser242Ala mutant and the Ser242Asp mutant, revealed that both mutants displayed a reduced kinase activity. This was observed for both, autophosphorylation as well as target phosphorylation of TPK1-N-Terminus. However, the phosphorylation of the N-terminal part of TPK1 was not so strongly affected by these mutations. This might be explained by the high affinity of the 14-3-3 binding motif in TPK1 to the kinase catalytic domain in CPK3 (figure 2.14).

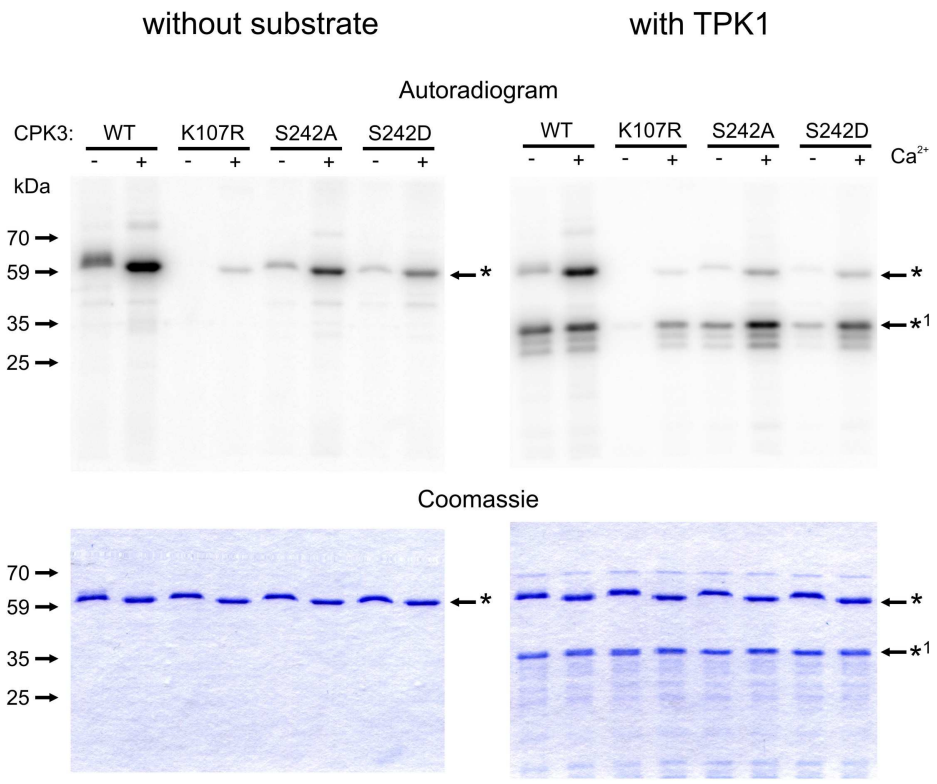

Figure 2.14: Phosphorylation of CPK3 WT, K107R, S242A and S242D without substrate (auto-phosphorylation *) and in the presence of TPK1-N-term (*[1]) as substrate. The corespoindng Coomassie stain is shown on the lower panels.

Proteomics approach to identify molecular targets of CPK3

As shown already in previous reports (Fig. 2.9), recombinant CPK3 could phosphorylate proteins in microsomal membranes highly specific in a Ca^{2+}-dependent manner *in vitro*. In these experiments it was found that CPK3 phosphorylates specifically proteins at molecular weights, corresponding to 25, 28, 40, 50, 55 and 120 kDa, respectively (Fig. 2.11c). Based on these results an unbiased proteomics based approach was performed aiming at the identification of further CPK3 targets in microsomal fractions. To this end a non-radioactive kinase assay was performed, and the samples were separated by SDS-PAGE.

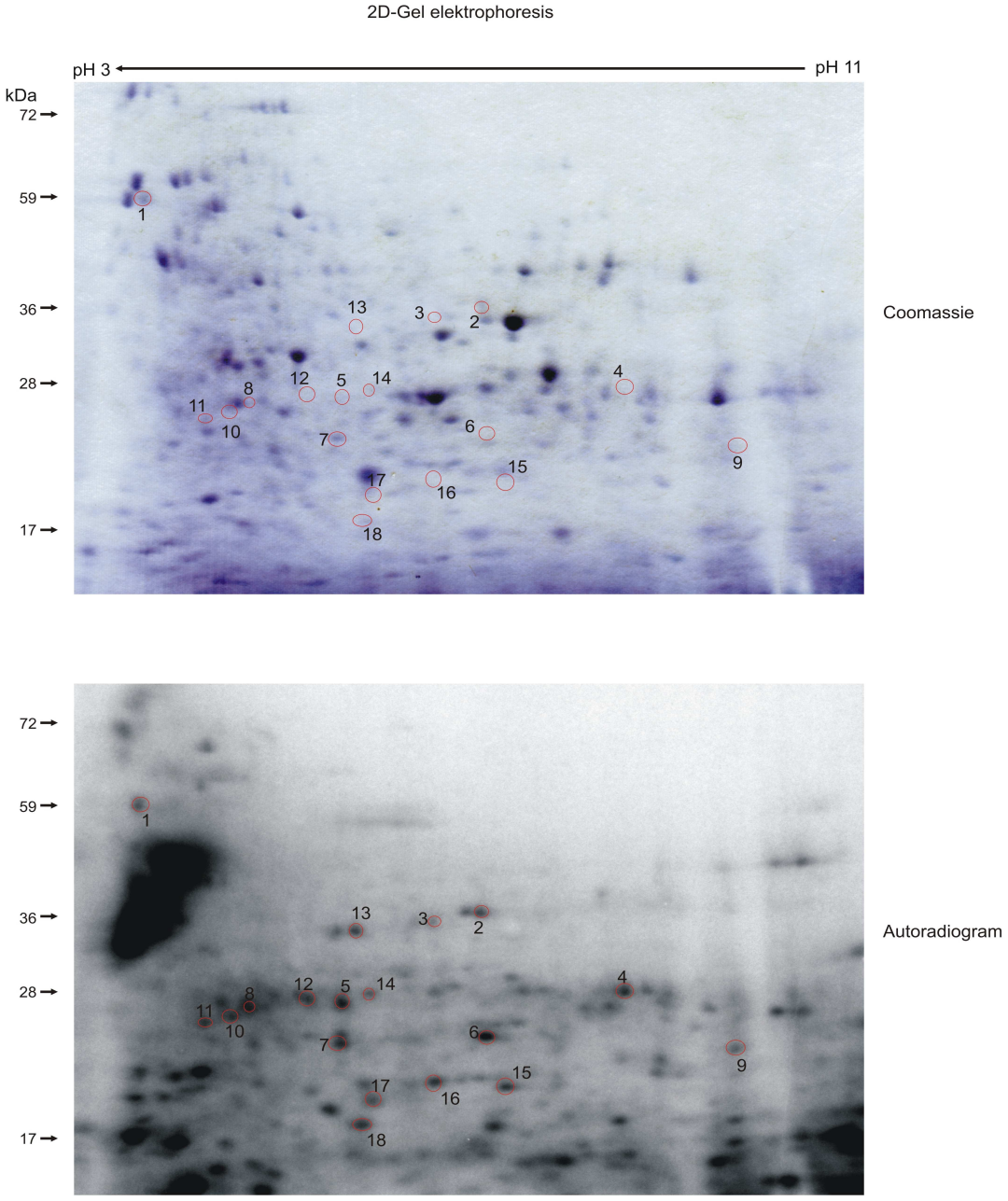

Figure 2.15: Coomassie stain and autoradiogram of the phosphorylation of microsomal membranes in the presence of recombinant CPK3 and γ^{32}-ATP. Excised spots which were analyzed by MS are marked with red cycles and are numbered.

Slices of the size of 28 and 40 kDa (figure 2.12 C marked with *) were excised from the gel and analysed by MS after tryptic in-gel digestion (table 1 and 2). Further samples were analyzed after 2D-gel separation of phosphorylated microsomal fractions (figure 2.15).

Based on a comparison of radioactively labelled samples, where microsomal membranes were phosphorylated by CPK3 in the presence of γ^{32}P-ATP, and a non-

radioactive 2D-gel, 18 different spots with high activity were selected for further analysis by MS resulting in the identification of following candidates (table 2). Identified proteins marked with * are also found in the "phosphate" database (http://phosphat.mpimp-golm.mpg.de/) which is a compilation of all *in vivo* identified phosphoproteins (Heazlewood, Durek et al. 2008). The selected proteins in bold should be analysed in future works because of there certain functions in ion homeostasis and signalling.

Table 1: MS results of the 28 kDa gel slice

Reference name	**NCBI (GI)**	**TAIR (GI)**
adenylate kinase	3746809	AT5G63400
ANAC071	15236556	AT4g23630*
AT5g58420/mqj2_10	17979233	AT5g58420*
ATPHB1 (PROHIBITIN 1)	15235317	AT4g28510
ATPHB3 (PROHIBITIN 3)	15237488	AT5g40770
ATPHB4 (PROHIBITIN 4)	15232129	AT3g27280
ATPHB5 (PROHIBITIN 5)	15241367	AT5g14300
ATPHB6 (PROHIBITIN 6)	15225374	AT2g20530
ATPUMP1	15232420	AT3g54110
band 7 family protein	18395770	AT3g01290*
Cytochrome c oxidase subunit 2	44887814	ATMg00160
EMB2296 (EMBRYO DEFECTIVE 2296)	15227954	AT2g18020
EMB2386 (EMBRYO DEFECTIVE 2386)	15218602	AT1g02780*
EMB3010 (EMBRYO DEFECTIVE 3010)	15238142	AT5g10360*
enoyl-CoA hydratase/isomerase family protein	30683577	AT4g16210
GRF9 (GENERAL REGULATORY FACTOR 9)	18406007	AT2g42590
multicatalytic endopeptidase complex	2511592	AT2g27020
phosphate-responsive protein	15242420	AT5g09440
plastid-lipid associated protein PAP	18403751	AT3g23400
porin	**15232074**	**AT3g01280**
porin	**15240765**	**AT5g67500**
porin	**15242210**	**AT5g15090**
porin	**15242146**	**AT5g57490**
putative protein	6562305	F13G24.110
putative ribosomal protein S4	6598334	AT2g17360
ribosomal protein L7A (RPL7aA)	15226635	AT2g47610
ribosomal protein L7A (RPL7aB)	15229338	AT3g62870
ribosomal protein L8 (RPL8C)	15234298	AT4g36130*
ribosomal protein L8-2	108860940	AT3g51190
ribosomal protein S6	2224751	AT5g10360
ribosomal protein S8 (RPS8A)	15241316	AT5g20290
RPS6 (RIBOSOMAL PROTEIN S6)	15236042	AT4g31700*
transducin family protein / WD-40 repeat	18421762	AT5g38480*
unknown protein	15227104	AT2g21870
v-ATPase subunit D	**5360953**	**AT3g58730***
VHA-E3	15222641	AT1G64200
V-type proton-ATPase	1143394	AT4g11150

Tabelle 2: MS results of the 40 kDa gel slice

Reference name	NCBI (GI)	TAIR (GI)
ANNAT1	**15220216**	**AT1g35720***
(S)-2-hydroxy-acid oxidase, peroxisomal	15231850	AT3g14420
AAC1 (ADP/ATP CARRIER 1)	15231937	AT3g08580
anion-transporting ATPase	18378897	AT1g01910
ATGSR1 (glutamine synthase)	**15240288**	**AT5G37600***
ATPDIL2-1/MEE30/UNE5 (PDI-LIKE 2-1)	15226610	AT2g47470
binding / catalytic/ coenzyme binding	18399328	AT2g20360
carbon-nitrogen hydrolase family protein	22326744	AT5g12040
CPK3	15236560	AT4g23650
F23N19.17	6630455	F23N19.17
GAPC	15229231	AT3g04120
GHMP kinase family protein	30678384	AT3g01640
GLN1;4 (Glutamine synthetase 1;4)	18418013	AT5g16570
glutamate-ammonia ligase (EC 6.3.1.2)	99698	AT5g37600
H^+-transporting two-sector ATPase	15229475	AT3g28715
membrane-associated salt-inducible protein like	2632061	AT4G36680
NADPH thioredoxin reductase	468524	AT2g17420
nucleotide-binding subunit of vacuolar ATPase	166627	AT1G76030
OEP37; ion channel	18406405	AT2g43950
protein kinase	**30695267**	**AT1g52540***
S-adenosyl-methionine-sterol-C-methyltransferase	2246456	AT1G76090
serine/threonine protein kinase	22331138	AT3g17410
SHS1 (SODIUM HYPERSENSITIVE 1)	15236783	AT4g32400
terpene cyclase/mutase-related	15234163	AT4g33360
UGE1	15222072	AT1g12780
unknown protein	22329857	AT1g29790
unknown protein	15233608	AT4g29520
uridine diphosphate glucose epimerase	12323247	AT1G63180
VACUOLAR ATP SYNTHASE	15233891	AT4g38510
VHA-A	15219234	AT1g78900

Table 3: MS results of the 18 spots from the 2d-gel PAGE

Reference name	NCBI (GI)	Tair (GI)	Found in spots
ACA1	**30690083**	**AT1G27770**	**11**
ACA2	**15235643**	**AT4G37640**	**12**
acetyl-CoA carboxylase beta subunit	7525042	ATCG00500	5,7,8,11,14,18
ADL6 (DYNAMIN-LIKE PROTEIN 6)	**15218486**	**AT1G10290***	**4,5,7,12**
AGO1 (ARGONAUTE 1)	15221177	AT1G48410*	9
ALDH3F1 (ALDEHYDE DEHYDROGENASE 3F1)	42567452	AT4G36250	8
ALDH3H1 (ALDEHYDE DEHYDROGENASE 4)	15219358	AT1G44170	15
APX3 (ASCORBATE PEROXIDASE 3)	15236239	AT4G35000	17
ATACP5 (acid phosphatase 5)	18401643	AT3G17790	11
ATBAG7	15241803	AT5G62390	17,18
ATCIMS	15238686	AT5G17920	2,6,13
ATGSTF10 (EARLY DEHYDRATION-INDUCED 13)	15224582	AT2G30870	7
ATPDR1/PDR1	18401096	AT3G16340	3,13
ATPHB3 (PROHIBITIN 3)	15237488	AT5G40770	4
ATRLI2 (RNase L inhibitor protein 2)	22328793	AT4G19210	14
calnexin, putative	15240773	AT5G07340	1
clathrin heavy chain, putative	30681617	AT3G11130	18
CLPC (HEAT SHOCK PROTEIN 93-V)	18423214	AT5G50920	2,3,6,10,13

CRT1 (CALRETICULIN 1); calcium ion binding	15223517	AT1G56340*	1
cytosolic tRNA-Ala synthetase	1673366	-	13
ECA4_ARATH Calcium-transporting ATPase 4	12643934	AT1G07670	10
FUS5 (FUSCA 5); MAP kinase kinase	18378920	AT1G02090	10
GAPC	15229231	AT3G04120	2,3,6,7
GF14chi isoform Arabidopsis thaliana GRF1	1255987	-	11
glutamate-tRNA ligase	30690281	AT5G26710	6,8,18
GRF2 (GENERAL REGULATORY FACTOR 2)	18411901	AT1G78300	10,11
GRF4 (GENERAL REGULATORY FACTOR 4)	**18399524**	**AT1G35160***	**10**
GRF5 (GENERAL REGULATORY FACTOR 5)	18417863	AT5G16050	11
GRF10 (GENERAL REGULATORY FACTOR 10)	18395103	AT1G22300	16,17
H^+-transporting two-sector ATPase	15229475	AT3G28715	11
HpcH/HpaI aldolase family protein	15236908	AT4G10750	5
HSP81-2 (EARLY-RESP. TO DEHYDRATION 8)	15241115	AT5G56030	5,8
KAB1 (POTASSIUM CHANNEL BETA SUBUNIT)	**15219795**	**AT1G04690**	**14**
KAPP (Kinase-associated protein phosphatase)	15239690	AT5G19280	12
kelch repeat-containing protein	30686755	AT2G36360	14
kinesin motor protein-related	15227596	AT2G36200	10
legume lectin family protein	15219173	AT1G53070	4
LOS1 (Low expression of osm. responsive gen. 1)	30696056	AT1G56070*	3,5,8,10,11,12
magnesium transporter CorA-like protein-related	22325463	AT2G04305	12
membrane-associated salt-inducible protein like	2632061	-	16
MFP2 (MULTIFUNCTIONAL PROTEIN)	15231317	AT3G06860	9
MgATP-energized glutathione S-conjugate pump	2909781	-	18
monooxygenase, putative (MO2)	15233923	AT4G38540	7
NDB1 (NAD(P)H DEHYDROGENASE B1)	18417151	AT4G28220	8
OMR1 (L-O-METHYLTHREONINE RESISTANT 1)	15232827	AT3G10050	13
PDR8/PEN3 (PLEIOTROPIC DRUG RESISTANCE8)	**15218936**	**AT1G59870***	**3,13,18**
porin	**15242210**	**AT5G15090**	**4,6,12,15,16,17**
porin	**15232074**	**AT3G01280**	**4,6,9,15,16,18**
porin	**15240765**	**AT5G67500**	**9**
protein phosphatase 2C, putative / PP2C	**15232538**	**AT3G15260**	**10,11**
protein phosphatase 2C, putative / PP2C	**18395099**	**AT1G22280***	**4**
putative ABC transporter	4581139	-	6,8,13
putative coated vesicle membrane protein	21595553	-	17,18
RPT4A (regulatory particle triple-A 4A)	15239140	AT5G43010	6,16
SDH2-1 (succinate dehydrogenase 2-1)	15232149	AT3G27380	5
sec34-like family protein	30698937	AT1G73430	8
signal recognition particle 54 kDa protein 2	11094805	-	5,12
sucrose synthase	**436792**	**-**	**2,11,15,17**
SUS4	**22331535**	**AT3G43190**	**2,7,13,16,17**
tetratricopeptide repeat (TPR)-containing protein	30690956	At4g37460	6
TOC75-III (translocon outer membrane complex 75-III)	15232625	AT3G46740	15
UbiE/COQ5 methyltransferase family protein	15242092	AT5G57300	7
Unknown protein	17065080	-	1
VACUOLAR ATP SYNTHASE SUBUNIT B2	15233891	AT4G38510	5
v-ATPase subunit D	5360953	-	18
VHA-A; ATP binding / H^+ transporting ATP synthase	15219234	AT1G78900	2,3,5,6,7,8,9,10,11, 12,13,14,15,17,18
VHA-A3 (VACUOLAR PROTON ATPASE A3)	18420373	AT4G39080	11,16
VPS45 (VACUOLAR PROTEIN SORTING 45)	18411376	AT1G77140	12
V-type proton-ATPase	1143394	-	5,8,9,12,14
Y5957_ARATH Uncharacterized protein At5g39570	75171219	At5g39570	1

2.2 Impact of N-myristoylation on localisation of CDPKs

The data presented in the previous chapters on the identification of potential CPK3 substrates in microsomal fractions illustrate already the great importance of the subcellular localization of components involved. Therefore a more general study should be performed to address the regulation of CDPK localization by N-terminal myristoylation and to elucidate general principles of this mode of protein targeting. To test whether the observed N-myristoylation of selected CDPKs influences their localization *in vivo*, I selected several different CDPKs, also including candidates, for which no modification had been predicted (Table 4). Two different experimental strategies were applied. The first needed the analysis of subcellular localization of CDPK-YFP-fusion proteins in isolated *Arabidopsis* protoplasts, and the second, analyzed subcellular localization in tobacco leaves after infiltration with *Agrobacteria*. The use either the wild type form of those proteins which should be myristoylated or the G2A mutant form which cannot be myristoylated *per se* should allow visualizing the effect of N-myristoylation *in vivo*.

Table 4: Prediction of CPK2, CPK6, CPK9 and CPK13

Protein	IMP predictor	PlantsP predictor
CPK2	TWILIGHT ZONE	Positive
CPK6	NO	Positive
CPK9	RELIABLE	Positive
CPK13	NO	Positive

Prediction of myristoylation using two prediction programs. The IMP prediction site: http://mendel.imp.ac.at/sat/myristate/SUPLpredictor.htm and the PlantsP predictor: http://plantsp.genomics.purdue.edu/plantsp/html/myrist.html.

Prediction and reality: Myristoylation of unpredicted candidates - *in vitro*

It was essential to demonstrate at the beginning that the investigated proteins are translated with the same efficiency and that they are indeed N-myristoylated or not, as expected for the respective protein or its mutant. This was done in an *in vitro* translation system as described already for CPK3. The results are shown below for CPK2, CPK3, CPK6, CPK9, CPK13, CPK16, CPK17 and CPK28, following the procedure described by Lu and Hrabak 2002. CPK2 was used as positive control for a myristoylated protein

(Lu and Hrabak 2002), and the G2A served as negative controls of all investigated CDPKs (figure 2.16).

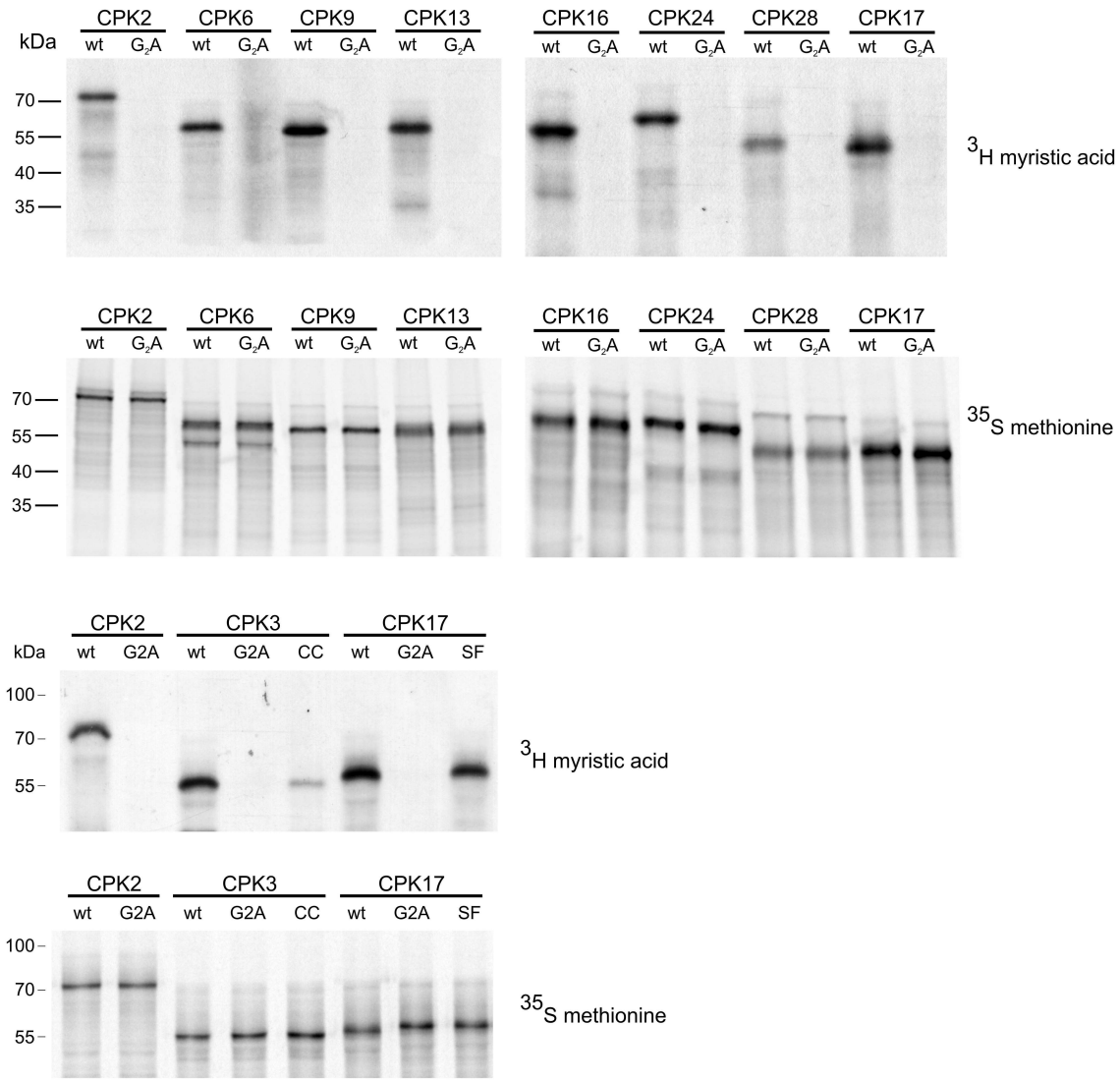

Figure 2.16: The wild type and the point mutants of the CDPKs were in vitro translated using coupled transcription/translation in wheat germ extracts. Translation to label total protein in the presence of [^{35}S] methionine. Translation in the presence of non radioactive amino acids but [^{3}H] labelled myristic acid to detect myristoylated proteins. CPK2 served as a positive control for a myristoylated protein in these experiments according to Lu and Hrabak 2002.

In summary, the N-myristoylation of all tested CDPKs could be shown in these experiments. Notably also those CDPKs which were not predicted by the IMP Predictor turned out to be myristoylated in-vitro. Therefore the next step was to test the effect of myristoylation on the localisation *in vivo* using YFP fusion proteins.

Prediction and reality: myristoylation of unpredicted candidates - in vivo

The localization of YFP-fusion proteins in transiently transformed *Arabidopsis* protoplasts and in tobacco leaf epidermal cells is shown in figure 2.17 (P1-P4 and T1-T4). The left panel shows always the localization of the wild type proteins as detected with the YFP filter, and the corresponding transmission light image of the same protoplast is shown in the adjacent picture to the right. The localization of the G2A mutants and the corresponding transmission light image of the protoplasts are shown in the right panel of the figure 2.17. For all investigated CDPKs, except for CPK6, the functional consequence of myristoylation on subcellular localisation could be clearly observed. The WT versions were always membrane bound whereas the G2A mutants showed a cytoplasmic localisation.

Additionally, young developing leaves of tobacco plants were infiltrated with constructs encoding YFP-fusion proteins of either the wild type forms of the selected CDPKs or their corresponding G2A mutants. The localization of the YFP-fusion proteins in epidermal leaf cells is shown in figure 2.17 T1-T4, two days after infiltration.

In agreement with the results obtained in protoplasts the wild type version of CPK2 was localized at membranes with clearly visible accumulation at distinct spots (figure 2.17 P1 and T1). Not even the nuclear membrane was visible in the YFP signal. In contrast, the G2A mutant of CPK2 showed a diffuse localization in the cytosol and also a strong signal from the surrounding of the nucleus (figure 2.17 P1 and T1). The CPK6-YFP fusion protein showed a different localization in epidermal leaf cells as compared to protoplasts. CPK6wt-YFP was localized to the plasma membrane and the nucleus. The localization of the CPK6G2A-YFP mutant was the same in principle; only the signal at the membranes was more diffuse (figure 2.17 P2 and T2). For CPK9 (figure 2.17 P3 and T3) and CPK13 (figure 2.17 P4 and T4) the results were the same as obtained from protoplasts.

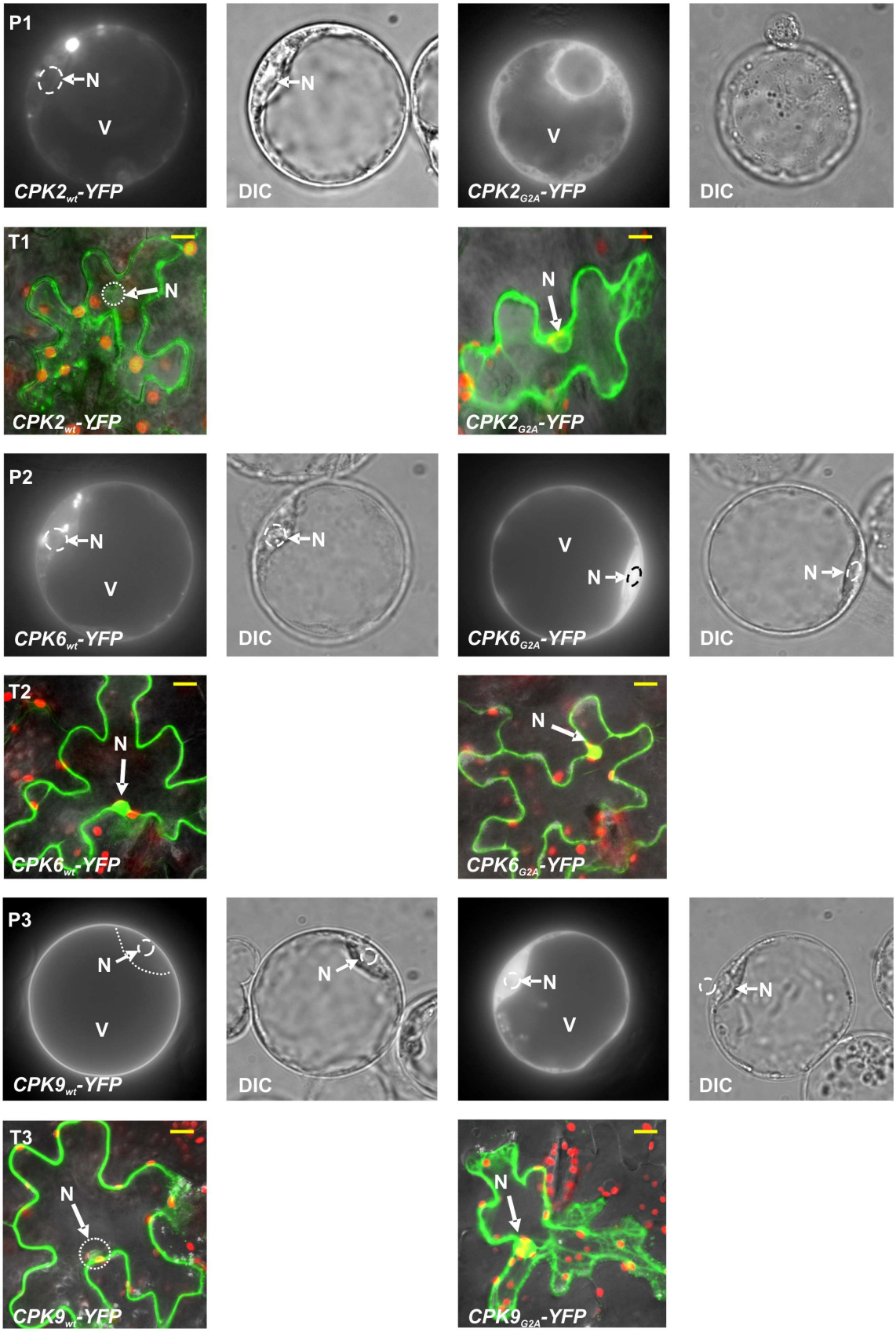

Figure 2.17: (continued next page)

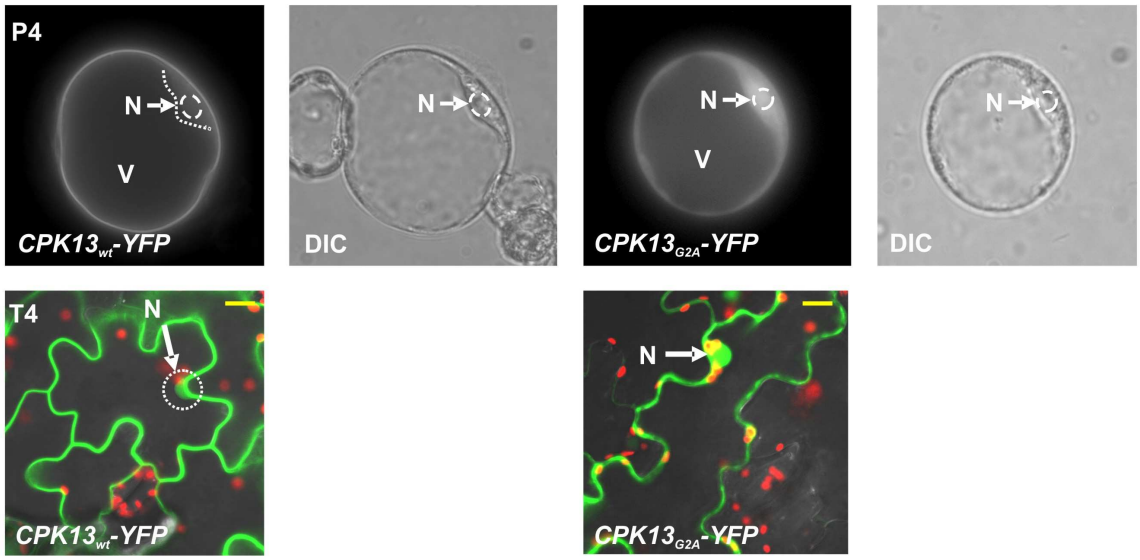

Figure 2.17: Transient expression of YFP-tagged WT/G2A mutants of CPK2, CPK6, CPK9 and CPK13 in Arabidopsis protoplasts (P1-P4) and in tobacco epidermal cells (T1-T4). P1-P4: For each CDPK the YFP signal resulting from a single protoplast is shown on the left, and the corresponding differential interference contrast (DIC) image of the whole cell is shown in the right. T1-T4: Confocal images which are merged from three channels. Red: auto-fluorescence of the chloroplasts; white: transmission light; green: YFP fluorescence of the expressed CDPK. The dashed white lines indicate the position of the nucleus and the borderline between the cytosol and the central vacuole. The pictures were taken the day after transformation for the protoplasts and two day after transformation the infiltrated tobacco leafs.

Addressing general principles of myristoylation and protein localization

CPK3 is the only CDPK from *Arabidopsis*, harbouring a "non-classical" N-myristoylation motif in its N-terminus, which does not also contain one or two cysteines in close vicinity. Because of this circumstance, CPK3 can undergo N-myristoylation but not palmitoylation which would require those cysteines. To test the effect of an artificially "forced" palmitoylation, the N-terminus of CPK3 was altered and two amino acids were replaced by cysteines (N-term: MGH**CC**SKSK). This modification should now enable also palmitoylation of CPK3. However, the *in vivo* expression of the YFP tagged mutant did not show increased attachments to membranes (figure 2.18 A and B). Interestingly the *in vitro* myristoylation of the generated CPK3 CC mutant showed also a severe reduction in N-myristoylation efficiency (figure 2.16).

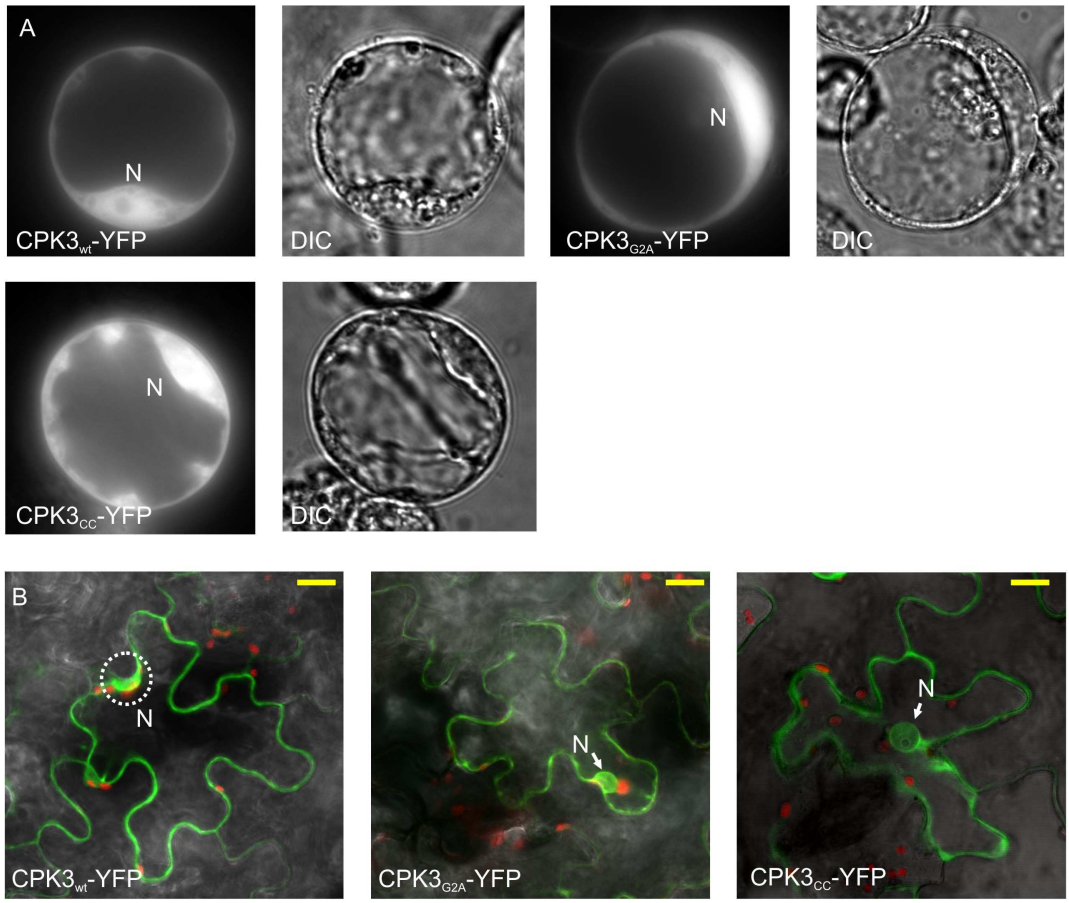

Figure 2.18: Transient expression of YFP-tagged WT/G2A/CC mutants of CPK3 in Arabidopsis protoplasts (A) and in tobacco epidermal cells (B). A: The YFP signal resulting from a single protoplast is shown on the left, and the corresponding differential interference contrast (DIC) image of the whole cell is shown on the right. Confocal images which are merged from three channels. Red: auto-fluorescence of the chloroplasts; white: transmission light; green: YFP fluorescence of CPK3. The pictures were taken the day after transformation of the protoplasts and two day after transformation of the infiltrated tobacco leafs.

CPK17 is an example for a CDPK with very clear and strong membrane localisation. Therefore CPK17 and the G2A mutant form of this CDPK which revealed a clear cytosolic localisation were used for further mutational studies on the N-terminal domain. The N-terminal domain of CPK17 contains the required glycine on the second position for N-myristoylation, and two additional cysteines for palmitoylation. To ask whether these cysteins are responsible for the palmitoylation and therefore for the membrane attachment, a mutant form of CPK17, without these two cysteines was analyzed. In this mutant both cysteins were replaced by one serine and a phenylalanine (N-Term: MGN**SF**SHGRD). The expression of this CPK17 SF in tobacco epidermal cells showed clear cytosolic localisation (figure 2.19 B). In contrast, the expression in protoplasts was different because most of the protein was localized to the surrounding

of the nucleus (figure 2.19 A). Interestingly, the *in-vitro* myristoylation of CPK17 SF showed only a weak reduction in myristoylation (figure 2.16) thus indicating that the observed difference in localization could indeed be attributed to the difference in palmitoylation.

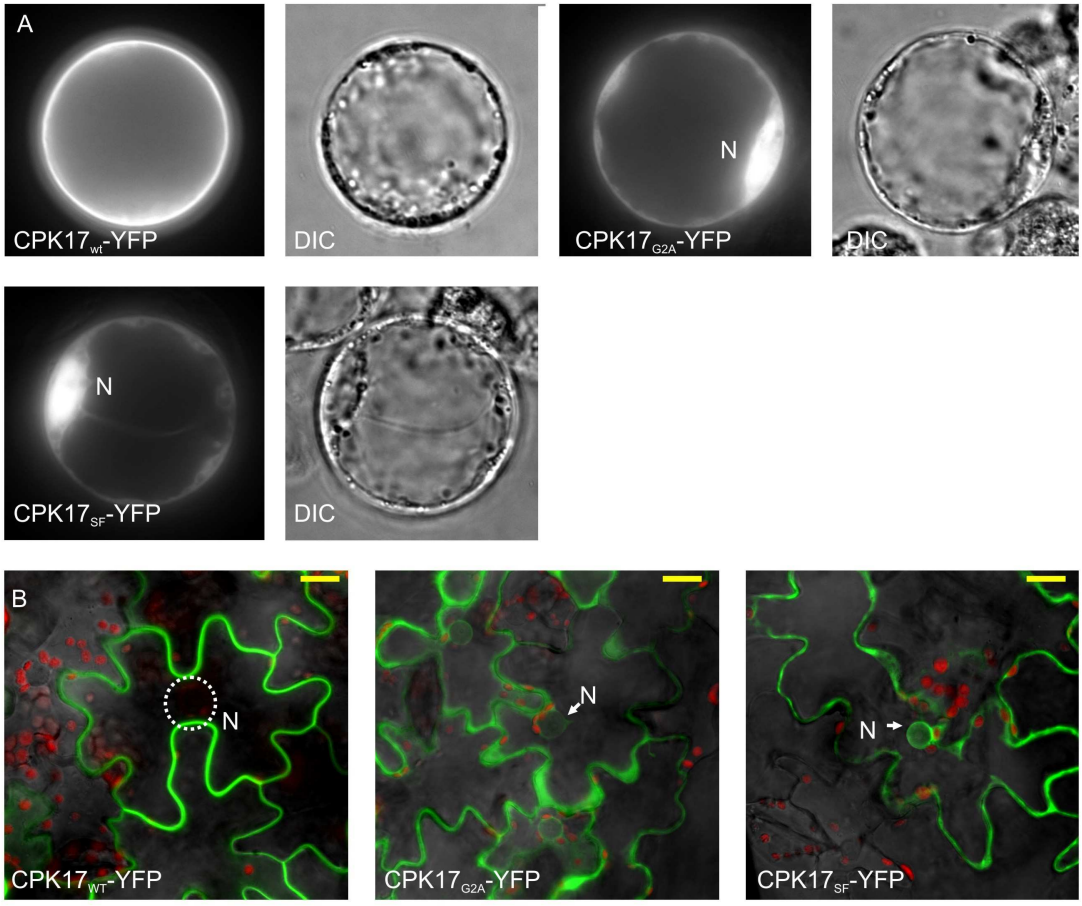

Figure 2.19: Transient expression of YFP-tagged WT/G2A mutants of CPK17 in Arabidopsis protoplasts (A) and in tobacco epidermal cells (B). A: The YFP signal resulting from a single protoplast is shown on the left, and the corresponding differential interference contrast (DIC) image of the whole cell is shown on the right. Confocal images which are merged from three channels. Red: auto-fluorescence of the chloroplasts; white: transmission light; green: YFP fluorescence of CPK17. The pictures were taken the day after transformation of the protoplasts and two day after transformation of the infiltrated tobacco leafs.

CPK16 was chosen for further studies because it is predicted as chloroplast-localized with a very high probability (i.e. in TAIR, based on TargetP prediction). The localisation of proteins to the chloroplast requires a chloroplast targeting sequence on the N-terminal domain which is cleaved of upon import into the chloroplast. The probability for a chloroplast localisation can be bioinformatically estimated by analysis of the N-terminal sequence (Emanuelsson, Nielsen et al. 2000). CPK16 contains also an N-myristoylation as well as a palmitoylation motif in its N-terminus. Using the

chloroplast prediction program Target P1.1 (http://www.cbs.dtu.dk/services/TargetP/) the score for CPK16 is 0.939 and the score for the G2A mutant CPK16 is 0.944. Nevertheless, the expression of CPK16 in tobacco epidermal leaf cells and protoplasts showed a membrane and nuclear attached localisation for the wild type version of this protein. However, if the G2A mutant of CPK16 was studied it turned out to be localized in chloroplasts and in the nucleus in tobacco epidermal leaf cells and in protoplasts (figure 2.20 A and B). An evidence for chloroplast localisation is the cleavage of the N-terminal sequence. To test the cleavage, the N-terminal domain of CPK16 and its G2A mutant was fused to YFP, expressed in tobacco leaf cells and analyzed by Western blotting using an antibody against GFP/YFP (figure 2.20 C). The detected size of the N-terminal domain of CPK16-YFP was at 40 kDa as expected, whereas the G2A mutant form of CPK16 had a reduced size of only about 30 kDa. This reduction in size is a strong evidence for a chloroplast localisation of CPK16 G2A due to processing of the N-terminal targeting peptide. However, it has to be kept in mind that this is an artificially created mutant form of the protein! Nothing is known about whether or not a given protein is actually N-myristoylated to 100 % in the cell.

To study the influence of palmitoylation of the chloroplast localized CPK16 G2A a loss-of palmitoylation CPK16 mutant was generated (C4F). In this mutant the single cystein residue was replaced by phenylalanine (MGL**F**FSSAAKS). The expression of this mutant in tobacco epidermal leaf cells revealed a loss-of membrane localization and a strong nuclear localisation. Again, the double mutant G2A C4F was localized to chloroplasts and the nucleus (figure 2.20 B).

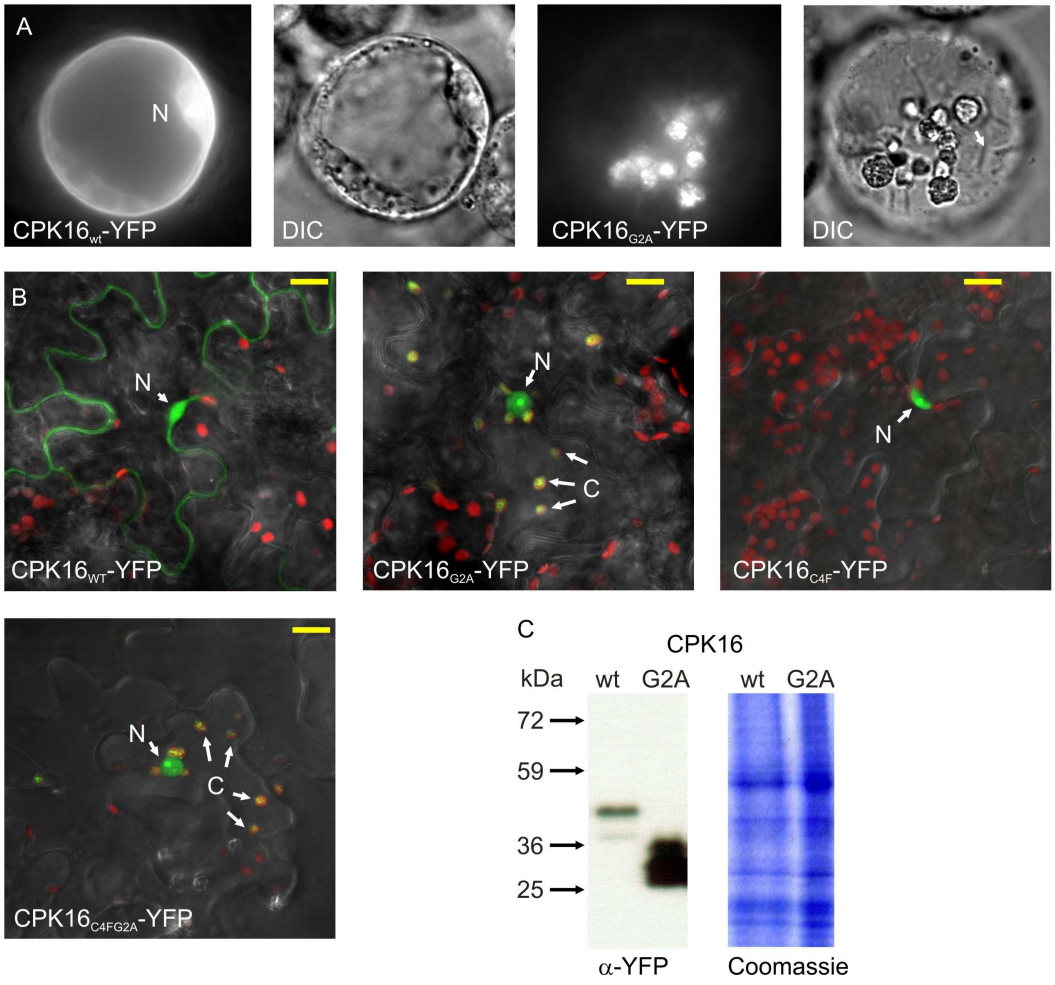

Figure 2.20: Transient expression of YFP-tagged WT/G2A mutants of CPK16 in Arabidopsis protoplasts (A) and in tobacco epidermal cells (B). A: The YFP signal resulting from a single protoplast is shown on the left, and the corresponding differential interference contrast (DIC) image of the whole cell is shown in the right. Confocal images which are merged from three channels. Red: auto-fluorescence of the chloroplasts; white: transmission light; green: YFP fluorescence of CPK17. The pictures were taken the day after transformation of the protoplasts and two day after transformation of the infiltrated tobacco leafs. C: Western blot (left) of samples from expressed wt/G2A N-term-CPK16-YFP in infiltrated tobacco leafs and the corresponding Coomassie stain.

CPK28 is the closest homolog of CPK16 in the Arabidopsis CDPK family revealing the highest sequence homology. Therefore its localization was studied and compared to CPK16. The wild type form of CPK28 was found to be membrane associated in tobacco epidermal leaf cells and in protoplasts, whereas the G2A CPK28 mutant was only visible in the nucleus (figure 2.21 A and B).

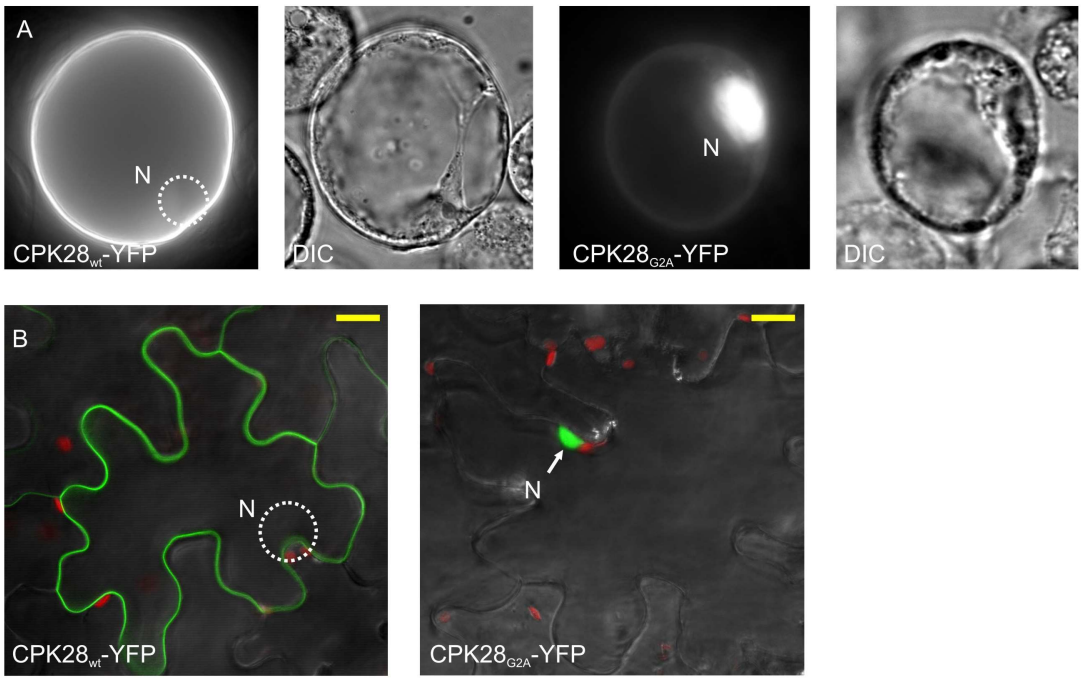

Figure 2.21: Transient expression of YFP-tagged WT/G2A mutants of CPK28 in Arabidopsis protoplasts (A) and in tobacco epidermal cells (B). A: The YFP signal resulting from a single protoplast is shown in the left, and the corresponding differential interference contrast (DIC) image of the whole cell is shown on the right. Confocal images which are merged from three channels. Red: auto-fluorescence of the chloroplasts; white: transmission light; green: YFP fluorescence of CPK28. The pictures were taken the day after transformation of the protoplasts and two day after transformation of the infiltrated tobacco leafs.

3 Discussion

Cellular signal transduction is a prerequisite for plants to be able to adapt to biotic or abiotic stress. Signal transduction involves first the recognition of a given signal and later on its processing into a cellular response, manifested for example in altered gene expression patterns or regulation of fluxes or even movements. A wide spectrum of stresses leads to the generation of free intracellular Ca^{2+} ions which act as secondary messenger not only in plants, but also in yeast or animal cells. In this context, the Ca^{2+} ions are used as fast signaling molecules. These signals are subsequently decoded by Ca^{2+}-sensing proteins such as CDPKs. They are immediately activated in the presence of Ca^{2+} and phosphorylate target proteins. A great number of proteins are known to be phosphorylated by CDPKs (Cheng, Willmann et al. 2002) and these target proteins are involved in biotic/abiotic stress response and regulation of metabolism (Cheng, Willmann et al. 2002). Notably, membrane associated or membrane localized proteins are overrepresent in this set of canonical CDPK targets, which could be explained by the large numbers of myristoylated and therefore membrane associated CDPKs.

3.1 CPK3 is an important regulator in the salt stress response

The analysis of the *cpk3* knock-out line revealed a salt sensitive phenotype on the one hand, and the over-expression of CPK3 remarkably increased salt tolerance in a dosage-dependent manner on the other hand. Furthermore, salt stress triggered CPK3-dependent changes in protein phosphorylation patterns as detected by Western blot of 2D-gel analysis using a phosphorylation specific antibody. Both hints are strong evidence for an important role of CPK3 in the salt stress response and adaptation in *Arabidopsis*. However, in the current literature only little is known about this particular CDPK. In 2003 Dammann et al. (Dammann, Ichida et al. 2003) showed a cytosolic/nuclear localization of CPK3-GFP fusion proteins in roots in an over-expression study. In 2006, Mori et al. (Mori, Murata et al. 2006) showed that *cpk3/cpk6* double knock-out plants displayed altered responses of vacuolar potassium channels in leaf guard cells in response to abscisic acid (ABA). But neither a direct phosphorylation of the channel itself, nor a phenotype of the knock-out plants in response to stress were shown in that work. The observed phenotype of *cpk3* mutants resembled that of MAPK mutants under similar stress conditions (Teige, Scheikl et al. 2004; Qiu, Zhou et al.

2008). This was the reason to extend the functional analysis of CPK3 in the salt stress response of Arabidopsis towards a deeper analysis of functional cross-talk between CDPK- and MAPK- dependent signaling.

3.2 CPK3 signaling upon salt stress does not cross-talk with MAP kinases pathways

Cross-talk between Ca^{2+} and MAPK signaling are well known for animal cells, where Ca^{2+} signals and calmodulines (CaMs) regulate the Ras/Raf/ERK-MAP kinase pathway(Agell, Bachs et al. 2002; Rozengurt 2007), but this general question has so far almost not been addressed in plants. In 2005 Ludwig et al.(Ludwig, Saitoh et al. 2005) reported ethylene-mediated cross-talk between CDPK and MAPK signaling. Ludwig et al. showed that elevated CDPK activities compromised stress-induced MAPK activities by over-expression of a truncated and thereby deregulated tobacco CDPK. Furthermore, this inhibition required ethylene synthesis and perception. In contrast to this work, no interference of CDPK and MAPK activities in the salt stress response could be observed for the investigated kinases. *Arabidopsis* MPK4 and 6, the major players in salt stress triggered MAPK pathways, showed normal activities in CPK3 knock-out and over-expressing lines, and vice versa, CPK3 activity was normally induced in MKK2 knock-out and over-expressing lines. Furthermore, the normal induction of MAPK dependent salt stress marker genes, performed in a *cpk3* knock-out and two independent CPK3 over-expressing lines, also indicated that these pathways act independently and in parallel. In this respect, it is important to note that even in animal cells quite different forms of cross-talk between Ca^{2+} and MAPK signaling pathways have been published. In the common view Ca^{2+} signals activate the MAPKs ERK and p38 in response to external signals (Agell, Bachs et al. 2002) but cases where Ca^{2+} and CaM have a clear inhibitory effect on ERK activation have also been reported (Agell, Bachs et al. 2002; Rozengurt 2007). The latter would be consistent with the observed inhibition of plant MAPK activities by expression of deregulated CDPKs in tobacco(Ludwig, Saitoh et al. 2005). However, the view that MAPK cascades are downstream of Ca^{2+} signaling pathways can certainly not be generalized, not even for the animal system, since it was also shown that the p38 MAPK pathway acts upstream of the Wnt/cyclic GMP/Ca^{2+} non-canonical pathway (Ma and Wang 2007). Therefore the conclusion is that cross-

talk between Ca^{2+}-signaling and MAPK pathways cannot be generalized but has to be specifically considered for each different stimulus and the involved kinases, as already pointed out for calcium activation of the ERK pathway in animal cells (Schmitt, Wayman et al. 2004).

3.3 CPK3 is rather involved in the immediate early response to salt stress

The result that the transcriptional response of a considerable number of known salt stress-responsive genes was not influenced in *cpk3* mutants or by CPK3 overexpression, raised the question how the observed salt sensitive phenotype could be explained. In this respect it is interesting to compare the cellular function of salt stress-activated protein kinases in different organisms. In yeast cells at least two signaling pathways are involved in regulating ion homeostasis and osmotic adjustment. One pathway involves the Ca^{2+}-dependent phosphatase calcineurin, which regulates the expression of ion transporters, e.g. ENA1, the major Na^+ efflux pump in the plasma membrane (Hohmann 2002; Matsumoto, Ellsmore et al. 2002). The second pathway activates the MAPK Hog1, which is required for transcriptional adaptation. But Hog1 is not only involved in transcriptional induction of stress response genes in yeast cells, it also regulates the activities of the Nha1 Na^+/H^+ antiporter and the Tok1 potassium channel by phosphorylation (Proft and Struhl 2004). This dual role of the MAPK Hog1 in yeast osmotic stress adaptation seems to have split in plants. Here mainly the MAPK pathway seems to be responsible for the transcriptional induction of the genes required for long-term adaptation, whereas the CDPK seems to have an important role in the immediate early response by phosphorylating target proteins. Again, this cannot be generalized for all CDPKs, since another Arabidopsis CDPK (CPK10) was shown to act as transcriptional inducer of a barley ABA-responsive promoter in maize leaf protoplasts (Sheen 1996). Additionally, a CDPK from the common ice plant *Mesembryanthemum crystallinum* phosphorylates a nuclear substrate protein in response to salt stress (Patharkar and Cushman 2000).

The number of 15 proteins which were found to be CPK3-dependent differentially phosphorylated on threonine residues after salt stress, presents a reasonable number of potential targets as can be deduced from current literature reviewing CDPK or MAPK

targets (Cheng, Willmann et al. 2002; Colcombet and Hirt 2008). Most importantly, the very few proteomic studies of plant salt stress response, which have been performed so far, focused rather on long-term changes in protein levels and did not address fast changes in post-translational protein modification like phosphorylation. In these studies it was reported that salt stress first causes a transient suppression of de novo protein synthesis (Ndimba, Chivasa et al. 2005), which is also known to occur in yeast cells (Teige, Scheikl et al. 2001), and that visible changes in the total protein patterns could be observed after several hours or even longer periods only (Ndimba, Chivasa et al. 2005; Jiang, Yang et al. 2007). It is clear that an additional and immediate mechanism of adaptation is required to enable plant survival in an acute stress situation. Only one single study did address fast changes in protein phosphorylation in response to salt stress in plants so far. In this study multiple phosphorylation of plant plasma membrane aquaporins was reported (Prak, Hem et al. 2008).

3.4 Subcellular localization of CPK3

A co-localization of the protein kinase and its targets would obviously favour fast and efficient signal transduction, particularly if the activating signal for the kinase is extremely transient and locally restricted, as it is known for CDPKs (Bootman, Lipp et al. 2001). Accordingly, the observed membrane localization of CPK3 would be consistent with a potential role in regulating channel proteins. Since the membrane localization of CPK3 seemed to contrast the published cytoplasmic and nuclear localization of CPK3 (Dammann, Ichida et al. 2003) the question was addressed by two independent approaches. The biochemical approach uses a CPK3-specific antibody for detection of the endogenous protein and confirmed the localization of CPK3-YFP fusion proteins in the nucleus and at cellular membranes in epidermal leaves. Analysis of these constructs in transgenic plants revealed that the CPK3-YFP fusion proteins were able to improve salt tolerance, thus proving these proteins to be functional. Further experiments revealed the membrane localization to be dependent on the N-terminal myristoylation of CPK3. However, a partial membrane association of CPK3 is also visible in the work performed by Dammann et al. in their figure 2 (Dammann, Ichida et al. 2003).

In contrast to CPK3, a nuclear/cytoplasmic localization has been reported for several plant MAPKs including *Arabidopsis* MPK4 and MPK6 (Schweighofer, Kazanaviciute

et al. 2007). The activation of gene expression through MAPK cascades involves dynamic changes of their subcellular localization, also reflecting the localization of their potential targets (Lee, Rudd et al. 2004). In this context, the observed N-myristoylation-dependent-membrane-localization of CPK3 would nicely provide a molecular basis for the different tasks of CDPK and MAPK pathways in plants' salt stress response.

3.5 Cellular targets of CPK3 in salt stress response

The findings that CPK3 is an important regulator of salt stress adaptation and that it is localized to membranes suggest that CPK3 regulates membrane associated proteins which are involved in salt stress adaptation. Regulation of membrane located channel proteins by phosphorylation is already a known mechanism for several proteins (Mahajan, Pandey et al. 2008). From literature it is known that the substrates of CDPKs contain a distinct phosphorylation motif, which is capable of binding of 14-3-3 proteins (Cheng, Willmann et al. 2002). 14-3-3 proteins (General Regulating Factors) recognize phosphorylation motifs in the phosphorylated state and subsequently modulate the function of the phosphorylated protein. The recently identified phosphorylation site in the vacuolar two-pore K^+ channel TPK1 (Latz, Becker et al. 2007) is a perfect CDPK target site for 14-3-3 protein binding after phosphorylation and it would therefore be an ideal candidate for CDPK-dependent regulation. CPK3 was clearly able to phosphorylate TPK1 in vitro (figure 2.12) and the phosphorylation of microsomal membranes further underpinned that TPK1 presents a valid CPK3 target *in vitro* as well as *in vivo*.

A large set of trans-membrane proteins, which are known to be important in the exchange of water and solute are the Major Intrinsic Proteins (MIPs) (Reizer, Reizer et al. 1993; Ishibashi and Sasaki 1995). MIPs that selectively transport water through membranes are called aquaporins. In *Arabidopsis* a family of 35 members of MIPs has been predicted, which can be classified into 4 groups according to their amino acid sequence homology: The plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), NOD26-like MIPs (NIPs), and the small basic intrinsic proteins (SIPs) (Johanson, Karlsson et al. 2001). It has been shown that some MIPs are regulated by phosphorylation. For example, Nodulin 26 (Nod26) from soy bean is phosphorylated on Ser262 by a CDPK (Weaver, Shomer et al. 1994). It was further shown that this phosphorylation enhances water permeability of the channel and it is

regulated developmentally and by osmotic signals (Guenther, Chanmanivone et al. 2003). The seed specific α-TIP is phosphorylated by a tonoplast localized Ca^{2+}-dependent kinase (Johnson and Chrispeels 1992). Three functional phosphorylation sites have been mapped in this channel (Ser7, Ser23 and Ser99) regulating its water permeability (Maurel, Kado et al. 1995).

In this work CPK3 was found to be associated with membranes *in vivo* and co-segregated with microsomal membranes in biochemical cell-fractionation experiments. For that reason the search for CPK3 substrates was focused on membrane-associated proteins. *In vitro* phosphorylation of isolated microsomal membranes revealed different phosphorylation patterns for the analyzed CDPKs (CPK3, CPK4, CPK5, CPK6 and CPK11). Interestingly, the phosphorylation pattern obtained with CPK3 differed most strongly from the phosphorylation patterns obtained with the other tested CDPKs. In particular, a strong phosphorylation was visible at proteins with molecular masses of about 25-28 kDa and 40 kDa, respectively.

In order to develop this approach for the detection of membrane-localized CPK3 targets further, a 2D-separation approach was used. Again, recombinant CPK3 was used to phosphorylate proteins in microsomal membranes before separation by 2D-gel electrophoresis. This approach resulted in the identification of a great number of proteins as potential targets by MS. Therefore all identified proteins were further analysed in two steps. The first selection represented the quality of the obtained spectra, thus reflecting the confidence, for the identification if this particular protein. Second, the identified proteins were analyzed for CDPK related phosphorylation motifs according to (Cheng, Willmann et al. 2002) in order to reduce the number of false positives. According to their function, the identified proteins could be classified in: transporter, metabolism, signalling and other/unknown (figure 3.1).

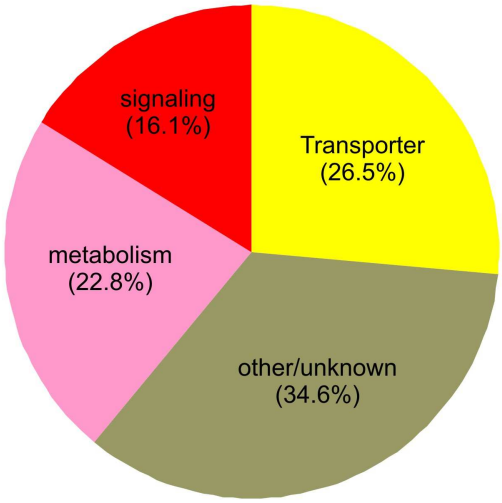

Figure 3.1: Functional classification of identified potential CPK3 targets in microsomal membranes.

Interestingly, there is a big overlap between the identified proteins and a recently published list of proteins identified in detergent-resistance membranes (DRM) which are also known as lipid rafts (Fauquenoy, Morelle et al. 2008). In this publication it was also shown that a subset of the identified proteins are Ca^{2+} dependent protein kinases. Moreover, these CDPKs (including CPK2) are also shown to be myristoylated and palmitoylated. *In vivo,* these lipid rafts are organized into discrete regions (microdomains) with distinct lipid and protein content.

To conclude, CPK3 is localized to membranes and phosphorylates Ca^{2+} dependently membrane bound proteins. The analysis of these proteins revealed that a major part is involved in transport of solutes/ions and signaling. Phosphorylation of these proteins could be a mechanism for there activation upon Ca^{2+} mediated stress response and would explain the salt sensitive phenotype of the *cpk3* knock-out line.

3.6 Activation of CPK3

The current model of the activation of CDPKs suggests that the binding of Ca^{2+} to the calmodulin domain of CDPKs results in an refolding of the protein which releases the auto-inhibitory domain from the kinase catalytic domain (Harper, Breton et al. 2004). This event makes the kinase catalytic domain accessible and opens the possibility for the interaction with the substrate. Therefore any changes, which would affect interaction between the kinase domain and the regulatory domain, would be expected to have a

great impact on kinase activity towards a given substrate. Autophosphorylation of CPK3 revealed the existence of five differentially phosphorylated forms (fig. 2.13 D). One of those sites could unambiguously be identified as Ser 242 in CPK3, which is localized within the substrate binding domain. Therefore this phosphorylation event was further studied by generation of loss-of-function (Ser to Ala) and gain-of-function (Ser to Asp) mutants of this site in CPK3. If the autophosphorylation at this site should be required to trigger the "dissociation" of the auto-inhibitory domain from the active kinase domain, the Ala mutation should have little effect on the activity, and the Asp mutation- mimicking the phosphorylated version – should render the kinase (more or less) Ca^{2+} independent. However, the functional analysis of these mutants showed that this not to be the case. Both mutants had a reduced activity as detected by autophosphorylation and phosphorylation of its substrate TPK1. Hence, Ser 242 seems not to be involved in this regulatory aspect of CPK3 and further studies are clearly required to solve this complex issue involving so many different phosphorylation sites.

3.7 General principles and consequences of protein N-Acylation

Most of the *Arabidopsis* CDPKs are thought to be N-terminally myristoylated and palmitoylated. The attachment of the myristic acid increases not only the hydrophobicity of the protein; it also influences the process of translation and the sub-cellular targeting of the protein. The data presented in this work underpin the significance of protein N-myristoylation for the appropriate sub-cellular localisation. The selected CDPKs, which were studied in more detail in this work, showed clearly that the loss of myristoylation was responsible for the loss of membrane association. Most of the analysed CDPKs displayed a membrane associated localisation in their wild type form which changed dramatically if the myristoylation sites were abolished by introducing point mutations. Such a localisation would therefore explain nicely the great number of known membrane localized target proteins for different CDPKs (Cheng, Willmann et al. 2002).

In agreement with other studies on CDPKs, the N-myristoylation of CPK2 has already been shown by Lu and Hrabak 2008. The N-myristoylation of CPK9 had never been demonstrated *in vitro* before, but a study by Dammann, Ichida et al. 2003 CPK9 was shown to be membrane associated in root cells, and (Lino, Carrillo-Rayas et al. 2006) isolated in the beetroot homologue of CPK9 from plasma membranes. Moreover, in

recent proteomic studies CPK9 was also found to be associated with membranes (Nuhse, Stensballe et al. 2003). In addition to these more or less "expected" results it was possible to demonstrate experimentally N-myristoylation of CPK6 and 13 *in vitro* despite the negative prediction for those candidates. Accordingly, those two CDPKs were listed as not myristoylated in literature (Cheng, Willmann et al. 2002). The biological relevance of this *in vitro* result is clearly visible *in vivo* as indicated by the different subcellular localization of those CDPKs (figure 2.17 P1-P4 and T1-T4). The difference between the different prediction programs and the experimental result might be explained by a different substrate specificity of the plant N-Myristoyl Transferase (NMT) in comparison to the well studied yeast or animal NMTs (Qi, Rajala et al. 2000; Thompson and Okuyama 2000; Boisson, Giglione et al. 2003), which serve as basis for most currently used prediction programs.

It is known that N-myristoylation and palmitoylation influences the localisation of proteins but the detailed mechanism, i.e. which modification influences which particular subcellular targeting is not well understood. To address this question I chose CPK3, CPK16, CPK17, and CPK28 to investigate general aspects of N-terminal myristoylation and palmitoylation. CPK3 was mutagenized to induce an artificial palmitoylation motif in order to test how the double acylation would influence localization. Of particular interest was the question whether this additional palmitoylation would prevent nuclear import of CPK3. Unfortunately no effect could be observed for the YFP-constructs in infiltrated tobacco leaves, but it remains to be tested if the mutated forms are indeed myristoylated and palmitoylated as expected.

CPK17 is N-myristoylated and palmitoylated in its wild type form and targeted to the plasma membrane. To test the effect of palmitoylation on plasma membrane targeting, two cysteins, which are localized in the N-terminal part of the protein, were replaced by serine and alanine. In this mutant the membrane associated localisation of the CPK17 wt was dramatically changed to the cytoplasma but notably not to the nucleus as it was seen for other CDPKs as CPK28 for example. Interestingly, this mutant showed also a similar localisation like the CPK17 G2A, which was clearly localized in the cytoplasm and not in the nucleus. For CPK17 it seems that both modifications, the N-myristoylation and palmitoylation, are necessary for the appropriate targeting to the plasma membrane.

The loss of N-myristoylation is not always connected with the loss of membrane attachment and the change to a cytoplasmatic localisation. The most striking example in

this context is certainly CPK16, which showed a membrane associated localisation as wt protein, but a chloroplast localization as G2A mutant. CPK16 also contains an N-terminal residue for palmitoylation, which could be responsible for an appropriate membrane localisation and to keep CPK16 out of chloroplasts. However, the loss of palmitoylation mutant C4F did not show a chloroplast localisation. It seems therefore that the palmitoylation of CPK16 is not the mechanism, which keeps CPK16 out of chloroplasts. On the other hand the C4F mutant also had a reduced membrane associated localisation and a strong nuclear localisation, which could be taken as evidence for a palmitoylation based mechanism of membrane attachment. A completely different explanation could be that the loss of membrane association of CPK16 C4F might also be explained by a fast brake-down of the mutant protein in the cytoplasm if the protein is not correctly localized to the membrane or nucleus. This effect could also be the reason why CPK28 showed a membrane associated localisation while the G2A mutant was exclusively localized in the nucleus. Still, it does not explain the chloroplast localisation of CPK16 G2A. CPK16 wt and G2A only differ in the N-myristoylation and a simple explanation for the chloroplast localisation of CPK16 could be that the G2A mutant has a reduced hydrophobic domain on the N-terminus.

In summary it became clear from these initial studies that the regulation of N-terminal myristoylation and palmitoylation is one of the key mechanisms for correct subcellular targeting of different CDPKs. Accordingly these studies should be continued using these CDPKs as ideal molecular tools to study general principles of subcellular targeting and the influence of N-terminal acylation. Moreover the localization of particular CDPKs at distinct membranes has high implications for its cellular functions, thus offering the possibility to test even phenotypes of these modifications *in planta*, as it was done for the myristoylated SOS3 (Ishitani, Liu et al. 2000) or CBL2 (Batistic, Sorek et al. 2008) for example.

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Curriculum Vitae

date of birth	24.01.1977 in Salzburg
father	Lothar Mehlmer
mother	Eva Mehlmer
1984-1986	elementary school
1986-1995	Rudolf-Steiner-Schule
1995-1998	school leaving examination (additional)
1998-1999	study of biology at the University of Salzburg
15. July 2002	6 weeks practical training at the Max-Planck-Institute for Molecular Genetics in Berlin
2002-2003	diploma theses at the Max-Planck-Institute for Molecular Genetics in Berlin
15. December	diploma examination
5. April 2004	4 weeks practical training at the Max-Planck-Institute of Biochemistry in Munich
10. May - July 2004	scientific work in the group of Prof. Koller (University of Salzburg)
1. November 2004	begin of my PhD theses at the MFPL in Vienna
18-28. September 2006	EMBO practical course on molecular genetics of Chlamydomonas

List of publications

1.) A CDPK pathway acts independently of MAPK signaling in the Arabidopsis salt-stress response
Norbert Mehlmer, Daniela Hofmann-Rodrigues, Bernhard Wurzinger and Markus Teige
J Biol Chem, JBC (in revision)

2.) CDPK dependent phosphorylation of the Arabidopsis vacuole tandem-pore K^+ channel TPK1 under salt stress
Latz* A., Mehlmer* N., Müller T, Csaszar E., Hedrich R., Teige M, and Becker D.
(Manuscript submitted)

3.) Functional Complementation of Yeast Mutants to Study Plant Signalling
Norbert Mehlmer, Elisabeth Scheikl-Pourkhalil and Markus Teige
Plant Signal Transduction Pathways. Methods and Protocols.
Series: Methods in Molecular Biology , Vol. 479, 2009, ISBN: 978-1-58829-943-7 (in press)

4.) Experimental testing of predicted myristoylation targets involved in asymmetric cell division and calcium-dependent signalling
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5.) Ubiquitin lysine 63 chain forming ligases regulate apical dominance in Arabidopsis.
Yin XJ, Volk S, Ljung K, Mehlmer N, Dolezal K, Ditengou F, Hanano S, Davis SJ, Schmelzer E, Sandberg G, Teige M, Palme K, Pickart C, Bachmair A.
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6.) Functional interaction in establishment of ribosomal integrity between small subunit protein rpS6 and translational regulator rpL10/Grc5p.
Karin Pachler Thomas Karl, Kerstin Kolmann, Norbert Mehlmer, Michaela Eder, Michael Loeffler, Kamil Oender, Elisabeth O. Hochleitner, Friedrich Lottspeich, Nikolaus Bresgen, Klaus Richter, Michael Breitenbach and Lore Koller.
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*with equal contribution