DIPLOMARBEIT

Titel der Diplomarbeit

Calcium dependent protein kinases as regulators of developmental processes in *Arabidopsis thaliana*

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag. rer.nat.)

Verfasserin:	Daniela Hofmann Rodrigues
Matrikel-Nummer:	0103923
Studienrichtung (lt. Studienblatt):	A490 Molekulare Biologie
Betreuer:	Univ. Doz. Dr. Markus Teige

Wien, am 23.10.2007

Zusammenfassung

Während ihres Lebenszyklus müssen sich Pflanzen konstant an sich verändernde äußere Bedingungen, wie Verfügbarkeit von Licht und Nährstoffen oder auch an abiotische und biotische Stresse anpasen. Der ubiquitäre second messenger Ca^{2+} spielt eine zentrale Rolle in der Transduktion externer Signale in molekulare Antworten. Die Vielzahl der räumlich-zeitlichen Ca^{2+} Wellenmuster ermöglicht die Spezifität der einzelnen Signale und wird durch zwei große Familien von Signaltransduktionsmolekülen, den Calcineurin B-like Protein interagierenden Proteinkinasen (CBL/CIPKs) und den Calcium-abhängigen Proteinkinasen (CDPKs) in die endogenen Netzwerke der metabolischen und Entwicklungsprozesse integriert.

Diese Arbeit konzentrierte sich auf die Calcium-abhängige Proteinkinase CPK3 aus *Arabidopsis thaliana*. Im ersten Teil wurden CPK3 Knockout- und Überexpressor-Linien auf Transkriptebene charakterisiert und phänotypische Differenzen unter bestimmten Wachstumsbedingungen analysiert. Von früheren Arbeiten war bekannt, dass CPK3 als positiver Regulator in Salzstressantwort wirkt. Hier wurde die Rolle von CPK3 in der transkriptionellen Regulation bekannter Salzstressantwortgene mittels RT-PCR analysiert. Der CPK3 Gehalt in den untersuchten Pflanzen hatte keinen Einfluss auf das transkriptionelle Induktionsmuster unter Salzstress. Daher wurde vermutet, dass CPK3 Salzstressantwort hauptsächlich auf post-transkriptioneller Ebene reguliert, ähnlich wie es bereits für Mitglieder der CBL/CIPK Familie gezeigt worden war, die den Plasmamembran Na^+/H^+ antiporter Salt overly sensitive (SOS) 1 oder den high-affinity K^+ Transporter AKT1 durch Ca^{2+}-abhängige Phosphorylierung aktivieren. Um den Einfluss von CPK3 auf die K^+-Homöostase durch Regulation von K^+-Kanälen zu evaluieren, wurden die CPK3-Linien auf Medium mit verschiedenen K^+-Konzentrationen angebaut. Ein weiteres Experiment wurde begonnen, um mögliche Kompensation der CPK3-Funktion in CPK3 Knockout Pflanzen durch CDPK-Redundanz zu untersuchen. Spezifische RT-PCR Primer wurden für alle übrigen *Arabidopsis* CDPKs designed um Transkript-Levels in Wildtyp und Knockout in verschiedenen Geweben und Entwicklungsstadien zu vergleichen, sowie um in silenced CPK3 Überexpressor Pflanzen mitausgeschaltete homologe Gene zu identifizieren.

Im zweiten Teil wurde die Interaktion von CPK3 mit Basic Leucine Zipper Transkriptionsfaktoren (bZIPs) weiter untersucht. C-Gruppen bZIPs, bZIP9 und 63 waren bereits in einem früheren Yeast-2-Hybrid Screen als Interaktionspartner von CPK3, sowie als *in vitro* Phosphorylierungs-argets identifiziert worden. Transiente Expression von bZIP9 und 63 als YFP-Fusionen zeigte deren ausschließliche Lokalisierung im Zellkern. Mit Hilfe von *in vitro* Kinase-Assays and MS Analyse konnten multiple CPK3-Phosphorylierungsstellen in beiden bZIPs nachgewiesen werden. Der Vergleich von Ergebnissen über Interaktion von CPK3 mit bZIP9 und 63 aus Yeast-2-Hybrid und Kinase-Assay zeigte, dass Yeast-2-Hybrid eine sub-optimale Methode für die Untersuchung der Interaktionen einer Proteinkinase ist. Die Analyse von bZIP Knockout-Linien zeigte einen möglicher Früh-Blüh-Phänotyp in *bzip63*.

Abstract

During their life cycle, plants constantly have to adapt their metabolism and development to varying external conditions, such as availability of light and nutrients as well as abiotic and biotic stresses. The ubiquitous second messenger Ca^{2+} plays a central role in transduction of external signals into molecular responses. The multitude of spatio-temporal Ca^{2+} wave patterns creates specificity for each signal and is integrated into the endogenous metabolic and developmental networks by two major families of signal transduction molecules, the Calcineurin B-like protein interacting protein kinases (CBL/CIPKs) and the Calcium dependent protein kinases (CDPKs).

This work focused on the calcium dependent protein kinase CPK3 of *Arabidopsis thaliana*. In the first part CPK3 mutant and overexpressor lines were characterized on transcript-level and analyzed on phenotypic differences under specific growth conditions. From previous works, CPK3 was known to act as positive regulator in salt stress response. Here, the role of CPK3 in transcriptional regulation of known salt stress response genes was analyzed via RT-PCR. CPK3 levels in the analyzed plants had no influence on the transcriptional induction pattern under salt stress. Therefore it was presumed that CPK3 mainly regulates salt stress response on the post-transcriptional level, similar to as it had been shown for members of the CBL/CIPK family, which activate the plasmamembrane Na^+/H^+ antiporter Salt overly sensitive (SOS) 1 or the high-affinity K^+ transporter AKT1 by Ca^{2+}-dependent phosphorylation. To evaluate the influence of CPK3 on K^+ homeostasis via regulation of K^+ channels, the CPK3 lines were grown on medium with different K^+ concentrations.

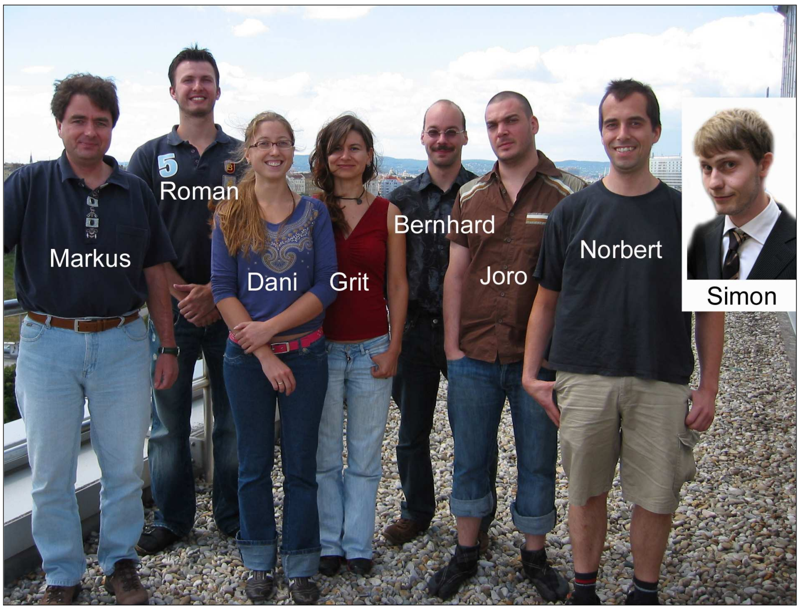
An additional experiment was started to investigate possible compensation of CPK3 function in CPK3 knock out plants through CDPK redundancy. Specific RT-PCR primers were designed for all remaining *Arabidopsis* CDPKs to compare transcript levels in wild type and knock out in different tissues and developmental stages, as well as in silenced CPK3 over-expressor plants to identify co-knocked down homologous genes.

In the second part, interaction with target proteins identified in a previous yeast-2-hybrid screen, the basic leucine zipper transcription factors (bZIPs) was further investigated. The C-group bZIPs, bZIP9 and 63, had already been identified as *in vitro* phosphorylation targets of CPK3. Transient expression of bZIP9 and 63 YFP-fusions revealed their exclusive localization to the nucleus. Using *in vitro* kinase assays and MS analysis, multiple CPK3 phosphorylation sites in both bZIPs were identified. Comparing results on interaction of CPK3 with bZIP9 and 63 in yeast-2-hybrid and kinase assay, it became evident that yeast-2-hybrid is a sub-optimal method for the analysis of protein kinase interaction. Analysis of bZIP knock out lines uncovered a possible early-flowering phenotype in *bzip63*.

Acknowledgements

Zu allererst möchte ich meinem Betreuer, Markus Teige, für die Möglichkeit an diesem Forschungsprojekt in seiner Arbeitsgruppe zu arbeiten, sowie für die geduldige und verständnisvolle Unterstützung auf die ich mich immer verlassen konnte. Mir ist klar, dass das nicht selbstverständlich ist und ich weiß es sehr zu schätzen.

Der gesamten Arbeitsgruppe, Norbert, Roman, Bernhard, Grit, Joro, Simon, Helga und Markus danke ich für die angenehme und konstruktive Athmosphäre, dank Euch werde ich immer gerne an die Zeit im Labor 1 zurückdenken.



Vorallem danke ich Norbert, für seine großzügige, endlose Hilfe mit den vielen Kleinigkeiten im Labor, der Optimierung sämtlicher Protokolle, Klonierungen, transgene Pflanzen etc., und nicht zuletzt für die unzähligen Gespräche, den offenen Gedankenaustausch und das Interesse an meiner Arbeit.

Bernhard, Grit und Simon für die regelmäßige Gesellschaft und Unterhaltung in den Mittagspausen.

Andi, Armin, Alois, Harald sowie all den anderen Menschen die mich während meines Studiums und meiner Diplomarbeit fachlich und moralisch unterstützt haben.

Und natürlich ganz besonders meinen Eltern und meinem Mann die immer für mich da waren und mir Kraft gegeben haben wenn ich sie am meisten gebraucht habe.

Table of contents

1. Introduction

1.1. Stress and signalling

Compared to animals, plants as sessile organisms are more sensitive to external conditions, as individuals generally are unable to leave a location with unfavourable conditions or actively fight an immediate threat. Hence, during the evolutionary process, plants have evolved different mechanisms of self-protection, primarily operating on the molecular level[1-3]. As a consequence, plants show a much greater genomic and epigenomic plasticity compared to animals[4], which becomes obvious in the increased number of events like hybridization[5], polyploidization[5,6], gene duplication[7], recombination[8], transcriptional[9] and post-transcriptional gene silencing[10,11], retro-transposition[12] etc.

During their life cycle plants have to face a variety of stressful conditions, such as low or high light, cold, drought, high osmolarity, heavy metal contamination, anoxia, pathogens, herbivores, as well as inherently generated stresses like reactive oxygen species[3,13]. Plant development and reproduction depend on a precise recognition of environmental conditions and the integration of this information within the endogenous metabolic and developmental networks[14]. For this, plants own a variety of signal transduction pathways and networks which translate the environmental signals into a molecular response.

1.2. The calcium signalling networks

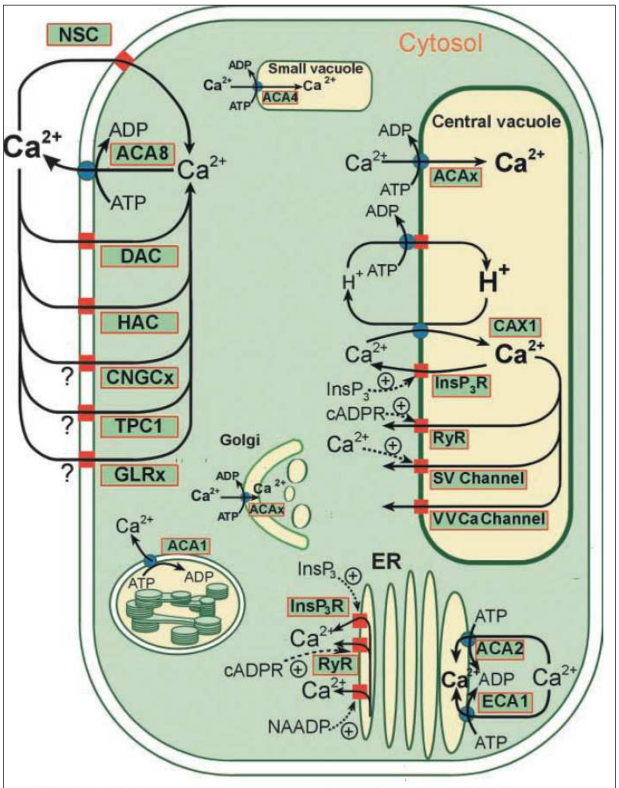


Fig 1.1: Schematic representation of identified Ca^{2+} transport pathways in plant cell membranes[16].

Calcium ions act as second messengers in a multitude of cellular processes including abiotic and biotic stress response, hormone signalling and physiological processes like guard cell regulation, root hair elongation and pollen tube growth[14-18]. The many calcium signals gain specificity by being released in the form of well defined spatial-temporal wave patterns[15-18] and the specific spatial-temporal expression patterns of sensor and effector molecules respectively[14-16]. Figure 1.1 shows the divers calcium transport pathways in plant cells.[16]

Calcium regulated protein kinases translate calcium signals into specific molecular responses[15,19]. Protein kinases have a crucial role in signal transduction as they act as molecular switches. Single and multisite phosphorylation can affect protein-protein interaction and induce global conformational changes in protein structure[20]. Phosphorylation of proteins can lead to their activation, deactivation, degradation as well as protection from it, a change in localization or in binding affinity[20]. There are four major gene families of protein kinases activated directly or indirectly by calcium: the calmodulin (CaM)-dependent protein kinases (CaMKs)[21], the calcium and calmodulin-dependent protein kinases (CCaMKs)[21], the calcineurin B-like protein (CBL) interacting protein kinases (CIPKs)[14,22] and the calcium-dependent protein kinases (CDPKs)[21,23,24].

CaMs and CBLs act as calcium sensors which bind to and activate their target proteins after binding of calcium ions via conserved motives called EF-hands[25,26]. CaMs belong to the most conserved proteins in the Eukaryot kingdom. Calmodulins and calmodulin-like proteins (CMLs) have diverse functions, their targets range from protein kinases to metabolic enzymes and cytoskeleton associated proteins.[26] In Arabidopsis 7 calmodulins and 50 calmodulin-like proteins have been identified.[25] CBLs show significant similarity to calcineurin B (CNB) and neuronal calcium sensors (NCS) from animals. They have been found to be exclusively targeted to CIPKs. The Arabidopsis CBL gene family consists of 10 members.[14]

CaMKs, CCaMKs, CIPKs and CDPKs belong to the CDPK-SnRK superfamily of protein kinases[14,21]. CaMKs, which form the predominant calcium sensor relays in animals, seem not to exist in plants, CCaMKs have been cloned from lily and tobacco, but appear to be absent from Arabidopsis[21]. The major calcium decoding network in plants is most obviously formed by CIPKs and CDPKs which are plant specific gene families. The Arabidopsis genome encodes 25 CIPKs and 34 CDPKs[14,21].

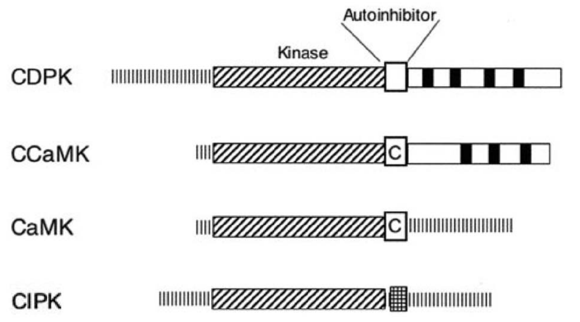


Fig. 1.2: Diagrammatic representation of the domain structures of calcium regulated protein kinases of the CDPK-SnRK superfamily. Broken lines at N or C termini indicate regions of variable lenght and function. Ca^{2+} binding EF hands are indicated as black boxes. The C in the autoinhibitory domain of CaMK and CCaMK indicates that it overlaps with a calmodulin-binding domain. The CIPK autoinhibitory domain (hatched) harbors a CBL binding domain (adapted from [14, 21]).

Figure 1.2 shows a schematic view of the domain structures of calcium regulated protein kinases[14,21]. All calcium regulated protein kinases dispose of an autoinhibitory domain which functions as pseudo-substrate to maintain the kinase inactive in the absence of Ca^{2+}/CaM/CBL. CaMKs and CIPKs are activated by the binding of CaM or CBL respectively to the autoinhibitory domain. This leads to a conformational change and exposure of the catalytic site of the kinase. CCaMKs are regulated more complex; autophosphorylation occurs upon binding of Ca^{2+} to the EF hands which increases the affinity for binding of the Ca^{2+}-CaM complex to the autoinhibitory domain and enhances substrate phosphorylation[21]. CDPKs are unique in that they don’t require an additional protein for activation but are able to auto-activate themselves by binding of Ca^{2+} to the EF hands in the C-terminal calmodulin-like domain which leads to a conformational change and exposure of the kinase domain.[21]

1.3. The Arabidopsis CDPK gene family

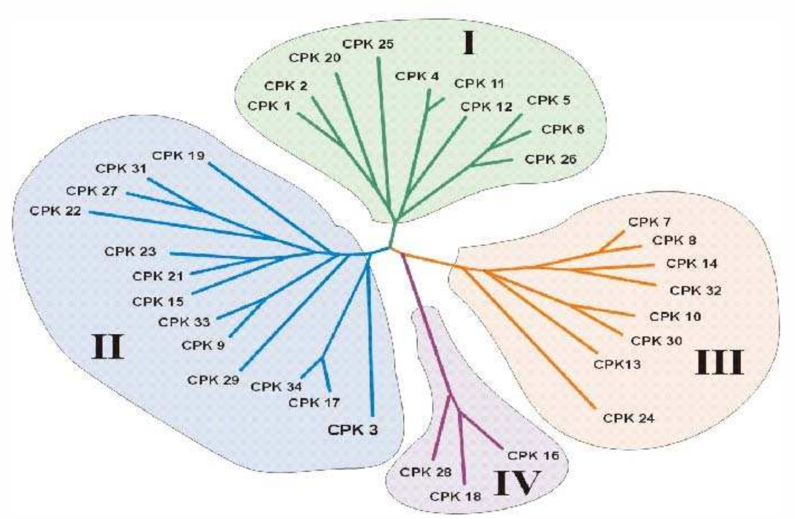


Fig. 1.3: Unrooted phylogenetic tree derived from the protein sequences of the 34 AtCDPKs

Based upon sequence homology, the 34 members of the Arabidopsis CDPK (or CPK) gene family can be divided into 4 subgroups (Figure 1.3). CDPK genes are highly homologous and are found on all 5 chromosomes. Probably CDPKs arose from a fusion of a kinase and a calmodulin and subsequent gene duplication[23,27]. Although there is not much known about functions of specific CDPKs, it is believed that they have both distinct and redundant functions which does not necessarily correlate with sequence homology. Functional specificity may be determined by spatial-temporal expression, post-translational modification, subcellular localization, as well as calcium sensitivity and substrate affinity[23].

All CDPKs consist of a variable N-terminal domain, a highly conserved serine/threonine kinase domain which itself is composed of various subdomains, a basic autoinhibitory linker and a CaM-like domain with 1-4 EF hands. The N-terminal domain hosts subcellular targeting information, such as predicted target peptides or sites for fatty acid attachment leading to membrane association[23,27].

1.3.1. Regulation of CDPK activity

CDPK activity is regulated diversely. The most important activator is calcium, but (auto)phosphorylation, phospholipids and 14-3-3 proteins have also been shown to regulate CDPKs positively and negatively[23].

Calcium is bound by the EF hands in the calmodulin domain which activates the kinase by release of the autoinhibitory pseudo-substrate (Figure 1.4)[28]. In most CDPKs the CaM-like domain consists of an N- and C-terminal lobe, each containing 2 EF-hands[27,28]. However, some CDPKs only own 1, 2 or 3 EF-hands whereas the positions of absence vary[23]. Moreover the EF-hands have a different effect on kinase activation depending on their position (N-terminal stronger than C-terminal) which is reflected in the higher conservation of the two N-terminal hands[23]. These variations may cause different allosteric properties and activation thresholds[23]. The range of known thresholds for Ca^{2+} activation of CDPK kinase activity lies approximately between 60nM and 1µM Ca^{2+} which reflects the physiological conditions of very low and high Ca^{2+} in the cytosol[27]. Calcium-binding constants of 0.6 mM and 30nM have been determined for

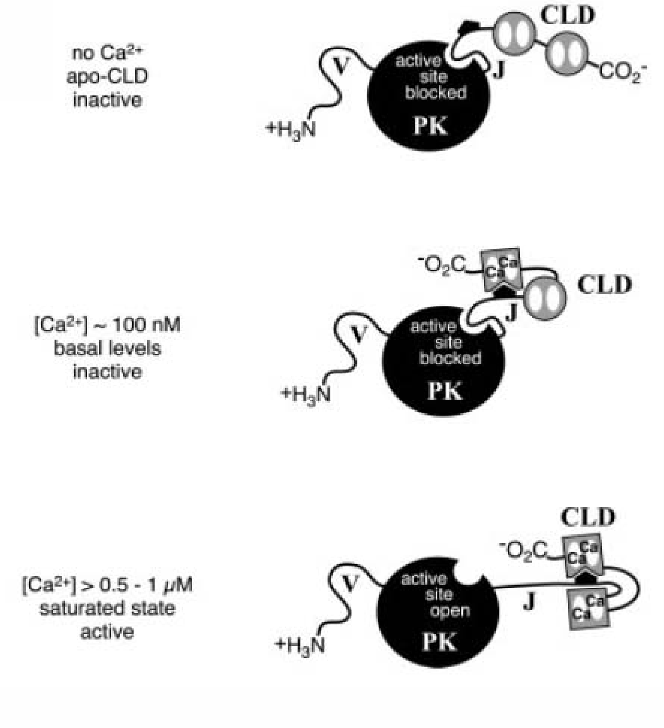


Fig. 1.4: Current model for calcium activation of CDPKs. Binding of calcium to both lobes of the CaM-like domain allows it to interact asymetrically with the junction domain blocking its autoinhibitory function[28].

N-terminal and C-terminal lobes, respectively, which means that under normal cytosolic conditions the C-terminal lobe has bound Ca^{2+} and is associated to the autoinhibitory domain[28].

Phosphorylation and autophosphorylation can enhance, activate or inhibit CDPK activity[23,27]. Among the Arabidopsis CDPKs, 16 contain a potential autophosphorylation site in the autoinhibitory domain[23]. Autophosphorylation at the analogous site of CaMKII leads to a constitutively active, Ca^{2+} insensitive enzyme. If this is the case for any of the CDPKs still needs to be investigated[23]. The autophosphorylation event is Ca^{2+} dependent in most studied cases[27]. Phosphorylation by other kinases allows crosstalk between the various signalling pathways which may act as important means of integration of environmental and developmental signals.

Phospholipids and 14-3-3 proteins have been shown to activate or enhance activity of certain CDPKs in the presence of calcium[23].

1.3.2. CDPK targets

CDPK substrates are diverse; they include proteins playing a role in carbon and nitrogen metabolism, phospholipid synthesis, defense responses, ion and water transport, cytoskeleton organization, transcription and hormone responses[27].

Carbon and nitrogen metabolism have to be tightly regulated in response to environmental conditions, especially between day and night. Two key enzymes of carbon metabolism modulated by CDPKs are Sucrose Synthase (SuSy) and Sucrose-Phosphate Synthase (SPS). SuSy is an essential enzyme of sink tissues and catalyzes the reversible cleavage of sucrose and UDP to UDP-glucose and fructose. CDPK dependent phosphorylation of SuSy reduces its membrane association increasing the cytosolic amount of SuSy which could influence the commitment of cleavage products to different biosynthetic pathways[23]. SPS mediates the synthesis of sucrose-6-phosphate, a key step in sucrose synthesis. SPS is inactivated in the dark through phosphorylation of Ser-158[29]. In spinach (*Spinacia oleracea*) SPS, this site was found initially to be phosphorylated *in vitro* by the spinach protein kinase activities of the chromatographic peaks PK_I and PK_{III}[23,29]. A CDPK with high homology to the Arabidopsis CPK3 was shown later to migrate in PK_I, PK_{III} contained the SNF1-related protein kinase SnRK1[30]. Nitrate Reductase (NR) is analogously inactivated by phosphorylation of Ser-543 and subsequent binding of 14-3-3 protein at night[23,29]. Interestingly, PK_I also phosphorylated spinach NR indicating simultaneous regulation of NR and SPS. Another kinase fraction, PK_{II} which also was identified as CDPK[30], phosphorylated NR but not SPS, providing a separate pathway of NR regulation[23,29,30].

Various examples exist on regulation of ion transporters by calcium signalling networks. Calcium signals are induced in response to hyperosmotic stress which indicates a role of calcium regulated kinases in transducing salt and drought stress response[23]. One of the first of these pathways elucidated was the SOS (salt overly sensitive) pathway. The plasmamembrane Na^+/H^+ antiporter SOS1 was shown to be activated by the CIPK SOS2 and the CBL SOS3 in response to calcium[31]. Maintaining a low Na^+ in the cytosol is essential for cellular function which makes regulation of the Na^+ homeostasis crucial especially in a high salt environment. But also other ions, like K^+ which is accumulated in most living cells, play an important role in ion homeostasis. In this respect, the high affinity K^+ transporter AKT1 was found to be regulated by a CBL/CIPK pathway. CBL1 and CBL9 activate CIPK23 which in turn phosphorylates AKT1. Plants lacking CIPK23 showed decreased, plants overexpressing CIPK23 increased resistance to low K^+ conditions[32]. Another K^+ channel regulated through Ca^{2+} and phosphorylation is KCO1 (TPK1). KCO1 is a fast-activating outward rectifying K^+-channel localized to the vacuolar membrane, which is activated directly by Ca^{2+} via its EF-hand motives[33,34]. A recent study showed that it is additionally activated by 14-3-3 proteins (regulatory proteins that bind to phosphorylated proteins), which is abolished through mutation of Ser42 to alanin[34].
Up to now there are very few examples of specific CDPK functions in plants. One of the reasons might be the lack of available phenotypes in single CDPK mutants, probably due to functional redundancies within the CDPK gene family[35]. Recently, CPK23 was found to be a negative regulator in osmotic stress response. *Cpk23* mutants showed a significant increase in drought resistance and slightly smaller stomatal aperture compared to wild type. Additionally it was found that *cpk23* mutant seedlings maintained K^+ content during NaCl treatment in contrast to the observed strong decrease in K^+ in the wild type[36]. Two other CDPKs, CPK3 and CPK6, have been shown to play redundant roles in the transduction of ABA and Ca^{2+} dependent stomatal closure[35]. *Cpk3cpk6* double mutants showed reduced activation of slow-type anion channels in response to ABA and Ca^{2+}. Fast Ca^{2+}-reactive stomatal closure was partially impaired.
These and other data indicate that regulation of plant ion transporters by phosphorylation might be a general response mechanism and calcium regulated kinases are widely believed to play a central role in these signal transduction pathways[23,32].

A broader way to regulate multiple cellular functions is the phosphorylation of transcription factors. Multisite phosphorylation of transcription factors appears to be a general mechanism of regulation[20]. Phosphorylation can influence DNA-binding ability, stability, migration from the cytosol to the nucleus, oligomerization and interaction with other proteins of the transcriptional machinery[20,27]. By now little is known about reversible phosphorylation of transcription factors in plants. Calcium dependent phosphorylation has been reported from the wheat (Triticum aestivum) bZIP (basic leucine zipper) transcription factor HBP-1a by a kinase present in the nuclear extract. Substitution of the identified serines to glutamates mimicking the phosphoralyted status inhibited DNA-binding[27]. In contrast, in the *trans*-acting factor GT1 from pea Ca^{2+}-dependent phosphorylation lead to a 10-20 fold stimulation of DNA-binding activity[27]. However, given the cytosolic Ca^{2+} increase in combination with transcriptional activation of many genes in response to a variety of environmental stresses and other signals, it is reasonable to hypothesize that CDPKs and/or CIPK/CBLs also play a role in transduction of these signals and transcription factor regulation[23,27].

1.3.3. CDPK phosphorylation motives

Apart from a few examples, little is known about the exact *in vivo* phosphorylation sites used by CDPKs. In contrast to MAP kinases, CDPKs appear to be targeted to variable motives. Table 1.1 gives an overview of known CDPK phosphorylation consensus sequences derived from target proteins and synthetic peptides[28]. Motif 1 and 4 are basically derivatives of the initially determined motif Simple 1 R-x-x-S/T. Motif 3 is the most different with two hydrophobic amino acids flanking the phosphorylated Ser/Thr.

Table 1.1: Motifs phosphorylated by CDPKs.[28] B: basic residue (R or K), Φ: hydrophobic residue, Z: any residue but not R.[37-42]

Motif	Position in sequence relative to site of phosphorylation (at 0)															Reference
	−9	−8	−7	−6	−5	−4	−3	−2	−1	0	+1	+2	+3	+4	+5	
Simple 1							B	X	X	S/T						[37]
Simple 2										S	X	B				[38]
Motif 1 minimal					Φ	X	B	X	X	S	X	X	X	Φ		[38, 39]
Motif 1 optimal				B	Φ	X	B	X	X	S	X	X	X	Φ	B	[38, 39]
Motif 2	B	B	X	B	Φ	X	X	X	X	S/T	X	B				[39, 40]
Motif 3									Φ	S/T	Φ	X	B	B		[41]
Motif 4					A/L	X	R	X	X	S	X	R	Z	R		[42]

1.3.4. AtCPK3 (At4g23650)

In the present study we focused on the characterization of AtCPK3. CPK3 is a 59,3 kDa, 529 amino acid polypeptide and belongs to subgroup II of CDPKs. The N-terminal region encodes a predicted chloroplast target peptide, however it was shown that CPK3 is not imported into the chloroplast. In contrast, it is myristoylated at G2 and localizes preferentially to the plasmamembrane and the nucleus (Mehlmer and Teige, unpublished). The kinase domain is composed of various conserved subdomains. K107 lies in the ATP-binding pocket and is essential for ATP-binding, the K107R mutant is inactive for phosphorylation (Teige, unpublished). Cleaving the C-terminus after S353 generates a constitutively active form, CPK3ΔC, lacking the autoinhibitory linker and the CaM-domain (Teige, unpublished). Below the mentioned features are depicted in the CPK3 protein sequence. The kinase domain and EF-hands are derived from smart and prosite prediction available on the TAIR database.

CPK3

```
  1 MGHRHSKSKS SDPPPSSSSS SSGNVVHHVK PAGERRGSSG SGTVGSSGSG TGGSRSTTST
 61 QQNGRILGRP MEEVRRTYEF GRELGRGQFG VTYLVTHKET KQQVACKSIP TRRLVHKDDI
121 EDVRREVQIM HHLSGHRNIV DLKGAYEDRH SVNLIMELCE GGELFDRIIS KGLYSERAAA
181 DLCRQMVMVV HSCHSMGVMH RDLKPENFLF LSKDENSPLK ATDFGLSVFF KPGDKFKDLV
241 GSAYYVAPEV LKRNYGPEAD IWSAGVILYI LLSGVPPFWG ENETGIFDAI LQGQLDFSAD
301 PWPALSDGAK DLVRKMLKYD PKDRLTAAEV LNHPWIREDG EASDKPLDNA VLSRMKQFRA
361 MNKLKKMALK VIAENLSEEE IIGLKEMFKS LDTDNNGIVT LEELRTGLPK LGSKISEAEI
421 RQLMEAADMD GDGSIDYLEF ISATMHMNRI EREDHLYTAF QFFDNDNSGY ITMEELELAM
481 KKYNMGDDKS IKEIIAEVDT DRDGKINYEE FVAMMKKGNP ELVPNRRRM
```

G N-myristoylated glycin

XX Kinase domain

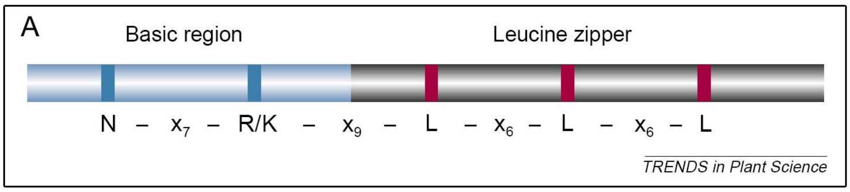
K Lysin in ATP-binding site

S S353, last amino acid in CPK3ΔC

XX EF-hand

1.4. The bZIP family of transcription factors

The bZIP proteins are a group of transcription factors that own a basic leucine zipper domain. This helical domain consists of a basic region for DNA-binding and a leucine zipper for dimerization (Figure 1.5)[43]. The basic region contains a nuclear localization signal and a conserved N-x_7-R/K motif that contacts the DNA. The leucine zipper starts exactly 9 aminoacids downstream from the conserved R/K and is made up by a heptad repeat of leucines or other hydrophobic aminoacids which creates a hydrophobic stretch on one side of the helix. On dimerization, bZIPs stick together along this hydrophobic stretch forming a coiled coil structure[43].



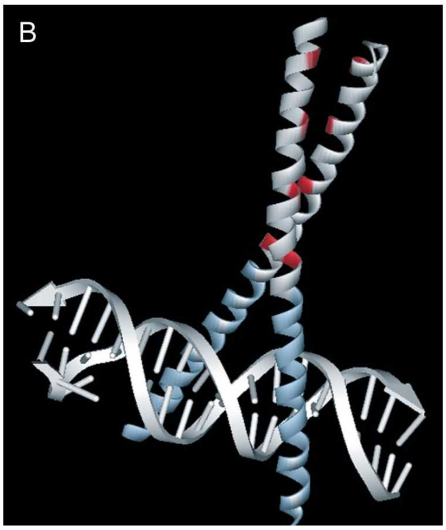


Fig. 1.5: Schematic representation of the bZIP domain.
A: Primary structure of the bZIP domain. The basic region is shaded in blue and the highly conserved residues are highlighted with blue and red boxes. A consensus sequence is given below. The leucines are sometimes replaced by other hydrophobic amino acids.
B: 3-dimensional structure of the GCN4 bZIP domain dimer bound to DNA. The leucine residues are positioned on one side of each helix and form coiled coils via van der Waals interactions[43].

75 bZIPs have been annotated from the Arabidopsis genome sequence which are about 4 times more than in yeast, worm and human[43]. According to sequence similarities of their basic region and other common features, like the length of the leucine zipper, the 75 Arabidopsis bZIP have been divided into 10 subgroups plus 3 bZIPs that could not be assigned to any group[43].
bZIPs emerged early in evolution which contributed to their structural and functional diversity. bZIPs are known to regulate divers processes, such as pathogen defence, light and stress signalling, seed maturation and flower development[43]. One example of a group of bZIPs regulated by multisite phosphorylation are the AREBs/ABFs (from the A-group) which bind an abscisic acid (ABA)-responsive cis-acting element named ABRE[44]. An ABA-dependent 42-kDa Ser/Thr kinase activity phosphorylated four R-x-x-S/T sites in three conserved regions of AREB1 (ABF2/bZIP36).[44] Substituting these four Ser/Thr residues by Asp lead to a constitutively active form of AREB1 which, when overexpressed in plants, caused ABA independent expression of target genes and increased drought resistance. Interestingly, the constitutively active form additionally induced expression of seed-specific genes which are normally not expressed in vegetative tissues in response to ABA[44], emphasizing the importance of transcription factor regulation through reversible phosphorylation.

1.4.1. The C-group bZIPs AtbZIP9 and AtbZIP63

The C-group of bZIPs is a highly homologous group of medium sized polypeptides (294-411 amino acids encoded in 6-7 exons). Their basic domain is located between the centre and the C-terminus and they own an extended leucine zipper with seven to nine heptad repeats. In addition, they share conserved potential target sites for protein modification such as phosphorylation sites that regulate nuclear translocation and DNA-binding properties.[43]
bZIP9 (At5g24800) is a 30,4 kDa, 277 amino acid protein also known as Basic Leucine Zipper O2 Homolog 2 (BZO2H2). bZIP63 (At5g28770) is of 33,5 kDa and 307 amino acids size and was former named BZO2H3. There are three splicing forms known of bZIP63 which differ in the second and last exon respectively. The transcript At5g28770.1, which lacks the first 7 amino acids of exon 2, has been isolated as interactor of CPK3ΔC in a yeast-two-hybrid-screen (Kolowrat and Teige, unpublished) and was used for all experiments of this work. bZIP9 and 63 expression is oppositionally regulated in response to light, bZIP9 transcript levels decrease whereas bZIP63 levels increase in the dark (Dröge-Laser and Vicente Carbajosa et al., unpublished)
In the sequences below, the basic leucine zipper domain is marked with red letters, the conserved N/R for DNA binding are highlighted in green, hydrophobic amino acids contributing to the leucine zipper in blue.

bZIP9

1-60	MDNHTAKDIG MKRSASELAL QEYLTTSPLD PCFDLMNRDY TCELRDSLLW SEGLFPAGPF
61-120	RDAQSSICEN LSADSPVSAN KPEVRGGVRR TTSGSSHVNS DDEDAETEAG QSEMTNDPND
121-180	LKRIRRMNSN RESAKRSRRR KQEYLVDLET QVDSLKGDNS TLYKQLIDAT QQFRSAGTNN
181-240	RVLKSDVETL RVKVKLAEDL VARGSLTSSL NQLLQTHLSP PSHSISSLHY TGNTSPAITV
241-277	HSDQSLFPGM TLSGQNSSPG LGNVSSEAVS CVSDIWP

bZIP63

1-60	MEKVFSDEEI SGNHHWSVNG MTSLNRSASE WAFNRFIQES SAAADDGEST TACGVSVSSP
61-120	PNVPVDSEEY RAFLKSKLNL ACAAVAMKRD TSGRSDNGGA NESEQASLAS SKATPMMSSA
121-180	ITSGSELSGD EEEADGETNM NPTNVKRVKR MLSNRESARR SRRRKQAHLS ELETQVSQLR
181-240	VENSKLMKGL TDVTQTFNDA SVENRVLKAN IETLRAKVKM AEETVKRLTG FNPMFHNMPQ
241-300	IVSTVSLPSE TSNSPDTTSS QVTTPEIISS GNKGKALIGC KMNRTASMRR VESLEHLQKR
301-307	IRSVGDQ

XX Basic leucine zipper domain

XX Basic DNA binding region

N R Conserved DNA binding amino acids

L Leucine

AFIMV Hydrophobic amino acids

1.4.2. CDPK phosphorylation of bZIP9 and 63

Using the available CDPK target sequences, candidate serines and threonines were determined in bZIP9 and 63. In the list below amino acids "conserved" between the phosphopeptide sequences are highlighted. In case of NIA2, SPS1, ACA2 and LeACS2 the phosphorylation has been experimentally confirmed.

In the bZIP sequences, candidate Ser/Thr and the flanking consensus sequences are indicated in the same colours. In bZIP9, the potential phosphorylation site S16 was mutated to an alanin; in bZIP63, the last 24 amino acids containing three possible target sites were cleaved to form the ΔC mutant. Additional truncation of the first 29 amino acids generated bZIP63ΔNC.

- Known CDPK target motives:

Simple 1[37]	+xx*
Simple 2[38]	Sx+
Motif 1 minimal[38,39]	•x+xxSxxx•
Motif 1 optimal[38,39]	+•x+xxSxxx•+
Motif 2[39,40]	++x+•xxxx*x+
Motif 3[41]	•*•x++
Motif 4[42]	A/LxRxxSxRzR
NIA2[29]	...APPSLKKSVSTPFMN...

SPS1[29] ...LRARIKRNVSCYGRF...

ACA2[41] ...RRFRFTANLSKRYEA...

LeACS2[41] ...KKNNLRLSFSKRM...

* Phosphorylated serine or threonine

\+ Basic residue

Φ Hydrophobic residue

X Any residue

z Any residue but not R

- Candidate serines / threonines in the bZIPs:

bZIP9

1-60	MDNHTAKDIG MKRSASELAL QEYLTTSPLD PCFDLMNRDY TCELRDSLLW SEGLFPAGPF
61-120	RDAQSSICEN LSADSPVSAN KPEVRGGVRR TTSGSSHVNS DDEDAETEAG QSEMTNDPND
121-180	LKRIRRMNSN RESAKRSRRR KQEYLVDLET QVDSLKGDNS TLYKQLIDAT QQFRSAGTNN
181-240	RVLKSDVETL RVKVKLAEDL VARGSLTSSL NQLLQTHLSP PSHSISSLHY TGNTSPAITV
241-277	HSDQSLFPGM TLSGQNSSPG LGNVSSEAVS CVSDIW

S S16, mutated to alanin in bZIP9 S16A

bZIP63

1-60	MEKVFSDEEI SGNHHWSVNG MTSLNRSASE WAFNRFIQES SAAADDGEST TACGVSVSSP
61-120	PNVPVDSEEY RAFLKSKLNL ACAAVAMKRD TSGRSDNGGA NESEQASLAS SKATPMMSSA
121-180	ITSGSELSGD EEEADGETNM NPTNVKRVKR MLSNRESARR SRRRKQAHLS ELETQVSQLR
181-240	VENSKLMKGL TDVTQTFNDA SVENRVLKAN IETLRAKVKM AEETVKRLTG FNPMFHNMPQ
241-300	IVSTVSLPSE TSNSPDTTSS QVTTPEIISS GNKGKALIGC KMNRTASMRR VESLEHLQKR
301-307	IRSVGDQ

E E30, first amino acid in the bZIP63ΔNC mutant

N N283, last amino acid in the bZIP63ΔC and ΔNC mutant

2. Material and methods

2.1. Strains and lines

2.1.1. Escherichia coli strains

DH5α	F-, ø80dlacZDM15, D(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk-, mk+), phoA, supE44, l-, thi-1, gyrA96, relA1; used for cloning.
BL21	*hsd*S *gal* (λcIts857 *ind*1 Sam7 *nin*5 *lac*UV5-T7 gene *1*); used for protein expression.

Unless indicated otherwise *E. coli* cultures were grown at 37℃ in LB liquid medium or on plates supplemented with the appropriate antibiotics.

2.1.2. Agrobacterium tumefaciens strains

AGL1	AGL0 (C58 pTiBo542) recA::bla, T-region deleted Mop(+) Cb(R) [AGL0 is an EHA101 with the T-region deleted, which also deletes the aph gene] [45]

Agrobacterium cultures were grown at 30℃ in YEB, L B or YPD liquid medium or on plates supplemented with the appropriate antibiotics.

2.1.3. Saccharomyces cerevisiae strains

L40	MATα *his∆200 trp1-900 leu2-3.112 ade*2 LYS2::(*lexA op*)$_4$HIS3 URA3::(*lexA op*)$_8$lacZ Gal4 gal 80

Yeast cultures were grown at 30℃ in YPD or SD with the appropriate auxotrophy selection (missing amino acids or nucleobases) in liquid medium or on plates.

2.1.4. Arabidopsis thaliana lines

Columbia	wild type ecotype
Wassilewskija	wild type ecotype
cpk3-2	Col-0 *cpk3* Kan^R, SALK_022862[46], obtained from *NASC*
35S::CPK3	Col-0 35S::CPK3-YFP Kan^R line A-D, obtained from Norbert Mehlmer
	Col-0 35S::CPK3-YFP Kan^R line #1-11, obtained from Norbert Mehlmer
bzip9	Col-0 *bzip9* SALK_093416[46], obtained from Wolfgang Dröge-Laser
bzip63	Ws *bzip63*, created by the Wisconsin Arabidopsis Knockout Facility[47], obtained from Jesús Vicente Carbajosa
35S::bZIP63	Col-0 35S::bZIP63, obtained from Jesús Vicente Carbajosa

2.1.5. Nicotiana tabacum lines

SR1 wild type ecotype

2.2. Plant cultivation

2.2.1. Arabidopsis cultivation

- Harvest of seeds

Well dried mature seeds were harvested from 12 - 14 weeks old plants. Seeds were separated from the debris by sieving and transferred to Eppendorf tubes (*Eppendorf*, Hamburg, Germany). If necessary seeds were dried in open tubes at 37℃ for 30 min before storage to ensure a low humidity which is essential for a good germination capacity. For long term storage the seeds were kept non-sterilized in a dark place at room temperature.

- Surface sterilization with chlorine gas

In a fume hood, opened Eppendorf tubes containing no more than 150 µl seeds were laid on green towels inside a transparent 35x25x12 cm plastic box with a tightly closable lid. In two beakers placed on the opposite side of the tubes' openings approximately 15 g $KMnO_4$ were mixed with 30 ml 5 M HCl, and 100 ml 2,5% HOCl with 50 ml 5 M HCl respectively and the lid closed immediately. Seeds were sterilized for 2 hours through the developing toxic chlorine gas which can be monitored by the bleaching of the green towels. To prolong the period of high germination capacity, seeds should be allowed to aerate for some minutes in a sterile bench before storing them. Sterilized seeds can be stored at room temperature for a few months.

- Growth conditions and transformant selection

<u>Soil grown plants</u>

Seeds of lines without selection were directly sown on soil. For better germination, trays were watered well and covered with plastic domes for 3-5 days. Plants were grown under long-day conditions (16h light / 8h dark) or alternatively kept for 2 - 3 weeks under short-day conditions (8h light / 16h dark) for a better biomass production and then transferred to long-day.
Growth chambers provided the following conditions: 22℃ constant temperature, light intensity of 200 µmol m^{-2} sec^{-1} (Osram (Munich, Germany) Lumilux Interna (yellow), Lumilux Plus (day light) and Fluora (violet) fluorescent tubes), 60% relative humidity. Percival AR-36L/LT incubators (*Percival Scientific*, Perry, IA, USA): 19℃ night/ 22℃ day, light intensity 200 µmol m^{-2} sec^{-1} (Phillips (Washington, DC, USA) F17T8/TL741 fluorescent tubes), 60% relative humidity.

Agar grown plants

Sterilized seeds were sown on ½ MS plates (dishes 145/20 with vent, #639103, Greiner, Kremsmünster, Austria) with or without kanamycin and the plates sealed with parafilm. After vernalization for 2 - 5 days at 4℃ in the dark, pl ates were transferred to a growth chamber with the following growth conditions: 16 h light / 8h dark, 200 µmol m^{-2} sec^{-1} (Osram Lumilux (cool white) fluorescent tubes), 23℃ constant temp. Afte r 3 days the parafilm was partly and after 7 days entirely removed to enable gas exchange. To grow kanamycin selected adult plants, seedlings were transferred to earth after 7 - 14 days. To increase the survival rate, the water-saturated soil was covered with quartz sand and plants were carefully placed into the wholes with their roots being aligned downwards by spraying with water. To maintain a high humidity, seedlings were covered with transparent plastic caps and the trays watered well.

Hydroponically grown plants

Arabidopsis hydroponic culture was done according to Tocquin et al[48]. 1 ml Gilson (Middleton, WI, USA) tip boxes with tip rack were filled with 400 ml of the desired ½ Hoagland medium and covered with aluminium foil. Seeds were sown on ½ MS agar plates or placed directly into 0,5 ml PCR tubes filled with ½ MS agar. Tubes were cut on the bottom, placed into a box filled with the desired ½ Hoagland medium and covered with a transparent plastic foil to maintain a high humidity. Seeds were vernalized for 2 days and put into the plant chamber for germination. After 5-7 days seedlings grown on plate were transferred to tubes and placed into medium boxes. Plants were kept with plastic cover for about 3 weeks, the cover was opened partly for 1 more week and then entirely removed. Plants were grown in short or long day (conditions see above) and watered with medium.

2.2.2. Tobacco cultivation

- Harvest of seeds

Tobacco seeds were harvested by transferring the content of one ripe, dry seed vessel into an Eppendorf tube.

- Growth conditions

Tobacco seeds were sown on soil pre-soaked with water and covered with aluminium foil for 5 days. Plants were grown under long-day conditions (16h light / 8h dark) at a light intensity of 200 µmol m^{-2} sec^{-1} (Osram Lumilux Interna (yellow light), Lumilux Plus (daylight) and Fluora (violet light) fluorescent tubes), 60% relative humidity and 22℃ constant temperature.

2.3. Media

All media were prepared as follows. Autoclave sterilization was done for 20 min at 120℃, sterile filtering through a 0,2 µm membrane. Antibiotics stock solutions were stored at -20℃, amino acid and glucose stock solutions at room temperature. Both were added after autoclaving at a temperature below 60℃. Media without antibiotics or amino acids were stored at room temperature, with antibiotics or amino acids at 4℃. All specifications are for 1 litre of media.

2.3.1. Bacterial media

All bacterial media were autoclave sterilized. For plates 3% Bacto-agar was added before autoclaving. Media components were purchased from BDMS/Difco, Sparks, MD, USA.

[1000x] Antibiotics stock solutions

Ampicillin:	[75 mg/ml] (in 50% Ethanol)
Kanamycin:	[50 mg/ml] (in water, sterile filtered)

- *E. coli* media

LB (Luria-Bertani) medium

10 g	Tryptone
5 g	Bacto-yeast extract
5 g	NaCl

- *A. tumefaciens* media

YEB medium

5 g	Tryptone
1 g	Bacto-yeast extract
5 g	Peptone
0,5 %	Sucrose
2 mM	$MgSO_4$

Induction medium

13,76 g	$K_2HPO_4.3H_2O$
4,5 g	KH_2PO_4
2,13 g	MES.H_2O (10 mM) pH 5.6
1 g	$(NH_4)_2SO_4$
0,246 g	$MgSO_4.7H_2O$ (1 mM)
0,2%	Glucose
50 µM	Acetosyringone (add 161,7 µl from 300 mM stock immediately before use)

Infiltration medium

5%	Sucrose or glucose
300 µM	Acetosyringon

½ MS (optionally, see “Plant Media”)

2.3.2. Yeast media

All yeast media were autoclaved. For plates 3% Bacto-agar was added before autoclaving. Amino acids and nucleobases were purchased from Sigma-Aldrich Corporation, St. Louis, MO, USA, all other media components from BDMS/Difco. All specifications are for 1 litre of media.

[100x] Amino acids stock solutions (sterilized by filtration)

Leucin:	8 g/l
Tryptophan:	4 g/l
Histidin:	2 g/l

Amino acid stock solution:

Amino Acid	[g/l]
Arginine	3
Aspartat	10
Isoleucine	2
Lysine	4
Methionine	2
Phenylalanine	6
Threonine	15
Valin	6,5

[20x] Glucose

40% Glucose in dH_2O

Sterilize by filtration or autoclaving

YNB + AUT

6,7 g	Yeast nitrogen base w/o amino acids incl. $(NH_4)_2SO_4$
40 mg	Adenine
40 mg	Tyrosin
40 mg	Uracil

SD (selective drop-out) medium

To YNB + AUT 2% Glucose and the appropriate amino acids for selection were added from stock solutions

YPD medium

20 g	Bacto-peptone
10 g	Bacto-yeast extract
40 mg	Adenine (optionally)

2% Glucose was added from stock solution

2.3.3. Plant media

Plant media components were purchased from Duchefa, Haarlem, Netherlands.

- MS (Murashige-Skoog) media

½ MS medium

2,2 g	MS medium with B5 vitamins

½ MS plates

2,2 g	MS medium with B5 vitamins
7 g	Plant agar

The pH was adjusted to 5,7 with NaOH or KOH prior to autuclaving

½ MS Kan + Suc plates

2,2 g	MS medium with B5 vitamins
10 g	Sucrose
7 g	Plant agar

The pH was adjusted to 5,7 with NaOH or KOH prior to autuclaving. 1 ml/l 1000x Kanamycin stock (50mg/ml, sterile filtered) was added before pouring the plates.

- Hoagland medium for hydroponic culture

Micronutrient stock solution

2,86 g	H_3BO_3
1,81 g	$MnCl_2.4H_2O$
0,22 g	$ZnSO_4.7H_2O$
0,08 g	$CuSO_4.5H_2O$
0,02 g	85% H_2MoO_4
ad 1 litre	dH_2O

When diluted 1:2000 the micronutrient stock solution provides the following in mg/litre:

Boron	0,25
Manganese	0,25
Zinc	0,025

Copper	0,01
Molybdenum	0,005

<u>1,4 g/l Fe-EDTA Chelat</u>

3,36 g	Na_2EDTA
2,79 g	$FeSO_4$
ad 400 ml	dH_2O

Stir at 70℃ until all the green $FeSO_4$ powder dissolved to form a clear orange solution

<u>½ Hoagland medium</u>

2,5 ml	1M $Ca(NO_3)_2$
1 ml	1M $MgSO_4$
0,5 ml	Micronutrient stock solution
1,25 ml	1,4 g/l Fe-EDTA Chelat

For:	add:	
Low K^+ (0,3 mM)	0,3 ml	1 M KH_2PO_4
	0,2 ml	1 M NaH_2PO_4
Standard K^+ (3 mM)	2,5 ml	1 M KNO_3
	0,5 ml	1 M KH_2PO_4
High K^+ (30 mM)	2,5 ml	1 M KNO_3
	0,5 ml	1 M KH_2PO_4
	27 ml	1 M KCl

2.3.4. Soil

<u>Earth</u>

Neuhaus N3 Huminsubstrat (Klasmann-Deilmann, Geeste-Groß Hesepe, Germany)

<u>Quarz sand</u>

Rasen-Quarz, Körnung 0,5 - 2,0 mm (Quarzwerke Österreich, Melk, Austria)

<u>Perlit</u>

Granuperl, 3 - 6mm, extra (GBC-Österreich e.Gen., Gartenbaucentrum, Wels, Österreich)

Soil components were mixed in the ratio 10:3:1 (earth:sand:perlit) and autoclave sterilized before use.

2.4. Bacteria methods

2.4.1. Competent *E. coli* cells

<u>TB Puffer</u>

10 mM	$CaCl_2$
10 mM	Pipes - NaOH
15 mM	KCl
Adjust pH to 6,7	
Add 55 mM	$MnCl_2$
Sterilize by filtration	

Manganese competent *E. coli* cells were prepared according to Inoue et al. (1990) [49].

DH5α cells from a glycerol stock were struck out on an LB or YPD plate in such a way that single colonies could be obtained and incubated at 37℃ over night.

A pre-culture was inoculated from a single colony in 100 ml LB 20 mM $MgSO_4$ and grown over night at 23℃. The next day 400 ml LB 20 mM $MgSO_4$ were inoculated with 2 ml pre-culture and incubated over night at 19℃. The OD_{600} should reach 0,6. Cells were transferred to 50 ml Greiner tubes and incubated on ice for 10 min before pelleting them for 10 min at 700g, 4℃. The supernatant should be clear, if it is not the procedure has to be repeated with a new culture. Pellets were resuspended in a total volume of 2x 32 ml ice cold TB buffer, pooled in 2 Greiner tubes and incubated for 10 - 30 min on ice. Competent cells were harvested by centrifugation for 10 min at 400g, 4℃.

For storage, pellets were resuspended in 2x 8 ml ice cold TB 7% DMSO and incubated for at least 20 min, better 1 - 2 h, on ice. Cells were aliquoted in Eppendorfs and frozen on -80℃.

2.4.2. *E. coli* plasmid transformation

Competent *E. coli* cells were thawed on ice. 50 µl cells were mixed with 10 µl ligation batch or 1 µg purified plasmid DNA (re-trafo) and incubated on ice for 5 - 30 min. Cells were heat shocked for 45 - 60 sec at 37 - 42℃ and put on ice for another 2 min. Then 800 µl LB without antibiotics was added and the cells allowed to recover for 20 - 60 min at 37℃, shaking. 100 - 800 µl cells were plated on LB plates supplemented with the appropriate antibiotic and grown at 37℃ over night.

2.4.3. Preparation of bacterial RNA for yeast transformation

<u>Buffers P1, P2 and P3</u>

See “Minipreparation of Plasmid DNA from *E. coli*”

PCI, Chloroform

See “Total RNA and Protein Isolation from Arabidopsis”

A crude mixture of bacterial RNAs was used as carrier for the plasmid DNA in yeast transformations. 600 ml of an *E. coli* overnight culture were harvested by centrifugation in GS3 tubes. The pellets were resuspended in 10 ml 0,5x TE and transferred to a 50 ml Greiner tube. Cells were pelleted again and resuspended in 5 ml buffer P1 without RNase. Cells were lysed by adding 5 ml lysis buffer P2 and 5 ml neutralization buffer P3 and the mixture left for 10 min on ice. After centrifugation for 10 min at 3000g, 4°C the supernatant was precipitated with 0,8 volumes of isopropanol at -20°C and centrifuged for 30 min at 3000g, 4°C. Pellets were resuspended in 0,5 ml 0,5x TE, transferred to Eppendorf tubes and extracted twice with 1 V PCI and once with 1 V chloroform. The aqueous phase was aliquoted in several tubes before precipitation with 0,8 V of isopropanol. After centrifugation for 30 min at 16.000g, at 4°C the pellets were washed with 70% ethanol and dissolved in a total volume of 4 ml 0,5x TE. The RNA concentration was measured at 260 and 280 nm and adjusted to 10 µg/µl (see “Spectrophotometric Measurement of Nucleic Acid Concentrations”)

2.4.4. Electrocompetent Agrobacterium cells

AGL1 cells from a glycerol stock were struck out on an LB or YPD plate in such a way that single colonies could be obtained and incubated at 30°C over night. 600 ml LB or YEB medium were inoculated with 3 single colonies and grown for 1,5 days at 30°C under shaking (an OD_{600} of 1 - 1,5 should be reached). Cultures were chilled on ice, transferred to sterile GS3 tubes and centrifuged for 10 min at 4000g, 4°C. Cell pellets were washed three times in 50 ml sterile 1 mM Hepes pH 7.0 and twice with 10% glycerol. Finally, pellets were resuspended in a total volume of 4 ml 10% glycerol. For storage, aliquots of 250 µl in 1,5 ml Eppendorf tubes were frozen in liquid nitrogen and stored at –80°C.

2.4.5. Agrobacterium plasmid transformation by electroporation

3 µg of plasmid DNA was placed into sterile electroporation cuvettes and put on ice. 50µl of competent AGL1 cells were added and mixed by pipeting. Electroporation was carried out with a Bio-Rad (Hercules, CA, USA) Pulse Controller electroporation device at 200 Ohm and 1,4 kV. Immediately afterwards cells were mixed with 800 µl LB medium, transferred to an Eppendorf and allowed to recover shaking for 30 min at 30°C. Cells were plated on LB plates supplemented with the appropriate antibiotics for selection and incubated overnight at 30°C.

2.5. Yeast methods

2.5.1. Yeast quick transformation

Trafo mix

800 µl	50% PEG (3.300) (sterile filtered)
100 µl	2 M lithium acetate (sterile filtered)
100 µl	1 M DTT (sterile filtered)
20 µl	bacterial RNA as carrier

About 50 µl of fresh yeast L40 cells from an agar plate were introduced into the mixture with a sterile tooth pick and mixed well by vortexing.

150 µl of trafo mix was added to 2 µg of DNA per plasmid and vortexed thoroughly. The samples were incubated at 30℃ and afterwards at 44℃ for 20 min each. 1 ml sterile ddH_2O was added and mixed carefully. Cells were spun down at 2500g for 1 min, resuspended in 100 µl sterile ddH_2O and plated on the appropriate SD medium. Colonies appeared after 2 - 3 days of incubation at 30℃.

2.5.2. Yeast protein extracts for Yeast-2-Hybrid

Enzyme-lysis buffer

25 mM	Tris.Cl pH 7.5
20 mM	NaCl
8 mM	$MgCl_2$
5 mM	DTT
0,1%	Tween 20

Sterile glass beads (1mm diameter)

Yeast colonies were struck out on SD plates and grown at 30℃ until sufficient cell material was produced. From each initial colony 3 cultures were inoculated in 3 ml SD medium and grown over night at 30℃ until the medium got strongly op aque. 2 ml culture was spun down, the pellet resuspended in 200 µl enzyme-lysis buffer and frozen in liquid N_2 immediately. Cells were thawed on ice, 50 - 100 µl glass beads were added and vortexed for 20 min at 4℃ in a Vibrax. The cell debris was removed by centrifugation for 10 min at 16.000g, 4℃ and the supernatant transferred to a new tube. Protein extracts were stored on ice until further analysis. Protein concentration was measured with Bradford assay (see “Determination of Protein Concentration with Bradford Assay“) using 1:20 dilutions of the extracts.

2.5.3. ß-galactosidase assays

Z-Buffer

161 g	$Na_2HPO_4.7H_2O$
5,5 g	$NaH_2PO_4.H_20$
0,75 g	KCl
0,246 g	$MgSO_4.7H_2O$
ad 1 litre	dH_2O

adjust pH to 7.0 with NaOH or H_3PO_4

Z-Buffer incl. DTT and X-Gal

To 10 ml Z-Buffer add 100 µl DTT (1M) and 156 µl X-Gal stock solution

X-Gal stock solution

20 mg/ml 5-Bromo-4-Chloro-3-Indolyl-β-D-galactoside (X-gal) in dimethylformamide (DMF)

ONPG (Ortho-nitrophenyl-β-D Galactopyranoside)

4 mg/ml in dH_2O

Nitrocellulose membrane

Protran BA 85, pore size 0.45 µm (Schleicher & Schuell, Dassel, Germany)

- ß-galactosidase filter lift assay

Several colonies of yeast cells carrying both bait and prey plasmids were struck out separately on SD plates and grown for 1 - 3 days until sufficient cell material was produced. It is important to use fresh, exponentially growing cells.

About 50 µl cells were scratched from the plate with a tooth pick and mixed with 10 µl ddH_2O in a tube. The cell suspension was spotted on a small area of a nitrocellulose membrane with a pipet. The membrane was frozen in liquid N_2 to break the cells and transferred into a Petri dish with the yeast colonies on the upper side. The membrane was covered with 1 ml Z-buffer incl. DTT and X-Gal. The dish was sealed with parafilm and incubated at 37℃ for 2 h to develop the blue colour.

- Liquid ß-galactosidase assay

650 µl Z-buffer incl. DTT and 150 µl ONPG were mixed with 50 µl protein extract from yeast L40 cells harbouring the desired plasmids. 2 reactions with Z-buffer were included as reference. Time was counted until the solution got moderately yellow and the reaction stopped with 400 µl of 1 M Na_2CO_3. Conversion of ONPG to the yellow ONP was quantified by measuring the extinction at 420 nm (absorption values should not exceed 0,9). The specific galactosidase activity was calculated with the following formula:

$U_{(ß\text{-galactosidase})}$ / mg protein = E_{420} * 25 * 1000 / 45 * t[min] * $c_{(protein)}$ [mg/ml]

2.6. Plant methods

2.6.1. Salt stress treatment of seedlings

For stress assays 12 - 20 days old seedlings grown on plates were transferred into liquid ½ MS medium and allowed to recover over night. The next day seedlings were transferred to ½ MS 150 mM NaCl and stressed for 30 min, 60 min, 3 h and 6 h. Immediately after the treatment seedlings were frozen in liquid nitrogen. A control without stress treatment was included.

2.6.2. Transient transformation of tobacco leaves by Agrobacterium infiltration

To analyse the subcellular localization of proteins, tobacco leaf epidermis cells were transiently transformed with a binary protein expression vector (pBIN19, see “Plasmid Constructs”) encoding a C-terminal YFP fusion of the protein of interest under control of a 35S promoter. To transform cells, tobacco leaves of were infiltrated with agrobacteria carrying the expression plasmid and analysed under the microscope.

An agrobacteria pre-culture was inoculated from a single colony or a glycerol stock in 15 ml YEB_{Kan} (alternatively LB_{Kan}) in a 100 ml Erlenmayer flask and incubated overnight at 30℃ on a shaker. The main culture was prepared by completing the pre-culture to 50 ml with YEB_{Kan} (or LB_{Kan}, the OD_{600} of the 50 ml suspension should be about 0,1) and incubated shaking for about 4h at 30℃ until an OD_{600} of about 0,6 was reached. Cells from 35 ml suspension were harvested by centrifugation for 15 min at 3500g, room temperature. The cell pellet should be slightly pink, but not red. The pellet was resuspended in 25 ml induction medium incl. acetosyringone and incubated shaking for another 60 min at 30℃.
Cells were pelleted by centrifugation for 15 min at 3500g, room temperature and resuspended in 10 - 15 ml infiltration medium. The agrobacteria suspension was infiltrated into leaves of 4 - 5 weeks old tobacco plants grown according to “Tobacco Cultivation”. Leaves were pierced with a needle and from the leaf bottom side the suspension was pressed through the wholes into the aerenchym using a 3 ml plastic syringe without needle. The infiltrated areas were marked with Edding. After infiltration plants were kept overnight in plastic bags with some water and afterwards transferred to the long-day chamber. 2 - 3 days after infiltration transfected leaf parts were analyzed under the fluorescence microscope.

2.6.3. Total RNA and protein isolation from Arabidopsis

<u>Phenol pH 4.0</u>
From AppliChem (Darmstadt, Germany) (A1624,0250) or saturated solution equilibrated in 100mM Tris.Cl pH 4.0

<u>Phenol pH 7.5</u>
ROTI® phenol pH 7.5 (Roth, Karlsruhe, Germany)

PCI

ROTI® 25:24:1 phenol:chloroform:isoamylalcohol (Roth)

RNA extraction buffer (REX)

1%	SDS
10 mM	Na_2EDTA
200 mM	Sodium acetate pH 5,2

Phenol extraction buffer (PEX)

0,7 M	Sucrose
0,1 M	KCl
0,5 M	Tris.Cl pH 7.5
50 mM	Na_2EDTA pH 8.0

add 100 µl / 30 ml of 12,8 M ß-mercaptoethanol immediately before use

ß-ME acetone

35µl ß-mercaptoethanol / 50ml acetone

DEPC ddH_2O

0,1 - 0,2 ml diethylpyrocarbonat (DEPC) were added to 100 ml ddH_2O, shaken vigorously and stored over night in a fume hood. Remaining DEPC was inactivated by autoclaving. DEPC treated water was stored at room temperature.

Fresh arabidopsis plant material was frozen in liquid N_2 and pestled until a fine homogeneous powder was obtained. In a 1,5 ml Eppendorf tube 100 - 250 mg of crashed plant material were mixed with 130 µl REX buffer, 130 µl phenol pH 4.0 and approximately 50 µl sea sand. The mixture was mortared with a pre-cooled glass driller until the material had thawed. Freezing and mortaring was repeated 3 - 4 times until the liquid appeared homogeneous. 400 µl REX buffer and 400 µl phenol pH 4.0 were added, the samples vortexed thoroughly and centrifuged for 10 min at 16.000g, 4℃.

- RNA isolation

The aqueous (upper) phase was extracted twice with 1 volume of PCI and once with 1 volume of chloroform. RNA was precipitated with two alternative methods:

- Isopropanol (also precipitates rests of DNA):
  The supernatant was precipitated for 20 min with 1 volume of isopropanol at -20℃. RNA was pelleted by centrifugation for 10 min at 16.000g, 4℃.
- LiCl (precipitates exclusively RNA but at lower yield than with isopropanol):
  The supernatant was precipitated over night with 1/3 volume of 10 M LiCl prepared with DEPC ddH_2O at 4℃. RNA was pelleted by centrifugation for 15

min at 16.000g, 4℃ and washed once with 2,5 M LiCl prepared with DEPC ddH_2O.

The pellet was washed with 70% EtOH prepared with DEPC ddH_2O and dried at 45℃ for 10 - 30 min. The RNA was resuspended in 25 - 50 µl DEPC ddH_2O and the concentration determined by measuring $A_{260/280}$ (see “Spectrophotometric Measurement of Nucleic Acid Concentration”)

- Protein isolation from the phenol phase of RNA isolation

The phenol (lower) phase was washed 2 - 3 times with an equal volume of PEX buffer until it had been reduced to about 200 µl (note that after washing the phenol phase stays on top). Samples were vortexed thoroughly and centrifuged for 5 - 10 min at 16.000g. Proteins were precipitated with 5 volumes of 0,1 M ammonium acetate in methanol over night at -20℃. Samples were centrifuged for 10 min and the pellet washed twice with ß-ME acetone (if necessary samples were frozen in liquid N_2 to facilitate resuspension at each washing step). The pellet was dried for 15 min in a speed vac. Dry pellets were stored at room temperature, after resuspension in 1x SDS loading buffer proteins were stored at -20℃.

- Protein isolation via acetone extraction

Plant tissue was harvested, immediately frozen in liquid nitrogen and pestled until a fine homogeneous powder was obtained. About 400 mg plant powder were mixed with 1,6 ml acetone in a 2 ml Eppendorf tube and vortexed at 4°C for 30 min in a Vibrax. Samples were centrifuged for 10 min at 16.000g, 4℃. The superna tant was discarded and the extraction repeated 3 - 4 times until the pellet appeared white. The pellet was then dried in a speed vac for 15 - 30 min at room temperature. The protein pellet was either used directly for experiments or further purified by phenol extraction.
For phenol extraction the pellet was resuspended in 500 - 600 µl phenol pH 7.5 by thorough vortexing and treated according to “Protein Isolation from the Phenol Phase of RNA Isolation”.

2.7. DNA methods

2.7.1. Agarose gel electrophoresis

<u>[50x] TAE</u>

242 g	Tris base
100 ml	0,5 M Na_2EDTA pH 8,0
57,1 ml	Acetic acid
ad 1 litre	dH_2O
pH 8.0	

[1x] TAE

40 mM Tris.Acetate, 1 mM Na_2EDTA, pH 8.0

5 mg/ml ethidium bromide

0,5 g ethidium bromide was added to 100ml of dH_2O, stirred for several hours to ensure that the dye had dissolved and stored protected from light at room temperature.

[6x] loading buffer

100 mM Tris.Cl pH 8.0, 50% glycerol, 0.05% bromphenol blue, 0.05% xylenecyanol

DNA molecular weight markers

1 kb DNA ladder plus and 100 bp DNA ladder (NEB New England Biolabs, Beverly, MA, USA)

0,6% - 2,5% agarose gels were prepared according to the size of the DNA molecules to be separated. 1x TAE was used for preparation of gels and as running buffer. For visualization of DNA fragments under UV light, 5 µl of a 5 mg/ml ethidium bromide stock were added per 100 ml of agarose. DNA was applied in 1x loading buffer, to estimate fragment sizes 0,5 µg molecular weight marker was applied as well. Gels were run at 100 - 130 V.

2.7.2. Minipreparation of plasmid DNA from *E. coli*

Resuspension buffer P1

50 mM Tris.Cl pH 8.0, 10 mM Na_2EDTA; after addition of 100 µg/ml RNase the buffer was stored at 4℃

Lysis buffer P2

200 mM NaOH, 1% SDS

Neutralization buffer P3

3 M potassium acetate pH 5.5

[10x] TE

100 mM Tris.Cl pH 8.0, 10 mM Na_2EDTA

[0,5x] TE

5 mM Tris.Cl pH 8.0, 0,5 mM Na_2EDTA

3 ml *Escherichia coli* culture inoculated from a single colony was grown in LB supplemented with the appropriate antibiotics. 2 ml culture was centrifuged for 2 min at 16.000g. The supernatant was removed and the pellet resuspended in 200 µl P1. 200 µl P2 was added and mixed by inverting. After addition of 200 µl P3 tubes were inverted immediately, stored on ice for 5 min and centrifuged for 10 min at 16.000 g, 4℃. The supernatant was transferred to a new tube, 400 µl isopropanol was added and incubated for 20 min at –20℃. After centrifugation for 15 min at 16.000 g, 4℃ the pellet was washed with 800 µl 70% ethanol and centrifuged again.

The supernatant was discarded, rests of liquid spinned down and removed with a pipet. The pellet was dried at 45℃ for 10 min and resuspended in 50 µl 0,5x TE.

2.7.3. Midipreparation of plasmid DNA from *E. coli*

Midipreparation of Plasmid DNA from *E. coli* was done with the Genomed JetStar 2.0 MIDI Prep Kit, using a slightly modified protocol.
100 - 200 ml of an *E. coli* over night culture was pelleted by centrifugation for 10 min at 3500g, 4℃ in 50 ml Greiner tubes. All traces of medium were removed with a pipet and cells were resuspended in 4 ml solution E1 with RNase. Cells were lysed by adding 4 ml solution E2, tubes were inverted several times and incubated for 5 min at room temperature. For neutralization 4 ml solution E3 was added and mixed immediately until a homogenous suspension was obtained. The mixture was centrifuged for 30 min at 3500g, 4℃.
The supernatant was applied to a JETSTAR column equilibrated with 10 ml equilibration solution E4. The column was washed twice with 10 ml solution E5. The plasmid DNA was eluted with 5 ml solution E6.
The DNA was precipitated with 1 volume of isopropanol for 20 min at -20℃ and pelleted by centrifugation for 30 min at 12000g, 4℃. After removing the supernatant, tubes were spinned down again and all traces of isopropanol were removed with a pipet. The pellet was resuspended in 150 µl 0,5x TE buffer and transferred to a 1,5 ml Eppendorf. The last step was repeated to get out all rests of DNA.
The DNA was precipitated again with 840 µl (2,8 V) 100% Ethanol and 2,5 µl 5 M NaCl for 10 min on ice and centrifuged for 15 min at 16000g, 4°C. The pellet was washed with 800 µl 70% Ethanol and dried at 45℃ for 10 min.
The DNA was resuspended in 50µl ddH_2O and the concentration determined by measuring A_{260}/A_{280}. Plasmid Midi preps were stored at -20℃ in ddH_2O at a concentration of 1 µg/µl.

2.7.4. Spectrophotometric measurement of nucleic acid concentrations

Nucleic acid concentrations were determined by measuring the absorbance of a 1:200 (DNA) or 1:250 (RNA) dilution in ddH_2O at 260/280 nm in a quartz cuvette. In these dilutions the nucleic acid concentrations in µg/µl correspond to $A_{260} * 10$.
Nucleic acid solutions were adjusted to a concentration of 1 µg/µl using the following formula:
$V\ ddH_2O\ \text{added} = A_{260} * 10 * V_{(\text{nucleic acid solution})} - V_{(\text{nucleic acid solution})}$

Pure DNA has an A_{260}/A_{280} ratio of ≥ 1,8. $A_{260}/A_{280} < 1.8$ indicates that the preparation is contaminated with proteins and/or aromatic substances (e.g. phenol), $A_{260}/A_{280} > 2$ indicates a possible contamination with RNA.

2.7.5. DNA purification

DNA was purified from contaminants, reaction additives or agarose gels (DNA bands cut out with a scalpel) using the Promega (Madison, WI, USA) Wizard® SV Gel and PCR Clean-Up System. DNA was purified with centrifugation according to the manufacturer's instructions and eluted in 25 - 50 µl DNase free ddH_2O.

2.7.6. Vectors

pACTII (Clontech, Takara Bio, Otsu, Shiga, Japan)
Yeast two-hybrid vector with Gal4 transcriptional activator domain; Amp^R

pACTIIJ: Created by Georgi Dermendjiev from pACTII, two NotI sites were reduced to one by deleting the sequence in between.

pBIN19
Created by Alois Schweighofer; binary vector for transient expression and stable transformation of plants; encodes Kan^R for bacteria, Kan^R or $Basta^R$ for plants, blue-white selection.

pBTM117
Created by Markus Teige from pBTM116[50], lacks an EcoRI site; yeast two-hybrid vector with lexA DNA-binding domain; Amp^R

pCRblunt (Invitrogen, Carlsbad, CA, USA)
Blunt end cloning vector, Kan^R, death gene for insert selection.

pGEX4T (GE Healthcare, Little Chalfont Buckinghamshire, UK)
IPTG-inducible *E. coli* expression vector for N-terminal GST-fusion proteins; Amp^R.

pTLT
Created by Norbert Mehlmer; intermediate vector for cloning into binary vectors, encodes an expression cassette with 1x 35S promoter, TEV leader and CaMV terminator for a C-terminal fusion to YFP or CFP; Amp^R

pTWIN1 (NEB)
IPTG-inducible *E. coli* expression vector for N- or C-terminal fusions with a chtitin binding domain linked by an auto-cleavable intein for the purification of untagged proteins; Amp^R

2.7.7. DNA restriction digests

1 - 2 µg plasmid DNA was digested using restriction enzymes and buffers from NEB or ROCHE (Mannheim, Germany) according to the manufacturer's instructions and analysed an a 0,8 - 2% agarose gel (see "DNA electrophoresis").

2.7.8. DpnI (NEB) digest of methylated DNA

PCR products were rid of the methylated template DNA (i.e. plasmids isolated from *E. coli*) by digest with DpnI.

15 µl	PCR product
3 µl	ddH_20
2 µl	10x buffer (supplied)
0,5 µl	DpnI (20 U/µl)

The reaction was incubated for 2 h at 37℃. Unmethylated DNA was extracted with the Promega Wizard® SV Gel and PCR Clean-Up System.

2.7.9. DNA ligation with T4 Ligase (*NEB*)

Fragments to be ligated were cut out from an agarose gel and purified with the Promega Wizard® SV Gel and PCR Clean-Up System. Relative molar quantities were estimated from the gel foto and the insert applied in approximately 5 time's excess in respect to the vector. In addition, a reaction without insert was performed to check for re-ligation of the vector.

1 µl	10x buffer (supplied)
5 - 8 µl	Insert
0,8 - 1,5 µl	Vector
0,2 µl	T4 ligase (400 U/µl)
ad 10 µl	ddH_20

The reaction was incubated for 30 min at room temperature and transformed into competent *E. coli* cells.

2.7.10. Polymerase chain reaction

PCR reactions were carried out in a total volume of 25 - 50 µl, using GoTaq (Promega), Vent (NEB) or Turbo Pfu (Stratagene, La Jolla, CA, USA) DNA polymerase. Oligonucleotides were purchased from VBC Genomics, Vienna, Austria and Microsynth, Balgach, Switzerland.

- PCR with GoTaq polymerase

30 - 250 ng	DNA template
5 µl	5x buffer (supplied)
0,5 µl	dNTP mix (10 mM each)
0,5 µl	5' oligo (10 pmol/µl)
0,5 µl	3' oligo (10 pmol/µl)
0,125 µl	Polymerase (5 U/µl)
ad 25 µl	ddH_2O

- PCR with Vent polymerase

1 µg	DNA template
5 µl	10x buffer (supplied)
2 µl	$MgSO_4$ (100 mM)
1 µl	dNTP mix (10 mM each)
1 µl	5' oligo (10 pmol/µl)
1 µl	3' oligo (10 pmol/µl)
0,2 µl	Polymerase (2 U/µl)
ad 50 µl	ddH_2O

- PCR with Turbo Pfu polymerase

30 - 500 ng	DNA template
5 µl	10x buffer (supplied)
1 µl	dNTP mix (10 mM each)
1 µl	5' oligo (10 pmol/µl)
1 µl	3' oligo (10 pmol/µl)
0,2 µl	Polymerase (2,5 U/µl)
ad 50 µl	ddH_2O

Reaction mixtures were prepared in 200 µl or 500 µl polypropylene tubes and covered with mineral oil when necessary. Denaturation of template DNA was carried out for 3 min at 95℃ in the first cycle. The following cycles (23 - 35) were carried out as described: 45 sec of denaturation at 95℃, 45 sec primer annealing at an appropriate temperature (50-70℃) and 1-8 min extension at 72℃. For completion of the polymerisation reaction an extra 2-10 min extension cycle was added after the last cycle.

2.7.11. DNase digest of RNA

Prior to RT-PCR RNA was digested with the Promega RQ1 RNase-Free DNase according to the manufacturer's instructions.

2.7.12. Reverse transcription (RT)-PCR

The RNA concentration was determined by measuring A_{260}/A_{280} (see "Spectrophotometric Measurement of Nucleic Acid Concentration"), absorption values were compared with the intensity of RNA bands on a 2,5% agarose gel and the approximate volume of 2 µg RNA estimated. Reverse transcription was performed using the Promega M-MLV reverse transcriptase according to the manufacturer's instructions.

x µl	RNA (2 µg)
1µl	Oligo $(dT)_{12-18}$ (0,5 µg/µl)
ad 17,75 µl	ddH_2O

The mixture was incubated at 70℃ for 5 min to denature RNA secondary structures and put on ice again for 5 min to enable oligo binding. Then the following reagents were added and the samples incubated at 40℃ for 60 min.

5 µl	5x buffer (supplied)
1,25 µl	dNTPs (10 mM each)
1 µl	Reverse Transcriptase (200 U/µl)

PCRs were performed with GoTag polymerase (see “PCR reaction”) at an annealing temperature of 50 - 54℃ and an extension for 1 min, using 1 - 2 µl of the reverse transcription reaction as template. To be able to compare relative quantities of the PCR products, reactions must not reach the saturated range which was regulated through the number of cylces run. To check success of the reverse transcription and to equalize total cDNA quantities, cDNA was amplified with Act3 primers for 23 - 24 cycles. 10 µl per reaction were separated on a 1,2 - 2% agarose gel.

2.7.13. Primers

- Primers used for cloning and mutagenesis: See Table 2.1.
- Primers used for RT-PCR: See Table 2.2.

Table 2.1.: Oligos used for cloning and mutagenesis

Gene/mutant	Id. Nr.	Vector	Purpose	Forward	Reverse
bZIP9 S16A	At5g24800	pGEX4T	site-directed mutagenesis	5'-AGT GCT GCT GAG CTC GCT CTA CAA GAG-3'	5'-GTA GAG CGA GCT CAG CAG CAC TTC TC-3'
CPK29ΔC	At1g76040	pBTM117	cloning / mutagenesis	5'-TAG GGC CCA TGC TTC AAA ACC AAC ATA A-3'	5'-ATG CGG CCG CGA ACA GCA CTA TTG ATT GG-3'

Table 2.2.: Oligos used for RT-PCR (a)

Gene		Id. Nr.	Forward	Reverse
ACS6	1-aminocyclopropane-1-carboxylate synthase 6	At4g11280	5'-GAG CGG CGG CGC AAC CGG AG-3'	5'-CCA CCC TGT CAT TGT AAG AG-3'
Act3	Actin	At2g37620	5'-ATG GTT AAG GCT GGT TTT GC-3'	5'-AGC ACA ATA CCG GTA GTA CG-3'
AHG3	ABA-hypersensitive germination 3 (AtPP2CA)	At3g11410	5'-GCT GCT CTC ATG TTG CGG AG-3'	5'-ATC ACC AAT TGC TCT AGA CAT GG-3'
ASN1	Asparagin synthetase 1	At3g47340	5'-AGC TTT CTC GCA GAT TGA GGC AC-3'	5'-CTT AAT CAC AGC GTT TTC AAA GGC-3'
bZIP9	Basic leucine zipper 9 (BZO2H2)	At5g24800	5'-CGA CCT CAT GAA CCG GGA TTA CAC-3'	5'-CGC TGA GTG ATC ATT ACA TGT TCT GAA G-3'
bZIP63	Basic leucine zipper 63 (BZO2H3)	At5g28770	5'-CTC CGA CGA AGA AAT CTC CGG TAA CC-3'	5'-CTT TAA CAG CTA CTG ATC CCC AAC GC-3'
ERD10	Early responsive to dehydration 10	At1g20450	5'-TCT TCC TCT TCG AGT GAT GAA G-3'	5'-TCT CTT CCT CTC CAG TGG-3'
ERD15	Early responsive to dehydration 15	At2g41430	5'-ATGGCGATGGTATCAGGAAG-3'	5'-CTTCCATCGAATCCTTGATC-3'
Erf6	Ethylene responsive element binding factor 6	At4g17490	5'-CCG TTG CCT ACT ACT GCC ACC-3'	5'-GCA CTT TCT CAA CCA CCG TC-3'
GolS2	Galactinolsynthase 2	At1g60470	5'-AAG GCT GTG TCG TGC GTG AG-3'	5'-GGC TTG GAT CCA GCT GCA CAG-3'
KC1	K+ inward rectifying channel protein 1	At4g32650	5'-CAT CAA ACT TCT ATG TGT AAC G-3'	5'-GCG AAA CCA GCT GGA CGA GG-3'
KCO1	Ca2+-activated outward rectifiying K+ channel 1 (TPK1)	At5g55630	5'-CCA GTG GTG TGG TAG ATG CTC TCT-3'	5'-ACT CTG CAG CTC CAA CAA CTC CAT C-3'
NHX1	NHE1 Na+/H+ exchanger 1	At5g27150	5'-CCT TGT TGA TTG GGC TAG GC-3'	5'-CTC AAG TCG AAA AGC TCA GC-3'
P5CS	Delta 1-pyrroline-5-carboxylate synthetase	At2g39800	5'-GGA GGA GCT AGA TCG TTC AC-3'	5'-TCA GTT CCA ACG CCA GTA GA-3'
PDH	Prolin dehydrogenase / oxidase	At3g30775	5'-CCA ACA TCT CAC TTT AGC TCA GTG G-3'	5'-CGA CGC AAG TCT CCC CGA ATC AGC G-3'
PP2C	Protein phosphatase type 2C	At1g07160	5'-AGA TCC CAA ACA GGC AAT ATT CGG-3'	5'-CTC CTA TGG TCA GAA GAG GTG GC-3'
Rd20	Responsive to desiccation 20	At2g33380	5'-CCA AAA CCA TAC ATG GCA AGA GC-3'	5'-TGA AAG CCA TCC AAA AGG ATC G-3'
Rd29a	Responsive to desiccation 29a	At5g52310	5'-AGC ACC CAG AAG AAG TTG AAC ATC-3'	5'-CGT TAC ATC CTC TGT TCC AG-3'
Rd29b	Responsive to desiccation 29b	At5g52300	5'-CGG CGC ACC AGT GTA TGA ATC C-3'	5'-TGA GAT AAT CCG ATC CTC CTC C-3'
SOS1	Salt overly sensitive 1	At2g01980	5'-GGA ATT CGT ATC TGG AAT GAG ATC G-3'	5'-CCC ACT CTT GAG CAG TGT AGT ATG C-3'
STZ	Cys2/His2-type zinc-finger protein	At1g27730	5'-ATG GCG CTC GAG GCT CTT AC-3'	5'-TCC TTC GTA GTG GCA CCG C-3'
T6PP	Trehalose-6-Phosphat Phosphatase	At2g18700	5'-CTG GGG AGT CGG ATT CGG TC-3'	5'-CCT TTG CTT ACT CCC TGA GGT-3'
TPS1	Trehalose-6-Phosphat Synthase 1	At4g22590	5'-GGC CTG GAT GCT CAA GTA TCC TTC-3'	5'-CAC CTC TAA AAC CTT TCT ACC GTG AG-3'
TPS11	Trehalose-6-Phosphat Synthase 11	At1g78580	5'-GTC AGT GCT CTC TTA GGT GTA AAG G-3'	5'-TGC CAA TTG GAA AAG CAG CTA CAC G-3'

Table 2.2.: Oligos used for RT-PCR (b)

Gene	Id. Nr.	Forward	Reverse
CPK1	At5g04870	5'-GGA GAG CTT CGA TCT CGA TTA TC-3'	5'-CAT TAT CTS AAT YTC TCT TCT CAC ATC-3'
CPK2	At3g10660	5'-CTT CTT CCC ATG GTA ATG GAC-3'	see CPK1
CPK3	At4g23650	5'-AGA TGT TCG CCG TGA AGT CC-3'	5'-ACG GAT GAT TTA GCA CTT CCG-3'
CPK4	At4g09570	5'-CAA AAA AAG GCA GAG ACT TTC G-3'	5'-TCT CTA TGC ATV ACA CCA AGM GAA TG-3'
CPK5	At4g35310	5'-GAT CCA GCT CTT GTT ATT CCT TTA AG-3'	see CPK4
CPK6	At2g17290	5'-GTG AAA AAA GTT GTG AAA TAG AGA GAG-3'	see CPK4
CPK7	At5g12480	5'-CAT TTT GGG CAG AGA CTG AGC-3'	5'-GTC TCT TGC TTT CTT AGG TCT CG-3'
CPK8	At5g19450	see CPK7	5'-GAA ACC CTG ATC TAC AAT TAT TGA GG-3'
CPK9	At3g20410	5'-CGA TCT TTC CTC TCT GGG TCG-3'	5'-AWC ACA CCC ATG AAA TGA CAA AT-3'
CPK10	At1g18890	5'-TGA TCT CCT TTC TCT GAG TTT GC-3'	5-CCD CCT TCA CAA AKC TCC AT-3'
CPK11	At1g35670	5'-CAA AGA AAA AGT CTG TTT ATC ATC TTC-3'	see CPK4
CPK12	At5g23580	5'-CAA CAG TTT CTC TTC TTC TTC CTC-3'	see CPK10
CPK13	At3g51850	5'-TCT ATT ACT TTC TAC TTC TGA AAC CCC-3'	see CPK10
CPK14	At2g41860	5'-GGA ACG GCT GGA TCG TTG-3'	see CPK10
CPK15	At4g21940	5'-CCC GTT AAT CAG CAA ATC G-3'	5'-TTC TCA GGC TTK AGA TCW CGA-3'
CPK16	At2g17890	5'-TGC AAC GGC TAA GAA AAC G-3'	5'-TGA TTT GAA CAR AAA RTT CTC TGG-3'
CPK17	At5g12180	5'-ATC TCC CCC ACC CTT TTA TGC-3'	5'-CCT TGA ATA CCT CTC CTG GCT TG-3'
CPK18	At4g36070	5'-GAG TGA GAA GGT TAG TAA TAA AAA CAA G-3'	see CPK16
CPK19	At1g61950	5'-CAA GAG CCA AGT TCC AGA TTG T-3'	see CPK9
CPK20	At2g38910	5'-CCG AAT CTG AAC CCT AAT GG-3'	see CPK1
CPK21	At4g04720	5'-AAC CCA TGA CTC AAC CAA TCC-3'	see CPK15
CPK22	At4g04710	5'-TTG CTG CGG AAG TAA ACC C-3'	see CPK15
CPK23	At4g04740	5'-ATC GTT GAT CAA GTC CCC G-3'	see CPK15
CPK24	At2g31500	5'-ATT CAA ACC GTT GGA TAT TAT TCC-3'	see CPK10
CPK25	At2g35890	5'-GGG AAT GTA TGT GTT CAT ATG GTC-3'	see CPK1
CPK26	At4g38230	5'-AAT TTG GAA GGG GAG GTG AA-3'	see CPK4
CPK27	At4g04700	5'-CTA ACC CGA AAG TGC GTT G-3'	see CPK15
CPK28	At5g66210	5'-GGT GTC TGT TTC TCC GCC AT-3'	see CPK16
CPK29	At1g76040	5'-CCT TGA AGG TGA TAG CGG A-3'	5'-GCA AAA GCA TTC GTC ACT GT-3'
CPK30	At1g74740	5'-GCC TGC GTC AAG TTT GAC C-3'	see CPK10
CPK31	At4g04695	5'-CGG TAT AAC TCG AAA ATG CG-3'	see CPK15
CPK32	At3g57530	5'-CTC AAT CTC TCA AGT TTC ATC GG-3'	see CPK10
CPK33	At1g50700	5'-CTG TTT TAT ATC CCT GAT TCT CTG C-3'	see CPK9
CPK34	At5g19360	5'-GAG GCG GCG GTG TTG GTG-3'	5'-AAT CCC ATT CTC TGA CTC AGC CC-3'

IUB Code for mixed base sites:

K = G, T
M = A, C
R = G, A
S = G, C
W = A, T
Y = T, C
D = G, A, T
V = G, A, C

2.8. Protein methods

2.8.1. Poly-acrylamid gel electrophoresis (PAGE)

- SDS acrylamid gels

[2x] separating gel buffer

1,5 M Tris.Cl pH 8.85

0,1% SDS

[2x] stacking gel buffer

0,25 M Tris.Cl pH 6.8

0,1% SDS

Acrylamid

30% 37,5:1 acrylamid:bisacrylamid (BioRad)

o Mini gels (BioRad Mini Protean® 3 System)

	12% Separating gel	4% Stacking gel
30% acrylamid	6 ml	0,8 ml
dH_2O	1,36 ml	2,15 ml
2x buffer	7,5 ml	3 ml
TEMED	30 µl	12 µl
10% APS	110 µl	42 µl
total volume	15 ml	6 ml

Specified volumes are for 4 gels.

o Midi gels (Whatman (Dassel, Germany) Biometra® Multigel-Long System)

	12% Separating gel	4% Stacking gel
30% acrylamid	12 ml	1 ml
dH2O	2,72 ml	2,68 ml
2x buffer	15 ml	3,75 ml
TEMED	60 µl	15 µl
10% APS	220 µl	54 µl
total volume	30 ml	7,5 ml

Specified volumes are for 2 gels.

10 - 17,5% Midi gradient gel		
	10%	17,50%
dH_2O	1,796 ml	-
2 M Tris pH 8.8	1,824 ml	1,824 ml
87,5% glycerol	613 µl	776 µl
30% acrylamid	2,17 ml	3,8 ml
10% SDS	65 µl	65 µl
10% APS	20 µl	20 µl
TEMED	2 µl	2 µl
total volume	6,5 ml	6,5 ml

Specified volumes are for 1 gel. The gel was poured using a Hoefer (San Francisco, CA, USA) SG30 gradient mixer. The 4% stacking gel was prepared as indicated above.

- Gel electrophoresis

[2x] SDS Sample Buffer

50% (v/v)	Glycerol
2% (w/v)	SDS
5% (v/v)	ß-mercaptoethanol
125 mM	Tris.Cl pH 6.8
0,05% (w/v)	Bromophenol blue

[10x] SDS Running Buffer

50 g	SDS
151,5 g	Tris base
720,5 g	Glycine
ad 5 litres	dH_2O

Protein molecular weight marker

PageRuler™ Prestained Protein Ladder #SM0671 (Fermentas, St. Leon-Rot, Germany)

Coomassie staining solution

2,5 g	Coomassie Brilliant Blue R-250
100 ml	Isopropanol
100 ml	Acetic acid
ad 1 litre	dH_2O

Boil briefly to dissolve Coomassie entirely

Destaining solution

10% isopropanol, 10% acetic acid

Samples were prepared by adding 1 volume of 2x sample buffer, incubated on 95℃ for 2 - 5 min and cooled on ice for 5 - 10 min. Gels were placed into the appropriate electrophoresis device, covered with 1x SDS running buffer and equilibrated for 15 min at 15 mA (optionally). To estimate protein sizes, 3 µl of PageRuler protein ladder was loaded together with the samples. Mini gels were run at 20 (stacking gel) respectively 40 mA (separating gel), Midi gels at 15 (stacking gel) respectively 30 mA (separating gel) per gel.
Proteins were visualized by staining the gel in hot coomassie staining solution for 10 min and destaining it several times with hot destaining solution for 10 - 30 min each. The destained gel was dried for 2 h at 70℃ in a SAVANT Slab Gel Drye r.

2.8.2. Determination of protein concentration with Bradford assay

Bradford reagent

Bio-Rad Protein Assay diluted 1:5 with dH_2O

950 µl Bradford reagent was mixed with 50 µl protein sample or buffer as a negative value. After incubation for 5 - 15 min at room temperature the absorption at 595 nm was measured. Concentrations were calculated from a BSA standard row with 500, 250, 125, 62,5, 31,2, 15,6 and 7,8 µg/ml which was measured under the same conditions.

2.8.3. GST-protein purification

[1x] GST-Buffer

50 mM	Tris.Cl pH 8.0
20 mM	$MgSO_4$
2 mM	DTT
5 mM	Na_2EDTA pH 8.0

¼ tablet/50ml Complete EDTA free Protease Inhibitor (Roche) was added immediately before use

Glutathione Sepharose™ 4B (GE-Healthcare)

150 µl of the bead stock suspension was spun down for 5 min at 200 - 400g, 4℃, washed 2x with 1 ml GST-buffer and resuspended in 500 µl cold GST-buffer before use.

Glutathione elution buffer (GEB)

10 mM	reduced Glutathione
50 mM	Tris.Cl pH 8.0

50 ml LB_{AMP} was inoculated with a single colony or a glycerol stock of *E. coli* BL21 carrying the desired pGEX plasmid and grown over night at 37℃. The culture was diluted to an OD_{600} of 0,1 - 0,2 in 300 ml LB_{AMP} and grown to an OD_{600} of 0,6 - 0,8. An aliquot of 800 µl (uninduced control) was taken and protein expression induced with 300 µl of 1 M IPTG (end concentration 1 mM). The culture was incubated at 30℃ for 4 h and anoth er aliquot was taken (induced control). Cells were harvested by centrifugation for 20 min at 2500g, 4℃, resuspended in 40 ml ice cold GST-buffer, transferred to Greiner tubes and centrifuged again for 10 min at 3000g, 4℃. The pellet was resuspended in 10 - 15 ml of ice cold GST-buffer, optionally it was stored at -20℃ until further processing.

Cells were broken by sonification on ice (3x 30sec, 100% intensity). 0,5 ml of 20% Triton-X 100 was added slowly and the lysate transferred to SS34 tubes. The cell debris was pelleted by centrifugation for 30 min at 17.000g, 4℃. The supe rnatant was transferred to a Greiner tube, 100 - 150 µl of glutathione sepharose was added and incubated for 30 - 60 min on a boogie wheel at 4℃. Sepharose was pelleted by centrifugat ion for 2 - 5 min at 400g, 4℃ and washed 2x with 10 ml cold GST-buffer. The fusion protein was eluted at least 2x with 300 µl GEB increasing incubation periods from 30 min to over night.

2.8.4. Protein purification with the NEB pTWIN system

<u>B1 Buffer</u>

20 mM	Tris.Cl pH 8.5
500 mM	NaCl
1 mM	Na_2EDTA (optional)

0,1% Tween 20 and ¼ tablet/50ml Complete EDTA free Protease Inhibitor (Roche) was added immediately before use

<u>B2 Buffer</u>

20 mM	Tris.Cl pH 7.0
500 mM	NaCl
1 mM	Na_2EDTA (optional)

<u>B3 Buffer</u>

20 mM	Tris.Cl pH 8.5
500 mM	NaCl
40 mM	DTT
1 mM	Na_2EDTA (optional)

<u>Chitin Sepharose</u>

NEB chitin beads

<u>Chitin Columns</u>

2,5 ml chitin sepharose was filled into Pierce Perbio (Rockford, IL, USA) 2 ml polystyrene columns and equilibrated with 25 ml of B1 buffer before use.

<u>10 kDa filter tubes</u>

Amicon Ultra MW 10.000 MWCO Centrifugal Filter Devices (Millipore, Billerica, MA, USA)

50 ml LB_{AMP} was inoculated with a single colony or a glycerol stock of *E. coli* BL21 carrying the desired pTWIN plasmid and grown over night at 37℃. The culture was diluted to an OD_{600} of 0,1 - 0,2 in 200 ml LB_{AMP} and grown to an OD_{600} of 0,5 - 0,7. An aliquot of 800 µl (uninduced control) was taken and protein expression induced with 70 µl of 1 M IPTG (end concentration 0,3 mM). The culture was incubated at 18℃ over nig ht or alternatively at 30℃ for 6 h and another aliquot was taken (induced control). Cells were harvested by centrifugation for 10 min at 2500g, 4℃, resuspended in 15 ml ice cold B1, trans ferred to Greiner tubes and centrifuged

again for 10 min at 3000g, 4℃. The pellet was resu spended in 10 ml of ice cold B1, optionally it was stored at -20℃ until further processing.

Cells were broken by sonification on ice (3x 30 sec, 100% intensity) and the crude cell extract* transferred to SS34 tubes. The cell debris was pelleted by centrifugation for 30 min at 17.000g, 4℃, the pellet* was stored at -20℃ for further an alysis and the clarified supernatant* was applied to the pre-cooled column in the 4℃ climate chamber. Optionally, the flow-through* was re-applied to the column to make sure all binding sites have been saturated. The column was washed* with 10-15 column volumes of B1, 500 - 1000 mM NaCl, flushed* with 3 column volumes B2 (for N-terminal fusion proteins) or B3 (for C-terminal fusion proteins) respectively. The column flow was stopped, columns were transferred to room temperature and incubated over night to allow cleavage.

Proteins were eluted with 3 volumes of cleavage buffer (B2 or B3 without DTT), whereas 1 - 2 ml fractions were taken (smaller volumes for the first fractions where most protein is present). The protein content of 50 µl pure elute was measured with Bradford assay (see “Determination of Protein Concentration with Bradford Assay”). If the protein content was satisfactory, elution was repeated after some hours. Elutes were concentrated to 200 - 500 µl with 10 kDa filter tubes and analysed on an SDS-PAGE together with aliquots taken during purification (see “Poly-Acrylamid Gel Electrophoresis”).

* As an internal control 40 µl aliquots were taken from each purification step marked with an asterisk.

2.8.5. Kinase assays

<u>[5x] Kinase buffer</u>

100 mM	HEPES pH 7,5
75 mM	$MgCl_2$
5 mM	DTT

For experiments without Ca^{2+} 10 mM EGTA was added

For experiments with Ca^{2+} 1 mM $CaCl_2$ was added

<u>Imager screen</u>

PhosphorImager® Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA, USA)

X µl	Substrate protein (1-5 µg)
4 µl	5x kinase buffer
0,1 µl	ATP (40 mM)
Y µl	Kinase (40-100 ng)
ad 20 µl	ddH_2O

The reaction was incubated for 10 - 30 min to enable auto-phosphorylation of the kinase with cold ATP (to analyse auto-phosphorylation of substrate proteins, neighter kinase nor cold ATP was added to the reaction). In the hot lab 1 µl [^{32}P]γ-ATP (8 mM) was added, mixed well and incubated for 20 - 30 min. The reaction was stopped by adding 20 µl 2x SDS sample buffer, 10 µl per reaction were loaded on a 12% SDS gel (see "SDS Acrylamid PAGE"). Radiation was measured by exposing the dry gel over night in an imager screen which was read out with a GE Healthcare Typhoon 8600 Variable Mode Imager.

2.8.6. Total dephosphorylation of proteins

Proteins were desphosphorylated sequence unspecifically using Lambda-phosphatase from NEB according to the manufactoresr's instructions.

2.8.7. Western blot

- Blotting on nitrocellulose membrane

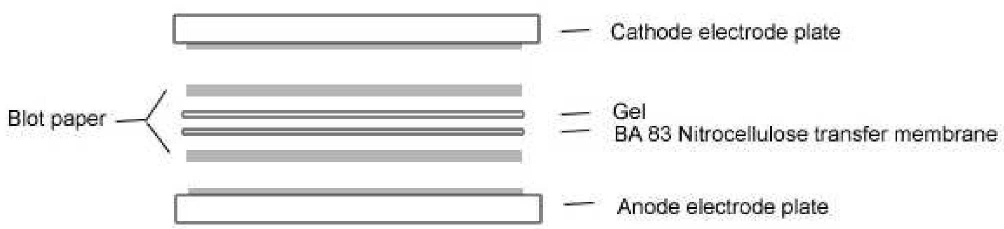
<u>Nitrocellulose membrane</u>
Whatman® Protran® BA 83 Nitrocellulose transfer membrane, pore size 0,2 µm

<u>Blot paper</u>
Bio-Rad Protean® II xi size extra thick blot paper

<u>Semi Dry Blot Puffer</u>

5,82 g	Trizma
2,93 g	Glycin
200 ml	Methanol
ad 1 litre	dH_2O

Membrane, acrylamid gel, as well as 2 layers of blot paper were equilibrated for a few minutes in semi dry blot buffer. The stack was assembled as indicated below.



- Blotting on PVDF membrane

<u>PVDF membrane</u>
Millipore Immobilon™-P transfer membrane, pore size 0,45 µm

Blot paper

Whatman® 3MM paper

Anode buffer I

0,3 M	Tris.Cl pH 10.4
10 %	Methanol

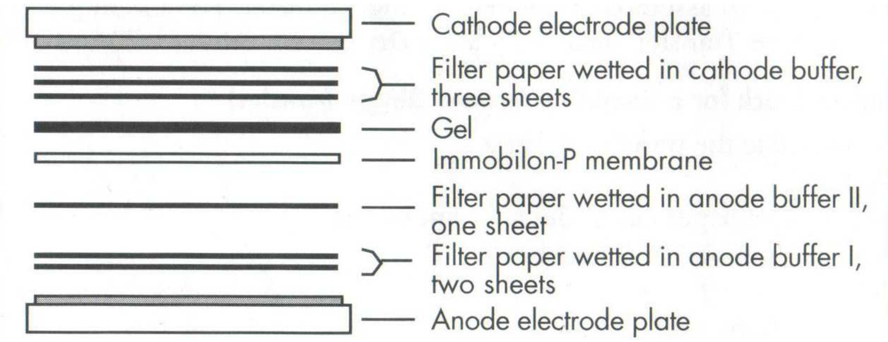
Anode buffer II

25 mM	Tris.Cl pH 10.4
10 %	Methanol

Cathode buffer

25 mM	Tris.Cl pH 9.4
40 mM	Glycine
10 %	Methanol

The membrane was wetted in 100% methanol, washed with dH_2O for 2 min and equilibrated in Anode buffer II for 5 min. The gel was equilibrated in Cathode buffer for 5 min. The blot paper was soaked with the respective buffers and the stack assembled as indicated below.



Air bubbles were removed by carefully rolling a centrifuge tube over the surface of the stack. Blotting was performed for 1 - 2 h, at 200 mA / 10 V per big gel (11x12 cm) gel and 70 mA / 5 V per standard gel respectively in a BioRad Trans-Blot® SD Semi-Dry Transfer Cell. After blotting the membrane was stained with Ponceau solution, washed with dH_2O and fixed with 10% acetic acid. If nitrocellulose was used the Ponceau stained membrane was scanned for documentation.

- Immunodetection

[10x] TBS

500 mM	Tris.Cl pH 7.4
1,5 M	NaCl

[1x] TBS-T

50 mM	Tris.Cl pH 7.4
150 mM	NaCl
0,1%	Tween.

HRP detection Kit

Pierce Perbio Super Signal® West Pico Chemiluminescent Substrate for HRP detection Kit:
Mix Stable Peroxide solution and Luminol / Enhancer solution 1:1

Exposure cassette

Kodak (Stuttgart, Germany) X-Omatic® Regular intensifying screen

X-ray films

Fujifilm (Tokyo, Japan) Fuji medical X-ray films 100 NIF 13x18

PVDF destaining solution

40 % Methanol
10 % Acetic acid

The membrane was washed with 1x TBS-T to remove Ponceau colour and blocked with TBS-T 5% milk powder for at least 20 min (shake at 40 - 50 rpm). If the 1st antibody was applied in milk, it was added directly in the appropriate dilution (1:500 – 1:2000). If it was applied in TBS-T 5% BSA, the membrane was washed 3 times with TBS-T after blocking. The 1st antibody was incubated for 1,5 h at RT or over night at 4℃ resp ectively. The membrane was washed 3x with TBS-T, the 2nd antibody was applied 1:5000 in milk powder solution and incubated for 45 min at room temperature. The membrane was washed 3x with TBS-T and transferred to a clean plate at each washing step to avoid unspecific binding of rests of 2nd antibody.
Excess liquid was drained before transferring the membrane to a glass plate. The membrane was covered with a 1:1 mixture of H_2O_2 and Luminol / Enhancer solution and incubated for 5 min. Excess liquid was drained the membrane transferred into an exposure cassette. X-ray films were exposed for 30 sec, 3 min as well as for shorter and / or longer periods. Films were developed in an AGFA (Mortsel, Belgium) Curix 60 X-ray film developing machine using AGFA G153 A and B developer solutions, G354 rapid fixer solution and tap water.
If PVDF membrane was used, the membrane was stained with Coomassie staining solution for 5 min and destained with PVDF destainer 3 - 4 times for 5 - 20 min each. The dry membrane was stored at RT.

2.8.8. Antibodies

Primary antibodies

Polyclonal peptide antibodies from rabbit purchased from Davids biotechnology, Regensburg, Germany:

Protein	Peptide antigene
AtCPK3	NPELVPNRRRM
AtbZIP9	CDNHTAKDIGMKRSA
AtbZIP63	CSLEHLQKRIRSVGD

GFP Polyclonal antibody from mouse (Roche)

Phospho-(Ser/Thr) PKD Substrates antibody from rabbit (Cell Signaling Technology, Danvers, MA, USA)

Secondary antibodies

IgG antibodies linked to Horseradish Peroxidase purchased from GE Healthcare:

ECL™ Anti-rabbit IgG from donkey

ECL™ Anti-mouse IgG from sheep

2.8.9. Mass spectrometry

Mass spectrometric measurements were performed at the MFPL Mass Spectrometry Facility in collaboration with Edina Czasar and Sonja Kolar. Proteins were treated with different proteases, such as trypsin, subtilisin, GluC and LysC and separated on a C18 column with acetonitril and formic acid as mobile phase. The MS was done in a in a linear ion trap with positive polarisation. Peptides were fragmented by CID fragmentation and measured with neutral loss scan and automatic ms3. Sequences were analyzed with Sequest and Mascot software.

2.9. Programs and databases

Vector NTI Advance™ 9.1 (Invitrogen)

ImageQuant 5.1 (Molecular Dynamics)

BioEdit Sequence Alignment Editor v7.0.5 (Tom Hall, Ibis Biosciences, Carlsbad, CA, USA)

Pubmed literature database: http://www.ncbi.nlm.nih.gov/sites/entrez/

NCBI BLAST: http://www.ncbi.nlm.nih.gov/BLAST/

Immunax antigenicity test: http://immunax.dfci.harvard.edu/Tools/antigenic.pl

Mips: http://mips.gsf.de/proj/plant/jsf/athal/index.jsp

TAIR (The Arabidopsis Information Resource): http://www.arabidopsis.org/

Arabidopsis eFP Browser http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi

Signal salk: http://signal.salk.edu/cgi-bin/tdnaexpress

NASC (The European Arabidopsis Stock Centre): http://arabidopsis.info/BasicForm

3. Results

3.1. CPK3 over-expression lines

3.1.1. The problem of silencing

CPK3 knock out and over-expressor plants were used to analyze the influence of CPK3 on the transcriptional expression pattern under stress conditions. The knock out line *cpk3-2* had already been analyzed and confirmed by Mori et al. (2006)[35]. As over-expressors, lines obtained from Norbert Mehlmer (35S::CPK3 A, B, C and D) were used first. Figure 3.1 shows the levels of CPK3 transcript in seedlings of different age after salt stress in the various lines. In the knock out there was no CPK3 product as expected, however there was a strong reduction of transcript in the 20-day-old over-expressor plants, especially in the stronger expressing line D, indicating a very fast and effective silencing. Accordingly, the next generation of constitutive over-expressor plants showed no more heterologous CPK3-YFP expression, which was monitored through YFP-fluorescence. This means that from constitutive over-expressor plants we could only use early seedlings of the T1 generation after dipping.

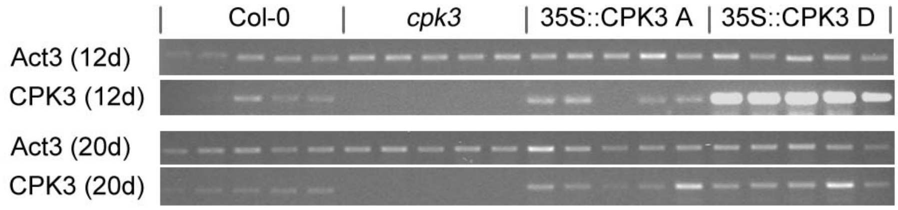


Fig 3.1: RT PCRs with RNA from 12 and 20 days old seedlings after 0 min, 30 min, 60 min, 3 h and 6 h stress with 150 mM NaCl in 1/2 MS

3.1.2. Searching for strong CPK3 over-expressors

11 35S::CPK3-YFP Kan^R lines of the T1 generation after dipping were obtained from Norbert Mehlmer and analyzed for CPK3-YFP expression levels. Tables 3.1-3.4 show the viability of the seedlings on kanamycin and the YFP-fluorescence after 7 days, when no silencing should yet occur, and 18 days, when silencing was already in progress. Silencing of Kan^R became appearent as bleaching of cotyledon margins and damage of second leaf meristem, silencing of CPK3-YFP expression as reduced YFP-fluorescence. Lines that could be used are marked in red, lines that were dismissed are marked in gray. Line 8 was not used at all due to insufficient seed material. From the segregation on Kan^R after 7 days, line #1 was considered homozygous, line #2 segregated 1:6 which is a pattern seen in heterozygous lines with multiple insertions. These two lines showed the highest viability combined with strongest average YFP-fluorescence and considerable amount of seeds and were used for all further experiments.

Table 3.1: Viability on 1/2 MS Kan after 7 days:

line	total germinated plants	not germinated seeds	dying	alive
1	140	35	5	
2	90	40	15	
3	280	65	15	
4	80	35	50	
5	120	30	10	
6	200	35	35	
7	200	20		25
9	200	20		30
10	120	80	40	
11	200	10	35	

Table 3.2: YFP-fluorescence after 7 days:

line	strong		weak	no fluorescence	total
	whole plant / leaf	root			
1	6	6	2	9	23
2	3	7	1	2	13
3	0	3	4	7	14
4	0	6	0	0	6
5	4	0	3	5	12
6	0	0	7	8	15
7	0	0	2	8	10
9	0	0	5	8	13
10	0	11	1	4	16
11	0	1	4	7	12

Table 3.3: Viability on 1/2 MS Kan after 18 days:

line	silencing of Kan^R
1	all but 1
2	all
3	90% weak
4	all
5	all
6	80% very weak
7	>90%
9	all
10	all strong
11	no

Table 3.4: YFP-fluorescence after 18 days:

line	strong				moderate				weak		no fluor.	total
	whole plant / leaf	part of plant	root	part of root	whole plant / leaf	part of plant	root	part of root	whole plant / leaf	root		
1	0	3	6	2	0	1	2	0	0	1	0	15
2	0	4	3	0	1	1	1	1	0	0	4	15
3	0	0	0	0	0	0	5	1	0	5	1	12
4	0	1	4	1	1	0	0	6	1	0	0	14
5	0	1	0	0	0	0	0	0	0	6	7	14
10	0	1	1	2	0	0	1	4	0	0	4	13

3.2. CPK3 in transcriptional regulation

Wild type, CPK3 knock out and over-expressor lines were analyzed on transcript level using semi-quantitative RT-PCR. As it was already known that CPK3 plays a role in salt stress response from the salt sensitivity phenotype of CPK3 mutant plants (Mehlmer and Teige, unpublished), we focused on studying transcriptional regulation under salt stress. RNA isolated from seedlings stressed for 0 min, 30 min, 60 min, 3 h and 6 h in liquid ½ MS containing 150 mM NaCl was used to compare wild type, the CPK3 knock out line *cpk3-2*, and the CPK3 overexpressor lines 1 and 2 in respect to mRNA expression of CPK3 itself as well as of various genes known to play a role in stress response, such as ion channels, osmotic stress induced genes, key enzymes for nitrogen metabolism and biosynthesis of signal molecules and compatible solutes (Figure 3.2). RT-PCR products were not sequenced, but all of them were of the expected size which is shown in Figure 3.3 and Table 3.5.

CPK3 mRNA expression itself was not induced through salt treatment, but transcript levels of another CDPK, CPK29, transiently increased. Expression of most of the analyzed genes was induced after a certain time of salt stress; however there was no difference in induction between the CPK3 lines. Apparently, CPK3 does not regulate transcriptional control of the analyzed genes under the applied conditions.

Interestingly, important post-translationally regulated sodium transporters such as SOS1 and NHX1 showed only slight induction after at least 60min of salt stress. The expression of the Ca^{2+}-activated outward rectifying K^+ channel KCO1 and the inward rectifying K^+ channel KC1 did not change significantly at all.

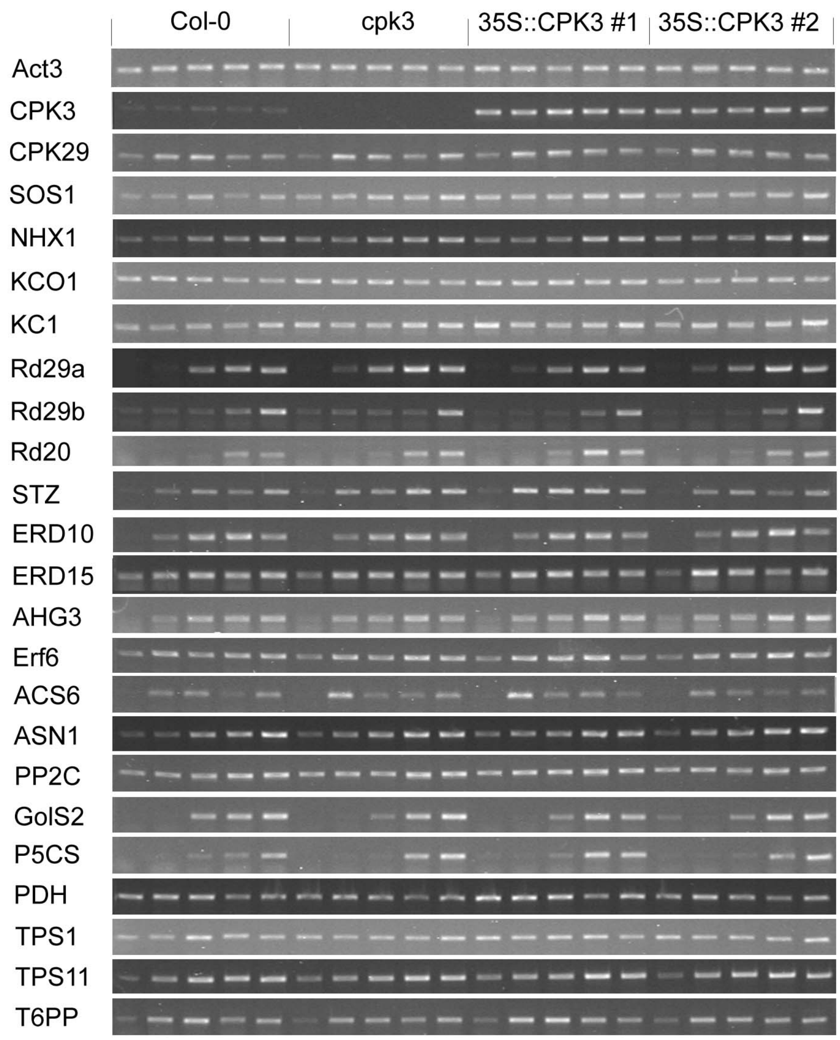


Fig 3.2: RT PCRs with RNA from 13 days old seedlings after 0 min, 30 min, 60 min, 3 h and 6 h stress with 150 mM NaCl in 1/2 MS

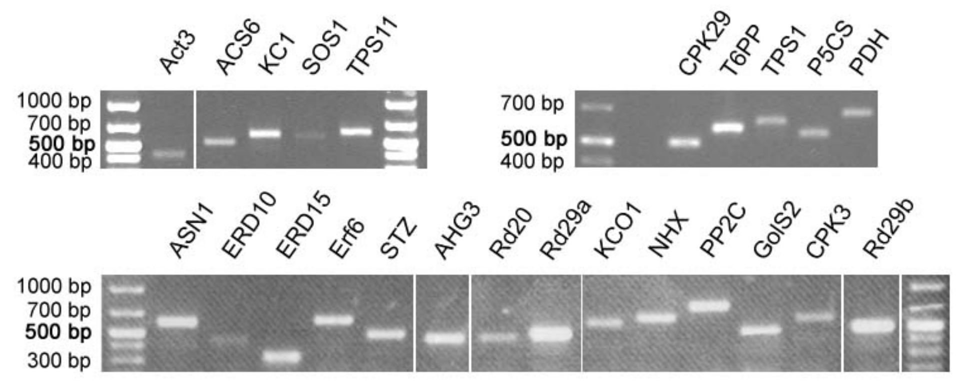


Fig. 3.3: RT-PCR fragment sizes

Table 3.5: Expected sizes of RT-PCR products.

Gene	Id.nr.	Expected fragment size
Act3	At2g37620	413
Acs6	At4g11280	518
AHG3	At3g11410	458
ASN1	At3g47340	593
CPK3	At4g23650	641
CPK29	At1g76040	518
ERD10	At1g20450	439
ERD15	At2g41430	311
Erf6	At4g17490	622
GolS2	At1g60470	576
KC1	At4g32650	581
KCO1	At5g55630	621
NHX1	At5g27150	658
P5CS	At2g39800	560
PDH	At3g30775	678
PP2C	At1g07160	712
Rd20	At2g33380	474
Rd29a	At5g52310	509
Rd29b	At5g52300	496
SOS1	At2g01980	574
STZ	At1g27730	483
T6PP	At4g22590	600
TPS1	At1g78580	631
TPS11	At2g18700	609

3.3. CPK3 as regulator of K^+ homeostasis

When Col-0 and *cpk3-2* plants were grown in hydroponic culture with standard ½ Hoagland medium, *cpk3* plants showed a stronger yellowing starting from the edges of older leaves after about 7 weeks (Mehlmer, unpublished). This is characteristic for deficiencies of macronutrients such as potassium or nitrogen and was another hint for a role of CPK3 in K^+ homeostasis. Indeed *cpk3* plants had a lower content of K^+ compared to the wild type. So, Col-0, *cpk3* and 35S::CPK3 plants were grown in hydroponic culture with different K^+ concentrations: 0,3 mM (low K^+), 3 mM (standard K^+) and 30 mM (high K^+). It was expected that high K^+ should rescue the *cpk3* phenotype.

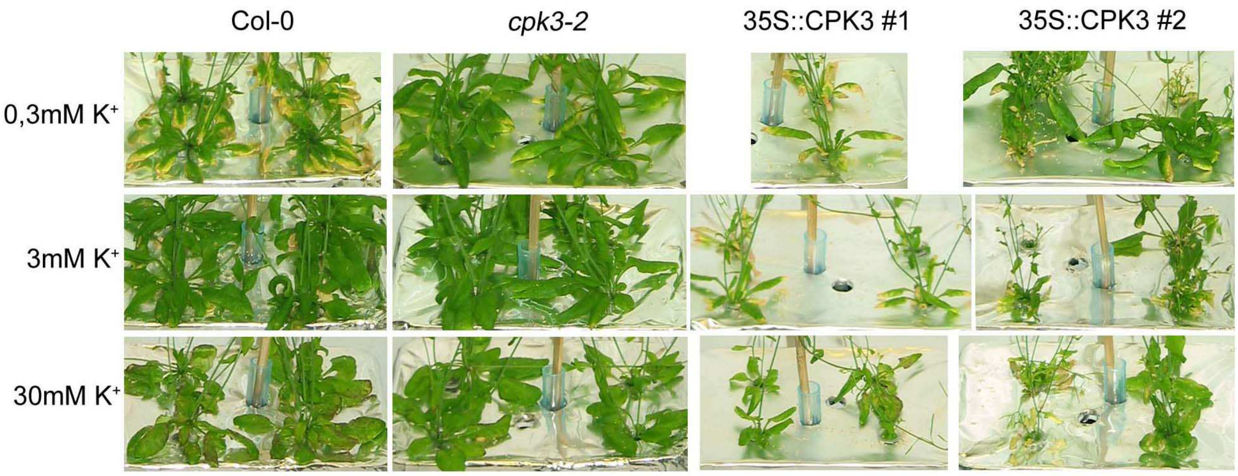


Fig. 3.4: 6 weeks old CPK3 plants grown in ½ Hoagland medium with varied K+ concentrations

Figure 3.4 shows the 6-week-old plants. In this experiment the yellowing phenotype of *cpk3* could not be reproduced, in contrast, at a concentration of 0,3 mM K^+, Col-0 shows a stronger yellowing than *cpk3*. At 30 mM K^+ plants did not show yellowing but developed brown dots. The homozygous over-expressor plants of line #1 showed reduced growth and viability and also a stronger yellowing. The line #2, which by its segregation pattern seemed to be heterozygous with multiple T-DNA insertions, showed a varied appearance, or even smaller than the plants of line #1, or bigger and healthier but of more bushy growth. Statistically, 1 out of 6 line #2 plants should be wild type (see “CPK3 over-expressor lines”). At this age, over-expressor plants have completely knocked down CPK3 and most probably also other homologous CDPKs which would lead to an amplified phenotype.

3.4. CDPK redundancy

To address the question whether in CPK3 knock out plants the CPK3 function was substituted by other homologous CDPKs, transcript levels of the remaining 33 CDPKs have to be analyzed in *cpk3* compared to Col-0 and also in silenced over-expressor plants. Specific RT-PCR oligos were designed for all CDPKs selecting variable regions in a multiple alignment of the available transcript sequences. For most CDPKs one oligo was ordered that bound in a conserved region and fitted for 2-5 CDPKs. The other oligo was designed in a variable region which was specific for the desired CDPK verified in an NCBI Blast search. If possible the specific oligos were set in the 5’ or 3’ UTR to avoid amplification of lab contaminations from cloning. As most oligos did not pass intron-exon borders, the RNA preparations were DNase digested prior to reverse transcription. Plant material was produced from different tissues (leaf, root, flower and green siliques) and developmental stages (seedling, adult plant) from Col-0 and *cpk3-2*. To investigate

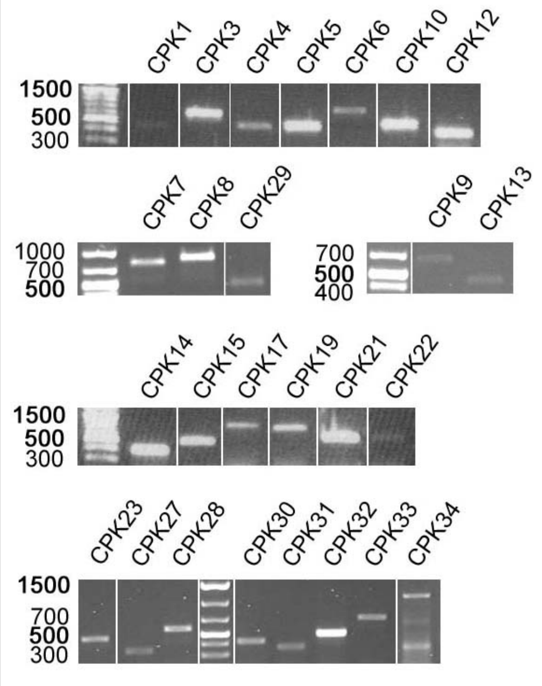


Fig. 3.5: RT-PCR with CDPK-oligos using cDNA from Col-0 adult leaf. CDPKs which could not be amplified within 37 cycles are not shown.

the multiple knock down during CPK3 silencing, material from silenced CPK3 over-expressor plants needs to be generated; also plants suffering salt stress have to be harvested to analyze transcript levels in response to hyper-osmotic stress.

The experiment is still under progress, up to now all oligos were tested on cDNA from adult leaf tissue. Figure 3.5 and Table 3.6 show the obtained and expected sizes of RT-PCR products respectively. CDPKs that were not visibly amplified after 37 cycles were possibly not expressed in the material used and are not shown. Data on expression in adult leaf from the Arabidopsis eFP browser are added in Table 3.6. Apart from CPK19 and 34 all CDPK oligos generated a product of the correct size.

Table 3.6: Expected fragment sizes and expression in leaf

Gene	Id.nr.	Expected fragment size	Annotated expression in adult leaf	RT-PCR product
CPK1	At5g04870	454	+	+
CPK2	At3g10660	611	-	-
CPK3	At4g23650	650	+	+
CPK4	At4g09570	480	+	+
CPK5	At4g35310	479	+	+
CPK6	At2g17290	659	(+)	+
CPK7	At5g12480	850	+	+
CPK8	At5g19450	950	+	+
CPK9	At3g20410	680	+	++
CPK10	At1g18890	482	+	+
CPK11	At1g35670	507	-	-
CPK12	At5g23580	396	-	+
CPK13	At3g51850	461	+	++
CPK14	At2g41860	398	-	+
CPK15	At4g21940	503	+	+
CPK16	At2g17890	562	-	-
CPK17	At5g12180	722	-	+
CPK18	At4g36070	531	-	-
CPK19	At1g61950	523	(+)	+
CPK20	At2g38910	537	-	-
CPK21	At4g04720	502	+	+
CPK22	At4g04710	456	+	+
CPK23	At4g04740	506	+	+
CPK24	At2g31500	497	-	-
CPK25	At2g35890	549	-	-
CPK26	At4g38230	441	-	-
CPK27	At4g04700	362	no data available	+

CPK28	At5g66210	591	+	+
CPK29	At1g76040	518	(+)	+
CPK30	At1g74740	413	(+)	+
CPK31	At4g04695	366	no data available	+
CPK32	At3g57530	491	(+)	+
CPK33	At1g50700	688	+	+
CPK34	At5g19360	761	-	

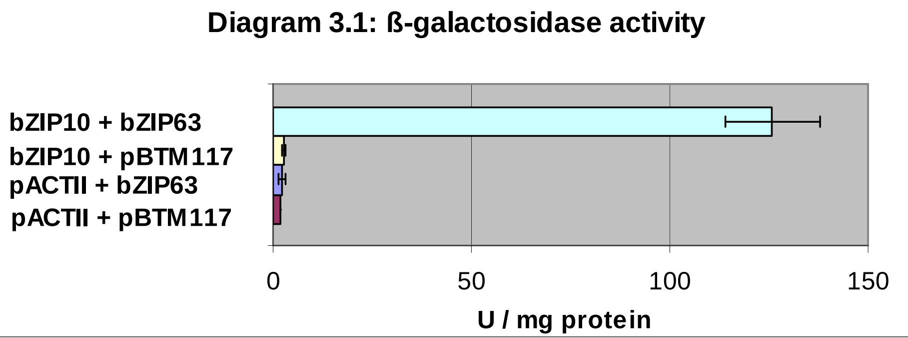
3.5. CPK3 targets: bZIP proteins

3.5.1. Yeast-2-hybrid interaction screen

In a previous yeast-2-hybrid screen bZIP63 had been identified as interaction partner of the constitutively active form CPK3ΔC. Now we wanted to test the specificity of this interaction by screening CPK3ΔC as well as CPK29ΔC against several bZIP proteins from different subgroups in a yeast-2-hybrid filter-lift-assay. For this, bZIPs were cloned into a modified Gal4 DNA-binding domain fusion vector pACTIIJ and the kinases fused to the LexA activator domain in pBTM117. The vector pACTIIJ containing bZIP10 obtained from Georgi Dermendjiev was tested using the published interaction between bZIP10 and bZIP63[51] as positive control. For each interaction, protein was isolated from 3 independent overnight cultures. Each sample of the positive control was measured twice. Table 3.7 and Diagram 3.1 show the total protein concentrations and specific ß-galactosidase activities of the samples. Obviously the positive control worked very well and the vector could be used for further experiments.

Table 3.7: Total protein concentration and ß-galactosidase activity

Sample	c [mg/ml]	U/mg protein
pACTII + pBTM117 (1)	2,46	1,93
pACTII + pBTM117 (2)	2,66	1,96
pACTII + pBTM117 (3)	2,80	1,99
pACTII + pBTM117		1,96
pACTII + pBTM117 bZIP63 (1)	2,96	1,43
pACTII + pBTM117 bZIP63 (2)	2,42	3,14
pACTII + pBTM117 bZIP63 (3)	2,60	2,71
pACTII + bZIP63		2,43
pACTIIJ bZIP10 + pBTM117 (1)	2,14	2,57
pACTIIJ bZIP10 + pBTM117 (2)	2,22	3,39
pACTIIJ bZIP10 + pBTM117 (3)	2,06	2,67
bZIP10 + pBTM117		2,88
pACTIIJ bZIP10 + pBTM117 bZIP63 (1)a	2,21	138,20
pACTIIJ bZIP10 + pBTM117 bZIP63 (1)b	2,21	114,57
pACTIIJ bZIP10 + pBTM117 bZIP63 (2)a	1,88	131,66
pACTIIJ bZIP10 + pBTM117 bZIP63 (2)b	1,88	113,82
pACTIIJ bZIP10 + pBTM117 bZIP63 (3)a	1,97	139,17
pACTIIJ bZIP10 + pBTM117 bZIP63 (3)b	1,97	117,19
bZIP10 + bZIP63		125,77



After the pACTIIJ vector had proofed to perform well, the bZIP screen was carried out. The chart below sums up which subgroup each bZIP belongs to. bZIP63ΔC is missing the last 24 amino acids including 3 potential phosphorylation sites.

C-group:	S1-group:	I-group:	H-group:
bZIP9	bZIP2	bZIP51	bZIP56
bZIP10	bZIP11		
bZIP25	bZIP53		
bZIP63 (ΔC)			

Figure 3.6 shows the results of the filter-lift-assays. The only interaction between kinase and bZIP visualizable in this Y-2-H assay was CPK3ΔC with the full-length bZIP63 which confirms previous results of our lab.

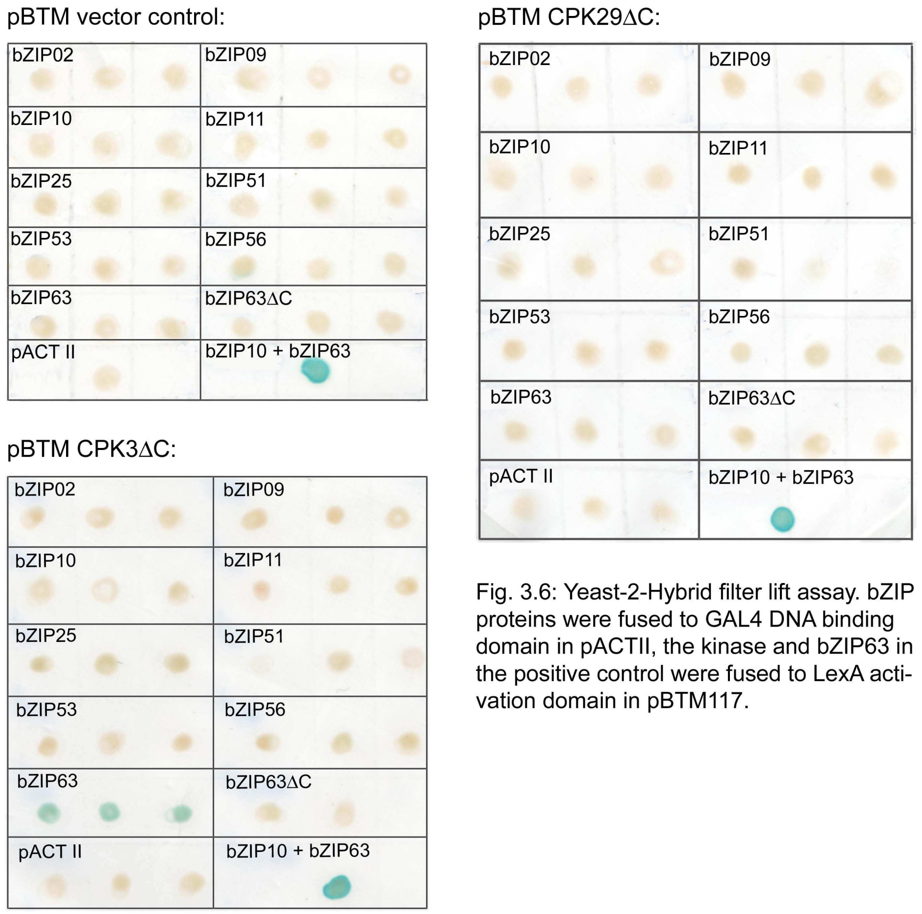


Fig. 3.6: Yeast-2-Hybrid filter lift assay. bZIP proteins were fused to GAL4 DNA binding domain in pACTII, the kinase and bZIP63 in the positive control were fused to LexA activation domain in pBTM117.

3.6. Subcellular localization of bZIP9 and 63 wt and mutants

The subcellular localization of bZIP9 and 63 was analyzed using *Agrobacterium* infiltration of tobacco leaves. bZIP9 and 63 wt as well as bZIP63 truncated mutants lacking regions with several putative phosphorylation sites were transiently expressed as YFP-fusions and analyzed under the fluorescence microscope (Figure 3.7). The GFP-filter shows YFP-fluorescence and chloroplast auto-fluorescence, the YFP-filter exclusively YFP-fluorescence. All bZIP proteins clearly localized to the nucleus. The truncations of bZIP63 had no influence on the subcellular targeting.

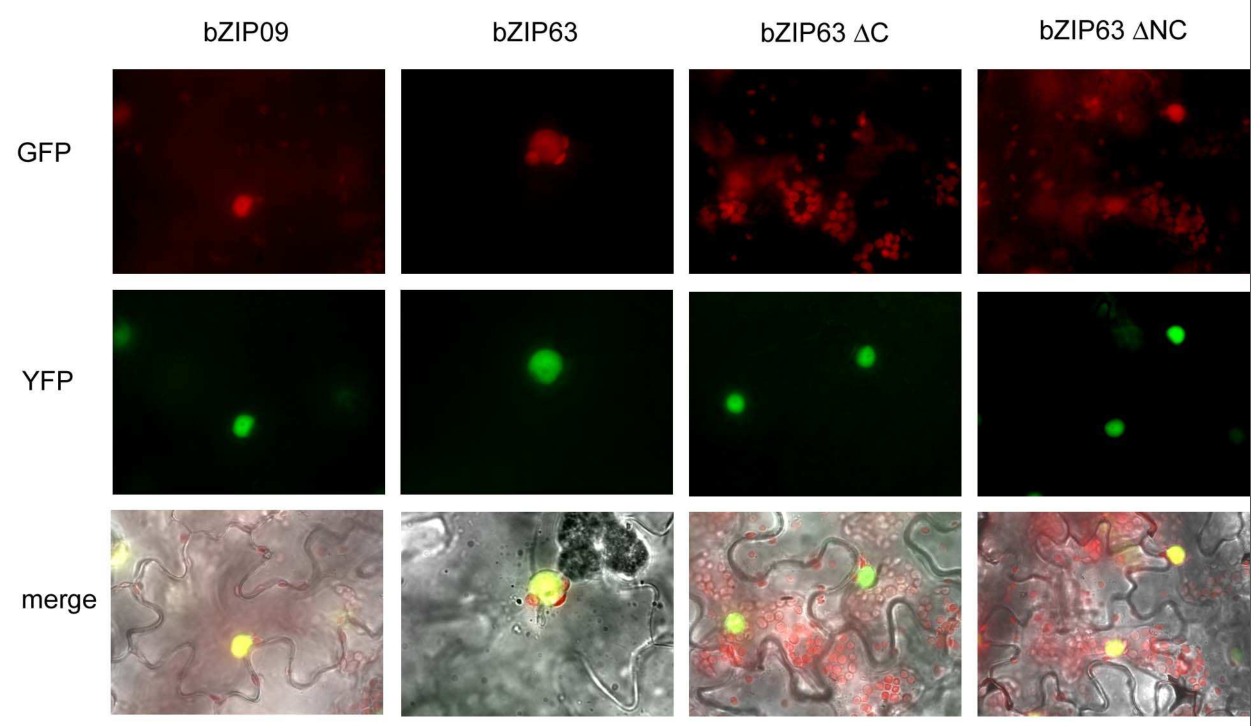


Fig. 3.7: Transient expression of wt and mutant bZIPs as YFP fusions in tobacco leaves (63x)

3.7. Phosphorylation of bZIP9 and bZIP63

3.7.1. CPK3 phosphorylation of bZIP9 and 63 wild type and mutant proteins

Within the C-group of bZIPs only bZIP 9 and 63 previously had been found to be phosphorylated by CPK3. In a radioactive kinase assay bZIP 9 and 63 wild type and mutant GST-fusion proteins were analyzed. Phosphorylation with or without calcium was compared to indicate the CPK3 dependence of phosphorylation. Proteins used in this assay had been purified and tested for autophosphorylation by Georgi Dermendjiev. Only bZIP9 S16A, which was newly generated by site directed mutagenesis, was tested for autophosphorylation here.

Figure 3.8 shows the phosphorylation of bZIP9 and 63 in the presence of CPK3 and the autophosphorylation control of bZIP9 S16A without CPK3 with and without calcium. Phosphorylation was clearly Ca^{2+} dependent in all samples. bZIP9 S16A showed no autophosphorylation, but a reduced signal compared to the wild type, as well as bZIP63ΔC.

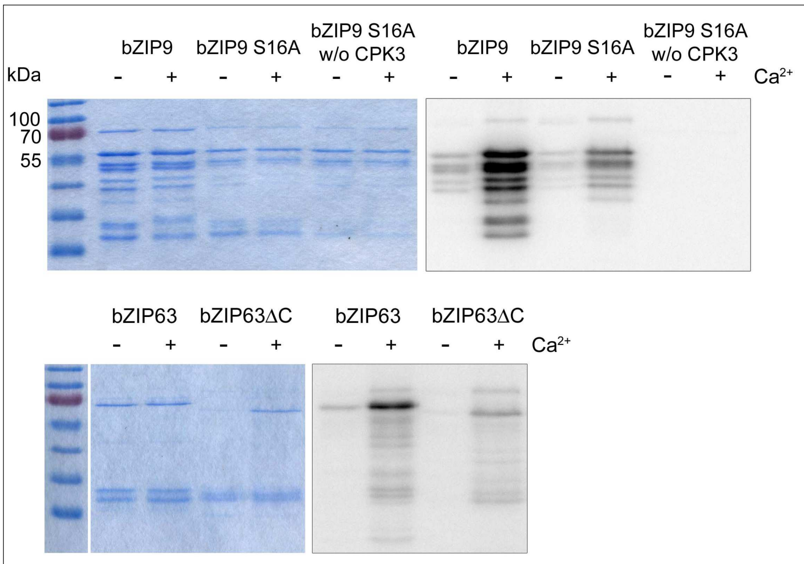


Fig. 3.8: ^{32}P-phosphorylation of bZIP9 and 63 wt and mutant GST-protein by CPK3 and bZIP9 S16A auto-phosphorylation with and without calcium.

3.7.2. Phosphorylation of untagged bZIPs provokes an upwards bandshift

bZIP9 and 63 were purified without tag from *E. coli* using the pTWIN intein system and treated with CPK3 and/or λ-phosphatase as indicated in Figure 3.9. "–" stands for the untreated control protein, "+ CPK3 + λ-phosphatase" means that an aliquot of the CPK3 treated sample was treated again with λ-phosphatase. There was no difference between control and λ-phosphatase treated proteins, but on incubation with CPK3 an additional band appeared above the normal level in both bZIPs which could be reversed through dephosphorylation.

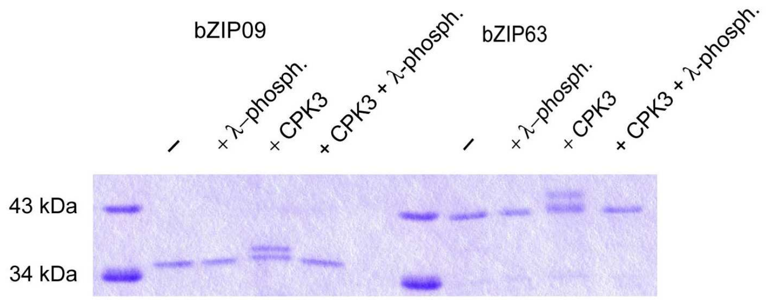


Fig. 3.9: Migration of untagged bZIP proteins on an SDS-acrylamid gel depending on their phosphorylation status.

3.7.3. Mass spectrometry analysis

bZIP9 and 63 were treated with λ-phosphatase or CPK3 as described above. Both upper and lower band from the CPK3 treated samples were cut out and submitted to MS (Figure 3.10). The samples were digested with trypsin. The phosphorylation data obtained from each digest are depicted in the sequences below. Red letters indicate covered regions, yellow marks indicate phosphorylated sites. Both bands of both proteins were found to be phosphorylated. In case of marked neighboured amino acids, such as T91, T92 and S93 of bZIP9, only one was phosphorylated but it was not known which of them.

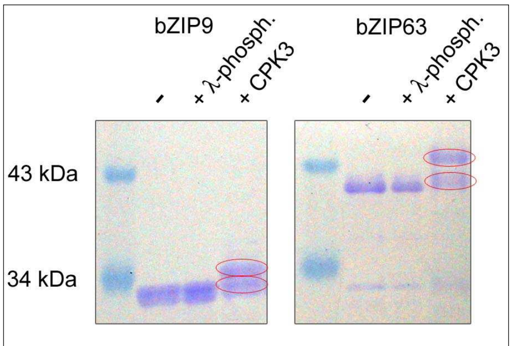


Fig. 3.10: Bands of CPK3 treated bZIP proteins cut out for MS analysis

- bZIP9

Lower band:

1-60 MDNHTAKDIG MKRSASELAL QEYLTTSPLD PCFDLMNRDY TCELRDSLLW SEGLFPAGPF
61-120 RDAQSSICEN LSADSPVSAN KPEVRGGVRR TTSGSSHVNS DDEDAETEAG QSEMTNDPND
121-180 LKRIRRMNSN RESAKRSRRR KQEYLVDLET QVDSLKGDNS TLYKQLIDAT QQFRSAGTNN
181-240 RVLKSDVETL RVKVKLAEDL VARGSLTSSL NQLLQTHLSP PSHSISSLHY TGNTSPAITV
241-277 HSDQSLFPGM TLSGQNSSPG LGNVSSEAVS CVSDIWP

T91, 92, S93	found 9x
S184	found 1x
T189	found 5x

Upper band:

1-60 MDNHTAKDIG MKRSASELAL QEYLTTSPLD PCFDLMNRDY TCELRDSLLW SEGLFPAGPF
61-120 RDAQSSICEN LSADSPVSAN KPEVRGGVRR TTSGSSHVNS DDEDAETEAG QSEMTNDPND
121-180 LKRIRRMNSN RESAKRSRRR KQEYLVDLET QVDSLKGDNS TLYKQLIDAT QQFRSAGTNN
181-240 RVLKSDVETL RVKVKLAEDL VARGSLTSSL NQLLQTHLSP PSHSISSLHY TGNTSPAITV
241-277 HSDQSLFPGM TLSGQNSSPG LGNVSSEAVS CVSDIWP

T91, 92, S93	found 3x
S184	found 2x
T189	found 8x

- bZIP63

Lower band:

1-60 MEKVFSDEEI SGNHHWSVNG MTSLNRSASE WAFNRFIQES SAAADDGEST TACGVSVSSP
61-120 PNVPVDSEEY RAFLKSKLNL ACAAVAMKRD TSGRSDNGGA NESEQASLAS SKATPMMSSA
121-180 ITSGSELSGD EEEADGETNM NPTNVKRVKR MLSNRESARR SRRRKQAHLS ELETQVSQLR
181-240 VENSKLMKGL TDVTQTFNDA SVENRVLKAN IETLRAKVKM AEETVKRLTG FNPMFHNMPQ
241-300 IVSTVSLPSE TSNSPDTTSS QVTTPEIISS GNKGKALIGC KMNRTASMRR VESLEHLQKR
301-307 IRSVGDQ

S29	found 2x
S177	found 3x
T196	found 2x
S293	found 20x

Upper band:

1-60	MEKVFSDEEI SGNHHWSVNG MTSLNRSASE WAFNRFIQES SAAADDGEST TACGVSVSSP
61-120	PNVPVDSEEY RAFLKSKLNL ACAAVAMKRD TSGRSDNGGA NESEQASLAS SKATPMMSSA
121-180	ITSGSELSGD EEEADGETNM NPTNVKRVKR MLSNRESARR SRRRKQAHLS ELETQVSQLR
181-240	VENSKLMKGL TDVTQTFNDA SVENRVLKAN IETLRAKVKM AEETVKRLTG FNPMFHNMPQ
241-300	IVSTVSLPSE TSNSPDTTSS QVTTPEIISS GNKGKALIGC KMNRTASMRR VESLEHLQKR
301-307	IRSVGDQ

S29	found 4x
S177	found 9x
T196	found 11x
S293	found 26x

To improve sequence coverage and to address the question if our bZIPs were already phosphorylated when purified from *E. coli*, bZIP9 and 63 untreated control and samples treated with CPK3 identically as in the former experiment prior to gel seperation were submitted to MS. bZIP9 was digested with GluC and subtilisin, bZIP63 with LysC and subtilisin. The phosphorylation data obtained from these digest were summarized together with the former trypsin digests in the sequences below. Red letters indicate covered regions, yellow marks indicate phosphorylated sites. Table 3.8 and 3.9 summarize how often amino acids were found phosphorylated and if they were also found unphosphorylated. In the untreated protein no phosphorylation was found.

- bZIP9 phosphorylated sites identified by MS

bZIP9 without CPK3:

1-60	MDNHTAKDIG MKRSASELAL QEYLTTSPLD PCFDLMNRDY TCELRDSLLW SEGLFPAGPF
61-120	RDAQSSICEN LSADSPVSAN KPEVRGGVRR TTSGSSHVNS DDEDAETEAG QSEMTNDPND
121-180	LKRIRRMNSN RESAKRSRRR KQEYLVDLET QVDSLKGDNS TLYKQLIDAT QQFRSAGTNN
181-240	RVLKSDVETL RVKVKLAEDL VARGSLTSSL NQLLQTHLSP PSHSISSLHY TGNTSPAITV
241-277	HSDQSLFPGM TLSGQNSSPG LGNVSSEAVS CVSDIWP

bZIP9 with CPK3:

1-60	MDNHTAKDIG MKRSASELAL QEYLTTSPLD PCFDLMNRDY TCELRDSLLW SEGLFPAGPF
61-120	RDAQSSICEN LSADSPVSAN KPEVRGGVRR TTSGSSHVNS DDEDAETEAG QSEMTNDPND
121-180	LKRIRRMNSN RESAKRSRRR KQEYLVDLET QVDSLKGDNS TLYKQLIDAT QQFRSAGTNN
181-240	RVLKSDVETL RVKVKLAEDL VARGSLTSSL NQLLQTHLSP PSHSISSLHY TGNTSPAITV
241-277	HSDQSLFPGM TLSGQNSSPG LGNVSSEAVS CVSDIWP

Table 3.8: Amino acids found phosphorylated in bZIP9

Aminoacid	found phosphorylated	found unphosphorylated
S47	3x (1 digest)	-
S65	8x (2 digests)	2 digests
T91, 92, S93	12x (2 digests)	-
S154	2x (1 digest)	-
S184	3x (2 digest)	-
T189	13x (2 digests)	2 digests
S226	30x (1 digest)	-

- bZIP63 phosphorylated sites identified by MS:

bZIP63 without CPK3:

```
1-60      MEKVFSDEEI SGNHHWSVNG MTSLNRSASE WAFNRFIQES SAAADDGEST TACGVSVSSP
61-120    PNVPVDSEEY RAFLKSKLNL ACAAVAMKRD TSGRSDNGGA NESEQASLAS SKATPMMSSA
121-180   ITSGSELSGD EEEADGETNM NPTNVKRVKR MLSNRESARR SRRRKQAHLS ELETQVSQLR
181-240   VENSKLMKGL TDVTQTFNDA SVENRVLKAN IETLRAKVKM AEETVKRLTG FNPMFHNMPQ
241-300   IVSTVSLPSE TSNSPDTTSS QVTTPEIISS GNKGKALIGC KMNRTASMRR VESLEHLQKR
301-307   IRSVGDQ
```

bZIP63 with CPK3:

```
1-60      MEKVFSDEEI SGNHHWSVNG MTSLNRSASE WAFNRFIQES SAAADDGEST TACGVSVSSP
61-120    PNVPVDSEEY RAFLKSKLNL ACAAVAMKRD TSGRSDNGGA NESEQASLAS SKATPMMSSA
121-180   ITSGSELSGD EEEADGETNM NPTNVKRVKR MLSNRESARR SRRRKQAHLS ELETQVSQLR
181-240   VENSKLMKGL TDVTQTFNDA SVENRVLKAN IETLRAKVKM AEETVKRLTG FNPMFHNMPQ
241-300   IVSTVSLPSE TSNSPDTTSS QVTTPEIISS GNKGKALIGC KMNRTASMRR VESLEHLQKR
301-307   IRSVGD
```

Table 3.9: Amino acids found phosphorylated in bZIP63

Aminoacid	found phosphorylated	also found unphosphorylated
S29	6x (2 digests)	1 digest
S177	12x (2 digests)	2 digests
T191	2x (1 digest)	2 digests
T196	13x (2 digests)	1 digest
T213	1x (1 digest)	2 digests
S246	4x (1 digest)	-
S293	57x (3 digests)	-

3.8. bZIP mutant lines

Knock out lines of bZIP9 and 63 and one untagged over-expressor line of bZIP63 were confirmed on RNA level by semi-quantitative RT-PCR (Figure 3.11). The obtained fragments match the expected sizes, which are 413bp for Act3, 772bp for bZIP9 and 919, 940 or 1007bp for the three annotated splicing forms of bZIP63.

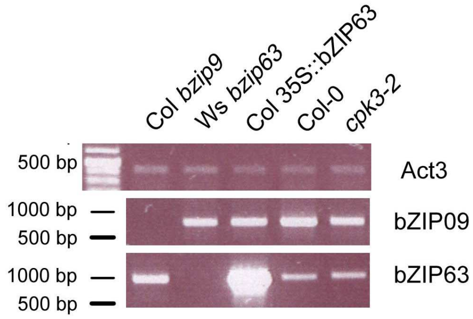


Fig. 3.11: mRNA levels of bZIP9 and 63 in wild type, knock out and overexpressor lines analyzed by RT-PCR. *cpk3-2* served as additional control.

3.9. bZIP antibodies

Antibodies were created against an N-terminal peptide of bZIP9 and a C-terminal peptide of bZIP63 (see Material and Methods). The antibodies were tested first on their ability to recognize both proteins in unphosphorylated and phosphorylated state. Therefore samples of bZIP proteins treated with λ-phosphatase or CPK3 were diluted 1:30 for the western blot. Additionally, a phospho-PKD antibody was tested on its specificity for the phosphorylated bZIPs (Figure 3.12). Both bZIP antibodies recognized the modified proteins as well, the phospho-PKD antibody only showed a signal for both bands of the CPK3 treated samples, indicating once more that there is also phosphorylation in the lower band.

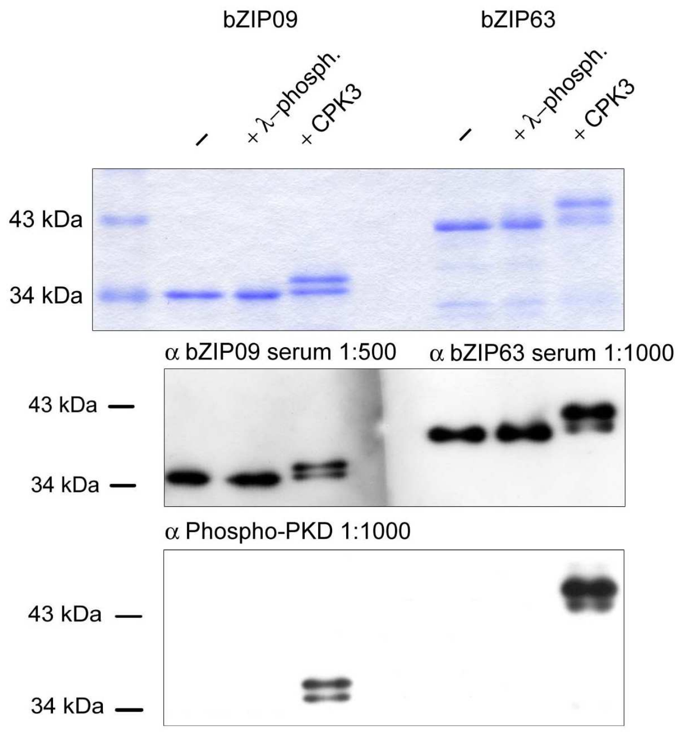


Fig. 3.12: bZIP 9 and 63 serum and phospho-PKD antibodies tested on recombinant protein

Then serum, pre-immune serum and affinity purified antibodies were tested on specificity in total leaf protein extracts (Figure 3.13). However, both antibodies recognized unspecific bands that did not appear in the pre-immune serum, which indicates that the peptide antigen might not have been sufficiently specific. The antibodies had to be reordered with different peptides.

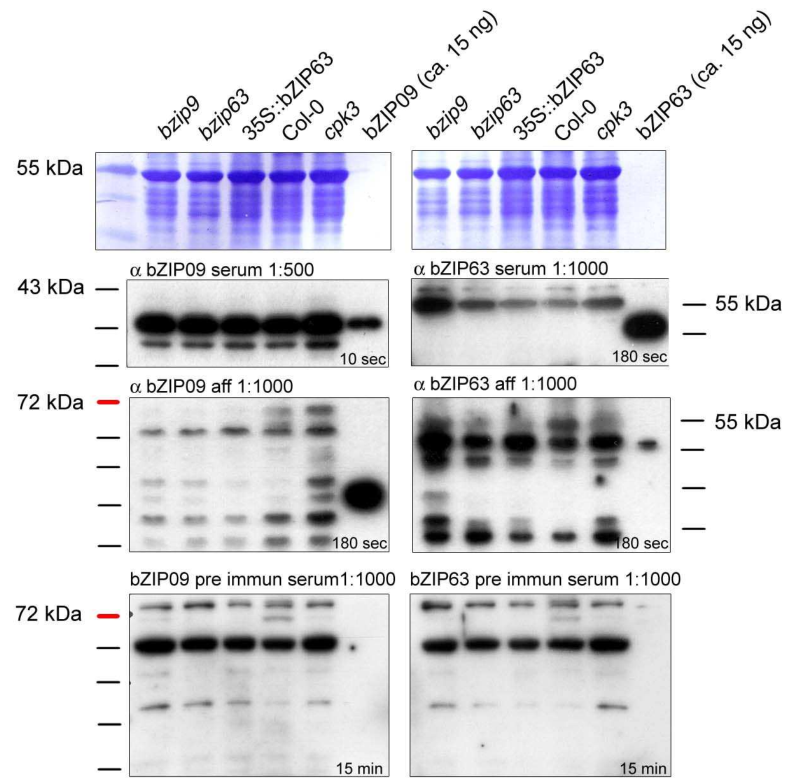


Fig. 3.13: bZIP 9 and 63 serum, pre-immun serum and affinity purified antibodies tested on bZIP mutant line leaf extreacts

3.10. bZIP mutant phenotype

bZIP mutant and over-expressor plants were grown on ½ Hoagland medium under short day conditions. After 4 weeks, an early flowering phenotype was observed in Ws *bzip63*. All other plants grown under the same conditions started flowering after 9-10 weeks. Figure 3.14 shows bZIP plants and Col-0 at the age of 6 weeks. Ws was not grown at the same time, however we had observed previously that Ws plants flowered even later than Col-0 plants when grown in hydroponic culture. Nevertheless, the experiment has to be reproduced with both Ws and Col-0 as wild type controls.

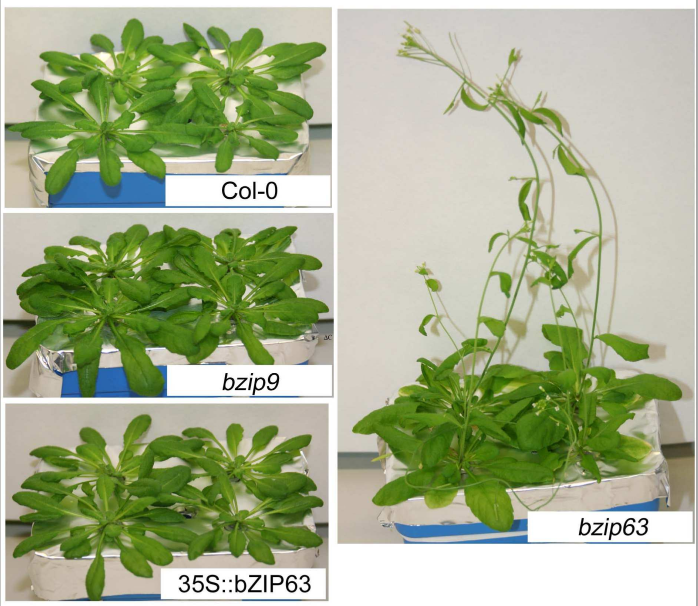


Fig 3.14: 6 weeks old plants grown in 1/2 Hoagland medium under short day conditions (8h light)

4. Discussion

4.1. CPK3 as transcriptional or post-transcriptional regulator

Under the applied experimental condition, CPK3 had no influence on the transcriptional activation of the analyzed salt-stress induced genes (Fig. 3.2). CPK3 is expressed at quite high levels in the wild type which could explain why it is not transcriptionally upregulated itself under salt stress even though it has a function in salt stress response. This is evident from the salt sensitive phenotype of *cpk3* mutant plants (Mehlmer and Teige, unpublished). On the other hand, the importance of SOS1 for salt tolerance together with its relatively unvariable transcript levels show the power of post-translational regulation[31]. Also expression of the other analyzed ion channels was not or only slightly changed. Post-transcriptional modification provides a fast and direct way of activation and there is evidence that these mechanisms, such as phosphorylation, are a general mechanism of regulation for ion transporters. The activation of SOS1 through a CBL/CIPK pathway is known since several years[31], which is true as well for the K^+ transporter AKT1[32]. But also KCO1, which is directly Ca^{2+}-activated via EF-hand motives, seems to be additionally activated by phosphorylation and subsequent interaction with 14-3-3 proteins[34]. NHX1, the major vacuolar Na^+/H^+ antiporter in Arabidopsis, seems not to be phosphorylated, but to be modulated by interaction with a CaM-like protein inside the vacuole in response to Ca^{2+} and pH[52], which is another example of the variety and abundance of post-expressional regulation.

We propose that CPK3 acts primarily as post-transcriptional regulator of salt stress response. Direct targets still remain to be identified; some of the principal candidates are ion transporters, but also metabolic enzymes for biosynthesis of compatible solutes or signalling molecules are likely modulated by post-translational modification. CPK3 might have additional functions that require transcriptional activation of target genes via activation of transcription factors (such as bZIPs). Most CDPKs are expressed in different cell types throughout the plant and are believed to

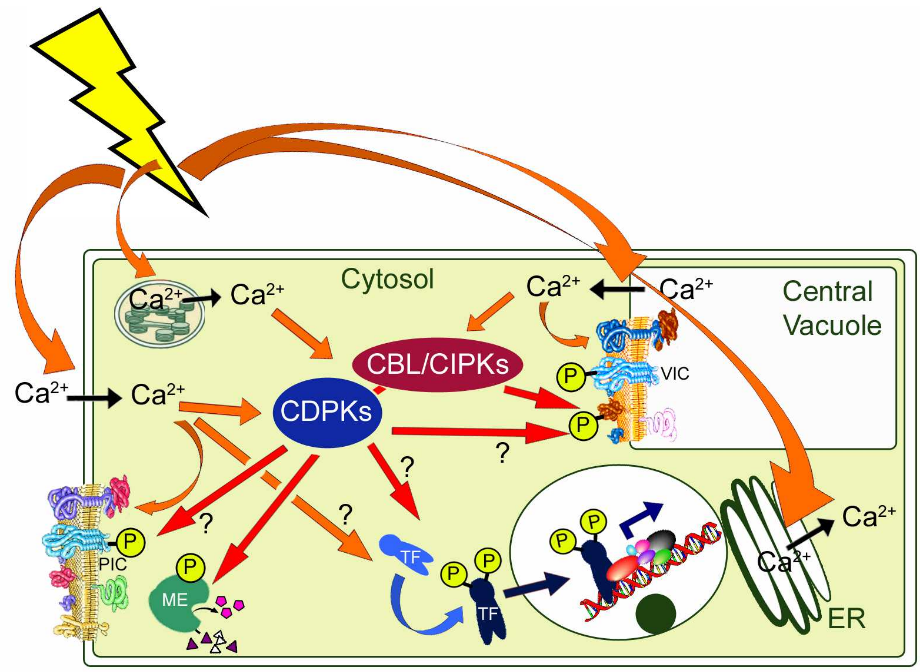


Fig. 4.1: Oversimplified model of calcium and especially hypothesized CDPK signalling pathways in response to an external stimulus. ME metabolic enzyme, PIC plasmamembrane ion channel, VIC vacuolar ion channel, TF transcription factor.

act in different signalling pathways in different tissues and under different conditions[35]. It is possible that CPK3 has mainly post-transcriptional function in salt stress response in roots and regulates e.g. flowering time through bZIP mediated transcriptional activation of target genes in the shoot. Figure 4.1 gives an overview on the possible action of CDPKs within the Ca^{2+}-signalling network in response to environmental signals.

4.2. CPK3 as regulator of K^+ homeostasis

The chlorotic deficiency phenotype previously observed in *cpk3* plants grown in hydroponic culture could not be reproduced. This can be due to slightly different growth conditions, but also because of repeated pathogen contaminations in the growth chamber. It was also observed that the used Col-0 wild type plants were of variable health and viability, which makes standardized phenotypic comparisons impossible. Nevertheless, K^+ homeostasis is one of the key requisites for high Na^+ tolerance[36] and should be further investigated.

Another interesting observation was the dwarf phenotype of 35S::CPK3 in hydroponic culture. It is likely that due to the high grade of homology among the CDPKs, other CDPKs will be co-knocked down in CPK3 silencing, leading to multiple silencing and an amplified phenotype. Interestingly 35S::CPK3 plants grow normally on earth, where many nutrients, such as potassium, are less limited than in hydroponic medium.

4.3. CDPK redundancy

In a primary experiment, all CDPK oligos were tested on specificity with Col-0 cDNA as template. Apart from CPK19 and 34 all fragments had the expected size. It is likely that with optimized PCR conditions these two oligos will still gain specific products, which will be checked by sequencing. CDPK expression did not always correlate with eFP browser data (Table 3.6). All CDPKs that could not be amplified are annotated as not expressed in leaf in the eFP browser, however various other genes (CPK12, 14 and 17) annotated as not expressed were amplified. Very weak expression is annotated for CPK6, 19, 29, 30 and 32, no data are available for CPK27 and 31. All of them were found to be transcribed at average levels. CPK9 and 13 transcripts were especially well amplified and showed products after 26 PCR cycles already whereas other CDPKs took at least 30 cycles. This indicates higher expression levels of these two CDPKs in the source material (adult leaf). However, cycle numbers are a very inaccurate means of quantification as amplification also depends on binding properties of each oligo.

Knowledge about CDPK transcript levels in various genotypes and tissues is certainly important for future investigations, although there is no guarantee that functional redundancies will be detected with this method. CDPKs can easily take over the function of a homologue by post-transcriptional activation, different sub-cellular targeting or simply by sufficient abundance at basal transcription levels which makes additional activation unnecessary. However it is out of temporal and financial possibilities to create specific antibodies against all 33 remaining CDPKs to investigate e.g. *in vivo* kinase activities in *cpk3* plants. Still transcript analysis can give a hint on dosage compensation and on the tissue-specific CDPK transcriptome and should not be left undone.

4.4. Interaction of CPKs with bZIP proteins in Yeast-2-Hybrid

In the Yeast-2-Hybrid analysis, only bZIP63 interacted with CPK3ΔC sufficiently to induce ß-galactosidase expression. None of the other analyzed bZIPs showed detectable interaction with CPK3ΔC or CPK29ΔC, not even bZIP9 or bZIP63ΔC even though they were phosphorylated by CPK3 in a Ca^{2+}-dependent manner *in vitro*. This phosphorylation is unlikely a mere unspecific reaction, as the other C-group bZIPs, 10 and 25, are no targets of CPK3 (Kolowrat and Teige, unpublished). However, phosphorylation is a very fast process and it is questionable if proteins will interact for sufficient time to induce ß-gal expression. It might be that the Yeast-2-Hybrid system is not the optimal method to study protein kinase interactions.

4.5. Subcellular localization of bZIP9 and 63

bZIP9 and 63, as well as the truncated mutants bZIP63ΔC and bZIP63ΔNC localized completely to the nucleus. bZIP proteins contain a conserved NLS in the central basic domain, so the nuclear localization would be expected even for the truncated proteins. It was possible that post-translational modification on the putative phosphorylation sites in the N- or C-terminus had an influence on subcellular targeting but this was clearly not the case.

4.6. Phosphorylation of bZIP9 and 63 by CPK3

In MS analysis, 7 sites were found phosphorylated in each bZIP9 and 63 after incubation with CPK3. It is eye-catching that most of these sites do not fit into any of the known consensi for CDPK target sequences. In the bZIP sequences below, consensus and identified sites are summarized. Red letters indicate covered regions, yellow marks: identified phosphorylation sites, frames: sites compatible with known consensus.

bZIP9 with CPK3:

1-60	MDNHTAKDIG MKRSASELAL QEYLTTSPLD PCFDLMNRDY TCELRDSLLW SEGLFPAGPF
61-120	RDAQSSICEN LSADSPVSAN KPEVRGGVRR TTSGSSHVNS DDEDAETEAG QSEMTNDPND
121-180	LKRIRRMNSN RESAKRSRRR KQEYLVDLET QVDSLKGDNS TLYKQLIDAT QQFRSAGTNN
181-240	RVLKSDVETL RVKVKLAEDL VARGSLTSSL NQLLQTHLSP PSHSISSLHY TGNTSPAITV
241-277	HSDQSLFPGM TLSGQNSSPG LGNVSSEAVS CVSDIWP

bZIP63 with CPK3:

1-60	MEKVFSDEEI SGNHHWSVNG MTSLNRSASE WAFNRFIQES SAAADDGEST TACGVSVSSP
61-120	PNVPVDSEEY RAFLKSKLNL ACAAVAMKRD TSGRSDNGGA NESEQASLAS SKATPMMSSA
121-180	ITSGSELSGD EEEADGETNM NPTNVKRVKR MLSNRESARR SRRRKQAHLS ELETQVSQLR
181-240	VENSKLMKGL TDVTQTFNDA SVENRVLKAN IETLRAKVKM AEETVKRLTG FNPMFHNMPQ
241-300	IVSTVSLPSE TSNSPDTTSS QVTTPEIISS GNKGKALIGC KMNRTASMRR VESLEHLQKR
301-307	IRSVGDQ

ST Site identified as phosphorylated in MS

$\boxed{\text{ST}}$ Potential phosphorylation site according to consensus sequences

Surprisingly, Ser 16 in bZIP9 was not found phosphorylated in MS. Ser 16 was a very hopeful candidate as it is conserved among the C-group bZIPs and flanked by a Motif 1 minimal consensus. Furthermore the phosphorylation signal was reduced in the S16A mutant compared to the wild type (Fig. 3.8), however it is possible that the difference in loaded protein amounts was underestimated from the coomassie stain. The only phosphorylated sites in bZIP9 that combined with any of the consensus sequences are the Thr 92/Ser 93 and Ser 226. Both sites were 12x and 30x respectively in two independent digests and never appeared unphosphorylated. Another considerable candidate is Thr 189 which does not fit into an available consensus but was found 13x in 2 digest, however also unphosphorylated.

In bZIP63, two already expected sites sharing a Motif 1 minimal consensus, Ser 29 and Ser 293, were phosphorylated. Ser 29 is the homolog of Ser 16 in bZIP9, Ser 293 is not present in the ΔC mutant which showed reduced phosphorylation. P-Ser 293 was found in all three digests with a total number of 57x. Unfortunately, Ser 187 and Ser 303 which also are part of the C-terminus and are surrounded by Motif 1 and Simple 1 consensus sequences respectively were not covered. Another identified site combining with a consensus is T191. However it was also found unphosphorylated in two independent digests indicating that only part of the protein was modified at this site. Candidates for further investigation are Ser 177 and Thr 196 which were found 12 and 13x respectively in two digests. Both do not share any of the consensus sequences but it would be interesting if phosphorylation is abolished in a quadruple mutant of S29, S177, T196 and S293 to alanin.

Another interesting observation was that even the lower band in the shifted bZIPs was phosphorylated at the same sites as the upper band. It is possible that phosphorylation at additional sites, which did not occur to completion, is responsible for the shift. Samples were incubated with kinase for 2h, so one would expected that all protein should be phosphorylated. Considering the complexity of multisite phosphorylation it might be that phosphorylation of certain residues influences phosphorylation of others which generates two populations of differentially modified proteins.

All data on CPK3 phosphorylation of bZIPs was generated *in vitro* with recombinant proteins purified from *E. coli*. Under the applied conditions CPK3 phosphorylates both bZIPs at multiple sites which even leaded to a shift in electrophoretic migration. The most urgent question is now if and under which conditions these modifications occur *in vivo*. One possibility to address this question is through immuno-precipitation and MS analysis of the target proteins from wild type and CPK3 mutant plants. However the ordered antibodies proved to be unspecific and could not be used for this purpose. Another difficulty in working with bZIPs is that they are extremely unstable and vanish from protein stocks after some time of storage. Huge amounts of protein were needed to achieve a decent coverage in MS. As it is likely that they are degraded in a protein extract as well, the chances are low to be able to purify them e.g. as a 2D-gel spot without antibody enrichment.

4.7. bZIP63 flowering phenotype

Similar to the CDPKs, also in the bZIP protein family mutant phenotypes turned out to be rare (Dröge-Laser et al., unpublished). A previously unnoticed early-flowering phenotype was observed in the line Ws *bzip63* when grown in hydroponic culture under short-day conditions. bZIP63 is known to be transcriptionally up-regulated during dark periods and it is possible that it has a function in measuring of night-length. Recently bZIP63 was brought into connection with energy signalling and starvation which, for a phototrophic organism, correlates with the dark period[53]. An early flowering phenotype of *bzip63* plants would make sense concerning these possible functions; plants could simply be unaware of the light conditions and continue their long-day developmental program. An early-flowering phenotype, however, was not observed in other labs when Ws *bzip63* was grown in short-day on earth. It is possible that hydroponic culture has an influence on the flowering phenotype in Wassilewskija, as in a previous experiment Ws-0 plants did not flower at all in hydroponic culture, neither in long- nor in short-day. Nevertheless the experiment needs to be reproduced with a direct control of Wassilewskija wild-type plants.

5. Outlook

5.1. Regulation of ion transporters by CPK3

Since no significant transcriptional activation of known Na^+ or K^+ channels could be detected in response to salt stress, phosphorylation studies of membrane proteins should be further persued. Cell-type specific analysis of root cells, were osmotic stress is most strongly perceived, is another possibility of more focused investigation in contrast to the whole plant systems, where phenotypes are often overlooked due to compensation through redundant signalling pathways[35].

5.2. CDPK redundancy

CDPK oligos were tested on amplification of a PCR product of the correct size. To additionally assure specificity, products will be gel eluated and sequenced. RNA will be isolated from the different samples (Col-0 and *cpk3-2*: seedling; leaf, root, flower and green silique from adult plant) and CDPK transcript levels analyzed. RNA isolation from flowers and green siliques still needs to be optimized as reverse transcription failed in previous experiments probably due to contaminations. Also samples from salt stressed Col-0 and *cpk3-2* seedlings will be generated and compared to unstressed seedlings. Various lines of silenced over-expressor plants will be examined on knock down of additional CDPKs and data compared with sequence homology of the affected genes. The aim is to generate an overview on the CDPK transcriptome in different genotypes and tissues during different developmental stages and under different conditions. It is possible that CDPKs functionally redundant to CPK3 show compensatory induction in cpk3 plants under certain conditions or in certain tissues, but lack of transcriptional induction does not indicate lack of redundancy.

5.3. bZIP peptide antibodies

Specific antibodies are essential for analysis of *in vivo* phosphorylation of the bZIP proteins. For new antibodies peptides were selected with high specificity according to Blast analysis. For bZIP9 only one specific peptide was found in the extreme C-terminus, for bZIP63 two peptides, N- and C-terminal, were selected.

bZIP9: LGNVSSEAVS CVSDIWP (C-terminal)

bZIP63: EKVFSDEEIS GNHHWSVNGM (N-terminal), EIISSGNKGK ALIGCKMNRT (C-terminal)

The bZIP sequences were analyzed for antigenicity with the Immunax program which predicts highly antigenic regions in a given protein. The peptide selected for bZIP9 showed the highest antigenicity for the whole protein, however the bZIP63 peptides had no predicted antigenicity at all. Nevertheless the peptides were ordered as there were no other specific sequences in the protein. The antibodies will be tested on sensitivity and specificity as before. In case they are not satisfactory other solutions have to be found. Sometimes antibodies against the entire protein are more specific than peptide antibodies. A last possibility could be the expression of epitope tagged bZIP proteins in knock out backgrounds.

5.4. bZIP *in vivo* phosphorylation

To confirm bZIP phosphorylation data *in vivo*, analysis of bZIP proteins purified from plants is absolutely essential. If the new peptide antibodies are sufficiently specific bZIPs can be immuno-precipitated from wild type and CPK3 knock out plants harvested under different conditions to examine their phosphorylation status through MS. Another possibility is to transform Col-0 and *cpk3* protoplasts with a construct encoding the His-, HA- or GFP-tagged bZIP under control of the CaMV 35S promoter and purify the heterologously over-expressed protein with an antibody against the tag. In the long-term run, stable *Arabidopsis* lines expressing tagged bZIP proteins at endogenous levels would be of great help.

6. References

1. Steinger, T., Roy, B.A. & Stanton, M.L. Evolution in stressful environments II: adaptive value and costs of plasticity in response to low light in Sinapis arvensis. *J Evol Biol* **16**, 313-23 (2003).
2. Agrawal, A.A., Conner, J.K., Johnson, M.T. & Wallsgrove, R. Ecological genetics of an induced plant defense against herbivores: additive genetic variance and costs of phenotypic plasticity. *Evolution Int J Org Evolution* **56**, 2206-13 (2002).
3. Shao, H.B. et al. Understanding molecular mechanism of higher plant plasticity under abiotic stress. *Colloids Surf B Biointerfaces* **54**, 37-45 (2007).
4. McKnight, T.D., Riha, K. & Shippen, D.E. Telomeres, telomerase, and stability of the plant genome. *Plant Mol Biol* **48**, 331-7 (2002).
5. Obbard, D.J., Harris, S.A., Buggs, R.J. & Pannell, J.R. Hybridization, polyploidy, and the evolution of sexual systems in Mercurialis (Euphorbiaceae). *Evolution Int J Org Evolution* **60**, 1801-15 (2006).
6. Henry, I.M. et al. Aneuploidy and genetic variation in the Arabidopsis thaliana triploid response. *Genetics* **170**, 1979-88 (2005).
7. Huber, D.P., Ralph, S. & Bohlmann, J. Genomic hardwiring and phenotypic plasticity of terpenoid-based defenses in conifers. *J Chem Ecol* **30**, 2399-418 (2004).
8. Gaut, B.S., Wright, S.I., Rizzon, C., Dvorak, J. & Anderson, L.K. Recombination: an underappreciated factor in the evolution of plant genomes. *Nat Rev Genet* **8**, 77-84 (2007).
9. Mathieu, O. & Bender, J. RNA-directed DNA methylation. *J Cell Sci* **117**, 4881-8 (2004).
10. Matzke, M.A., Mette, M.F. & Matzke, A.J. Transgene silencing by the host genome defense: implications for the evolution of epigenetic control mechanisms in plants and vertebrates. *Plant Mol Biol* **43**, 401-15 (2000).
11. Matzke, M.A., Aufsatz, W., Kanno, T., Mette, M.F. & Matzke, A.J. Homology-dependent gene silencing and host defense in plants. *Adv Genet* **46**, 235-75 (2002).
12. Piegu, B. et al. Doubling genome size without polyploidization: dynamics of retrotransposition-driven genomic expansions in Oryza australiensis, a wild relative of rice. *Genome Res* **16**, 1262-9 (2006).
13. Blokhina, O., Virolainen, E. & Fagerstedt, K.V. Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Ann Bot (Lond)* **91 Spec No**, 179-94 (2003).
14. Batistic, O. & Kudla, J. Integration and channeling of calcium signaling through the CBL calcium sensor/CIPK protein kinase network. *Planta* **219**, 915-24 (2004).
15. Sanders, D., Brownlee, C. & Harper, J.F. Communicating with calcium. *Plant Cell* **11**, 691-706 (1999).
16. Sanders, D., Pelloux, J., Brownlee, C. & Harper, J.F. Calcium at the crossroads of signaling. *Plant Cell* **14 Suppl**, S401-17 (2002).
17. Allen, G.J. & Schroeder, J.I. Combining genetics and cell biology to crack the code of plant cell calcium signaling. *Sci STKE* **2001**, RE13 (2001).
18. Thomas, A.P., Bird, G.S., Hajnoczky, G., Robb-Gaspers, L.D. & Putney, J.W., Jr. Spatial and temporal aspects of cellular calcium signaling. *Faseb J* **10**, 1505-17 (1996).
19. Harper, J.F., Breton, G. & Harmon, A. Decoding Ca(2+) signals through plant protein kinases. *Annu Rev Plant Biol* **55**, 263-88 (2004).
20. Holmberg, C.I., Tran, S.E., Eriksson, J.E. & Sistonen, L. Multisite phosphorylation provides sophisticated regulation of transcription factors. *Trends Biochem Sci* **27**, 619-27 (2002).
21. Hrabak, E.M. et al. The Arabidopsis CDPK-SnRK superfamily of protein kinases. *Plant Physiol* **132**, 666-80 (2003).

22. Kolukisaoglu, U., Weinl, S., Blazevic, D., Batistic, O. & Kudla, J. Calcium sensors and their interacting protein kinases: genomics of the Arabidopsis and rice CBL-CIPK signaling networks. *Plant Physiol* **134**, 43-58 (2004).
23. Cheng, S.H., Willmann, M.R., Chen, H.C. & Sheen, J. Calcium signaling through protein kinases. The Arabidopsis calcium-dependent protein kinase gene family. *Plant Physiol* **129**, 469-85 (2002).
24. Harmon, A.C., Gribskov, M. & Harper, J.F. CDPKs - a kinase for every Ca2+ signal? *Trends Plant Sci* **5**, 154-9 (2000).
25. McCormack, E., Tsai, Y.C. & Braam, J. Handling calcium signaling: Arabidopsis CaMs and CMLs. *Trends Plant Sci* **10**, 383-9 (2005).
26. Luan, S., Kudla, J., Rodriguez-Concepcion, M., Yalovsky, S. & Gruissem, W. Calmodulins and calcineurin B-like proteins: calcium sensors for specific signal response coupling in plants. *Plant Cell* **14 Suppl**, S389-400 (2002).
27. Klimecka, M. & Muszynska, G. Structure and functions of plant calcium-dependent protein kinases. *Acta Biochim Pol* **54**, 219-33 (2007).
28. Hegeman, A.D. et al. A phyloproteomic characterization of in vitro autophosphorylation in calcium-dependent protein kinases. *Proteomics* **6**, 3649-64 (2006).
29. McMichael, R.W., Jr., Bachmann, M. & Huber, S.C. Spinach Leaf Sucrose-Phosphate Synthase and Nitrate Reductase Are Phosphorylated/Inactivated by Multiple Protein Kinases in Vitro. *Plant Physiol* **108**, 1077-1082 (1995).
30. Douglas, P., Moorhead, G., Hong, Y., Morrice, N. & MacKintosh, C. Purification of a nitrate reductase kinase from Spinacea oleracea leaves, and its identification as a calmodulin-domain protein kinase. *Planta* **206**, 435-42 (1998).
31. Qiu, Q.S., Guo, Y., Dietrich, M.A., Schumaker, K.S. & Zhu, J.K. Regulation of SOS1, a plasma membrane Na+/H+ exchanger in Arabidopsis thaliana, by SOS2 and SOS3. *Proc Natl Acad Sci U S A* **99**, 8436-41 (2002).
32. Xu, J. et al. A protein kinase, interacting with two calcineurin B-like proteins, regulates K+ transporter AKT1 in Arabidopsis. *Cell* **125**, 1347-60 (2006).
33. Czempinski, K., Zimmermann, S., Ehrhardt, T. & Muller-Rober, B. New structure and function in plant K+ channels: KCO1, an outward rectifier with a steep Ca2+ dependency. *Embo J* **16**, 2565-75 (1997).
34. Latz, A. et al. TPK1, a Ca(2+)-regulated Arabidopsis vacuole two-pore K(+) channel is activated by 14-3-3 proteins. *Plant J* (2007).
35. Mori, I.C. et al. CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S-type anion- and Ca(2+)-permeable channels and stomatal closure. *PLoS Biol* **4**, e327 (2006).
36. Ma, S.Y. & Wu, W.H. AtCPK23 functions in Arabidopsis responses to drought and salt stresses. *Plant Mol Biol* (2007).
37. Roberts DM, H.A. Calcium-modulated protein-targets of intracellular calcium signals in higher plants. *Annu Rev Plant Physiol Mol Biol* **43**, 375–414 (1992).
38. Neumann GM, T.I., Polya GM. Identification of the site on potato carboxy peptidase inhibitor that is phosphorylated by plant calcium-dependent protein kinase. *Plant Science* **114**, 45–51 (1996).
39. Huang, J.Z. & Huber, S.C. Phosphorylation of synthetic peptides by a CDPK and plant SNF1-related protein kinase. Influence of proline and basic amino acid residues at selected positions. *Plant Cell Physiol* **42**, 1079-87 (2001).
40. Huang, J.Z., Hardin, S.C. & Huber, S.C. Identification of a novel phosphorylation motif for CDPKs: phosphorylation of synthetic peptides lacking basic residues at P-3/P-4. *Arch Biochem Biophys* **393**, 61-6 (2001).
41. Hernandez Sebastia, C., Hardin, S.C., Clouse, S.D., Kieber, J.J. & Huber, S.C. Identification of a new motif for CDPK phosphorylation in vitro that suggests ACC synthase may be a CDPK substrate. *Arch Biochem Biophys* **428**, 81-91 (2004).

42. Loog, M. et al. Peptide phosphorylation by calcium-dependent protein kinase from maize seedlings. *Eur J Biochem* **267**, 337-43 (2000).
43. Jakoby, M. et al. bZIP transcription factors in Arabidopsis. *Trends Plant Sci* **7**, 106-11 (2002).
44. Furihata, T. et al. Abscisic acid-dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. *Proc Natl Acad Sci U S A* **103**, 1988-93 (2006).
45. Lazo, G.R., Stein, P.A. & Ludwig, R.A. A DNA transformation-competent Arabidopsis genomic library in Agrobacterium. *Biotechnology (N Y)* **9**, 963-7 (1991).
46. Alonso, J.M. et al. Genome-wide insertional mutagenesis of Arabidopsis thaliana. *Science* **301**, 653-7 (2003).
47. Sussman, M.R., Amasino, R.M., Young, J.C., Krysan, P.J. & Austin-Phillips, S. The Arabidopsis knockout facility at the University of Wisconsin-Madison. *Plant Physiol* **124**, 1465-7 (2000).
48. Tocquin, P. et al. A novel high efficiency, low maintenance, hydroponic system for synchronous growth and flowering of Arabidopsis thaliana. *BMC Plant Biol* **3**, 2 (2003).
49. Inoue, H., Nojima, H. & Okayama, H. High efficiency transformation of Escherichia coli with plasmids. *Gene* **96**, 23-8 (1990).
50. Bartel, P., Chien, C.T., Sternglanz, R. & Fields, S. Elimination of false positives that arise in using the two-hybrid system. *Biotechniques* **14**, 920-4 (1993).
51. Ehlert, A. et al. Two-hybrid protein-protein interaction analysis in Arabidopsis protoplasts: establishment of a heterodimerization map of group C and group S bZIP transcription factors. *Plant J* **46**, 890-900 (2006).
52. Yamaguchi, T., Aharon, G.S., Sottosanto, J.B. & Blumwald, E. Vacuolar Na+/H+ antiporter cation selectivity is regulated by calmodulin from within the vacuole in a Ca2+- and pH-dependent manner. *Proc Natl Acad Sci U S A* **102**, 16107-12 (2005).
53. Baena-Gonzalez, E., Rolland, F., Thevelein, J.M. & Sheen, J. A central integrator of transcription networks in plant stress and energy signalling. *Nature* **448**, 938-42 (2007).

Curriculum vitae

Personal information

Name	**Daniela Hofmann Rodrigues**
Date of birth	01.12.1892
Place of birth	Vienna, Austria
Nationality	Austrian
Family status	Married since 02.06.2004 with Ivan Koppen Rodrigues
Adress	Schüttelstraße 85/20, 1020 Vienna
E-mail	daniela.hofmann.rodrigues@univie.ac.at

Education

1989 - 1993	Primary school (Volksschule), Vienna
1993 - 2001	High school (Realgymnasium), Vienna
2001 - 2007	Study of **Molecular Biology** at the University of Vienna with specialization on plant biology, genetics and microbiology; Diploma thesis at the Department of Biochemistry, research group of Markus Teige

Professional activities and skills

05/2005 - 06/2006	**Technical assistant** in the Drosophila RNAi library at the Institute of Molecular Biotechnology of the Austrian Academy of Sience (IMBA), Vienna
2006 - 2007	**Tutorship** for student practical courses of Biochemistry and Molecular Biology
09/2007	**Poster presentation** at the 4th Tri-national Arabidopsis meeting in Vienna; Mehlmer N, Hofmann-Rodrigues D, Wurzinger B, Teige M: Role of the calcium dependent protein kinase CPK3 in *Arabidopsis* salt stress response
Languages	German (mother tongue), English and Portuguese (fluent in speech and writing)