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DIPLOMARBEIT

Titel der Diplomarbeit

Regulation of bZIP Transcription Factors by Phosphorylation – Identification of Kinases and *in vivo* Phosphorylation sites

angestrebter akademischer Grad

Magistra der Naturwissenschaften

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Zusammenfassung

Genexpressionsmuster sind einer ständigen Veränderung unterworfen, einerseits aufgrund von Stresssituation, andererseits aber auch als Teil des normalen Entwicklungszyklus. Eine schnelle Änderung der Aktivität eines Transkriptionsfaktors (TF) ist notwendig, um schnelle Reaktion auf veränderte Umweltbedingungen oder Zustände in der Zelle zu ermöglichen. Ein Mechanismus um die Aktivität schnell anzupassen ist Phosphorylierung. Sie kann Einfluss auf die Lokalisierung, Stabilität, DNA Bindung oder Protein-Protein Interaktion des TF haben. BZIP (basic leucine zipper) TF sind an der Regulation diverser Prozesse beteiligt und wurden in allen Eukaryoten gefunden. Das Genom von *Arabidopsis thaliana* codiert für ca. 75 davon. C/S1 Gruppen Mitglieder könnten eine Rolle bei der Regulation der Genexpression als Reaktion auf Energie Mangel und Stress aber auch in der Samenreifung spielen. Im Zusammenhang mit Energie Mangel wurden sie bereits mit AKIN10 und AKIN11 in Verbindung gebracht, 2 Kinasen für die eine zentrale Rolle in der Energie-Mangel-Signaltransduktion vorgeschlagen wird. Das legt nahe, dass Phosphorylierung eine Rolle für die Aktivität des TF spielt.

Hier zeige ich, dass bZIP63, ein Mitglied der C-Gruppe, *in planta* phosphoryliert ist. Dafür wurden bZIP63-GFP Überexpressor Pflanzen in zwei unterschiedlichen Ansätzen verwendet: 2D gel Elektrophorese in Kombination mit Western blots und Immunpräzipitation gefolgt von Massenspektrometrie (MS) Analyse. Auf Western blots von Protein Extrakten nach 2D Elektrophorese kann man einen Shift des Signals von bZIP63-GFP zum höheren pH Bereich beobachten, was auf *in vivo* Phosphorylierung hindeutet. Immunpräzipitation und MS Analyse von bZIP63-GFP aus im Dunklen geernteten Blättern führte zur Identifikation von 4 *in vivo* Phosphorylierungsstellen auf dem Protein unter diesen Bedingungen. In-Gel Kinase Assays wurden verwendet um potentielle bZIP63 spezifische Kinasen zu identifizieren. Es gab 3 starke Signale, was auf Beteiligung von mindestens 3 Kinasen hinweist. Zur Identifikation der Kinasen wurden Proteine mit einer hohen Affinität zu bZIP63 durch Affinitätsreinigung im Proteinextrakte angereichert, bevor dieser in In-Gel Kinase Assays verwendet wurde. Im Bereich des Signals wurden Gelstücke ausgeschnitten und darin enthaltene Proteine durch MS identifiziert. 3 Gruppen von Kinasen wurden gefunden. 2 Mitglieder der SnRK1 Familie, AKIN10 und AKIN11, wurden identifiziert. CDPKs (calcium dependent protein kinases) waren mit CPK3 und einigen anderen Mitgliedern vertreten, die jedoch aufgrund schlechter Proteinabdeckung nicht eindeutig identifiziert werden konnten. Die dritte Gruppe von Kinasen ist Casein Kinase II, von der 2 katalytische Untereinheiten, CKA1 und CKA2, gefunden wurden. Zusammengefasst war es mir möglich mehrere *in vivo* Phosphorylierungsstellen und potentielle bZIP63 spezifische Kinasen zu identifizieren und die Basis für weitere Untersuchungen über die Funktion der Phosphorylierung zu legen.

Abstract

Gene expression patterns change constantly in response to stress, but also as part of the normal developmental cycle. Rapid change in the activity of a TF (transcription factor) is essential to allow fast responses to changing environmental or intracellular conditions. One mechanism to modulate TF activity rapidly is phosphorylation, which can affect localisation, stability, DNA binding or protein–protein interaction of the TF. BZIP (basic leucine zipper) transcription factors are involved in regulation of numerous processes and can be found in all eukaryotes. The *Arabidopsis thaliana* genome encodes about 75 of them. The C/S1 group members are proposed to play a role in reprogramming gene expression in response to energy deprivation and stress as well as in seed maturation. In the context of energy deprivation they were already associated with AKIN10 and AKIN11, 2 kinases proposed to be central regulators of energy deprivation signalling. This suggested a role for protein phosphorylation in the activity of these transcription factors.

Here I show that bZIP63, a member of the C–group, is phosphorylated *in planta* using bZIP63–GFP over–expressor plants in two different approaches: 2D gel electrophoresis in combination with Western blotting and Immunprecipitation followed by Mass spectrometry analysis. On Western blots of protein extracts after 2D separation the signal from bZIP63–GFP shifts to higher pH ranges when the extract is treated with phosphatase, indicating *in vivo* phosphorylation of the protein. Immunprecipitation and Mass spectrometry analysis of bZIP63–GFP from leaves collected in the dark revealed 4 *in vivo* phosphorylation sites on the protein under these conditions. In–gel kinase assays were used to identify potential bZIP63 specific kinases. 3 strong signals were obtained from total protein extracts, indicating a role for at least 3 kinases. For identification of the kinases protein extracts were enriched for proteins with a high affinity for bZIP63 via affinity purification before use in the in–gel kinase assay. Gel slices were cut out in the range of the signal and proteins identified by Mass spectrometry. 3 groups of protein kinases were found in the samples. Two members of the SnRK1 (SNF1 (Sucrose non–fermenting1) related kinases) family, AKIN10 and AKIN11 were identified. CDPKs (calcium dependent protein kinases) were represented by CPK3 and some other members, but protein coverage for these was too low for clear identification. The third group of kinases is Casein Kinase II, of which two catalytic subunits, CKA1 and CKA2 were found. Taken together, I could identify several in vivo phosphorylation sites on bZIP63 and potential bZIP63 specific kinases clearing the way for further investigation on function of bZIP63 phosphorylation.

Table of Contents

1. Introduction

1.1.   Transcription factor regulation – aim of the project

Regulation of gene expression is crucial for the life of every organism on earth. The major part of regulation happens on gene transcription level, by the action of transcription factors. These are proteins that bind to specific, regulatory DNA sequences in the promoter or enhancer region of genes and alter their transcription positively or negatively (Latchman, 1997). This can happen by one of several strategies: stimulating or inhibiting RNA polymerase activity, altering DNA condensation by directing histone acetyltransferases (HAT) or histone deactetylase (HDAC) to the DNA and recruiting of coactivators or corepressors (Holmberg et al., 2002).

Transcription factor activity is regulated on transcriptional as well as on posttranscriptional level. Protein synthesis, stability and subcellular localisation determine the general availability of the transcription factor. Whether it can act as an activator or repressor depends on additional factors like DNA binding activity and binding of cofactors. Posttranscriptional regulation has an impact on several of these regulatory levels. One of the most important modifications is phosphorylation, which can rapidly alter the activity of a transcription factor, making quick responses to changing demands in the cell possible (Holmberg et al., 2002).

My diploma work focuses on AtbZIP63, a transcription factor of the bZIP (basic leucine zipper) family in the plant *Arabidopsis thaliana*. It is regulated on transcriptional level by the day/night cycle and by sugars and a function in energy deprivation response was proposed. There are hints that this transcription factor's activity is regulated by phosphorylation (Baena-Gonzalez et al., 2007; Usadel et al., 2008). Prediction programs provide a big number of potential phosphorylation sites along the protein, including sequences responsible for nuclear import, protein–protein interaction and DNA binding.

The two big goals of this diploma thesis were identification of *in vivo* phosphorylation sites and of the kinases phosphorylating bZIP63. This will make it possible to analyse the impact of every individual phosphorylation event on activity and localisation of the transcription factor and help to gain a better understanding of the significance of transcription factor phosphorylation. Knowledge of upstream acting kinases will help to resolve the function of bZIP63 in the cell.

1.2. BZIP (basic leucine zipper) transcription factors in *Arabidopsis thaliana*

Transcription factors of the bZIP family are found in all eukaryotes analysed to date (Jakoby et al., 2002). In plants they are involved in regulating numerous processes like tissue differentiation, cell elongation, pathogen defence, light and stress signalling, energy homeostasis, seed maturation, flower development etc (Correa et al., 2008; Jakoby et al., 2002; Schutze et al., 2008).

1.2.1. Characteristics

BZIP transcription factors are characterized by 2 structural features located adjacent to each other on the protein: the basic region, responsible for translocation and DNA binding and the leucine zipper for dimerization. These two form a contiguous, amphipathic α–helix (Jakoby et al., 2002; Vinson et al., 2006).

The basic region is a ~ 16aa (amino acid) long stretch containing a bipartite NLS (nuclear localisation signal) followed by an invariant $N–x_7–R/K$ motif that interacts with the major groove of the DNA (Jakoby et al., 2002; Vinson et al., 2006). This basic region and the following linker region are responsible for recognizing the target site on the DNA, preferentially a sequence with an ACGT in the core. Preferred target sites in plants are A–(TACGTA), C– (GACGTC) or G–boxes (CACGTG) (Jakoby et al., 2002; Siberil et al., 2001; Vinson et al., 2006).

The NLS regulates translocation of the protein to the nucleus. In several bZIP transcription factors protein phosphorylation sites are predicted inside and around the NLS (Siberil et al., 2001), indicating a potential function for phosphorylation in protein localisation.
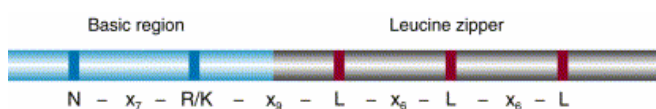
Fig 1.1 Scheme of the bZIP domain. The basic region is shaded in blue, the leucine zipper in grey. Highly conserved positions are highlighted. A consensus sequence is give below.
Figure from (Jakoby et al., 2002)
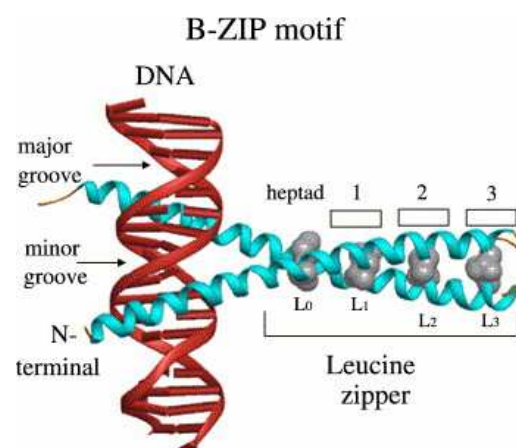
Fig 1.2 X-ray structure of a GCN4 bZIP dimer bound to double stranded DNA. The DNA is red, the bZIP proteins blue with leucines shown in grey.
Figure from (Vinson et al., 2006)

9aa C–terminal to the basic region lies the leucine zipper, a heptad repeat with leucine or other bulky hydrophobic amino acids every 7 residues, which forms the amphipathic helix. Along this stretch 2 bZIP proteins can dimerize, the leucine zipper domains forming a coiled

coil structure with the hydrophobic side of the helices acting as an interaction interface (Jakoby et al., 2002). In this coiled coil the two helices take on a supercoiled structure so that all leucines are aligned and the number of amino acids per turn is reduced from 3.6 to 3.5. As a consequence the helices are bent to a curve forming a flexible fork with the basic regions at the open end and free to interact with the DNA (Siberil et al., 2001).

The minimum number of heptad repeats required for coiled coil formation is 4 – 5. Whereas human bZIP transcription factors often have a quite low number, plant bZIP proteins are characterized by unusually long zipper domains, often consisting of 8 or 9 heptad repeats (Deppmann et al., 2004; Vinson et al., 2006). The sequence of the heptad plays a crucial role in determining potential interaction partners among bZIP transcription factors, which can either form homodimers or heterodimers. As shown in Fig 1.3 positions a and d of the helix interact directly. These are typically hydrophobic amino acids, position d preferentially a leucine (Vinson et al., 2006). In *Arabidopsis thaliana* 56% of d are leucines, in human the percentage is even higher with 66% (Deppmann et al., 2004). Positions g and e play a major role in stability and specificity of interactions. These positions often contain charged amino acids. Depending of the kind of charge this can lead to attraction and thus stabilisation of the coiled coil or repulsion and destabilization (Vinson et al., 2006).
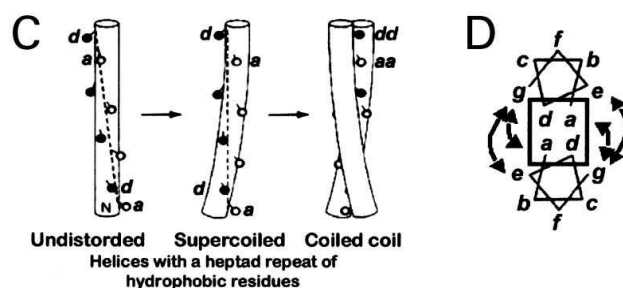
Fig 1.3 (**C**) Formation of a coiled coil; (**D**) Helical wheel representation of the positions in the heptad repeat. Amino acids of the hydrophobic core (a, d) interact directly, adjacent ones (e, g) via electrostatic interactions.
Figure from (Siberil et al., 2001)

1.2.2. Dimerization:

As already mentioned bZIP transcription factors can either form homodimers or heterodimers on the DNA. This dimerization is likely to be crucial for bZIP activity (Siberil et al., 2001). Binding of the DNA stabilizes the dimer. Supposedly the general mechanism is that a meta–stable dimer forms, that can bind to the DNA, thereby stabilizing it. Alternatively some proteins might be able to bind the DNA as monomers and form dimers afterwards, as has been shown for the human bZIP transcription factors Fos and Jun (Siberil et al., 2001; Vinson et al., 2006).

Assuming the number of bZIP transcription factors in *Arabidopsis thaliana* is 77 (Correa et al., 2008) a potential number of 77 homodimers and 2888 heterodimers could exist. However, many of these dimers will never be formed *in vivo*. The composition of the individual leucine zipper domains can favour or prevent interaction due to electrostatic interactions as described above (Jakoby et al., 2002; Siberil et al., 2001; Vinson et al., 2006).

Apart from that, different expression pattern and subcellular localisation, posttranslational modification and degradation restrict possible interactions (Vinson et al., 2006).

1.2.3. bZIP transcription factors in *Arabidopsis thaliana* – grouping and evolution

The *Arabidopsis thaliana* genome contains about 75 – 77 putative genes coding for bZIP transcription factors (Correa et al., 2008; Jakoby et al., 2002). Different authors have predicted quite a varying number of these genes ranging from lower numbers like 67 (Deppmann et al., 2004; Vinson et al., 2006) to as much as 81 (Riechmann et al., 2000). The exact number though is not clear, as many of the putative genes have not been analysed yet. Jakoby *et al.*, 2002 predicted 75 genes coding for bZIP transcription factors and subdivided them into 10 groups (A – I and S) according to sequence similarities in the basic region and domains shared by some bZIP factors.

A more recent approach by Corrêa *et al.*, 2008, analyzing the evolutionary relationship of green plant bZIP transcription factors, worked with 77 genes in *Arabidopsis thaliana* and found 13 groups of homologues, 3 more than before (A – L and S). Still, some genes could not be classified.

In *Arabidopsis thaliana* the number of bZIP transcription factors is about 4 times the number in human, worm or fly (Jakoby et al., 2002). A comparison of genes from algae, mosses, gymnosperms and angiosperms suggests that the ancestor of green plants contained only 4 genes coding for bZIP proteins, functioning in oxidative stress, UPR (unfolded protein response) and light signalling (Correa et al., 2008). Early in evolution the bZIP genes must have been amplified and developed new functions explaining the bigger number and diversity in plants and allowing the colonization of land (Correa et al., 2008; Jakoby et al., 2002).

The common ancestor of today's eukaryotes might even have had less bZIP proteins. Comparison of plant, animals and fungi revealed only one common ancestor of bZIP genes. This finding is supported by the fact that the primitive eukaryote *Giardia lamblia* codes for only one bZIP transcription factor playing a role in both UPR and oxidative stress response. Corrêa *et al.*, 2008 proposed a model how the 4 ancestral plant bZIP genes have evolved from the single gene in the common eukaryotic ancestor. This ancestral gene combined all basic functions: oxidative stress response, regulation of energy metabolism, UPR and light response. By and by the 4 founder genes evolved and specialized in function (see Fig 1.4).
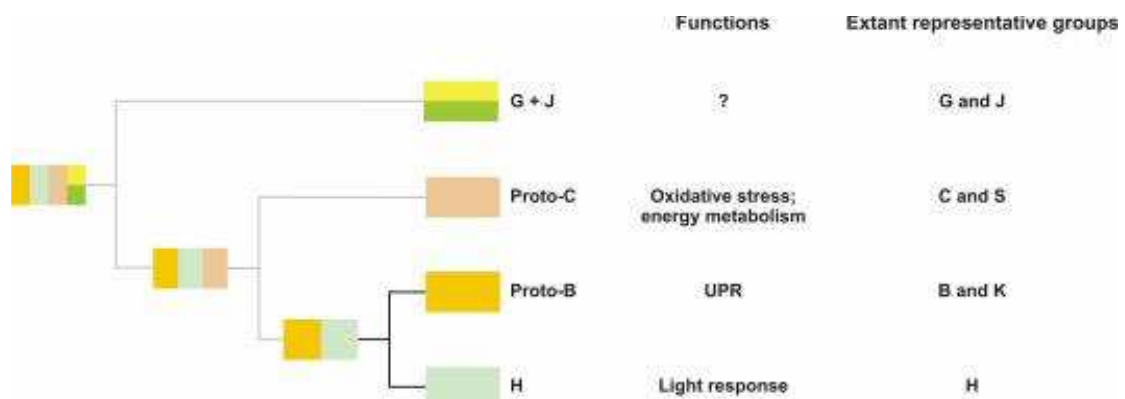
Fig 1.4 Model for the emergence of the four founder bZIP genes in plants. The four founder genes in group G + J, Proto–C, Proto–B and H derived from a single ancestral gene common to all eukaryotes, which combined all functions.
Figure from (Corrêa et al., 2008)

Further gene duplication and specialisation events of these 4 founder genes then would have led to the formation of the numerous bZIP genes found in plants today. Table 1.1 summarizes our current knowledge about the function of the individual bZIP groups.

1.2.4. C and S1 bZIP transcription factors

The model of plant bZIP transcription factor evolution from Corrêa *et al.*, 2008 proposes a function for group C and S in oxidative stress response and energy metabolism (see Fig 1.4). This seems to hold true. Members of the C and S1 class of bZIP transcription factors are proposed to play a role in transcriptional reprogramming of plant

Group	Function of some members in
A	ABA response, seed formation, salt stress signalling
C	seed storage protein production, N/C (nitrogen/carbon) balance
D	pathogen response and development (SA and ethylene signalling)
G	UV and blue light signal transduction, seed maturation, N/C balance
H	promotion of photomorphogenesis
I	vascular development
K	UPR
S	sucrose and stress signalling

Table 1.1 Possible function for some genes in the 13 groups of bZIP transcription factors in *Arabidopsis thaliana* (Correa et al.. 2008: Jakobv et al.. 2002)

growth in response to energy deprivation and stress as well as in seed maturation (Baena-Gonzalez et al., 2007; Baena-Gonzalez and Sheen, 2008; Weltmeier et al., 2009).

BZIP53 and bZIP10 were shown to act in abiotic stress and oxidative stress respectively. BZIP53 together with bZIP10 and 25 seem to play a role in late seed development inducing seed storage proteins and bZIP11 and 44 could play a role in embryogenesis (Weltmeier et al., 2009).

In respect to energy deprivation signalling S1 (and C) group bZIPs were linked to AKIN10 and AKIN11. These two kinases were recently proposed as central signalling joint in adaptation to energy deprivation and stress (Baena-Gonzalez et al., 2007; Baena-Gonzalez and Sheen, 2008; Weltmeier et al., 2009).

Group C bZIP proteins: AtbZIP9, AtbZIP10, AtbZIP28 and AtbZIP63

Proteins of this group are characterized by a long leucine zipper of 7 to 9 heptad repeats in a central or C–terminal position on the protein. Additionally they have an N–terminal hydrophobic or acidic domain, a Ser/Thr cluster followed by an acidic stretch and several putative phosphorylation sites. Especially AtbZIP10 and AtbZIP28 show significant similarity to Opaque2 from maize, which is involved in seed storage protein production (Jakoby et al., 2002) and is regulated by phosphorylation (Ciceri et al., 1997).

Group S1 bZIP proteins: AtbZIP1, AtbZIP2, AtbZIP11, AtbZIP44 and AtbZIP53 (Ehlert et al., 2006; Weltmeier et al., 2009)

Group S bZIP proteins are shorter with a long leucine zipper of 8 to 9 repeats and are characterized by the absence of introns in the genes (Correa et al., 2008; Jakoby et al., 2002). Members of the S1 group have an unusually long 5' leader containing conserved small upstream ORFs (open reading frames), which are involved in posttranscriptional repression (Jakoby et al., 2002; Weltmeier et al., 2009) by sucrose via the SIRT (sucrose induced repression of transcription) mechanism (Weltmeier et al., 2009). Involvement in sugar signalling is proposed (Baena-Gonzalez et al., 2007; Baena-Gonzalez and Sheen, 2008; Correa et al., 2008; Jakoby et al., 2002).

From the amino acid composition of the leucine zipper both class C and S1 (except AtbZIP1) are predicted to form homodimers (Deppmann et al., 2004). In contrast to this, data from Y2H (yeast–2–hybrid) and P2H (protoplast–2–hybrid) experiments suggest specific heterodimerization between C and S1 group members as well as some intra–group interactions and could hardly observe any homodimerization (Ehlert et al., 2006). The contradiction might be explained by a stabilizing effect of the DNA on the unfavourable interaction (Deppmann et al., 2004).

In addition to available microarray data on expression a recent approach using promoter – GUS fusions of all C and S1 group bZIP proteins gives insight into expression and localisation pattern. Expression was high in sink tissue (young leaves, pollen, anthers) and roots in comparison to source tissue (old leaves). But individual proteins have quite a distinct expression pattern. As C/S1 group members per se have overlapping transcription factor properties this limitation in working points and interaction partners enables distinct functions of each transcription factor pair.

14

1.2.5. bZIP63 (BZ02H3) – At5g28770

bZIP63 is a bZIP transcription factor of the C group.

The gene codes for 3 different splicing forms. Splicing form 1 and 2 differ only in the second exon, where splicing form 1 lacks the first 7aa (amino acids) compared to splicing form 2. The third splicing form is much shorter, including the 5th intron, but lacking most of the 6th exon. SqRT–PCR data with splicing form specific primers show, that all 3 of them are expressed. Splicing form 1 and 2 are equally expressed, but splicing form 3 is low abundant (data from Daniela Hofmann Rodrigues and Bernhard Wurzinger).
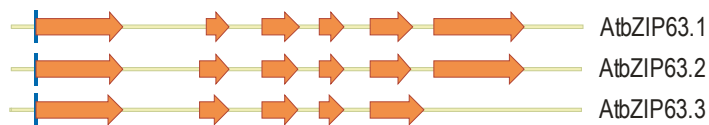
Fig 1.5 Splicing forms of bZIP63; orange arrows depict the exons

Expression data from chip experiments suggest expression in all organs, with higher levels in leaves and some parts of the flower and lower levels in roots (GeneVestigator).

This contradicts our own sqRT-PCR data, which suggest a higher expression in roots than in leaves (data from Bernhard Wurzinger). Promoter – GUS fusion experiments also suggest high expression of bZIP63 in roots as well as in sink tissue like young leaves and flowers, and lower expression in old leaves (Weltmeier et al., 2009).

Microarray data show expressional changes to some stimuli. Induction can be observed in response to senescence and some extent to high CO_2 concentrations. Weak repression can be observed for high light, sucrose and osmotic stress. The strongest repressor though is glucose (GeneVestigator). In carbon starved seedlings addition of sugars leads to a rapid repression of bZIP63 (Usadel et al., 2008). Also the expression level changes during the day/night cycle reflecting the changing energy/carbon supply under light/dark conditions. Early during the night bZIP63 is induced, levels increasing during the dark period. Soon after the onset of light bZIP63 levels decrease again (Usadel et al., 2008) (data from Bernhard Wurzinger and Daniela Hofmann Rodrigues).

Concerning the subcellular localisation, bZIP63 was exclusively detected in the nucleus (YFP and GFP fusion).

Y2H and P2H data suggest a heterodimerization with the C group transcription factor bZIP10 (also: data from Daniela Hofmann Rodrigues) as well as with S1 group bZIP transcription factors, especially bZIP11 and bZIP2 (Ehlert et al., 2006).

Taken together the experimental data available to date suggest a role for bZIP63 in energy deprivation signalling. Furthermore phosphorylation of bZIP63 could play a crucial role in its

activity. S1 group bZIP transcription factors as well as bZIP63 were associated with the signalling function of protein kinase AKIN10 (Baena-Gonzalez et al., 2007; Baena-Gonzalez and Sheen, 2008). Together with AKIN10 bZIP63 can induce expression of ASN1, a marker gene for low energy conditions (Baena-Gonzalez et al., 2007). The web–based phosphorylation prediction programs PhosPhAt (http://phosphat.mpimp-golm.mpg.de/) and NetPhos2.0 (http://www.cbs.dtu.dk/services/NetPhos/) reveal a number of potential phosphorylation sites on bZIP63 (see Fig 1.6). Identification of phosphorylation sites *in vivo* and of the kinases responsible for the modification were the two big goals of this diploma thesis.

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  1 MEKVFSDEEI SGNHHWSVNG MTSLNRSASE WAFNRFIQES SAAADDGEST TACGVSVSSP

 61 PNVPVDSEEY RAFLKSKLNL ACAAVAMKRG TFIKPQDTSG RSDNGGANES EQASLASSKA

121 TPMMSSAITS GSELSGDEEE ADGETNMNPT NVKRVKRMLS NRESARRSRR RKQAHLSELE

181 TQVSQLRVEN SKLMKGLTDV TQTFNDASVE NRVLKANIET LRAKVKMAEE TVKRLTGFNP

241 MFHNMPQIVS TVSLPSETSN SPDTTSSQVT TPEIISSGNK GKALIGCKMN RTASMRRVES

301 LEHLQKRIRS VGDQ*
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Fig 1.6 Position of the bZIP domain and potential phosphorylation sites on bZIP63.2.

bZIP domain bZIP-basic domain (DNA binding) bipartate NLS (nuclear import)
PhosPhAt prediction: ▼ general phosphorylation site
NetPhos2.0 prediction: ▲ CKII target site, ▲ general phosphorylation site

1.3.   Energy metabolism and bZIP transcription factors

Plants are exposed to different kinds of stress on a regular basis. Even the simple cycle of day and night challenges plants to adapt to changing conditions constantly, as the lack of light leads to energy deprivation at night (Usadel et al., 2008; Weltmeier et al., 2009). Energy deprivation seems to be a consequence of many different stresses, not solely the result of sugar deprivation or darkness. SnRK1 (SNF1 [sucrose non–fermenting1]–related kinases) group members, namely AKIN10 and AKIN11, are proposed to be central integrators of energy deprivation signals, leading to changes in enzyme activity as well as global transcriptional changes (Baena-Gonzalez et al., 2007; Baena-Gonzalez and Sheen, 2008).

Energy deprivation leads to downregulation of anabolism and upregulation of catabolism and alternative energy generating pathways. This happens both on the level of enzyme activity changes and transcriptional reprogramming. One marker gene for energy deprivation is *DIN6*

= *ASN1* (asparagine synthase), which is upregulated in the dark and under energy depriving conditions like hypoxia to shift nitrogen storage from glutamine to the less carbon consuming asparagine. Overexpression of AKIN10 and AKIN11 induces ASN1 expression in a phosphorylation dependent manner implying a role for these two kinases in energy deprivation signalling, which is independent from the known HXK1 (Hexokinase 1) pathway (see Fig 1.7). Global gene expression analysis revealed ~ 600 target genes for AKIN10, many correlating with chip datasets from different starvation experiments (Baena-Gonzalez et al., 2007; Baena-Gonzalez and Sheen, 2008). Most of these genes are affected during a normal dark phase. Only very few respond to extreme C depletion like in an extended night. This supports the idea that AKIN10 contributes to regulation of expression in response to small C changes (Usadel et al., 2008).
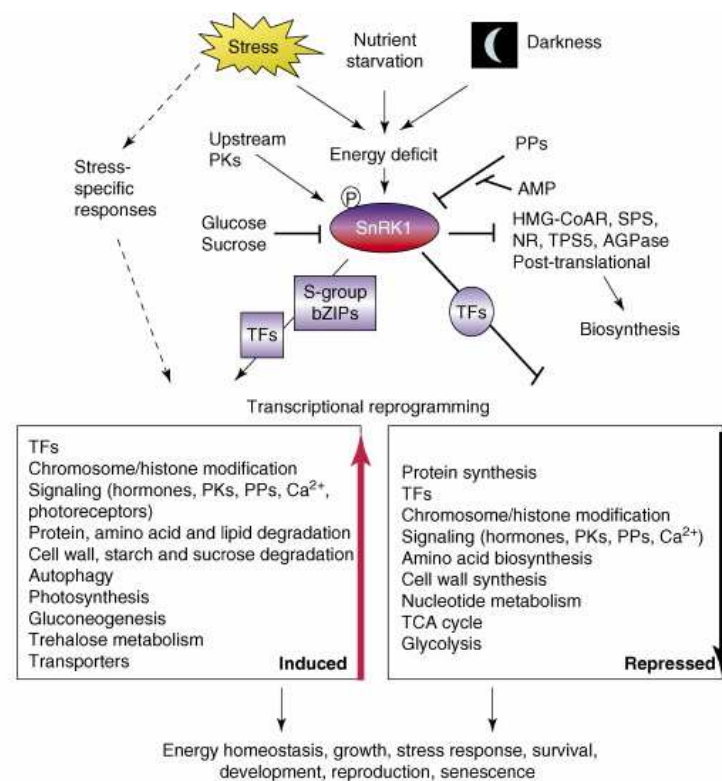
Fig 1.7 Model for the role of SnRK1s in decoding energy signals from Baena–Gonzáles *et al.* 2008 (Baena-Gonzalez and Sheen, 2008)
Abbreviations: AGPase, ADP-glucose pyrophosphorylase; HMGCoAR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; NR, nitrate reductase; SPS, sucrose phosphate synthase; TCA, tricarboxylic acid cycle; TPS5, trehalose-6-phosphate synthase 5.

As one group of downstream mediators of AKIN10 and AKIN11, bZIP transcription factors of the S1 group were proposed. *ASN1* contains a G–box in its promoter, a typical target for bZIP transcription factors. Presence of the G–box is essential for AKIN10 dependent expression. Several known G–box binding bZIP transcription factors were tested, but only S1 group factors and the C group factor bZIP63 were shown to induce expression of ASN1

together with AKIN10 (Baena-Gonzalez et al., 2007). Again, these findings support a role for the C/S1 group in sugar signalling.

1.4. BZIP transcription factor activity – the role of posttranslational regulation

Expression and interaction patterns alone do not suffice to explain the transcription factor activities of the complex network of bZIP transcription factors. Several examples are known where posttranslational modification plays a major role in availability and activity of transcription factors. Some examples for regulation of bZIP activity are given below.

1.4.1. Protein–protein interactions and processing

Interaction with other proteins can have a great influence on bZIP activity. FD (flowering locus D = AtbZIP27) regulates expression of flowering genes. Depending on the cofactor binding to FD it can either act as a strong activator or repressor of expression.

TGAs (group D bZIPs) work in pathogen defence. SA signalling leads to nuclear import of NPR1 (non–expressor–of–pathogenesis–related–genes–1) = NIM1, which can then bind to the TGAs and influence transcription of PR (pathogen response) genes. NIMIN1 (NIM1– interacting protein 1) binds NPR1 and counteracts its actions.

Subcellular localisation of *AtbZIP10* is regulated by binding of LSD1, which masks the NLS and keeps the transcription factor in the cytoplasm. In response to SA treatment or pathogen attack bZIP10 is released and translocated to the nucleus where it can alter gene expression (Schutze et al., 2008).

An example where processing of bZIP proteins influences their activity are the B group proteins AtbZIP17 and AtbZIP28. BZIP17 activates genes in response to salt stress, whereas bZIP28 is associated with UPR (unfolded protein response), an other ER stress condition. Both proteins have transmembrane domains and are kept in the ER under normal conditions. Stress leads to cleavage of the protein by a site–1 protease (S1P) and translocation of the N–terminal fragment to the nucleus where stress response genes are induced (Liu et al., 2007a; Liu et al., 2007b).

1.4.2. Phosphorylation

Since the discovery of the first kinases in the 1950s our awareness of their importance has changed dramatically. We now know that a great number of proteins is phosphorylated and reversible phosphorylation has proven to be a major mechanism for regulating many cellular processes at different levels (Cohen, 2002). It is one mechanism to rapidly change activity of transcription factors in response to changes in the cells demands (Holmberg et al., 2002).

This can happen in at least 6 different ways. Phosphorylation can affect activity of the transcription factor, stability and subcellular localisation, protein–protein interaction, DNA binding activity and can lead to activation induced inactivation (Cohen, 2002; Holmberg et al., 2002). Multisite phosphorylation and phosphorylation by several different kinases provide possibilities to integrate different signals from separate signal pathways (Holmberg et al., 2002).

Several bZIP transcription factors were shown to be phosphorylated. Group A bZIP factors, which play a role in ABA (abscisic acid) and abiotic stress signalling are phosphorylated in response to ABA and some stress situations. In rice, mediators of this phosphorylation were identified as members of the SnRK2 family of protein kinases. Group G as well as the group I bZIP factor show nuclear relocation in response to phosphorylation (Schutze et al., 2008). CKII (Casein kinase II) dependent phosphorylation was shown to alter the DNA binding activity of several bZIP transcription factors from human, maize and *Arabidopsis thaliana* either positively (c–Fos, EmBP–2, AtGBF1) or negatively (ZmBZ–1, AtHY5) (Hardtke et al., 2000; Klimczak et al., 1995; Klimczak et al., 1992; Schutze et al., 2008). Also stability of the *Arabidopsis* bZIP proteins ABI5 and HY5 and the bHLH (basis helix loop helix) transcription factor HFR1 is increased by phosphorylation via CKII (Hardtke et al., 2000; Park et al., 2008; Schutze et al., 2008).

1.5. AKIN10/AKIN11 – members of the SnRK (SNF–related kinase) superfamily

1.5.1. The SnRK family of protein kinases

Arabidopsis thaliana codes for 38 protein kinases of the SnRK family. These are Ser/Thr protein kinases. In plants, 3 subgroups can be distinguished, which differ in respect to their C–terminal region, implicated in protein–protein interaction (see Fig 1.8).

Fig 1.8 Domain structure of the SnRK subfamilies. Striped box = kinase domain, broken lines at C– and N–termini = variable regions, D/E box in SnRK = acidic patch, checked box = autoinhibitory domain
Figure modified from (Hrabak et al., 2003)

Group 1 consists of 3 members and shows highest homology to yeast *SNF1* (sucrose non–fermenting 1) and mammalian *AMPK* (AMP–activated protein kinase). Group 2, consisting of 10 members, and group 3, consisting of 25, are unique to the plant kingdom. The latter two are characterized by an acidic patch and by an autoinhibitory domain

binding Ca^{2+} binding proteins respectively. Group 3 SnRKs are involved in salt stress response (Halford and Hardie, 1998; Hrabak et al., 2003).

1.5.2. Proposed function of SnRK1 group members: AKIN10 (= SnRK1.1) and AKIN11 (= SnRK1.2)

Yeast Snf1 is involved in response to nutritional and environmental stress. It is essential for derepression of genes repressed by glucose, turning on alternative energy metabolism pathways to enable growth on carbon sources like glycerol or ethanol (Halford and Hardie, 1998).

Data achieved to date indicate a similar role for SnRK1 members in plants. Key metabolic enzymes like HMG–CoA reductase, SPS (sucrose phosphate synthase) and NR (nitrate reductase) were identified as substrates for spinach SnRK1s (Halford and Hardie, 1998; Sugden et al., 1999b). Also, global expressional changes in response to carbon deprivation are mediated by the *Arabidopsis* SnRK1s AKIN10 and AKIN11 (Baena-Gonzalez and Sheen, 2008; Usadel et al., 2008). As already mentioned these kinases are proposed to be central regulators of energy deprivation response (See Fig 1.7). Many of the genes downregulated by AKIN10 under energy deprivation conditions are involved in biosynthetic processes, while genes upregulated work in catabolic and alternative energy generating pathways (Baena-Gonzalez et al., 2007; Baena-Gonzalez and Sheen, 2008).

AKIN10 and 11 might also play a role in vegetative and reproductive growth. Double knockout is lethal and knock down leads to an impaired transcriptional switch between dark and light as well as to impaired starch mobilization, growth defects and senescence. Overexpression of AKIN10 improves resistance to sugar deprivation and life span, but alters flower morphology and leads to delayed flowering (Baena-Gonzalez et al., 2007).

Both genes have 3 different splicing forms and are expressed in all tissues of the plant. Different stimuli have only little effect on expression (TAIR, GeneVestigator).
A proposed recognition motif for SnRK1 group members is H(X/B)(B/X)XX(Ser)XXXH, with H representing hydrophobic and B basic amino acids. While plant SnRKs seem to prefer a Ser as target, mammalian and yeast enzymes also phosphorylate Thr (Halford and Hardie, 1998; Halford et al., 2003).

1.5.3. SnRK1s – activity and interaction partners

Like in mammals, where activity of AMPK is dependent on the upstream kinase AMPKK (AMPK kinase), SnRK1s are believed to be activated by phosphorylation of a Thr in their T–loop. This theory is supported by the fact that phosphorylation by animal AMPKK and other

kinases can increase activity of some plant SnRK1s (Hrabak et al., 2003; Sugden et al., 1999a; Sugden et al., 1999b). Also dephosphorylation of the Thr by mammalian PP2C (Phosphatase 2C) decreased activity of 2 spinach SnRK1s (Sugden et al., 1999a).

In mammals AMP is the intracellular messenger regulating activation and inactivation. This happens by allosteric activation of AMPK and AMPKK and by positive and negative influence of AMP–AMPK interaction on AMPKK and PP2C respectively (Sugden et al., 1999a). Although there seems to be no allosteric activation of SnRKs or Snf1, AMP also plays an essential role in plants and yeast (Halford and Hardie, 1998; Hrabak et al., 2003; Sugden et al., 1999a). Already low concentrations of AMP inhibit dephosphorylation of spinach SnRK1s by PP2C, indicating that also in plants SnRK1s react to small changes in AMP/ATP levels in the cell (Sugden et al., 1999a). This goes well with the assumption that SnRK1 members work in sugar deprivation signalling as the AMP/ATP ratio rises under starvation conditions (Baena-Gonzalez and Sheen, 2008).

A model for activation/inactivation of SnRKs comes from yeast. There, the activating subunit Snf4 binds to phosphorylated Snf1 under glucose starvation conditions, thus enabling interaction with additional proteins and activity of Snf1. Under high glucose conditions Snf1 is dephosphorylated, which leads to autoinhibition of Snf1 by interaction of it's C– and N–terminus (See Fig 1.9) (Kleinow et al., 2000).

Both positive and negative regulators of SnRK1s have been found in *Arabidopsis*. AtSNF4 (a functional homologue to yeast Snf4 (Kleinow et al., 2000)) has been identified and another homologue, AKINγ, has been proposed, but fails to suppress the yeast *snfΔ4* phenotype (Kleinow et al., 2000).

A potent inactivator of AKIN10 and 11 is PRL1 (pleiotropic regulatory locus 1). It binds to the SnRKs near the SNF4 binding site and inhibits their activity *in vitro* (Bhalerao et al., 1999).

In their active form SNF1–like proteins form a heterotrimeric complex. Yeast Snf1 interacts with its activator Snf4 and one protein of the SIP1/SIP2/Gal83 subfamily, acting as a scaffold to bring Snf1 and 4 together. An additional function for this third component could be targeting of the complex to its substrates in the cell. In mammals the according subunits of the complex are termed α, γ and β subunits (Halford and Hardie, 1998; Halford et al., 2003). In plants, the composition possibilities might be more complex. In addition to homologues to the α, β and γ subunits a class of AKINβγ proteins was discovered combining SNF4 features with a KIS (kinase interaction sequence) domain typical for a β subunit. These could interact with SnRKs without the third interaction partner (See Fig 1.9). One example is AtSNF4 (Lumbreras et al., 2001).
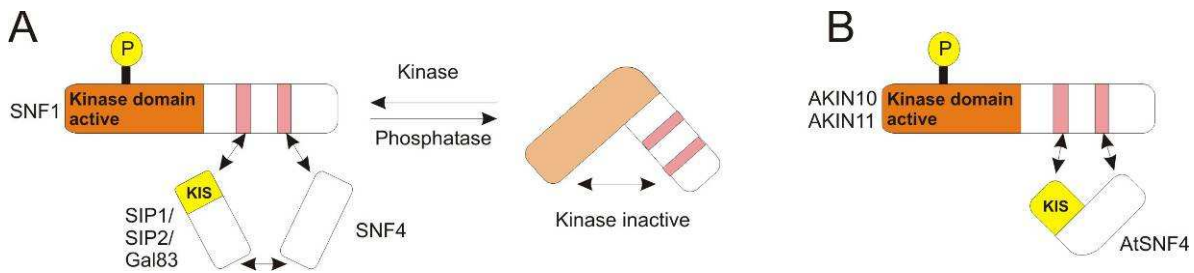
Fig 1.9 (**A**) Model for activation/inactivation of yeast Snf1. In the phosphorylated state Snf1 can form a heterotrimeric complex with Snf4 and SIP1/SIP2/Gal83 and is active. Dephosphorylation prevents formation of the complex and leads to inactivation of the kinase. (**B**) Alternative complex formation in plants. In addition to heterotrimeric complexes dimeric complexes are possible. KIS = kinase interaction sequence

In addition to the already mentioned proteins some other interaction partners for SnRK1s were identified. AtPTPKIS1, a protein Tyr phosphatase containing a KIS domain was shown to interact with AKIN11 in a Y2H. Whether SnRK1s themselves or other proteins are substrates for this phosphatase remains to be revealed (Fordham-Skelton et al., 2002). Also interaction with the proteasomal subunit α4/PAD1 and the SCF E3 ubiquitin ligase component SKP1/ASK1 were found. These interactions are not competitive but are reduced by PRL1 binding. AKIN10 and AKIN11 are not ubiquitylated, but could possibly phsophorylate substrates of the E3 ligase prior to ubiquitylation. A potential role for this SnRK–E3 ligase–proteasome complex was proposed (Farras et al., 2001).

1.6. Calcium Dependent Protein Kinases (CDPKs)

1.6.1. CDPK properties

Calcium is a very important secondary messenger in cell signalling. In plants, the major part of calcium dependent signal transduction via kinases is decoded by the group of CDPKs. These Ser/Thr kinases are unique to plants, green algae and some protists and cannot be found in animals, where CaMK (Calmodulin dependent protein kinases) and protein kinase C are major integrators of calcium signals (Hrabak et al., 2003).

They contain three characteristic domains (see fig 1.10): a Ser/Thr

Fig 1.10 Domain structure of the CDPK family of protein kinases. Broken lines = N–terminal variable region, striped box = kinase domain, white box = autoinhibitory domain, black boxes = calcium binding EF hands. Figure modified from (Hrabak et al., 2003)
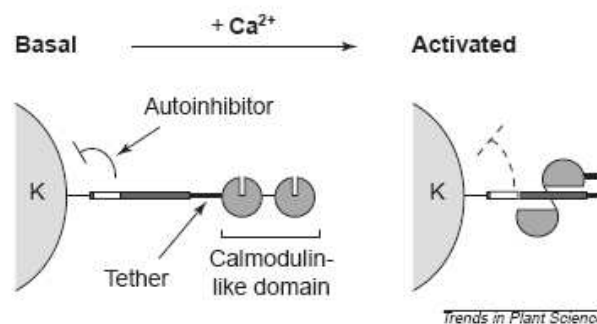
Fig 1.11 Release of inhibition model for activation of CDPKs by calcium. The kinase undergoes a conformational change in response to calcium, leading to displacement of the interaction between autoinhibitory domain and kinase domain. Figure from (Harmon et al., 2000).

kinase domain, followed by an autoinhibitory domain and a calmodulin like regulatory domain containing 4 EF hands for calcium binding. The N–terminus is variable and in many cases contains a myristoylation and palmitoylation motif, leading to membrane localisation of many CDPKs (Dammann et al., 2003). Regulation is thought to be similar to CaMKs. Under low calcium conditions the autoinhibitory domain might act as a pseudosubstrate (Harmon et al., 1994) and bind to the kinase domain, leading to a low basal activity of CDPKs. When calcium concentrations rise, the EF hands bind calcium and then bind to or near the autoinhibitory domain, triggering a conformational change which leads to release of the kinase domain (see fig 1.11). *Arabidopsis thaliana* codes for 34 CDPKs, which can be separated into four subgroups (Cheng et al., 2002; Harmon et al., 2000; Hrabak et al., 2003).

1.6.2. Function of CPK3

CPK3 and its close homologue CPK6 were shown to function in ABA dependent stomatal closure by regulation of a guard cell ion channel (Mori et al., 2006). A role in regulating SPS (sucrose synthase) and NR (nitrate reductase) in response to darkness were proposed (Douglas et al., 1998; Harmon et al., 2000). CPK3 also plays a role in salt stress response. KO plants show a salt sensitive phenotype and CPK3 activity is stimulated by salt stress (Mehlmer and Teige, unpublished). In the cell the protein is located at membranes as well as in the cytoplasm and the nucleus (Dammann et al., 2003) (Mehlmer and Teige, unpublished). A role for CPK3 in different signalling pathways is thus possible, because substrates could be found in different compartments and calcium signals are spatially restricted, which allows specific activation of CDPKs.

1.7.   CKII (Casein Kinase II)

1.7.1. General description of CKII and its properties

Casein kinase II (CKII), along with Casein kinase I, was the first kinase identified in history. It was described in 1954 as an enzyme in rat liver mitochondria, which could phosphorylate the artificial substrate casein (Burnett and Kennedy, 1954; Pinna, 1994). Since then hundreds of substrates for CKII have been found in various different organisms pointing to a crucial role for CKII in various mechanisms in the cell. Indeed, CKII seems to be essential in many aspects of cellular life. Knock out of CKII in yeast is lethal (Moreno-Romero et al., 2008; Riera et al., 2001). So it is in *Arabidopsis* (Moreno-Romero et al., 2008). In plants substrates for CKII are involved in many different pathways (see Fig 1.12) (Hardtke et al., 2000; Hidalgo et al., 2001; Moreno-Romero et al., 2008; Riera et al., 2001). A knock–down approach in *Arabidopsis* showed an impact on chloroplast development, cell cycle progression, cell division and cell expansion (Moreno-Romero et al., 2008).
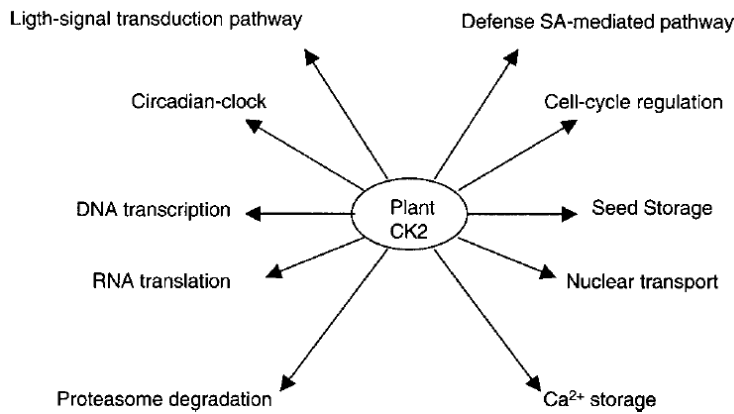
Fig 1.12 Schematic representation of the physiological roles of CKII in plants
Figure from Riera et al 2001 (Riera et al., 2001)

CKII is a Ser/Thr (serine/threonine) kinase with a minimal consencus target site of (S/T)XX(D/E) (Park et al., 2008). Homologues are found in all eukaryotes analysed to date, but not in prokaryotes (Riera et al., 2001) and they are highly conserved between species. In the cell it is usually found as a holoenzyme, consisting of 2 catalytic α subunits and 2 regulatory β subunits (Riera et al., 2001; Salinas et al., 2006), which can increase the α subunits' activity up to 100–fold (Klimczak et al., 1995). In plants monomers of one α subunit were also observed, but enzymatic properties differ from that of the holoenzyme (Klimczak et al., 1995).

The *Arabidopsis thaliana* genome encodes 4 α and 4 β subunits, more than human and yeast. This would allow formation of several different holoenzymes with potentially different functions. Unfortunately up to date no study about the interaction pattern of the individual subunits has been done (Riera et al., 2001). However, expression and subcellular localisation of all subunits was analysed some years ago. They are expressed in all tissues at a similar level, but expression level differs between the subunits. All α subunits were detected in the nucleus but one, which is localized in the chloroplast. The distribution of the β subunits is more diverse. They are found either in the nucleus or the cytoplasm or in both. None is found in the chloroplast, yet chloroplasts show kinase activity characteristic for CKII leaving the question whether an unknown chloroplast β subunit exists or the α subunit acts alone (Salinas et al., 2006).

name	expression	subcellular localisation
αA	medium	nucleus (nucleolus)
αB	medium	nucleus (nucleolus)
αC	low	nucleus
αcp	high	chloroplast
β1	low	nucleus + cytoplasm
β2	medium	nucleus (nucleolus)
β3	medium	nucleus + cytoplasm
β4	low	(nucleus) + cytoplsam

Table 1.2 expression pattern of *Arabidopsis* CKII subunits
Data from Salinas et al 2006 (Salinas et al., 2006)

Several characteristics typical for CKII have been reported and used to identify CKII activity in cell extracts. In contrast to other kinases CKII can use GTP in addition to ATP as a phosphodonor, although with less efficiency. Also preference for acidic protein substrates like casein, stimulation by polycations and salt dependent binding to phosphocellulose and deaggregation have been observed (Klimczak et al., 1992). One important tool in analysing CKII dependent phosphorylation are specific inhibitors. The most widely used inhibitors in experiments are heparin, as a competitor to the substrate, as well as DRB (5,6-dichloro-1-(b-D-ribofuranosyl) benzimidazole) and TBB (4,5,6,7-tetrabromo-2-azabenzimidazole) as competitors to the phosphodonor (Hathaway et al., 1980; Sarno et al., 2002).

1.7.2. bZIP regulation by CKII

Many of the CKII substrates identified to date are transcription factors. As already mentioned some of them are bZIP transcription factors (GBF1 = AtbZIP41, HY5 = AtbZIP56, ABI5 = AtbZIP39). Yamaguchi *et al.*, 1998 mapped the interaction site between CKII α subunits and the human bZIP transcription factor ATF1 and found that interaction depends mainly on the basic domain. Considering the relatively high conservation of this domain a lot of bZIP proteins might be targeted by CKII.

Analysis of the bZIP domain of several bZIP proteins from different species showed a potential CKII target site within the NLS. Phosphorylation of this site could regulate nuclear translocation of some bZIP transcription factors (Siberil et al., 2001). Phosphorylation on other sites of the protein can alter protein–protein interaction with other effectors like it is the case with HY5, where phosphorylation prevents COP1 binding and subsequent degradation by the 26S proteasome (Hardtke et al., 2000). It was also proposed that bZIP proteins could act as scaffold for CKII, bringing the kinases onto the DNA where it could phosphorylate other nearby proteins like polymerase II (Yamaguchi et al., 1998).

2. Material and Methods

2.1. DNA Methods

2.1.1. Agarose gel electrophoresis

<u>50x TAE (stock)</u>
242g Tris base
100ml 0.5M Na_2EDTA pH = 8,0
57.1ml acetic acid
ad 1l with ddH_2O

<u>1x TAE</u>
40mM Tris acteate, 1mM Na_2EDTA, pH = 8.0

<u>Ethidium bromide stock solution (5mg/ml)</u>
Ethidium bromide was dissolved in ddH_2O by stirring for several hours and stored protected from light at room temperature.

<u>6x loading buffer</u>
100 mM Tris.Cl pH = 8.0, 50% glycerol, 0.05% bromphenol blue, 0.05% xylenecyanol

<u>DNA molecular weight marker</u>
1kb DNA ladder plus (NEB New England Biolabs, Beverly, MA, USA)

This method was used to separate DNA or RNA fragments of different size for various purposes.

According to the size of the nucleotide fragments gels containing 0.8 – 2% agarose were prepared. Agarose was dissolved in 1x TAE by heating in the microwave and 4 – 7µl of a ethidium bromide stock solution (5mg/ml) per 100ml were added before pouring to enable DNA/RNA visualisation with UV light.

Samples were mixed with 6x DNA loading buffer before loading. Gels were run at 100 – 130V with 1x TAE as a running buffer.

<u>recommended agarose concentration:</u>

fragment size (bp)	% agarose
100 – 500	2
500 – 1000	1.5
> 1000	1 – 0.7

2.1.2. Determination of DNA/RNA concentration via comparison with the DNA marker

To estimate plasmid concentrations the plasmid was first digested with a suitable restriction enzyme and then loaded on an agarose gel. GeneRuler^{TM} 1kb DNA Ladder Plus was used as a maker. Intensity of single bands was compared to the intensity of marker bands roughly equal in size with known DNA amount. The so obtained DNA amount of the DNA fragment was used to calculate the amount/concentration of the whole plasmid.

2.1.3. Determination of DNA/RNA concentration via spectroscopy

To estimate DNA and RNA concentrations in solution absorbance of a suitable dilution in ddH$_2$O was measured at 260 and 280nm in a quartz cuvette. When A$_{260}$ = 1 DNA/RNA concentrations are as indicated in the table below.

dsDNA	50µg/ml
ssDNA	40µg/ml
RNA	33µg/ml

pure DNA solution: A260/A280 = 1.8
pure RNA solution: A260/A280 = 2.0

If the solution is still contaminated with proteins or phenol the ratio is considerably lower than 1.8% (50% DNA/protein solution: A260/A280 = 1.5), if it is contaminated with RNA the ratio is higher.

2.1.4. Dephosphorylation of vector backbones

To reduce re-ligation of the plasmid backbone during a ligation reaction of plasmid and insert which were cut with the same restriction enzyme at both ends the plasmid backbone was dephosphorylated. The fragment was cut out from an agarose gel and purified with the Promega (Madison, WI, USA) Wizard$^{®}$ SV Gel and PCR Clean–Up System. Then it was dephosphorylated for 30min at 37℃ using 2µl Arctic phosphatase from NEB with the according buffer and used in a ligation reaction.

2.1.5. DNA ligation with T4 ligase

Fragments to be ligated were cut out of an agarose gel and purified with the Promega (Madison, WI, USA) Wizard$^{®}$ SV Gel and PCR Clean–Up System. When both ends were cut with the same restriction enzyme the vector backbone was dephosphorylated with the Arctic

Phosphatase (NEB) before ligation. Concentrations were estimated from the gel photo. Plasmid backbones and inserts were ligated with T4 ligase (NEB) using a 5-fold excess of insert. A control ligation without insert was included to check for re-ligation frequency.

0.5 – 2µl plasmid
1.5 – 8µl insert
1µl 10x T4 ligase buffer
0.2µl T4 ligase
ad 10µl ddH$_2$O

The ligation reaction mix was incubated at room temperature for 25min and then used to transform competent *E. coli* cels.

2.1.6. DNA purification

DNA fragments were purified from contaminations, reaction additives (enzymes) or agarose (DNA cut out from a gel with scalpel) using the Promega (Madison, WI, USA) Wizard® SV Gel and PCR Clean–Up System. The "DNA Purification by Centrifugation" protocol was used and DNA was eluted from the column using 30 – 50µl nuclease free water.

2.1.7. DNA restriction digest

DNA restriction digests were performed using enzymes from NEB or Roche.
Plasmid DNA was digested for 1 – 2h in a total volume of 30 – 50µl using 0.5 – 1.5µl of each restriction enzyme under the conditions recommended by the enzymes manufacturer. Restriction products were analysed on a 0.8 – 2% agarose gel.

2.1.8. Fill in with Klenow fragment (NEB)

Products of a restriction digest which were to filled up with Klenow fragment were purified from an agarose gel using the Promega (Madison, WI, USA) Wizard® SV Gel and PCR Clean–Up System. The DNA was eluted in 88µl ddH$_2$O and mixed with 10µl buffer 2 (NEB) 1µl 10mM dNTPs and 1µl Klenow fragment and incubated at 37℃ for 20min. The reaction was stopped using the Promega (Madison, WI, USA) Wizard® SV Gel and PCR Clean–Up System again and the DNA eluted in 25µl ddH$_2$O.

2.1.9. Isolation of genomic DNA from *Arabidopsis thaliana*

Genomic DNA from *Arabidopsis thaliana* was extracted using the peqGOLD Plant DNA Mini Kit (peQLAB).

2.1.10.　　　Mini prep 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°C

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

A single E. coli colony was inoculated in LB containing the appropriate antibiotics and grown over night at 37°C. 2 – 3ml of this over night cult ure were harvested by 2min centrifugation in an eppendorf tube at top speed (16 000rcf). The supernatant was removed and the cells were resuspended in 200µl buffer P1. 200µl P2 were added and the tubes inverted immediately. After addition of 200µl P3 the tubes were vortexted and incubated on ice for 20 – 30min. The cell debris was pelleted by 10min centrifugation at top speed and the supernatant was transferred to a new tube. 0.7vol isopropanole (~ 420µl) were added. Alternatively the tubes were incubated on ice or −20°C for another 20min to support precipitation. After 10min centrifugation at top speed the supernatant was removed and the DNA pellet was washed with 70% EtOH and centrifuged again. The supernatant was removed and the remaining liquid was spinned down and removed. The pellet was dried at room temperature or at 37°C until it was transparen t and resuspended in 50µl 0.5x TE.

2.1.11.   Midi prep of plasmid DNA from *E. coli*

Midi prep 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 10min at 4000g at 4°C in 50ml Greiner tubes. The supernatant was r emoved and cells were resuspended in 4ml solution E1 with RNase. Cells were lysed by adding 4ml solution E2, inverting the tubes inverted several times and incubation at room temperature for 5min. For neutralization 4ml solution E3 were added and mixed immediately until a homogenous suspension was obtained. The mixture was centrifuged for 30 min at 4000g at 4°C.

The supernatant was applied to a JETSTAR column equilibrated with 10ml equilibration solution E4. The column was washed twice with 10ml solution E5. The plasmid DNA was eluted with 5ml solution E6, precipitated with 1 volume of isopropanol for 20min at -20°C and pelleted by centrifugation for 30min at 12000g at 4°C. After removing the supernatant, tubes were spinned down again and all traces of isopropanol were removed with a pipet. The pellet was resuspended in 150µl 0.5x TE buffer and transferred to a 1,5ml Eppendorf tube: This step was repeated to get out all rests of DNA.

The DNA was precipitated again with 840µl (2,8vol) 100% Ethanol, incubated for 10 min on ice and centrifuged for 15 min at 16000g at 4°C. Th e pellet was washed with 700µl 70% Ethanol and dried at 45°C 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°C in ddH_2O at a concentration of 1µg/µl.

2.1.12.   Polymerase Chain Reaction (PCR)

PCR mix for GoTaq (Promega)
30 – 250µg DNA
5µl GoTaq buffer (5x)
0.5µl dNTPs (10mM each)
0.5µl primer 1 (10pmol/µl)
0.5µl primer 2 (10pmol/µl)
0.125µl GoTaq
ad 25µl ddH_2O

PCR reaction mixes were prepared on ice in 200 or 500µl PCR tubes. DNA and polymerase were pipetted at the wall of the tube separate from each other, the rest at the bottom of the tube, spinned down just before PCR run and the mix was covered with mineral oil when necessary.

The first cycle always was a 5min denaturation step at 95°C. All following cycles consisted of

a 1min denaturation step at 95℃, a 45sec – 1min pr imer annealing step at the appropriate temperature (52 – 62℃) and a 2 – 10min elongation step at 72℃. A last cycle of 7min at 72℃ was added to ensure complete elongation of all fragments.

When samples were removed during the PCR run this was done during the primer annealing step.

Single colony PCR was perfomed by picking colonies from the LB plate with a pipette tip, introducing the tip in the PCR tube containing PCR mix without DNA and stirring. The tips were used to inoculate cultures and the PCR was performed like described above.

2.2. RNA methods

2.2.1. Total RNA preparation of plants (*Arabidopsis thaliana*)

All buffers and solutions used after the RNA precipitation step were prepared with DEPC water.

DEPC water
1 – 2ml DEPC was added to 1l ddH$_2$O and stirred on a magnetic stirrer over night followed by autoclaving

Acidic phenol (Roth)
pH = 4

RNA extraction buffer
1% SDS, 10mM EDTA, 200mM NaAc pH = 5.2

PCI (Roth)
Phenol:Chloroform:Isomylalcohol = 25:24:21

Fresh plant material was frozen in liquid nitrogen and mashed with a spatula. 100 – 150mg of the grinded material were mixed with ~20mg of sea sand treated with PVPP, 130µl of RNA extraction buffer and 130µl of acidic phenol and were further grinded using a glass drill. The plant material was frozen in liquid nitrogen and drilled until the sample reached room temperature. Then it was frozen again and the procedure repeated 2 – 4 times. 400µl of RNA extraction buffer and 400µl of acidic phenol were added and the sample vortexed for one minute and put on ice until procedure. The sample was then centrifuged for 10min at top speed at 4℃ in a table centrifuge, the aqueous pha se was transferred to a new tube and mixed with an equal volume of PCI by 30sec vortexing. The phases were separated by 2min centrifugation and the PCI extraction was repeated up to 3 times. To remove traces of PCI

the aqueous phase was extracted with an equal volume of Chloroform 1 – 2 times. Then the aqueous phase was transferred to a new tube and mixed with 1/3 volume 10M LiCl. To precipitate RNA the sample was incubated at 4°C for 2h – over night and then centrifuged for 15min at top speed at 4°C. The supernatant was disc arded and the RNA pellet washed twice with 2.5M LiCl. Then the pellet was washed twice with 70% EtOH, dried at room temperature and resuspended in 25 – 50µl DEPC water. RNA concentration was measured photometrically and the RNA solution was stored at –80°C

2.2.2. Reverse transcription PCR (RT–PCR)

Prior to RT–PCR RNA was submitted to DNAse digest using RQ1 RNAse (Promega). RNA concentration in the sample was estimated measuring the $A_{260/280}$. 8µl RNA suspension containing 2µg RNA were mixed with 1µl RQ1 buffer and 1µl RQ1 DNAse ad incubated at 37°C for 1h. The reaction was stopped by addition o f 1µl stop solution and incubation at 65°C for 15min.

Reverse transcription of the mRNA was performed using the Promega MSV reverse transcriptase. 16.75µl RNA suspension containing 8µg RNA were mixed with 1µl oligo dTs (100µM) and incubated at 70°C for 5min allowing pri mer annealing. Then 1µl MSV reverse transcriptase, 5µl MSV buffer and 1.25µl dNTPs (10mM each) were added and the reaction mix incubated at 40°C for 1h. cDNA was stored at 4° C for up to 2 weeks.

For semi quantitative RT–PCR equal amounts (≥ 1µl) of cDNA of each sample were used in a PCR reaction with GoTaq polymerase. The PCR program included 24 – 26 cycles of 45'' primer melting at 94°C, 45'' primer annealing at 52 °C and 1min extension at 72°C. To ba able to compare the signals the PCR reaction must not reach the saturation phase. This was regulated by the number of cycles in the PCR program. To control success of the PCR and adapt or compare cDNA amounts 10µl of the PCR products were loaded on a 2% agarose gel and the signal intensities were quantified using ImageJ. The first PCR run was done with primers amplifying the house keeping gene Act3 and the signal intensities were compared and used to equalize the cDNA amounts in each single PCR reaction.

2.3. Protein methods

2.3.1. 2D gel electrophoresis

Immobiline^{TM} DryStrip (GE Healthcare)

pH = 3 – 11 NL, 7cm

pH = 4 – 7, 7cm

1x Rehydration stock solution (Thiourea rehydration buffer)

7M Urea

2M Thiourea

2% (w/v) CHAPS

2% (v/v) IPG buffer (pH = 3–11)

for stock solution: 7mg DTT/2.5ml

Before focussing 0.1 – 0.2µl Bromphenol blue stock solution were added

Bromphenol blue stock solution

100µl 1x Rehydration stock solution + 0,002% Bromphenol blue

IPGphor (GE Healthcare)

SDS Equilibration buffer

6M Urea

75mM TrisCl pH = 8.8

29.3% glycerol

2% SDS

1% Bromphenol blue stock solution

DTT SDS Equilibration buffer

100mg DTT + 10ml SDS Equilibration buffer

Iodacetamide SDS Equilibration buffer

250mg Iodacetamide + 10ml SDS Equilibration buffer

SDS Equilibration Agarose

1% Agarose + SDS Equilibration buffer + additional Bromphenol blue (darker blue)

Acetone or phenol extracted proteins from 500mg plant material were resuspended in 300µl Rehydration stock solution. Protein concentrations were compared on SDS PAGE gels. 127µl Rehydration stock solution with Bromphenol blue containing equal amounts of protein were applied to a 7cm Immobiline^{TM} DryStrip (GE Healthcare). The strip was covered with Silicon oil and incubated over night (Rehydration loading). Isoelectric focussing was

performed with a IPGphor from GE Healthcare according to the manufacturer's instructions. Then the strip was equilibrated in 10ml DTT SDS Equilibration buffer for 15min and in 10ml Iodacetamide SDS Equilibration buffer for another 15min. For separation in the second dimension 12% SDS PAGE gels were used.

2.3.2. Affinity purification of bZI63 binding proteins

For affinity purification of proteins binding to bZIP63 GST tagged bZIP63 was expressed and coupled to a GSTrap^{TM} column. Whole protein extracts were loaded on this column. Wash and elution of bZIP63 binding proteins was performed with rising concentrations of NaCl.

GSTrap^{TM} FF columns 1ml (GE Healthcare)
Columns were stored at 4℃ in 20% EtOH. Before use they were washed with 5ml ddH2O and equilibrated with 5 – 10ml cold GST binding buffer. Alternatively columns were washed with 5ml 70% EtOH and 5 – 10ml ddH2O before equilibration to remove hydrophobically bound substances.

BioRad Econo Pump
protein extracts were loaded very slowly (~ 0.1ml/min), washing and elution at ~ 0.2 – 0.5ml/min

1x GST binding buffer
50mM TrisCl pH = 8, 20mM MgSO4, 2mM DTT, 5mM Na2EDTA
alternatively ¼ tablet/50ml Complete Mini EDTA–free Protease inhibitor (Roche) and 10µl β–Mercaptoethanol were added before use

Glutathione elution buffer (GEB)
10mM reduced Glutathione, 50mM TrisCl pH = 8

Lacus buffer = protein extraction buffer
see "Media and Buffers"

GST–bZIP63 was expressed in *E. coli* like described in "Purification of GST–tagged proteins". The cells were harvested by 30min centrifugation at 2500g at 4℃ and the pellet was resuspended in 5ml or 10ml ice cold GST binding buffer per 100ml cell culture and sonicated 2 times for 30sec. Tween20 was added to a final concentration of ~ 0,1% and the cell debris pelleted by 15min centrifugation at 13000 – 15000rcf at 4℃. The supernatant was filtered through a 0.45µm filter before loading on the column.

Protein extracts were prepared from leaves, seedlings or root cell culture with Lacus buffer according to the quick method and filter sterilized.

4 – 5ml of the *E. coli* protein extract containing GST tagged bZIP63 were loaded slowly onto the column and the column was washed with 5ml GST binding buffer. Alternatively the column was then washed with 5ml GST binding buffer containing 0.5M NaCl and 5ml GST binding buffer without NaCl to disassemble and remove bZi63 homodimers. Then the column was equilibrated with 5ml Lacus buffer and 2 – 5ml of the plant protein extract was loaded. The column was washed and eluted with Lacus buffer containing 0.075 – 1M NaCl. Finally the column was eluted with Gluthathion elution buffer and washed with dH_2O and 20% EtOH.

The elutions were then concentrated using Amicon® Ultra Centrifugal Filter Devices (10.000Da) (Millipore).

2.3.3. Buffer exchange with PD-10 desalting columns

PD-10 Desalting Columns (GE Healthcare)

The column was equilibrated with 20ml target buffer, 2.5ml protein solution were loaded and proteins were eluted with 3.5ml target buffer.

2.3.4. Dephosphorylation of protein extracts with λ Phosphatase

λ Protein Phosphatase (NEB)

λ Protein Phosphatase buffer (NEB)
400µl 10x λ PP buffer (NEB) + 400µl 10x $MnCl_2$ (NEB) + 3.2ml dH_2O

Proteins from "3ml" grinded leaf material were extracted with 4ml λ Protein Phosphatase buffer, the extract spinned and filtered through Miracloth. To one half of the extract 24µl λ Protein Phosphatase were added. Both mixes were incubated at 15℃ for 30min. Proteins were extracted with phenol.

2.3.5. Determination of protein concentrations via Bradford assay

Bradford reagens
BioRad Protein Assay diluted 1:5 with ddH_2O

950µl Bradford reagens were mixed with 50µl protein solution (suitable dilution) or buffer as a blank and incubated for 5 – 15min at room temperature. OD_{595} was measured in plastic cuvettes and compared to a standard row of BSA with 500, 300, 200, 100, 50, 25 and 12.5µg/ml measured under the same conditions.

2.3.6. Immunprecipitation

Anti–GFP (Roche)
anti–GFP IgG monoclonal antibody (Cat. No. 11 814 460 001)
dissolved in ddH_2O to reach a final concentration of 0.4mg/ml

Protein A Sepharose^{TM} CL–4B (GE Healthcare)
Dry beads were soaked in 1ml cold Lysis buffer for 10min,centrifuged at 5000rpm for 30sec without brake. The supernatant was removed and the beads taken up in 1ml cold Lysis buffer.
Beadvolume after quelling: 50µl/8µg Anti–GFP

Lysis buffer:
modified Lacus buffer with EDTA and 150mM NaCl:
25mM TrisCl pH = 7.8, 10mM MgCl2, 15mM EDTA, 150mM NaCl, 1mM DTT, 1mM NaF, 0.5mM Na3VO4, 15mM β-glycerophosphate, 0.1%Tween–20, Complete Mini EDTA–free Protease inhibitor (Roche) tablette (2/50ml)

Wash buffer
50mM TrisCl pH = 7.5, 250mM NaCl, 0.1% NP–40, 0.05% Na deoxycholate

Immunprecipitation was basically performed as recommended by Roche.

Protein extracts from "15 – 20ml" grinded plant material were done with the quick method using cold Lysis buffer. 100µl Protein A Sepharose beads in 1ml Lysis buffer were mixed with 16µg (= 40µl) Anti–GFP (Roche) in a 1.5ml reaction tube and incubated on a rotor wheel at 4°C for 2.5h. The beads were transferred to a 15ml greiner tube and spinned down for 1min at 4000rcf at 4°C without brake. The supernatant wa s removed and the beads resuspended in 10ml protein extract and incubated on the rotor wheel for another 60 – 70min. Then the beads were washed two times with Lysis buffer and two times with Wash buffer by spinning down the pellets, resuspending in 8ml buffer and rotating for 8min at 4°C. Finally the beads were taken up in 100µl 2x SDS Sample buffer and boiled for 5min before loading on a PAGE gel.

2 control samples were done. 8µg Anti–GFP (Roche) probe were coupled to 50µl Protein A Sepharose and incubated with 1ml Lysis buffer instead of protein extract to identify the antibody bands on the PAGE gel. 50µl Protein A Sepharose not coupled to Anti–GFP (Roche) probe were incubated with 5ml protein extract to identify proteins binding to the Sepharose instead of the probe.

2.3.7. In-gel kinase assay

In gel kinase assays were performed similar as described in basic protocol 6 from "Assays of protein kinases using exogenous substrates." (Carter, 2001) and basic protocol 3 from "The detection of MAPK signaling." (Shaul and Seger, 2006).

[γ^{32}P] ATP (DuPont)

Storage Phosphor Screen (GE Healthcare)
00314338aa; 20x 25cm
exposed to light for 15 – 30min before use

Typhoon 8600 Variable Mode Imager (Amersham Biosciences)

4x SDS loading buffer
40% (v/v) glycerol, 8% (w/v) SDS, 8% (v/v) β–mercaptoethanole, 200mM TrisCl pH = 6.5, 0.2% bromphenol blue

Wash buffer I
50mM TrisCl pH = 8, 20% Isopropanol

Wash buffer II
50mM TrisCl pH = 8, 1mM DTT

Denaturation buffer
50mM TrisCl pH = 8, 1mM DTT, 6M Guanidinium HCl

Renaturation buffer
50mM TrisCl pH = 8, 0.05% Tween 20

Kinase buffer
20mM HEPES pH = 7.5, 20mM MgCl2, 50µM CaCl2, 1mM DTT, 0.05% Tween 20

Kinase reaction solution
20ml Kinase buffer + 50µM ATP (total ATP amount) + 100µCi [γ32P] ATP

<u>Wash buffer III</u>

5% TCA

<u>Wash buffer IV</u>

5% TCA, 1% sodium pyrophosphate

<u>Protein extracts</u>:

Simple protein extracts were prepared either by extraction with Acetone or with Lacus buffer. Affinity purified samples were prepared as described in "Affinity purification of bZIP63 binding proteins". Protein concentration in the extracts was determined with Bradford assay and comparison of the samples by SDS PAGE. A 4x SDS loading buffer was used to avoid diluting the protein extracts too much. Samples were not boiled before loading. Up to 35µg protein extract were applied.

<u>Gel preparation and run:</u>

0.75mm mini SDS polyacrylamide gels (BioRad MiniProtean[®] 3 System) with 1mg/ml substrate (recombinant bZip63.2) directly polymerized into the gel or without substrate were prepared according to one of the recipes descibed below.

Gels were loaded and run at 4°C at low voltage (20V per gel) to avoid heating of the gel.

<u>Gel recipe 1 (used for the first gels)</u>

4ml 12% separating gel:

1mg/ml substrate

1ml 1.5M TrisCl pH = 8.8

1.7ml 30% acrylamide 0.8% bisacrylamide

ad H_2O to get a final volume of 4ml

50µl 10% APS

3µl TEMED

2.5ml 3% stacking gel:

1.85ml H_2O

312.5µl 0.5M TrisCl pH = 6.8

275µl 30% acrylamide 0.8% bisacrylamide

60µl 10% APS

2.5µl TEMED

Gel recipe 2 (used for later gels)

4ml 12% separating gel:

1mg/ml substrate

2ml separating gel buffer (1.5M TrisCl pH = 8.85, 0.1% SDS)

1.6ml 30% acrylamide 0.8% bisacrylamide

ad H_2O to get a final volume of 4ml

37.5µl 10% APS

8µl TEMED

1.5ml 4% stacking gel:

0.5ml H_2O

0.75ml stacking gel buffer (0.5M TrisCl pH = 6.8, 0.1% SDS)

0.2ml 30% acrylamide 0.8% bisacrylamide

10µl 10% APS

3µl TEMED

Wash/denaturation/renaturation:

The gel was incubated 3x 20min in Wash buffer I, shaking moderately to remove SDS from the gel, 3x 20min in Wash buffer II to remove the Isopropanol and 3x 20min in Denaturation buffer at room temperature. To renature the proteins the gel was incubated in Renaturation buffer at 4°C over a period of 12 – 18h changing th e buffer 10 times after at least 30min. This step was performed over night.

Kinase assay:

The gel was incubated 2x 30min in Kinase buffer, shaking moderately at room temperature and then incubated in 20ml Kinase reaction solution for 30min. Then the gel was washed 2x for 15min with Wash buffer III and several times with Wash buffer IV until the wash buffer contained only very little radioactivity. Gels were dried on a slab gel drier and put on a Phosphor Screen over night. Alternatively gels were stained with Coomassie staining solution and destained (see SDS PAGE) before drying. Signals were recorded using a Typhoon 8600 imager.

2.3.8. Mass spectrometry

Mass spectrometric measurements (MS/MS) were performed at the MFPL Mass Spectrometry Facility in collaboration with Edina Czasar and Sonja Kolar. Samples were prepared as described in "Sample preparation and In–gel digest of proteins for Mass Spectrometry analysis".

2.3.9. Protein extraction from plants (*Arabidopsis thaliana*) – Acetone method

β–mercaptoethanol acetone (β–MeAc)
50ml acetone + 35µl β–mercaptoethanol

For purposes not requiring a maximum purity of the protein extract, such as for Western blots, the acetone extraction method was used.

Fresh plant leaf material frozen in liquid nitrogen or plant material stored at –80℃ was grinded into a fine powder using a spatula or a mortar. 100 – 400mg were transferred into a tube and mixed with 1 – 1.6ml β–MeAc. The suspension was sonicated briefly followed by 10min centrifugation at top speed at 4℃ in a desk centrifuge. The supernatant was removed and 1ml β–MeAc was added and mixed with the plant material by vortexing for 30sec – 1min. Centrifugation, removal of supernatant and vortexing was repeated until the pellet was completely white and the supernatant didn't turn green anymore. When the plant material was grinded very well or the sonication step was considered to be harmful sonication was substituted by vortexing for 30min at 4℃. Also the other sonication steps were longer in this case. The plant cell pellet was then dried in a speed vac for 15 – 30min and could be stored at room temperature, 4℃ or –20℃. When the protein extract was used for Western blotting it was taken up in a suitable amount (50 – 200µl) 1x SDS sample buffer and vortexed at 95℃ for 5min. Samples were centrifuged for 5min before every use and stored at –20℃

2.3.10. Protein extraction from plants (*Arabidopsis thaliana*) – Phenol method

Phenol (pH = 7.5)

PEX = Phenol extraction buffer (pH = 7.5)
0.7M sucrose
0.1M KCl
0.5M TrisCl pH = 7.5

50mM EDTA

10µl β–mercaptoethanol per 50ml added before use

<u>Ammonium acetate</u>

100mM Ammonium acetate in MetOH, −20℃

Proteins were extracted from liquid solutions by addition of an equal volume of phenol, followed by vortexing for 1min and centrifugation for 10min at 3500rcf. The upper, aqueous phase was removed and an equal volume of PEX was added to the phenol phase. After vortexing and centrifugation the upper, phenol phase was transferred to a new tube and extraction with PEX repeated 2 times. Proteins were precipitated over night at −20℃ by addition of 5x volume of cold Ammonium acetate in MetOH. Proteins were pelleted by 5min centrifugation at 16100rcf and washed MetOH and 2 times with Acetone (+β–mercaptoethanol). The pellet was dried in the SpeedVac.

2.3.11.   Protein extraction from plants (*Arabidopsis thaliana*) – quick method (Lacus buffer)

Protein extracts that were not used in western blots were mostly done with Lacus buffer. Plant material frozen in liquid nitrogen was grinded together with sea sand + PVPP using a mortar and the resulting powder mixed with at least an equal volume of cold Lacus buffer. The suspension was vortexed for 1min and centrifuged for 5 – 15min at up to 13000rcf at 4℃. The supernatant containing the soluble protein s was then used in the experiments.

2.3.12.   Protein precipitation with MeOH

Proteins were precipitated by mixing up to 200µl protein suspension with 1ml MeOH, 1M NH4Ac and incubating at -20℃ for at least 30min. T he proteins were then pelleted by 10min centrifugation, the supernatant removed and the pellet was resuspended in the desirable buffer.

2.3.13.   Purification of GST-tagged proteins

GST–tagged proteins were isolated using either Glutathione Sepharose beads or GSTrap columns.

<u>Glutathione Sepharose^{TM}4B (GE Healthcare)</u>

150µl of the bead suspension solution was centrifuged for 5min at 200 – 400rcf at 4°C, the supernatant removed and the beads washed twice with 1ml GST binding buffer and finally resuspended in 500µl cold GST binding buffer.

<u>GSTrap^{TM} FF columns 1ml (GE Healthcare)</u>

Columns were stored at 4°C in 20% EtOH. Before use they were washed with 5ml ddH2O and equilibrated with 5 – 10ml cold GST binding buffer. Alternatively columns were washed with 5ml 70% EtOH and 5 – 10ml ddH2O before equilibration to remove hydrophobically bound substances.

<u>BioRad Econo Pump</u>

protein extracts were loaded very slowly (~ 0.1ml/min), washing and elution at ~ 0.2 – 0.5ml/min

<u>1x GST binding buffer</u>

50mM TrisCl pH = 8, 20mM MgSO4, 2mM DTT, 5mM Na2EDTA

alternatively ¼ tablet/50ml Complete Mini EDTA–free Protease inhibitor (Roche) and 10µl β–Mercaptoethanol were added before use

<u>Glutathione elution buffer (GEB)</u>

10mM reduced Glutathione, 50mM TrisCl pH = 8

A single colony or a small amount of a glycerol stock containing *E. coli* with the pGEX vector with the desired construct was inoculated in LB containing the appropriate antibiotics and grown over night at 37°C. This preculture then was diluted to OD_{600} = 0.1 – 0.2 in 200ml selective LB and grown further until the OD_{600} was ~ 0.6. Expression was induced by addition of IPTG (Isopropyl-β-D-thiogalactopyranosid) to reach a final concentration of 1mM and the culture was further incubated at 30°C for 3 – 4h. A small aliquot of 1ml was taken before induction and after the end of the incubation to control expression efficiency. These were centrifuged for 30sec, the pellet was resuspended in 1x SDS loading buffer boiled for 5min and loaded on a SDS PAGE gel. The rest of the culture was cooled down immediately and the cells were harvested by 30min centrifugation at 2500rcf at 4°C. The supernatant was discarded and the pellet used for further purification immediately or frozen at –20°C for later use. The pellet was then resuspended in 10 – 20m ice cold GST binding buffer and sonicated 3x for 30sec at 100% intensity. Tween 20 was added to a final concentration of 0.1 – 0.5% and the cell debris pelleted by 15 – 30min centrifugation at 13000 – 17000rcf at 4°C.

<u>Glutathione Sepharose beads:</u>

For purification with the Gutathione Sepharose beads the supernatant was transferred to a greiner tube, 200µl of the Glutathione Sepharose bead suspension was added and the suspension then was incubated for 30 – 60min an a boogie wheel at 4°C. Then the sepharose was pelleted by 2 – 5min centrifugation at 400rcf at 4°C and washed 2 times with 10ml cold GST binding buffer before transferring into a small tube and spinning down in a swing out centrifuge without brake to remove the supernatant completely. To elute bound proteins 100µl cold GEB were mixed with the beads and incubated on ice for at least 5min. The suspension was centrifuged and the supernatant collected. Another 100µl were mixed with the beads and incubated on ice for a longer time period before proceeding like with the first elution. Purification efficiency was analysed by SDS PAGE. Elutions were mixed with glycerol to a final concentration of 20 – 25% and stored at –80°C.

<u>GSTrap FF columns:</u>

For purification with the GSTrap columns the supernatant wassterile filtered or sucked through a 0.45µm membrane filter to remove cell debris which would plug the column. The supernatant was then applied slowly (0.1 – 0.2ml/min) to the equilibrated column and the column was washed with 5 – 10ml GST binding buffer (1 – 2ml/min). Bound proteins were eluted with 5 – 10ml GEB (1 – 2ml/min) and the purification efficiency was analysed by SDS PAGE. Elutions were mixed with glycerol to a final concentration of 20 – 25% and stored at –80°C.

2.3.14. Purification of His–tagged proteins with a HiTrap™ Chelating column (Amersham) under denaturing conditions

<u>1ml HiTrap™ cheating column (Amersham)</u>
columns were stored and washed like described below

<u>BioRad Econo Pump</u>
loading, washing and elution was performed with a flow rate of ~ 1ml/min if possible; when the pressure was too high flow rate was decreased

<u>Binding buffer</u>
0.5M NaCl, 5mM imidazole, 1mM beta–mercaptoethanole, 6M guanidine hydrochloride, 20mM phosphate, pH = 8

<u>Elution buffer</u>
= wash buffer + 20 – 500mM imidazole

<u>Stripping buffer</u>

= wash buffer + 50mM EDTA

<u>Wash buffer</u>

0.5M NaCl, 5mM imidazole, 1mM beta–mercaptoethanole, 8M urea, 20mM phosphate, pH = 8

All buffers (and later also the protein extract) were filtered through a 0.45µm membrane filter to prevent clogging of the column.

This method was used to purify proteins at large scale which could not be purified under native conditions due to the formation of inclusion bodies.

6xHis–tagged proteins were expressed in *E. coli* using different expression vectors. A single colony was inoculated in LB containing the appropriate antibiotics and grown over night at 37℃. This preculture then was diluted to $OD_{600} = 0.1 – 0.2$ and grown further at 37℃ until the OD_{600} was ~ 0.6. Expression was induced by addition of IPTG (Isopropyl-β-D-thiogalactopyranosid) to reach a final concentration of 1mM in the culture and further incubation at 37℃ for 2 – 4h. A small aliquot of 1 ml was taken before induction and after the end of the incubation to control expression efficiency. These were centrifuged for 30sec, the pellet was resuspended in 1x SDS loading buffer boiled for 5min and loaded on a SDS PAGE gel. The rest of the culture was cooled down immediately and the cells were harvested by 30min centrifugation at 2500rcf at 4℃. The supernatant was discarded and the pellet frozen at –20℃ for later use.

The cell pellet was then resuspended in 5ml/100ml culture Binding buffer containing 20mM imidazole and sonicated 3x 10sec to lysate the cells and shear the DNA. The cell debris was pelleted by 20min centrifugation at 25000rcf at 4℃. Alternatively smaller amounts were also pelleted with the table centrifuge at top speed and room temperature for 5 – 10min. The supernatant was then filtered through a 0.45µm membrane filter before application on the column.

Columns were long–term stored in 20% EtOH at 4℃. Before loading the protein extract they had to be washed with ddH$_2$O and equilibrated. When a column was expected to be clogged or contaminated from a previous purification run the Ni^{2+} was stripped from the chelating groups with 5ml stripping buffer. The column then was washed with 10ml ddH$_2$O. Alternatively the column was washed with 5ml 1M NaOH to remove precipitated proteins. Here it is crucial that no Ni^{2+} is on the column anymore which would immediately form precipitates otherwise. Afterwards the column was washed with ddH$_2$O or Wash buffer until the flowthrough had pH = 7 – 7.5. Then the column was reloaded using 2 – 5ml 0.1M NiSO$_4$

and washed again with 5ml ddH$_2$O. The column was equilibrated with 5ml Binding buffer containing 20mM inimdazole. Then the protein extract was loaded and the column washed with 5ml Binding buffer (20mM imidazole) and 5ml Wash buffer containing 20mM imidazole. The amount of imidazole needed for elution has to be tested for each protein. It was found that the following conditions were suitable for 6xHis–bZip63.2: To wash off proteins weakly binding to the column another wash step with 5ml Wash buffer containing 40mM imidazole was performed. The His–tagged protein was eluted with Wash buffer containing 150mM imidazole. To remove remaining proteins from the column an elution step with 500mM imidazole was included followed by washing with ddH$_2$O and 20% EtOH for storage of the column. Elutions were collected in 1 – 1.5ml aliquots and analysed on a SDS PAGE.

The elutions containing protein were then concentrated using Amicon® Ultra Centrifugal Filter Devices (10.000Da) (Millipore) and the protein was stored at –80℃ in 20% glycerol.

2.3.15.   Purification of proteins with the pTWIN system (NEB)

Chitin Sepharose
NEB chitin beads

Chitin Columns
Pierce Perbio (Rockford, IL, USA) 2ml polystyrene columns

10kDa filter tubes
Amicon Ultra MW 10.000 MWCO Centrifugal Filter Devices (Millipore, Billerica, MA, USA)

Buffer B1
20mM TrisCl pH= 8.5, 1M NaCl, 1mM Na2EDTA (optional), 1mM β–mercaptoehanole (for purification with pTwin vector)

Buffer B2 (for N-terminal fusions; pTwin vector)
20mM TrisCl pH = 7, 1M NaCl, 500µM Na2EDTA (optional)

Buffer B3 (for C–terminal fusions; pTxb3 vector)
20mM TrisCl pH = 8.5, 1M NaCl, 500µM Na2EDTA (optional), 40mM DTT

Kinase buffer + DTT
20mM Hepes pH = 7.5, 50mM MgCl2, 1mM DTT

pTxb3 was used to express and purify bZip63 under native conditions. This vector can be used to express proteins with a C–terminal Intein–tag for purification with Chitin Sepharose.

To prepare the Chitin Sepharose column 6ml Chitin Sepharose suspension per 200ml *E. coli* culture were transferred to a 50ml greiner tube and the tube was filled up to 50ml with buffer B1. The beads were pelleted by 1min centrifugation at 1500rpm at 4°C. Brakes were turned off at 350rpm to prevent resuspension of the pellet during the braking process. The supernatant was removed, the beads resuspended in 40ml B1 and centrifuged again. Again the supernatant was removed and the beads were ready for sample application.

A single colony or a tiny amount of a glycerol stock of *E. coli* BL21 (ER2566) containing the expression vector was inoculated in LB containing Ampicillin and grown over night at 37°C. The culture was diluted to $OD_{600} = 0.1 - 0.2$ in a final volume of $200 - 250$ml and grown to $OD_{600} = 0.5 - 0.7$. Expression was induced by adding IPTG to a final concentration of 0.4mM and shaking at 16°C over night. After some hours of induction 50% of the previously used amount Ampicillin was added to the culture to replace the degraded antibiotic and improve expression efficiency. Samples were taken before induction and after the end of the incubation time to control expression efficiency. These were centrifuged for 30sec, the pellet was resuspended in 1x SDS loading buffer boiled for 5min and loaded on a SDS PAGE gel. The rest of the culture was cooled down immediately and the cells were harvested by 20min centrifugation at 1500rcf at 4°C. The supernatant was discarded and the pellet could be used immediately or frozen at –20°C for later use.

All following steps were performed at 4°C. The cell –pellet was resuspended in 10ml cold B1 and sonicated 3 times for 30sec with 100% intensity. 0.1% Tween 20 (Sigma) was added to the crude cell extract, which was centrifuged again for 20min at 1500rcf to separate the cell debris and insoluble components from the soluble proteins. The supernatant was applied to the equilibrated Chitin Sepharose beads and rotated on a boogie wheel for 30min. Then the greiner tube was filled to 50ml with B1 and the beads were washed 3 times by 1min centrifugation at 1500rcf, removal of the supernatant and resuspension in fresh B1. Finally the beads were resuspended in 6ml B1 and applied to 2 columns (3ml per column) and the packed columns were washed with 3 column volumes of B1. 3 column volumes B3 were applied to the column and the flow was stopped by closing the column at bottom and top. The column was transferred to room temperature and incubated over night. Then proteins were eluted with 2 volumes of buffer B3. The flowthrough was collected directly in 10kDa filter tubes. After another hour incubation at room temperature remaining proteins were eluted with another 2ml B3 without DTT. Proteins in the elution were concentrated by centrifugation at 4000rcf at 4°C until the volume was reduced to $0.5 - 1$ml. The buffer was exchanged for kinase buffer by filling up the tube with 4ml kinase buffer and centrifugation to reduce the volume several times. Finally the volume was reduced to $200 - 500$µl and protein concentration was determined by Bradford assay. Glycerol was added to a final concentration of 10% and the protein suspension was stored at –80°C. Samples of 40µl were

taken at every step of the purification process and were loaded on a SDS PAGE together with a small aliquote of the final protein suspension to control purification efficiency.

2.3.16. Sample preparation and in-gel digest of proteins for Mass spectrometry analysis

Samples for Mass Spectrometry analysis from Immunprecipitation and In–gel kinase assay experiments were loaded on PAGE gels. Gels were prepared and run as described in "SDS PAGE (poly–acrylamide gel electrophoresis)" followed by Coomassie and/or Blum silver staining. Gels were placed between clean transparent foil for scanning. Interesting parts of the gel were cut out with a clean razor blade, transferred to a 1.5ml reaction tube and frozen in liquid nitrogen. The blade was rinsed with dH_2O between 2 samples. Frozen gel pieces were either stored at –80°C or further prepared according to the In–gel digest protocol.

In–gel digest of Proteins for Mass Spectrometry analysis:

0.6ml Gen–X–Press reaction tubes

50mM NH_4HCO_3
0.4g/100ml; prepared freshly every day

Acetonitrile
34967 Lo Riedl–de Haen
specific for LC–MS (CHROMASOL V)

Reduction buffer
10mM DTT in 50mM NH4HCO3 (Fluka)

Iodacetamide solution
0.01g/ml in 50mM NH4HCO3
light sensitive; prepared just before use and stored in the dark, prevents Cys oxidation

Trypsin (Roche, 03708985001) solution
1µg Trypsin/4µl 1mM HCl; stored at –80°C

Trypsin (Roche, 03708985001) dilution
4µl Trypsin solution + 96µl 50mM NH4HCO3

Subtilisin (Fluca, 85968-25MG) solution:
10µg/µl in 1mM HCl; prepare fresh every time (ca be used for 2 – 3d only)

<u>Subtilisin (Fluca, 85968-25MG) dilution:</u>

dilute Subtilisin solution 1:100 with Urea:Tris (10:1)

Urea:Tris = 9ml 6M Urea + 1ml 1M TrisCl pH = 8.5

<u>6M Urea</u>

Amresco, purity > 99.5%

<u>1M TrisCl pH = 8.5</u>

Amresco, purity >99.5%

<u>10% HCOOH</u>

Merck, Suprapure

All steps were performed under a flow hood if possible. The bench and utensils were cleaned with dH_2O and pure EtOH before use. Clean tips were used.

<u>Preparation of the gel for enzymatic digest:</u>

The gel piece was thawed and placed on a glass slide with 150µl dH2O and cut in smaller pieces of ~ 1x1mm using a razor blade. The pieces were then transferred to a 0.6ml reaction tube with 150µl dH2O. Glass plate, razor and forceps were cleaned with 50% pure EtOH before proceeding with the next sample. The gels were washed 3 times with dH2O by shaking at room temperature for 10min, brief spinning, removal of the liquid and addition of 200µl fresh dH2O. In case the gels were stained with Coomassie a destaining procedure was included: The liquid was removed and 200µl 50mM NH4HCO3 and 160µl Acetonitrile were added and after brief vortexing the gels were incubated on a shaker for 15min. Then the liquid was removed and the gel pieces were mixed with 160µl Acetonitrile for 5min, followed by removal of the liquid and drying in a vacuum centrifuge for 5 – 15min. In the reduction step the dried gel pieces were mixed with 200µl reduction buffer and shaken at 56℃ for 30 – 60min. After brief spinning the reduction buffer was removed and 200µl Acetonitrile were added. After 5min shaking the Acetonitrile was removed again, the gel pieces were taken up in 100µl Iodacetamide solution and incubated in the dark for 20min. The Iodacetamide solution was removed and the gel pieces were washed 3 times by shaking in 200µl 50mM NH4HCO3. Then the 50mM NH4HCO3 was removed and the gel pieces mixed in 200µl Acetonitrile for 5min, followed again by removal of the liquid and drying in a vacuum centrifuge for 5 – 15min. After this step the gel pieces were either used for enzymatic digest immediately or stored at –80℃ for later use.

<u>Trypsin digest:</u>

Aliquots of the Trypsin solution were thawed on ice and diluted to 10ng/µl (Trypsin dilution). The gel pieces were covered with 20 – 60µl of the Trypsin dilution and incubated at 4℃ for

10min for quelling. When the gel was not covered completely more Trypsin dilution was added and the quelling step was repeated. Then the Trypsin dilution was removed, the gel covered with 50mM NH4HCO3 and incubated at 37℃ ove r night.

<u>Subtilisin digest:</u>
The gel pieces were covered with 20 – 60µl of the Subtilisin dilution and incubated at 4℃ for 10min for quelling. When the gel was not covered completely more Subtilisin dilution was added and the quelling step was repeated. Then the Subtilisin dilution was removed, the gel covered with 50mM NH4HCO3 and mixed with 300rcf for 30min – 2h.

<u>Stop of the digest and peptide extraction:</u>
The enzymatic digest was stopped by addition of 10% HCOOC to reach a final concentration of 1% in the solution. The samples were sonicated in a sonication bath for 5 – 10min. Then the liquid was transferred to a 200µl reaction tube using gel loading tips to prevent contamination with gel pieces. The gel was covered with ~ 20µl 5% HCOOH, sonicated for 10min and the liquid transferred to the reaction tube again. The last extraction step was repeated one more time. The extract was centrifuged for 1 min at 16000rcf and the liquid transferred to a new reaction tube to remove any contamination from the gel. Samples were stored at 4℃ or –20℃ until measurement.

2.3.17.   SDS PAGE (poly-acrylamide gel electrophoresis)

<u>Acrylamide</u>
30% 37.5:1 Acrylamide:Bisacrylamaide (BioRad)

<u>seperating gel buffer</u>
1.5M TrisCl pH = 8.85, 0.1% SDS

<u>stacking gel buffer</u>
0.5M TrisCl pH = 6.8, 0.1% SDS

<u>mini gels</u> (BioRad MiniProtean® 3 System)
amounts indicated are for 4 mini gels (0.75mm)

	12% separating gel	**4% stacking gel**
30% acrylamide	6ml	0.8ml
ddH$_2$O	1.36ml	2.15ml
2x buffer	7.5ml	3ml
TEMED	30µl	12µl
10% APS	110µl	42µl
total	15ml	6ml

<u>10x SDS running buffer</u>
50g SDS
151.1g Tris base
720.5g glycine
ad 5l with ddH2O

<u>2x SDS sample buffer</u>
50% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β–mercaptoethanole, 125mM TrisCl pH = 6.5, 0.05% bromphenol blue

<u>Protein molecular weight marker</u>
PageRuler™ Prestained Protein Ladder #SM0671 (Fermentas)

<u>Coomassie staining solution</u>
2.5g coomassie brilliant blue R–250/G–250 (R:G = 4:1), 100ml isopropanole, 100ml acetic acid, dissolve coomassie completely by heating and ad to 1l with ddH2O

<u>Destaining solution</u>
10% isopropanole, 10% acetic acid

Samples were prepared by mixing with SDS sample buffer, heating to 95℃ for 2 – 5min and centrifugation at 13000g for 5min. Gels were placed in the gel running chamber and covered with 1x SDS running buffer; slots were washed with running buffer. 3μl protein marker as well as the desired amount of the samples were loaded into the slots. The gels were run at 20mA/gel until the coloured running front was in the separating gel and then current intensity was raised to 40mA/gel. Gels were stained by incubation in hot Coomassie staining solution for about 5 – 10min at a shaker. Then staining solution was exchanged for hot destaining solution and the gel was incubated until the background of the gel was white changing the destaining solution several times. The destained gels were dried on a slab gel dryer at 65 – 70℃ for 1.5 – 2h.

2.3.18. Silver staining of SDS gels for Mass spectrometry

Original protocol: (Shevchenko et al., 1996)

<u>Fixing solution</u>
50% MeOH, 5% Acetic acid

<u>Washing solution</u>
50% MeOH

<u>Sensitizing solution (prepare freshly)</u>
0.3g/l Na2S2O3 x 5H2O

<u>Silver solution (prepare freshly)</u>
1g/l AgNO3

<u>Developing solution (prepare freshly)</u>
20g/l Na2CO3 x 10H2O, 400µl/l 35% formaldehyde

<u>Stop solution</u>
5% Acetic acid

After gel run slab gels are fixed by 20min incubation in Fixing solution followed by 10min washing in Washing solution and 2h to over night washing in dH$_2$O. For gels that were already fixed in SDS destaining solution (10% Acetic acid, 10% Isopropanol) the Fixing and first wash step were omitted. Gels were washed in dH$_2$O by repeated steps of heating in the microwave followed by 10min incubation and change of dH$_2$O until the Acetic acid was removed from the gel completely. Then the gel was incubated in Sensitizing solution for 1min and washed with dH$_2$O two times for one minute. It was incubated in Silver solution for 20min at 4°C and washed again with dH$_2$O twice. To visualize the stain the gel was incubated for up to 10min in Developing solution and the reaction was stopped by changing the solution for Stop solution and incubating 3 times for 1min.

2.3.19. Size exclusion chromatography with superdex 75 column (FPLC)

FPLC was performed with the Pharmacia FPLC System (LKB Pump P–500, LKB Controller LCC–501 Plus, VWM 2141, FRAC–100) using a SuperDex 75 (Amersham Pharmacia/GE Healthcare) column according to the manufacturers instructions in order to separate proteins of different size.

All buffers and solutions applied to the column were filtered through a 0.45µm membrane filter. Before use the column was equilibrated with 2vol running buffer with a flow rate of 0.4ml/min. The sample was injected with 0.3ml/min and loaded with 0.4ml/min. 0.4ml fractions were collected and analysed on a SDS gel. After use the column was washed with 2vol 20% EtOH.

2.3.20. Western blot

Blotting aperture

Trans–Blot® SD Semi–Dry Transfer Cell (BioRad)

PVDF membrane

Millipore ImmobilonTM-P Transfer Membrane

Anode buffer I

0.3M TrisCl pH = 10.4, 10% methanol

Anode buffer II

25mM TrisCl pH = 10.4, 10% methanol

Cathode buffer

25mM TrisCl pH = 9.4, 40mM glycine, 10% methanol

Ponceau staining solution

2g Ponceau S

30g TCA

30g sulfosalicylic acid

ad 100ml ddH2O

10x TBS

500mM TrisCl pH = 7.4, 1.5M NaCl

1x(1.5x) TBS-T

100ml (150ml) 10x TBS + 1ml (1.5ml) Tween® 20 (Sigma) per 1l

Blocking solution (milk powder solution)

5% skim milk powder solution in 1x (1.5x) TBS–T

Antibody solutions

antibodies were diluted 1:500 – 1:5000 (depending on the antibody) in Blocking solution

Immunostaining reagents for detection of HRP

SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Prod #34080): SuperSignal® West Pico Stable Peroxide Solution + SuperSignal® West Pico Luminol/Enhancer Solution; a 1:1 mixture of the two solutions was prepared just before use

Exposure cassette

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

Fujifilm (Tokyo, Japan) Fuji medical X-ray films 100 NIF 13x18; 18x24

Coomassie staining solution for membranes

2.5g coomassie brilliant blue R–250/g–250 (R:G = 4:1), 100ml EtOH, 100ml acetic acid
dissolve coomassie completely by heating and ad to 1l with ddH2O

Samples and a protein molecular weight marker were loaded on an SDS gel and run as described in the protocol for SDS PAGE. After the run the gel was equilibrated by 5 – 10min incubation in Anode buffer II. The anode was moistened with Anode buffer I and 2 layers of 3mm whatman paper soaked in Anode buffer I followed by 2 layers of whatman paper soaked in Anode buffer II were placed on it. The PVDF membrane was moistened by shaking in methanol for 10sec and in ddH$_2$O for 5min and then equilibrated by shaking in Anode buffer II for 5 – 10min. It was then placed onto the whatman paper stack followed by the equilibrated acrylamide gel and 3 layers of whatman paper soaked in Cathode buffer. Air bubbles were removed by carefully rolling a centrifugation tube over the stack before covering with the cathode moistened with Cathode buffer. Proteins were blotted onto the membrane for 1.5h at 16V and 150mA for one mini gel.

Cathode
whatman paper in Cathode buffer
acrylamide gel
Immobilon–P membrane
whatman paper in Anode buffer II
whatman paper in Ande buffer I
Anode
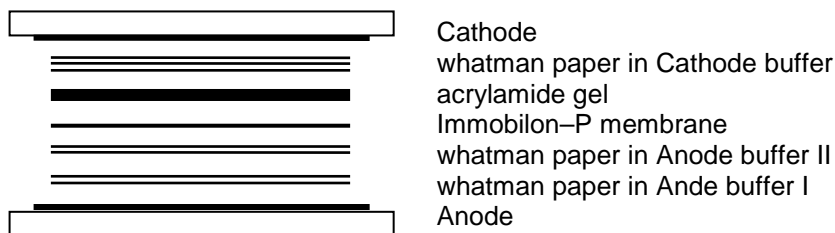
After the blotting process was finished the membrane was stained with Ponceau stain to control the transfer of the proteins and mark the positions of the lanes on the membrane with a pencil. The membrane was briefly incubated in Ponceau staining solution and rinsed with ddH$_2$O several times. To destain the membrane completely it was further incubated in TBS–T or ddH$_2$O. TBS–T mostly was used with 1.5x concentration instead of 1x. During the whole staining procedure one of the two concentrations was used exclusively, but never a mixture of both.

For immunostaining the membrane was blocked by 20min – 1h incubation in Blocking solution before incubating with the primary antibody solution over night at 4°C or 1.5h at room temperature. The antibody solution was removed and the membrane was washed by shaking up to 5min in TBS–T for 3 times and incubated with the secondary antibody solution for 45min – 1h at room temperature. Again the antibody solution was discarded and the membrane washed 3 times with TBS–T. Then the membrane was put on a stack of tissues to

remove the liquid and placed on a glass plate, removed and placed on the plate again until the membrane was just dry enough that it would not stick to the glass. It was covered with HRP detection solution, incubated for 5min and placed on tissues again to remove excess liquid. Then the membrane was positioned in an exposure cassette. An X–ray film was laid onto the membrane in the cassette, exposed and 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.

Membranes were then stained with membrane Coomassie staining solution and destained with destaining solution or a special destaining solution conaining 50% methanol and 10% acetic acid and dried and stored at room temperature.

2.4. Bacterial methods

2.4.1. Chemically competent *E. coli*

TB buffer (pH = 6.7)
10mM CaCl2
10mM Pipes-NaOH
15mM KCl2
55mM MnCl2 (adjust pH before adding MnCl2)
filter sterilize

A culture of the strain to be made competent was streaked on selective LB plates and grown over night at 37℃. 400ml LB with 20mM MgSO4were in oculated with 2 – 4 single colonies and grown to OD600 = 0.5 – 0.6 at room temperature. Then the culture was incubated on ice for 10 – 20min and centrifuged for 10min at 2000rpm at 4℃. The pellet was resuspended in 80ml TB buffer, incubated on ice for 20 – 30min and centrifuged again. The pellet was then resuspended in 16ml TB buffer with DMSO at a final concentration of 7%, incubated on ice for 1h and aliquots of 440µl were frozen on dry ice and stored at –80℃.

2.4.2. Transformation of chemically competent bacteria

DNA for transformation:
Retransformation of a plasmid: 2µl of a plasmid midi prep (1µg/µl)
Transformation of a ligation product: all 10µl of the reaction mix

Competent bacterial cells were stored with 25% glycerol at –80℃. Before transformation they were thawed slowly on ice and 50µl per reaction were transferred into a new tube. The

DNA was added and mixed with the cells by carefully tipping at the side of the tube and incubated on ice for 15min. Heat shock was performed by incubating at 42°C for 45'' – 1min. The tube was put back on ice and 800µl LB medium was added. Then the cells were shaken at 37°C for 1h and an appropriate amount was streak ed onto LB plates containing the according antibiotics. For streaking all of the cells onto the plate the cells were pelleted by brief centrifugation, the supernatant was poured off and the pellet resuspended in the remaining liquid. The LB plates were incubated at 37°C over night. Plates were then stored at 4°C.

2.4.3. Transformation of electrocometent bacteria via electroporation

Electroporation cuvettes (Cell projects), 2mm

GenePulser® (BioRad)
settings for 2mm cuvettes and 40 – 50µl cells:
restistor: 200Ω (up to 400Ω are possible)
capacity: 25µF
voltage: 2.5V

Competent cells were thawed on ice for 30min. Cuvettes were cooled on ice and 100 – 500ng (1 – 2µl of a plasmid mini prep) plasmid DNA were pipetted into the gap between the electrodes. 60µl competent cells were put in the gap carefully, the electrical pulse was set and 1ml LB medium was added. After pipetting up and down for 3 times the suspension was transferred to a 1.5ml reaction tube and incubated at 37°C for 1h shaking vigorously. Cells were then plates on selective LB and grown at 37°C over night.

2.5. Plant methods

2.5.1. Plant cell culture

Plant cell suspension culture and plant callus culture were grown at 22°C in the dark on a shaker in 1x MS medium for cell suspension culture. Medium was exchanged under sterile conditions every week in case of cell suspension culture and every second week in case of callus culture.

To exchange the medium the culture was transferred to a 50ml greiner tube and the cells were allowed to settle down. 20 – 30ml of the medium were removed leaving 15ml cell culture in the tube and fresh ell culture medium was added to reach a final volume of 40ml.

The new suspension culture was then transferred into a glass flask, which was covered with aluminium foil and sterilized at 200℃ for 2 – 3h b efore use, and tightly closed.

Cell culture was collected by filtering the culture through a 0.45μm membrane filter until the cell material was comparatively dry. Cells were either used immediately or stored at –80℃.

2.5.2. Plant cultivation on soil

Seeds sterilized with clorine gas were sown on ½ MS agar plates and sealed with Parafilm (Pechiney Plastic Packaging) and incubated at 4℃ i n the dark for at least 2 days before transferring them to a growth chamber with 12h/12h light/dark cycle. Less than 2 week–old seedlings were planted in soil, covered with a plastic hood and put into a long day or short day growth chamber. The hood was removed after some days. Alternatively to the germination on MS plates seeds were also sown on soil directly.

For seed production plants were grown in a long day chamber. Flowering shoots were covered with paper bags to make seed collection easier. Dried seeds were collected and sieved through a kitchen sieve to remove leaves and dried parts of the flower. Seeds were stored in small tubes at room temperature in the dark.

2.5.3. Plant cultivation in hydroponic culture

Seeds sterilized with clorine gas were sown on ½ MS agar plates and sealed with parafilm (Pechiney Plastic Packaging) and incubated at 4℃ i n the dark for at least 2 days before transferring them to a growth chamber with 12h/12h light/dark cycle. Some day–old seedlings were transferred to big PCR tubes filled with ½ MS agar and a cut off bottom by carefully pressing the root into the soft agar with forceps. Clean tip boxes without lid were filled with ½ Hoagland medium and covered with aluminium foil. The PCR tubes containing the seedlings were pushed through holes in the aluminium foil so that the bottom of the tube is in the medium. The boxes were put in a long or short day plant growth chamber and covered with transparent foil for several days before cutting it open and finally removing it. Hoagland medium was refilled when the level dropped too low.

2.5.4. Seed sterilisation with chlorine gas

Dry seeds in an opened small tube together with one beaker containing some potassium permanganate and another containing 100ml diluted (~ 20 – 50%) hydrochloride were put in a plastic box lined with green tissues. 30 – 50ml 5M HCl was added to both beakers and the

box was closed immediately. Seeds were incubated for ~ 2h until the green tissues turned yellow, the tubes were closed and seeds stored at room temperature. Only so much seeds as needed were sterilized as germination may be affected by the procedure.

2.6. Media and buffers

2.6.1. ½ Hoagland medium (for 1l)

2.5ml 1M $Ca(NO_3)_2$
1ml 1M $MgSO_4$
2.5ml 1M KNO_3
0.5ml 1M $KHPO_4$
0.5ml micronutrient stock solution
1.25ml 1.4g/l Fe-EDTA

2.6.2. Lacus buffer (protein extraction buffer)

25mM TrisCl pH = 7.8
10mM $MgCl_2$
15mM EGTA
75mM NaCl
1mM DTT or β-Mercaptoethanol
1mM NaF
0.5mM Na_3VO_4
15mM β-glycerophosphate
0.1%Tween–20
 0.25mM PMSF
 5μg/ml Leupeptin
 5 Aprotinine
 = Complete Mini EDTA–free Protease inhibitor (Roche) tablette (1/50ml)

store at –20°C in greiner tubes
add just before use:
complete tablette (PMSF has a very short half life!)
DTT or β-Mercaptoethanole

2.6.3. LB (Luria-Bertani) medium (for 1l)

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

2.6.4. 1x MS medium/MS agar

MS medium was used for agar plates and cell suspension or callus culture

<u>1x MS medium</u>
4.4g/l MS base + vitamins (Duchefa)
pH = 5.8 (with KOH)

<u>MS agar plates</u>
0.5x MS + 0.68 – 0.7% plant agar (Duchefa)
alternatively add: 30g/l sucrose; antibiotics

<u>1x MS for cell suspension culture</u>
1x MS + 30g/l sucrose + 1µg/ml 2,4D (~ 5µM)
filter sterilized

2.6.5. SDS sample buffer

<u>2x SDS sample buffer</u>

50% (v/v) glycerol	25ml
2% (w/v) SDS	10ml 10% stock (pH = 7.2 with HCl)
5% (v/v) β-mercaptoehanol	2.5ml
125mM Tris-Cl pH = 6.8	6.25ml 1M stock
0.05% bromphenol blue	9mg fill to 50ml with ddH2O

<u>4x SDS sample buffer</u>

40% (v/v) glycerol	8ml
8% (w/v) SDS	1.6g
8% (v/v) β-mercaptoehanol	1.6ml
200mM Tris-Cl pH = 6.8	4ml 1M stock
0.2% bromphenol blue	40mg fill to 50ml with ddH2O

2.6.6. SOB medium

20g Tryptone
5g Bacto-Yeast extract
0.5g NaCl
2.5ml 1M KCl
pH = 7
before use add 10ml sterile 1M $MgCl_2$

2.6.7. YEB medium (for 1l)

10g Tryptone
5g Bacto-Yeast extract
5g Peptone
0.5% sucrose
2mM MgSO4

2.7. Strains and lines

2.7.1. E. coli strains

E. coli strains were used for cloning (DH5α, ER2566) or protein expression (ER2566, BL21(DE3)pLysS)

DH5α   .F-, ø80dlacZDM15, D(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk-, mk+), phoA, supE44, l-, thi-1, gyrA96, relA1

BL21(DE3)pLysS   F-, ompT hsdSB (rB- mB-) gal dcm (DE3) pLysS (CamR)

ER 2566   fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10--TetS)2 [dcm] R(zgb-210::Tn10--TetS) endA1 Δ(mcrC-mrr)114::IS10

2.7.2. *Arabidopsis thaliana* lines

Col–0   Columbia wild type ecotype

cpk3-2   Col-0 *cpk3* knock–out, Kan[R], SALK_022862 (Alonso et al., 2003), obtained from *NASC*

Ws–2   Wassilewskija wild type ecotype

bzip63	Ws *bzip63* knock–out, Kan[R], created by the Wisconsin Arabidopsis Knockout Facility (Sussman et al., 2000), obtained from Jesús Vicente Carbajosa	
35S::*bZIP36*	Col-0 bZip63 over–expressor, obtained from Jesús Vicente Carbajosa	
35S::*bZIP63–GFP*	Col–0 bZip63–GFP over–expressor, Hyg[R], transformation with pH7FWG2.0, line #3 (T3 generation) obtained from Christina Chaban	
35S::*HA–bZIP63*	Col–0 HA–bZip63 over–expressor, line 1.1.1 and daughter line 1.1.1.5, obtained from Wolfgang Dröge–Laser	
bzip09	Col-0 *bzip9*, SALK_093416 (Alonso et al., 2003), obtained from Wolfgang Dröge-Laser	

2.8.   Vectors and PCR primers

2.8.1. Vectors

Expression vector for GST fusion proteins: pGEX–4T (GE Healthcare)

Expression vectors for Intein tagged fusion proteins: pTxb3, pTwin (NEB)

Expression vectors for His tagged fusion proteins:

        pET–DEST42 (Gateway[TM], Invitrogen)
        pDEST17 (Gateway[TM], Invitrogen)
        pRSetA (Invitrogen)
        pCoolH (pTxb3 backbone with pRSetA expression cassette; vector map see supplementary figure 3)

Vector for cloning: pCR Blunt (Invitrogen)

2.8.2. Primers

Primers were ordered from Microsynth. Sequencing was done by Microsynth or Agowa.

oligos used for sequencing, cloning and sqRT–PCR			
ID	name/description	used for	sequence from 5' to 3'
127	ACT3 forw	sqRT–PCR	ATGGTTAAGGCTGGTTTTGC
128	ACT3 rev	sqRT–PCR	AGCACAATACCGGTAGTACG

182	bZIP63 N–terminus	sequ. PCR	AACCATGGAAAAGTTTTCTCCGACGAAGAAATCTCC
183	bZIP63 C–terminus	sequ. PCR	TTGCGGCCGCCCTGATCCCCAACGCTTCGAATACG
703	bZIP63 RT-fwd	sqRT–PCR	CTCCGACGAAGAAATCTCCGGTAACC
704	bZIP63 RT-rev	sqRT–PCR	CTTTAACAGCTACTGATCCCCAACGC
829	bZIP63.2+3 RT fw	bZIP63.2	GGAACTTTCATCAAACCTCAGG
830	bZIP63.1 RT fw	bZIP63.1	CGCCATGAAAAGGGATACTTCTGG
831	bZIP63.1+2 RT rev	bZIP63.1 + 2	CAGCCATTTTCACCTTTGCTCG
834	pD991AP3-RB	sequ. PCR	TGGCGA ATGAGACCTCAATTGCGAGCTTT
835	pD991AP3-LB	sequ. PCR	CATTTTATAATAACGCTGCGGACATCTAC

2.9.   Antibodies

2.9.1. Primary antibodies

<u>Anti bZIP peptide antibodies</u>: polyclonal antibodies from rabbit (Davis biotechnology, Regensburg, Germany)

>   bZIP63–C1 (old): SLEHLQKRIRSVGDQ
>   bZIP63–C2 (new): EIISSGNKGKALIGCKMNRT
>   bZIP63–N: EKVFSDEEISGNHHWSVNGM
>   bZIP09 (new, E459): LGNVSSEAVSCVSDIWP

bZIP63 protein sequence:

```
  1 MEKVFSDEEI SGNHHWSVNG MTSLNRSASE WAFNRFIQES SAAADDGEST TACGVSVSSP
 61 PNVPVDSEEY RAFLKSKLNL ACAAVAMKRG TFIKPQDTSG RSDNGGANES EQASLASSKA
121 TPMMSSAITS GSELSGDEEE ADGETNMNPT NVKRVKRMLS NRESARRSRR RKQHALSELE
181 TQVSQLRVEN SKLMKGLTDV TQTFNDASVE NRVLKANIET LRAKVKMAEE TVKRLTGFNP
241 MFHNMPQIVS TVSLPSETSN SPDTTSSQVT TPEIISSGNK GKALIGCKMN RTASMRRVES
301 LEHLQKRIRS VGDQ*
```

bZIP09 protein sequence:

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

<u>Mouse anti GFP</u> (Roche)

<u>Rat anti HA</u> (Santa Cruz: HA–probe (153): sc–53516)

<u>Mouse anti HA</u> (from cell culture supernatant)

2.9.2. Secondary antibodies

Anti rabbit IgG HRP conjugated (GE Healthcare: NA934V)

Anti mouse IgG HRP conjugated (GE Healthcare: NA931V)

Anti rat IgG HRP conjugated (Jackson Immuno Research)

2.10. Programs and databases

2.10.1. Programs

Vector NTI Advance 9 (Invitrogen)

Adobe photoshop CS3

ImageJ 1.39u

2.10.2. Web-based programs, databases and tools

Pubmed/NCBI http://www.ncbi.nlm.nih.gov/pubmed/

TAIR (The *Arabidopsis* Information Resource) www.arabidopsis.org

Genevestigator https://www.genevestigator.ethz.ch/gv/index.jsp

EMBL–EBI (PICR - Protein Identifier Cross-Reference Service) http://www.ebi.ac.uk/Tools/picr/

ExPASy (Peptide cutter + domain search programs)http://www.expasy.ch/

Pfam domain search http://pfam.sanger.ac.uk/search?tab=searchSequenceBlock

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

PhosPhAt http://phosphat.mpimp-golm.mpg.de/

NetPhos2.0 http://www.cbs.dtu.dk/services/NetPhos/

Mascot (used by Mass spectrometry facility)

Sequest (used by Mass spectrometry facility)

3. Results

3.1.   Identification of Kinases phosphorylating bZIP63

Kinases phosphorylating bZIP63 specifically were identified with the help of in–gel kinase assays and Mass spectrometry.

In an in–gel kinase assay the protein for which kinases should be identified (substrate) is polymerized into a PAGE (poly acrylamide gel electrophoresis) gel. The protein extract, from which the kinase should be identified, is loaded on the gel and proteins are separated according to their MW. Then all proteins are denatured and renatured slowly in the gel to re-establish their native conformation. The gel is then incubated with $\gamma^{32}P$ ATP as a phosphodonor for kinases. In a gel without substrate, kinases can only phosphorylate themselves and proteins running at the same height in the gel. This results in weak background signals. In a "substrate–gel", where the kinase substrate is distributed equally throughout the gel, a strong signal is seen at the position of the kinase.
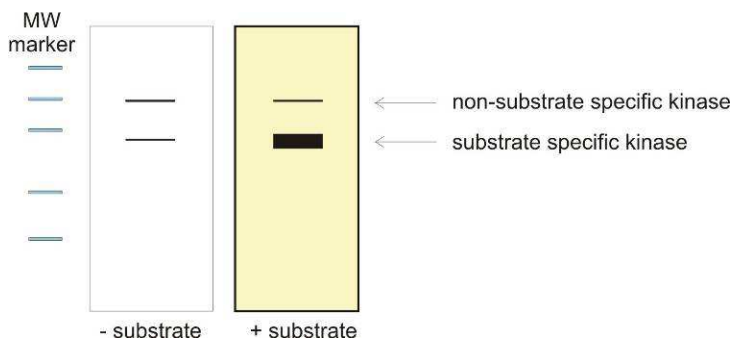

Fig 3.1 Scheme of an in–gel kinase assay readout: autophosphorylation signal (left) vs. substrate phosphorylation signal (right)

3.1.1. Recombinant bZIP63.2

As a substrate for the in–gel kinase assay splicing form 2 of bZIP63 was chosen. Splicing form 1 could easily be expressed and purified under native conditions with the pTwin system (NEB) (see fig 3.2). This was not possible with splicing form 2, which had to be purified under denaturing conditions.
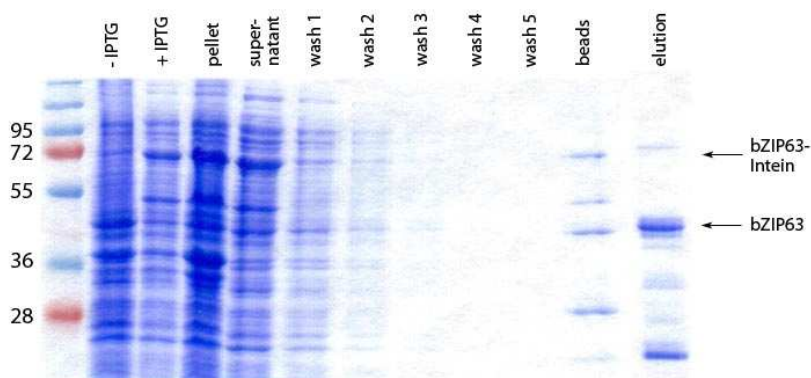

Fig 3.2 Induction of bZip63.1–Intein expression and purification. *BZip63.1* in pTxb3 was transformed into the *E. coli* expression strain ER2566 and expression was induced by addition of IPTG and incubation at 16℃ over night.

3.1.1.1. Native purification of bZIP63.2 with the pTwin system (NEB)

Expression of splicing form 2 was first tried with the same vector system as had been used for splicing form 1. For this, *bZIP63.2* had been cloned into pTxb3, which creates a C–terminal Intein tag. None of the clones tested showed an additional band at 63kDa from induction and a test–purification from one of the clones resulted in no recombinant bZIP63.2 (not shown).

To exclude the possibility that the expression vector was defective, *bZIP63.2* was cloned into the vector backbone of pTxb3–*bZIP09*, which had worked well previously. Additionally *bZIP63.2* was cloned into pTwin, which has an N–terminal Intein tag. As a control for the expression conditions pTxb3–*bZIP63.1* was used, which can easily be induced. A strong induced band could be seen for the positive control Intein–*bZIP63.1*, but again no strong induction could be observed for any of the clones tested from splicing form 2 (see fig 3.3).
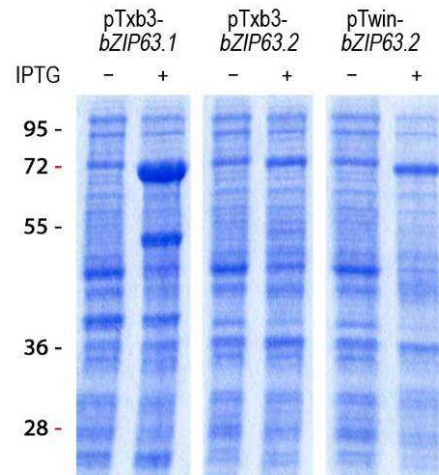

Fig 3.3 Testexpression of pTxb3–*bZIP63.1*, pTxb3–*bZIP63.2* and pTwin–*bZIP63.2*. Expression was induced by addition of IPTG and 4h incubation at 30℃. Expected size of the proteins: pTxb3–*bZIP63.2* (63kDa), pTwin–*bZIP63.2* (60kDa).

3.1.1.2. Denaturing purification of bZIP63.2 with His tag

As the in–gel kinase assay requires big amounts of recombinant protein, purification of His tagged bZIP63.2 from inclusion bodies under denaturing conditions was necessary. For this, several different vectors were tried and the best one chosen for expression and purification of the protein over a Ni^{2+} chelating column (HiTrap^R column, GE Heathcare).

pET–DEST42–*bZIP63.1* and pET–DEST42–*63.2* as well as pDEST17–*bZIP63.2* constructs were obtained from Christina Chaban (University of Tuebingen), transformed and tested for expression of the recombinant protein. The pET–DEST42 constructs have a C–terminal, the pDEST17 construct an N–terminal His tag. All three constructs showed good induction (see fig 3.4). PET–DEST42–*bZIP63.2* was chosen for testing and optimizing purification
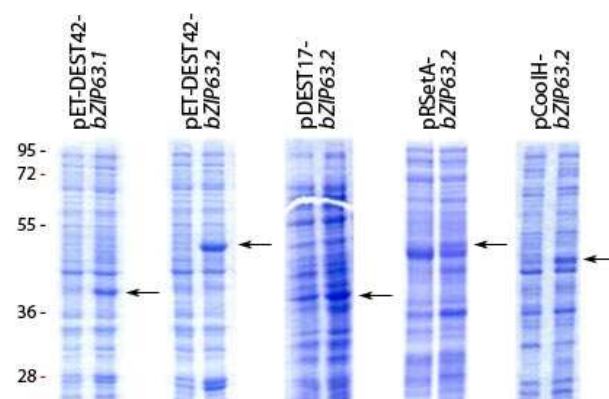

Fig 3.4 Induction of His tagges bZip63 protein in different vectors. Arrows indicate positions of the tagged protein.
pET–DEST42: C–terminal His tag
pDEST17, pRSetA, pCoolH: N–terminal His tag

conditions with the Ni^{2+} chelating column.

First, different protein extraction procedures were tested. To see whether the recombinant protein is soluble or in inclusion bodies, cells harvested after induction were either resuspended in native or denaturing buffer (binding buffer), followed by sonication and centrifugation. In the denaturing buffer all proteins were in the supernatant and none in the pellet. The native buffer could not resuspend most of bZIP63. This showed that bZIP63.2 clearly was in inclusion bodies (see fig 3.5).

Then it was tested whether extraction of soluble proteins with native buffer before applying the denaturing buffer would result in pre–purification of the protein extract. For this, the pellet after protein extraction with native buffer was resuspended in denaturing buffer with or without previous sonicaiton before centrifugation. In both cases the complexity of the protein extract was reduced but still some bZIP63 could not be resuspended. This pre–purification step was not used again because the benefit is low and too much of the recombinant protein is lost in the first step.
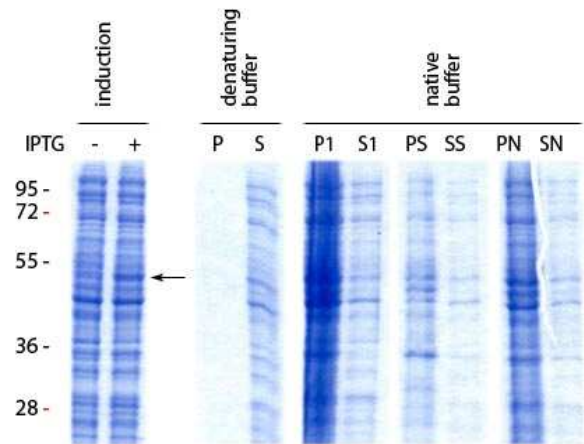

Fig 3.5 Test of different protein extraction procedures for purification of His tagged bZIP63.2. Abbreviations: P (pellet), S (supernatant), P1 (pellet in native buffer), S1 (supernatant in native buffer), PS (pellet from: P1 resuspended in denturing buffer, sonicated), SS (supernatant from: P1 resuspended in denturing buffer, sonicated), PN (pellet from: P1 resuspended in denaturing buffer), SN (supernatant from: P1 resuspended in denturing buffer)

His tagged bZIP63.2 was then purified using the Ni^{2+} chelating column. A step gradient of Imidazole was used to find the best concentrations for washing and elution (see fig 3.6 A). With 100mM most proteins binding unspecifically could be eluted. BZIP63.2 came of at 100mM and 150mM. To prove that the protein isolated was bZIP63.2, a Western blot with peptide antibodies recognizing the C–terminus and N–terminus of recombinant bZIP63 was done. Apart from the full length protein, degradation products of bZIP63.2 were purified. Separation of the full length protein from the smaller degradation products with size exclusion chromatography with a superdex 75 column was tried. This was unsuccessful, as the two most prominent bands could not be separated completely (see fig 3.6 B).

To get rid of the degradation products other expression vectors were tried. *BZIP63.2* was cloned into pRSetA, creating an N–terminal His tag. Purification of bZIP63.2 with this vector improved the quality of the recombinant protein (see fig 3.6 C), but the yield was decreased.

To improve the expression level, the expression cassette from pRSetA was cut out with SalI/XbaI and cloned into pTxb3 cut with BamHI/XbaI. The resulting vector, pCoolH–*bZIP63.2*, was then used for all further purifications of recombinant bZIP63.2. The yield was much better than with pRSetA, but also degradation products increased again. Before using the recombinant protein for the in–gel kinase assay identity of the protein and degradation products was again confirmed by Western blot (see fig 3.6 E).
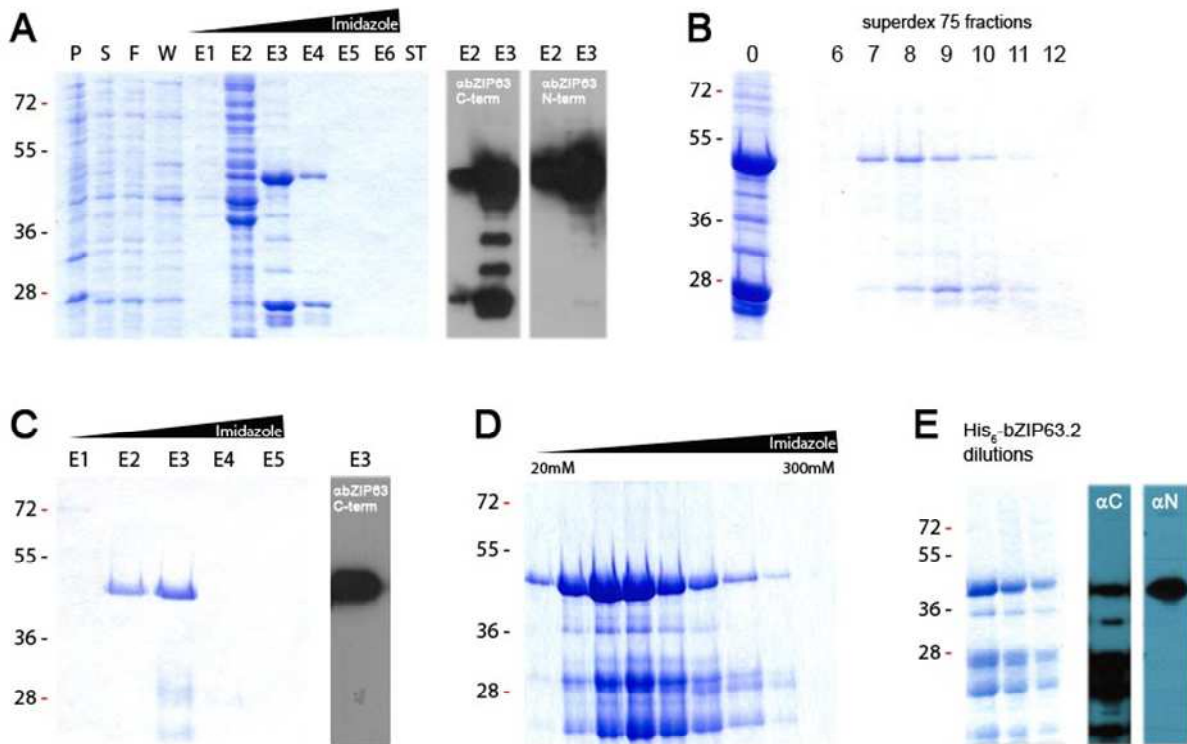

Fig 3.6 Purification of His tagged bZIP63.2 under denaturing conditions with the HiTrap[R] chelating column.
A: Expression vector pET–DEST42–*bZIP63.2*; column purification (left) and Western blot of elutions 2 and 3 with αbZIP63–C1 and αbZIP63–N probes (against C and N–terminus of bZIP63) (right). Abbreviations: P (pellet after induction), S (supernatant after induction), F (column flowthrough), W (wash fraction), E (elution), ST (strip fraction)
B: Fractions 6 – 7 from the superdex 75 size exclusion chromatography of elution 3 (0) from A
C: Expression vector pRSetA–*bZIP63.2*; Imidazole elutions (left) and Western blot with αbZIP63–C1 probe (right)
D: Expression vector pCoolH–*bZIP63.2*; Imidazole elution with a linear gradient from 20mM to 300mM
E: Dilutions of the recombinant His_6–bZIP63.2 protein and Western blot with αbZIP63–C1 and αbZIP63–N probes
Imidazole concentrations: E1 (50mM), E2 (100mM), E3 (150mM), E4 (200mM), E5 (250mM), E6 (300mM)

3.1.2. Affinity purification of plant cell extracts

Affinity purification of crude protein extracts was done to isolate proteins with a high affinity to bZIP63 for identification of kinases in the in–gel kinase assay. The complexity of the sample is clearly decreased by this step (see fig 3.7). This makes identification of low abundant proteins like kinases by Mass spectrometry easier and reduces the chance to identifying unspecific kinases of the same size as the kinase giving the signal in the assay.

Affinity purification was done by coupling GST tagged bZIP63 to a column, loading of the protein extract and washing with low salt concentration. Proteins were eluted with rising salt concentrations of up to 1MNaCl and proteins binding to the column with Glutathione (GSH). NaCl elutions, but not GSH elutions gave a signal in the kinase assay.

For affinity purification, N–terminally tagged bZIP63.1 and C–terminally tagged bZIP63.1 and bZIP63.2 were used.
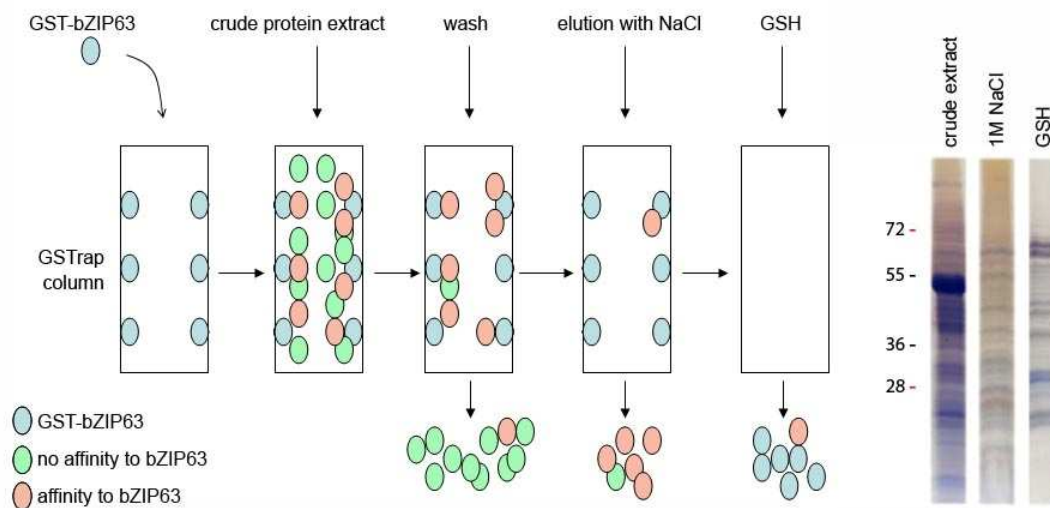

Fig 3.7 Left: Scheme for affinity purification of bZIP63 binding proteins; **Right**: Comparison of crude protein extract from seedlings with the affinity purified sample (1M NaCl elution) and the Glutathione (GSH) elution of the column. Complexity of the protein extract is decreased by affinity purification. Only little protein binds to the column too tight for NaCl elution.

3.1.3. Establishment of the in–gel kinase assay – Test–assays with Histone III–S

To test the conditions for the in–gel kinase assay a series of test assays was done with Histone III–S from calf thymus (Sigma). 1mg/ml Histone was polymerized in PAGE gels and different plant protein extracts and recombinant CPK3 (Calcium dependent protein kinase 3) were loaded. The recombinant kinase gave a strong signal in the kinase assay (see fig 3.8 A), and a very weak signal from autophosphorylation on a gel without substrate (not shown). The leaf protein extracts only gave a signal when big amounts of protein (30 – 45µg) were loaded.

One concern about this assay was that the substrate (Histone) would run out of the gel during the gel run. This actually happens, but during the washing/denaturing/renaturing steps the substrate gets redistributed equally throughout the gel by diffusion (see fig 3.8 B). Unfortunately, also low molecular weight proteins tend to diffuse out of the gel as can be seen by the vanishing lower marker bands. Altogether diffusion of bigger proteins is not a problem. Coomassie staining shows no remarkable diffusion within the gel.
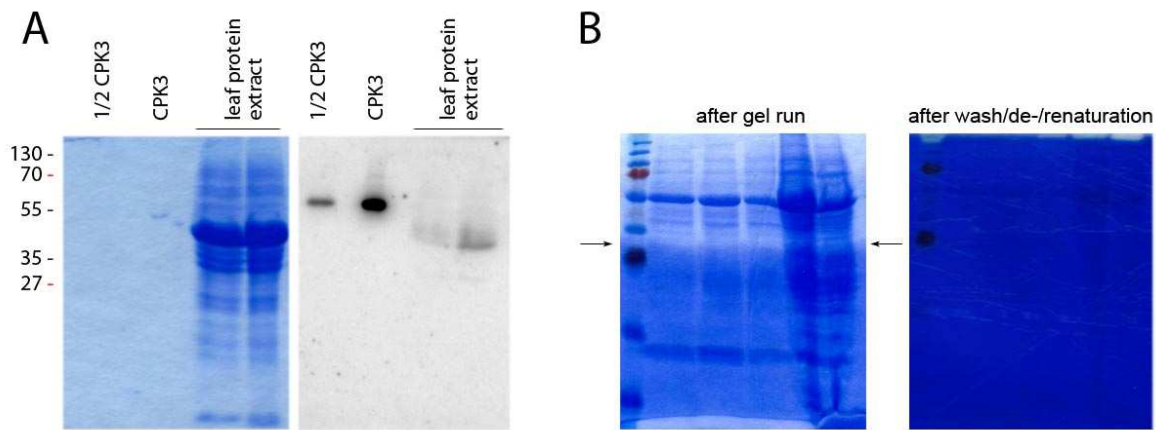
Fig 3.8 In–gel kinase assay with 1mg/ml Histone III–S
A: Coomassie stain of a gel without substrate and radiography signal from a gel with Histone. Recombinant protein kinase CPK3 gives a strong signal, 40 – 50µg protein from leaf protein extracts give a weak signal.
B: Coomassie stain of gels with 1mg/ml Histone III–S after gel run (left) and after in–gel kinase assay (right). Arrows indicate the front of Histone III–S running out of the gel.

3.1.4. Identification of Kinases by in–gel kinase assays with bZIP63.2

To identify kinases phosphorylating bZIP63, in–gel kinase assays with 1mg/ml recombinant His–tagged bZIP63.2 as substrate were done. Recombinant CPK3 was used as a positive control. This kinase was chosen, because it interacts with bZIP63 in a Y2H assay (Kolowrat, 2005) and it can phosphorylate the transcription factor *in vitro* (Hofmann Rodrigues, 2007). Crude protein extracts or affinity purified extracts were loaded.

Comparison between gels with and without substrate (bZIP63.2) showed that substrate phosphorylation gives a much stronger signal than autophosphorylation. On gels without substrate signals were barely visible (see fig 3.9 A). Therefore later assays were only done in the presence of substrate.

In–gel kinase assays with crude protein extracts from roots and leaves of plants grown in hydroponic culture and from root cell culture gave 3 strong signals (see fig 3.9 B and fig 3.10), 2 bands at 50 – 60kDa and one at about 35kDa. Although the protein amount in the root and leaf samples was about equal, the signals are much stronger in protein extracts from root material or cell culture. This could be due to lower expression of these kinases in leaves than in roots. Also in the leaf samples the two upper bands are shifted up to 70kDa and down to 45kDa. It is probable that these two bands represent the same proteins like in the root samples, but they are pushed up or down in the gel by the huge masses of RuBisCO running at about 50kDa.

Samples from Col–0, taken after a dark or light period, were compared to a sample from *cpk3* KO plants. This was done, because bZIP63 expression is regulated during the dark/light cycle, increasing in the dark and decreasing in the light. No difference could be

detected between these 3. Small variations in signal strength are not significant, because they were not reproducible. Although expression of bZIP63 is regulated by light, this seems not to be true for the kinases. The fact that the same 3 bands could be detected in the sample from the *cpk3* KO questions the role of CPK3 as a specific kinase for bZIP63.

Root cell culture was grown in medium without sugars for 2 and 4 days and transferred to medium with 30g/l glucose for 30min before harvesting. These samples were subjected to an in–gel kinase assay (see fig 3.9 B). This was done, because bZIP63 expression is inhibited by sugars, especially by glucose (data from Bernhard Wurzinger), and a role for bZIP63 in energy deprivation signalling was proposed (Baena-Gonzalez et al., 2007). All samples of the starved root cell culture show no change in intensity of the lower band at 35kDa, but they display only one upper band at 50kDa, which is much stronger than in non–starved cell culture (see fig 3.10). Addition of glucose for a short time period had no effect. This would indicate that expression of kinases representing one of the upper bands is increases, while the others are not expressed under long–term starvation conditions.

To identify the kinases responsible for the 3 strong signals, in–gel kinase assays with affinity purified protein extracts from root cell culture or roots from hydroponic culture were done (see fig 3.9 C – D). Proteins binding to bZIP63 were eluted from the affinity column with different NaCl concentrations. In the wash fraction with 75mM NaCl all 3 bands are present. With rising salt concentrations first the kinases representing the upper bands and then the lower band come off. Obviously affinity to bZIP63 is higher for the smaller kinase. Also, the bigger kinases seem to bind bZIP63 dimers better than monomers. When the affinity column is loaded with GST–bZIP63 the transcription factors presumably form dimers at the column due to the high concentration of the protein. Washing of the column with 500mM NaCl before application of the protein extract should remove bZIP63 not binding to the column and thus should break up dimers. In an affinity purification sample with a monomer–column the upper bands lack in the elution fractions (see fig 3.9 C). However, this could also be a result of the lower bZIP63 concentration on the column.

Affinity purification was done with N–terminally tagged bZIP63.1 (see fig 3.9 C) as well as C–terminally tagged bZIP63.1 and bZIP63.2 (see fig 3.9 D). This was done to see whether the tag would interfere with interaction with some partners and whether there is a difference between the two splicing forms.

Samples after affinity purification with N– and C–terminally tagged bZIP63 show no big difference. Both the lower and the upper bands are present, showing that the position of the tag does not interfere with binding of these kinases. A weak additional band arises at ~ 27kDa when C–terminally tagged bZIP63 is used. This signal is not visible from crude protein extract.

When splicing form 2 is used for affinity purification an additional band arises at 40kDa in the 300mM NaCl elution fraction. This signal also cannot be seen from a crude protein extract. Splicing form 1 seems to have a higher affinity for the bigger kinases than splicing form 2. The signal from the upper bands is stronger for splicing form 1 and can still be seen in the 300mM NaCl elution, whereas most of the kinase is washed off with 75mM in the case of splicing form 2.
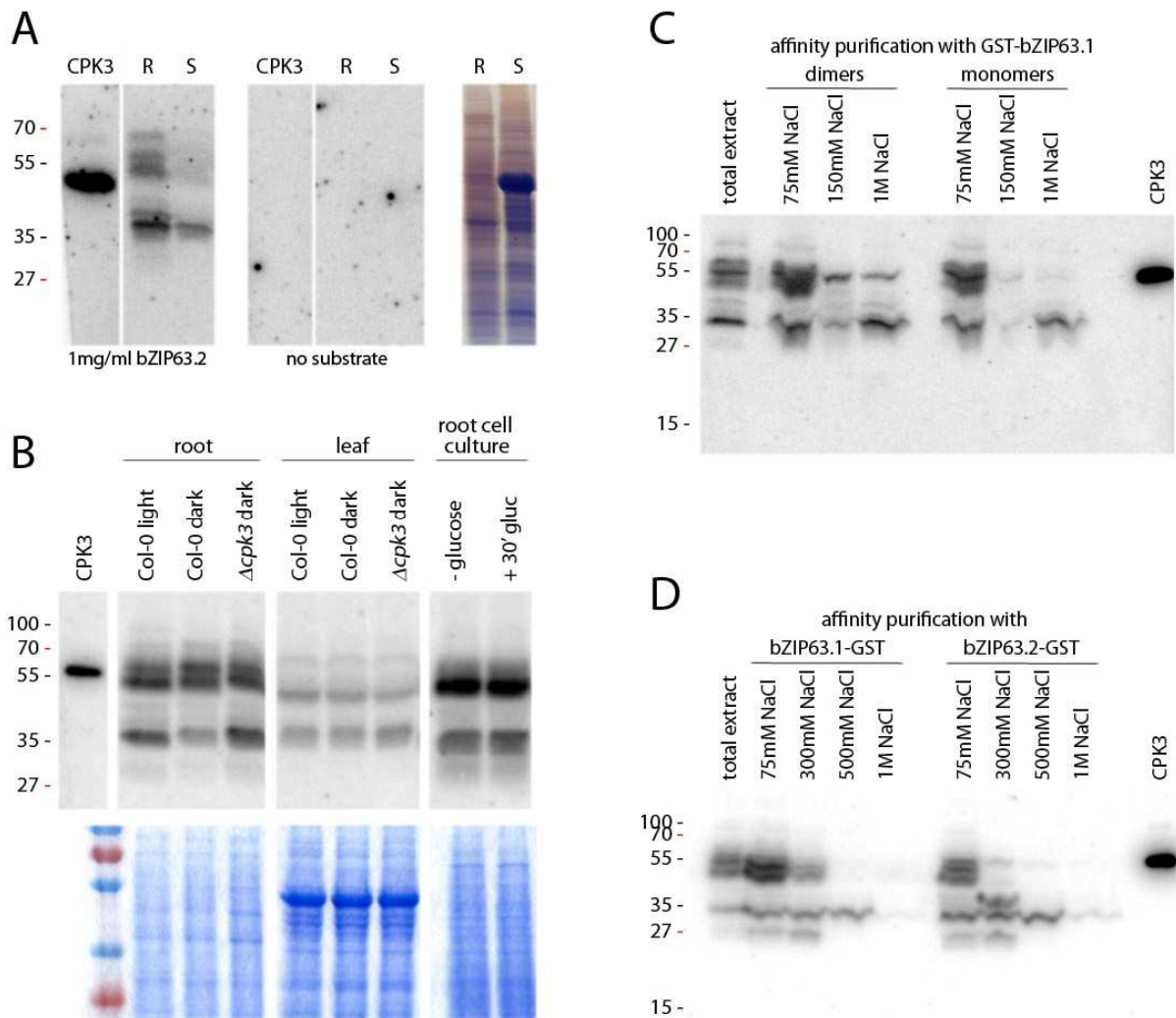

Fig 3.9 In–gel- kinase assay with 1mg/ml bZIP63.2 polymerized into the gel
A: Comparison between substrate– (left) and autophosphorylation (middle). Recombinant CPK3 and crude protein extracts from roots from hydroponic culture (R; 15µg) and from 2–week old seedlings grown on ½ MS plates (S; 25µg) were loaded on gels with and without bZIP63.2. Coomassie stain of the protein extracts is shown right.
B: In–gel kinase assay with crude protein extracts. Root and leaf protein extracts from Col–0 and *cpk3* KO (*Δcpk3*), collected after a light or dark period, as well as protein extracts from root cell culture grown without sugar for 2 days and re–fed with glucose for 30min were loaded. Root extracts contained 8.2µg protein, leaf extracts 16.9µg.
C: In–gel kinase assay with affinity purified protein extracts from root cell culture. GST–bZIP63.1 was used for purification. Samples on the left side (dimers) were prepared like ordinary. To purify bZIP63 binding proteins with GST–bZIP63.1 monomers (right side), the column was washed with 500mM NaCl before loading of the crude protein extract to wash off GST–bZIP63.1 binding to other GST–bZIP63.1 rather than to the column.
D: In–gel kinase assay with affinity purified protein extracts from roots from hydroponic culture. BZIP63.1–GST (left) and bZIP63.2–GST (right) were used for purification.

Table of all protein kinases found by MS

Accession nr.	Name	Description	MW (TAIR)	Localisation
(A) 50–65kDa (splicing form 1 and 2)				
At3g01090	**AKIN10**	***Arabidopsis* SNF1 kinase homologue 10**	**61**	**nucleus**
At3g29160	**AKIN11**	***Arabidopsis* SNF1 kinase homologue 11**	**59**	
AT4G23650	**CPK3**	**Calcium dependent protein kinase 3**	**59**	**cytoplasm, membrane, nucleus**
AT1G35670	CPK11	Calcium dependent protein kinase 2	56	plasma membrane
At4g35310	CPK5	Calcium dependent protein kinase 5	62	membrane
At3g20410	*CPK9*	*Calcium dependent protein kinase 9*	*60*	*plasma membrane*
At1g72710	*CKL2*	*Casein kinase I like*	*52*	*cytoplasm, nucleus*
At3g13670	*MMM17.17*	*protein kinase family protein*	*80*	
At5g64960	*CDKC2*	*cycline dependent kinase group C 2*	*57*	*nucleus*
At5g19010	*MPK16*	*MAP kinase 16*	*65*	*plasma m., vacuole*
At2g40730	*T7D17.9*	*HEAT repeat-containing protein*	*86*	
AT5G24710	*MXC17.10*	*similar to protein kinase family protein*	*148*	*plasma membrane*
AT1G51850	*T14L22.6*	*leucine-rich repeat protein kinase*	*96*	*plasma membrane*
At1g67840	*CSK*	*Chloroplast sensor kinase*	*67*	*chloroplast*
At2g28990	*T9I4.7*	*leucine-rich repeat protein kinase*	*99*	*endomembrane*
(B) 40–50kDa (splicing form 2)				
At3g01090	AKIN10	*Arabidopsis* SNF1 kinase homologue 10	61	nucleus
At3g29160	AKIN11	*Arabidopsis* SNF1 kinase homologue 11	59	
AT2G18170	*MPK7*	*MAP kinase 7*	*42*	
(C) 30–40kDa (splicing form 1 and 2)				
At3g50000	**CKA2**	**Casein kinase II subunit alpha 2**	**47**	**nucleus**
At5g67380	**CKA1**	**Casein kinase II subunit alpha 1**	**48**	**nucleus**
At2g23070	*CKAcp*	*Casein kinase II chloroplast alpha subunit*	*50*	*chloroplast*
(D) 25–30kDa (splicing form 2)				
At2g11520	*CRCK3*	*Calmodulin binding receptor–like kinase*	*57*	*endomembrane*
AT3G21630	*CERK1*	*Chitin elicitor receptor kinase 1*	*67*	*plasma membrane*
(E) regulatory subunits				
At1g09020	SNF4	activator subunit for SNF1–related kinases	53	
At5g47080	CKB1	Casein kinase II subunit beta 1	32	nucleus
At2g44680	CKB4	Casein kinase II subunit beta 4	32	nucleus, cytoplasm

Table 3.1: Table of all protein kinases found by Mass spectrometry of gel slices from affinity purified samples. Data from several affinity purification and MS runs are summarized. Significant findings are shown bold. Kinases that are suspected to be artefacts due to bad protein coverage, wrong size or subcellular localisation are shown in italic.
(**A**): gel slices covering the upper bands (50–65kDa); samples from root cell culture and root protein extract purification with splicing form 1 and 2
(**B**): gel slice covering the additional band in the root extract purification with bZIP63.2 (40–50kDa)
(**C**): gel slices covering the lower band (30–40kDa); samples from root cell culture and root protein extract purification with splicing form 1 and 2
(**D**): gel slice covering the range of 25–30kDa; samples from root extract purification with bZIP63.2
(**E**) regulatory subunits of the CKII (Casein kinase II) or AKIN complex, also found in the affinity purified samples

To identify the individual kinases the affinity purified samples were loaded on PAGE gels without substrate and gel slices were cut out from the regions covering the signal in the in–gel kinase assay. Proteins were extracted from the gel, digested with Trypsin and analysed by Mass spectrometry (MS/MS). In a typical gel slice 50 – 100 different proteins were identified. Several protein kinases could be found (see table 3.1).

In the range of the two upper bands from the in–gel kinase assay, 2 kinases of the SnRK1 superfamily were identified repeatedly: AKIN10 and AKIN11. Also a regulatory subunit, AtSNF4, was found in the affinity purified samples. It is thus highly probable that at least one of the two bands results from phosphorylation of bZIP63 through AKIN10 and AKIN11.

CPK3 was also found in the range of the upper bands when C–terminally tagged bZIP63 was used for affinity purification of root protein extracts. Other Calcium dependent protein kinases were identified too (CPK11, CPK5), but the protein coverage for these was not so good. From only one experiment it is impossible to say which CDPKs, if any, could phosphorylate bZIP63.

In the range of the lower band, three catalytic subunits of Casein kinase II were found: CKA1, CKA2 and the chloroplast localized CKAcp. CKA1 and CKA2 are significant findings, CKAcp is supposed to be an artefact. Also regulatory beta subunits CKB1 and CKB2 were found in the affinity purified samples.

Several other protein kinases were found, but were excluded because of bad protein coverage, wrong subcellular localisation (chloroplast) or wrong size. Most of them were only found once and are represented by one or two peptides. Generally protein kinases were low abundant in the affinity purified samples, indicating that this step successfully removed unspecific kinases.

3.1.5. Confirmation of CKII identity

Unlike other kinases Casein kinase II can use GTP in addition to ATP as a phosphodonor. This trait was used to show that the band appearing in in–gel kinase assays at 35kDa represents CKII. In an in–gel kinase assay with crude protein extract from root cell culture and roots from hydroponic culture the upper bands vanish completely, but the lower band at 35kDa remains when GTP is used instead of ATP. This further supports the assumption that this kinase is CKII.

In parallel, an in–gel kinase assay without Ca^{2+} was done to show whether any Ca^{2+}–dependent kinases are responsible for one of the signals. When Ca^{2+} was omitted throughout protein extraction and kinase assay the lower band weakens to about half intensity. The uppermost band at 60kDa as well as recombinant CPK3 show only a very weak signal and the band at
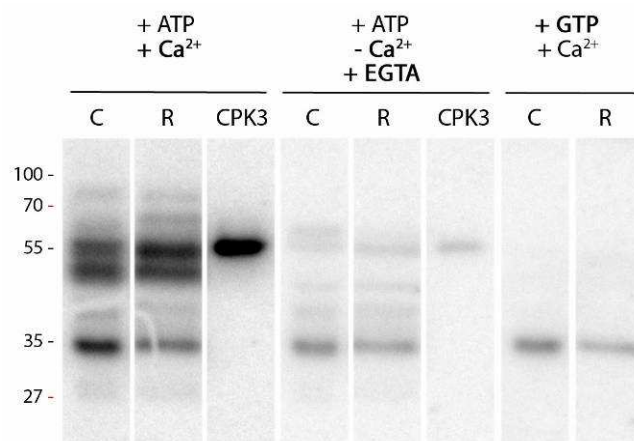

Fig 3.10 In–gel kinase assay with root cell culture (C) and roots from hydroponic culture (R) under different conditions. Left: ordinary in–gel kinase assay with 50µM CaCl$_2$. Middle: in–gel kinase assay without CaCl$_2$ and with 5mM EGTA. Right: in–gel kinase assay with 100µCi γ^{32}P–GTP.

50kDa vanishes completely. This indicates that a Ca^{2+}–dependent protein kinase is responsible for one of the upper bands. To prove this, kinase assays with lower EGTA concentrations will have to be done as 5mM might already interfere with the signal from the Ca^{2+} independent CKII.

3.2. Identification of in vivo phosphorylation sites on bZIP63

Information about the *in vivo* phosphorylation state of bZIP63 was obtained by Immunprecipitation (IP) of bZIP63 from protein extracts followed by Mass spectrometry analysis. Several antibodies were tested for specificity and plant lines were tested for bZIP63 expression to choose the best strategy. IP was then done on bZIP63.2–GFP OE plants using an anti GFP probe.

3.2.1. Characterization of *bzip63* KO in Ws (Wassilewskija)

As a negative control in the Western blots with bZIP63 peptide antibodies, a *bzip63* KO line in the background of the Wassilewskija (Ws) ecotype was used. This line had been obtained from the Wisconsin University stock collection and KO had been confirmed by RT–PCR (Data from Daniela Hofmann Rodrigues).
In order to determine the T–DNA insertion site, genomic DNA was isolated from Col–0, Ws–2 (Wassilewskija wild type) and *bzip63* KO plants in the background of Ws. A PCR with primers for *bZIP63* showed a band at ~ 1500bp in the wt plants and a ~ 6kb bigger product in the KO plant representing the gene with the insertion cassette (see fig 3.11 A). A second PCR with *bZIP63* primers and primers for left and right border of the cassette gave a product of 220bp with the 5' *bZIP63* and the LB (left border) cassette primer. This PCR product was sequenced to show the exact insertion site within the first exon (see fig 3.11 B).

The PCR product from Col–0 is slightly bigger than from Ws–2 (see fig 3.11 A). This observation led to the question whether the Ws–2 gene is shorter and this would have an effect on the protein sequence. The PCR product from Ws–2 was sequenced showing several base changes throughout the gene in comparison to the Col–0 sequence (TAIR). Also both sequences lack several parts compared to the other explaining the size difference of the PCR products (see Supplementary fig 2). These do not affect the protein sequence, because all but one are in intron sequences. At the end of the 5th exon the Ws–2 sequence has an additional T. Assuming that this T is spliced as part of the intron the protein sequence of Col–0 and Ws–2 shows only 6 amino acid changes (see fig 3.12) for splicing form 1 and 2. Splicing form 3 though would be affected as in this splicing form this intron is not spliced out. This would lead to an earlier stop codon in Ws–2, shortening the protein from 250 to 228 amino acids.
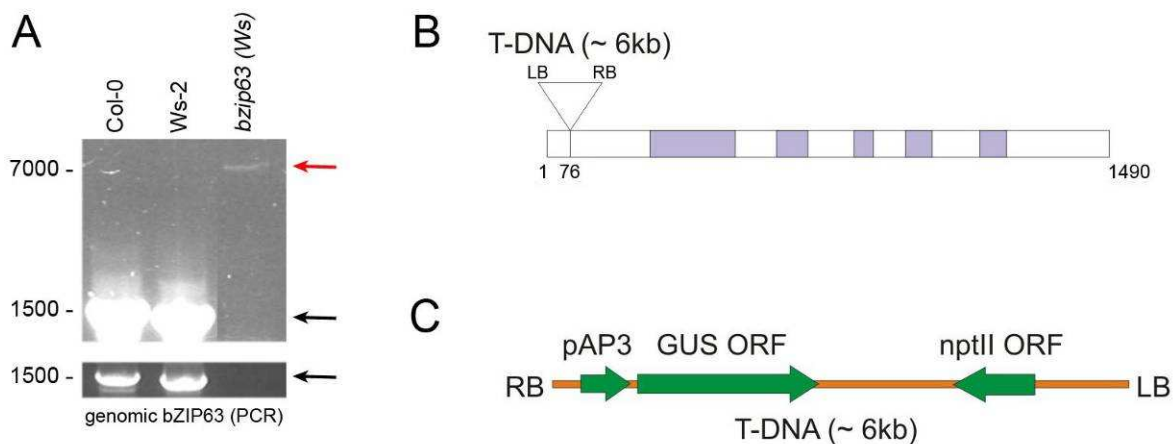
Fig 3.11 A: PCR of full length *bZIP63* from genomic DNA of Col–0, Ws–2 and *bzip63* KO plants in the background of Ws. Red arrow indicates *bZIP63* with T–DNA cassette; black arrows indicate bZIP63 without insert. The gene product is slightly bigger in Col–0 than in Ws–2.
B: Position and orientation of the T–DNA cassette in the *bZIP63* gene in the KO.
C: T–DNA cassette contains a GUS ORF under control of the Apetala 3 promoter (pAP3) and a kanamycin resistance gene (nptII ORF).

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Col-0  MEKVFSDEEI SGNHHWSVNG MTSLNRSASE WAFNRFIQES SAAADDGEST TACGVSVSSP
Ws-2   MEKVFSDEEI SGNHHWSVNG MTSLNRSASE GAFNRFIQES SAAADDGESA TACGVSVSSP

  61   PNVPVDSEEY RAFLKSKLNL ACAAVAMKRG TFIKPQDTSG RSDNGGANES EQASLASSKA
  61   PNVPVDSEEY RAFLKSKLNL ACAAVAMKRG TFIKPQDTSG RSDNGGANES EQGSLASSKA

 121   TPMMSSAITS GSELSGDEEE ADGETNMNPT NVKRVKRMLS NRESARRSRR RKQAHLSELE
 121   TPMMSSAITS GSELSGDEEE ADGETNMNPT NVKRVKRMLS NRESARRSRR RKQAHLSELE

 181   TQVSQLRVEN SKLMKGLTDV TQTFNDASVE NRVLKANIET LRAKVKMAEE TVKRLTGFNP
 181   TQVSQLRVEN SKLMKGLTDV TQTFNDASVE NRVLKANIET LRAKVKMAEE TVKRLTGFNP

 241   MFHNMPQIVS TVSLPSETSN SPDTTSSQVT TPEIISSGNK GKALIGCKMN RTASMRRVES
 241   MFHTMPQIVS TVSLPSETSN SLDTTSSQVT TPEIISSGNK SKALIGCKMN RTASMRRVRA

 301   LEHLQKRIRS VGDQ*
 301   LEHLQKRIRS VGDQ*
```

Fig 3.12 Comparison of the protein sequences of bZIP63.2 in Col–0 (line 1) and Ws–2 (line 2) excluding the T at the end of exon 5 in the Ws–2 sequence.
altered protein sequence bZIP domain bZIP-basic domain (DNA binding)

3.2.2. bZIP63 and bZIP09 peptide antibodies

Peptide antibodies against bZIP63 and bZIP09 had been designed and obtained from rabbit to detect the untagged bZIP proteins in plant protein extracts (Hofmann Rodrigues, 2007). As all bZIP transcription factors are very conserved in their central part the peptides had been chosen from the N– and C–terminal part of the proteins. Previously tested peptide antibodies could detect the recombinant protein but failed to be specific (Hofmann Rodrigues, 2007).

Specificity of one αbZIP09 and two αbZIP63 probes were tested in Western blots using recombinant bZIP protein and different plant protein extracts. The expected size of endogenous bZIP09 and bZIP63 is 31kDa and 34kDa respectively.

The αbZIP09 probe recognized recombinant bZIP09 (see fig 3.13). As previously observed, a bandshift could be seen between bZIP09 phosphorylated *in vitro* by CPK3 (Calcium dependent protein kinase 3) and unphosphorylated protein after treatment with λ–Phosphatase. In the protein extracts a single band was detected at ~ 40kDa both in Col–0 and *bzip09* KO. This shows, that the bZIP09 antibody is quite specific, but recognizes some protein other than bZIP09 in plant protein extracts. A Western blot with protein extracts of Col–0 and *cpk3* KO from different tissues shows that the protein recognized is not present in roots (see fig 3.13). To clarify the identity of this protein an Immunprecipitation and Mass spectrometry analysis should be done.
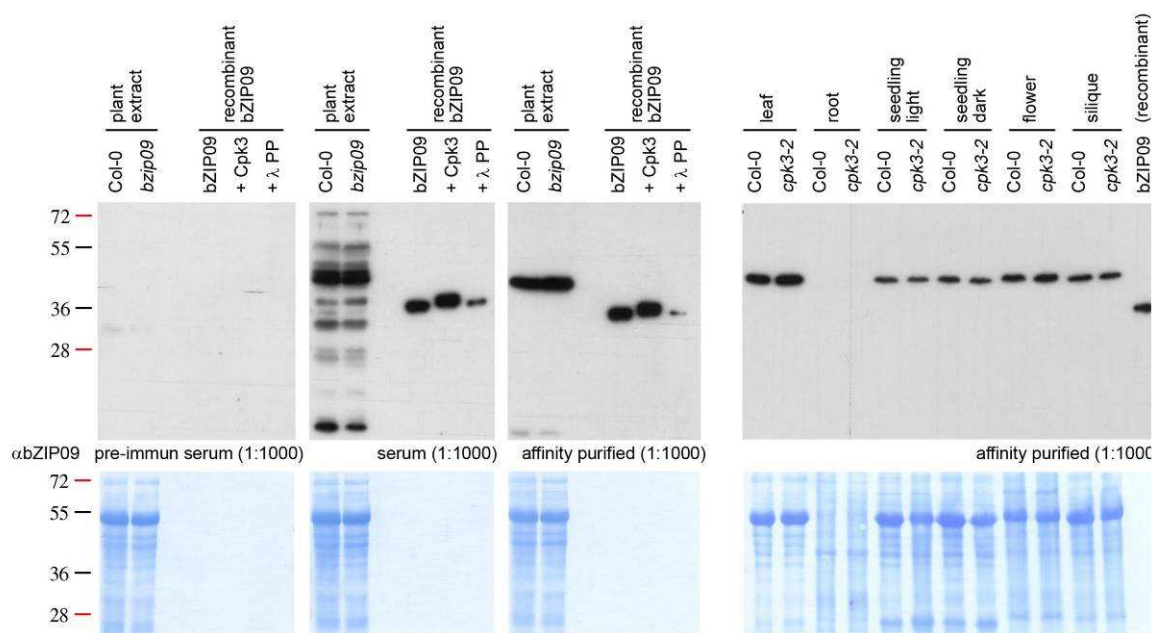

Fig 3.13 Western blot with pre–immune serum, serum and affinity purified αbZIP09 peptide antibody.
Left: Antibodies were tested on protein extracts from wt (Col–0) and *bzip09* KO extracts as well as on recombinant bZIP09 and recombinant bZIP09 treated with CPK3 (Calcium dependent protein kinase 3) or λ Phosphatase (λ PP).
Right: Western blot on protein extracts from different tissues of wt (Col–0) and CPK3 KO plants (*cpk3–2*).

63

strongly (see fig 3.14), but not exclusively as several other bands appeared. Again, a bandshift between phosphorylated and unphosphorylated protein could be observed. In plant protein extracts from Col–0 and bZIP63 OE the probe fails to recognize bZIP63.

The C–terminal bZIP63 peptide antibody (bZIP63–C2) recognized phoshorylated and unphosphorylated recombinant bZIP63 specifically, but very weakly (see fig 3.14) when the antibody was subtracted with *bZIP63* KO protein extract before use. Again no signal from the endogenous protein could be detected in the plant protein extracts.

In contrast to the new antibodies the old C–terminal antibody (bZIP63–C1, (Hofmann Rodrigues, 2007)) does recognize bZIP63 in whole protein extracts. A Western blot with protein extracts from Col–0 and bZIP63–GFP OE plants showed a signal from the GFP–

tagged bZIP protein (see fig 1.14 left). Unfortunately this antibody too is unspecific and strongly recognizes an other protein at ~ 34kDa.
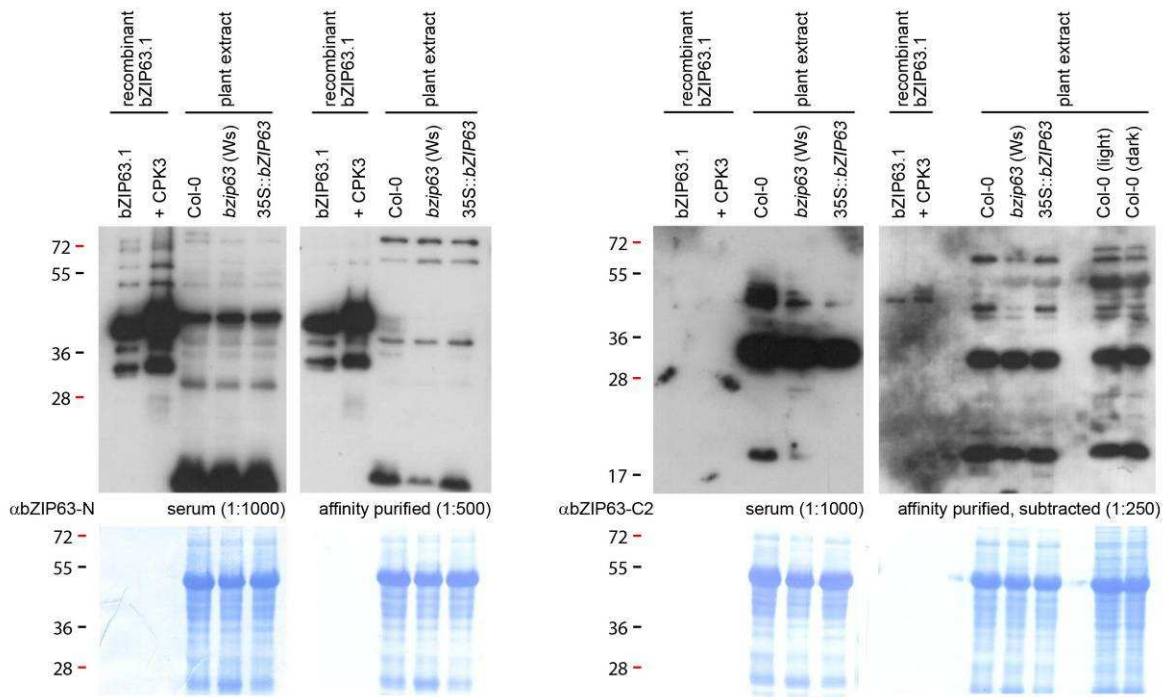

Fig 3.14 Top
Western blot with serum and affinity purified αbZIP63 peptide antibodies. Antibodies were tested on recombinant bZIP63.1 and bZIP63.1 treated with CPK3 (Calcium dependent protein kinase 3) as well as protein extracts from wt (Col–0), *bzip63* KO (*bzip63*, ecotype Ws) and bZIP63 OE (35S::*bZIP63*) plants.
Top left: peptide antibody against the N–terminus of bZIP63
Top right: 2[nd] peptide antibody against the C–terminus of bZIP63. (light) samples taken during day, (dark) samples taken during night
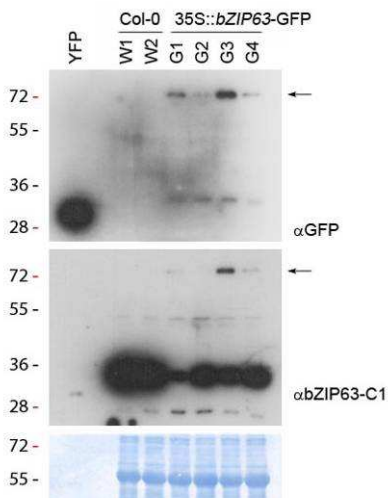
Fig 3.14 Left
Western blot on protein extracts from wt (Col-0) and bZIP63-GFP OE plants. Arrows indicate the position of bZIP63–GFP.
Above: antibody against GFP (Roche)
Below: 1[st] peptide antibody against the C–terminus

3.2.3. BZIP63 OE lines

As the αbZIP63 peptide antibodies are not specific it was impossible to use wt plants for Immunprecipitation of bZIP63. Therefore, plants overexpressing tagged bZIP63 were used. For this, 35S::*HA–bZIP63* and 35S::*bZIP63–GFP* plants were available. Expression of the tagged protein and specificity of the antibodies was tested before the GFP tagged line was chosen for bZIP63 purification.

3.2.3.1. HA tagged lines

To identify plants with a high expression level for bZIP63 sqRT–PCR with primers for *bZIP63* was done on leaf samples from 35S::*HA–bZIP63* plants. All individuals tested showed increased expression of the mRNA, except 2 individuals (number 18 and 21), which seemed to silence *bZIP63* (see fig 3.15).

To see whether the rat anti HA antibody would be specific and give no background signals in plant protein extracts, western blots were done with protein extracts from Col–0 and the HA–bZIP63 OE plants. As a positive control a yeast extract containing a HA tagged protein was used. Unfortunately the antibody failed to recognize the positive control and gave no signal at all for the plant protein extracts. As the secondary antibody did recognize the primary antibody quite well it was concluded that the rat anti HA probe was defective.
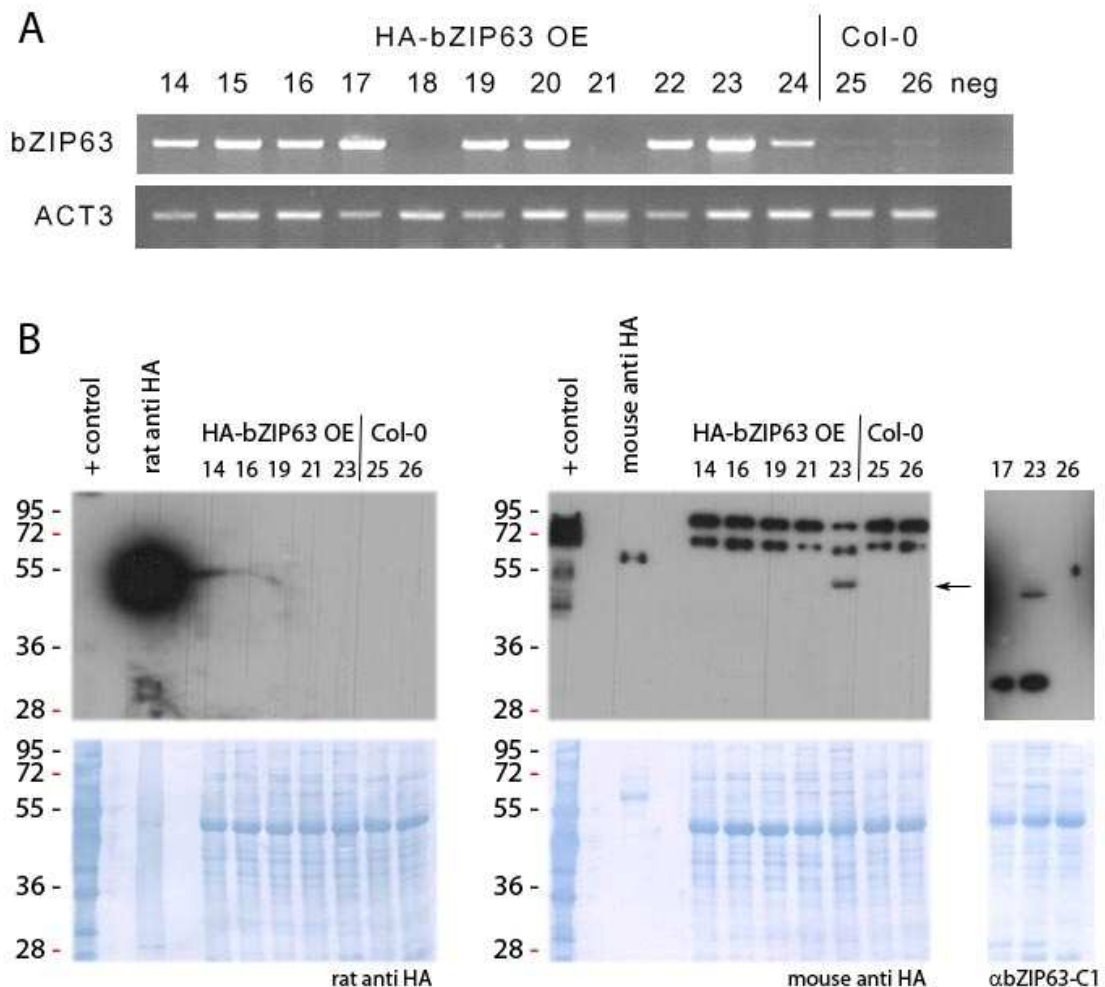
Fig 3.15 sqRT–PCR and Western blots with 35S::*HA–bZIP63* plants
A: sqRT–PCR of total *bZIP63* (tagged and native) in bZIP63 OE plants and Col–0; Actin 3 was used as control.
B: Western blots of protein extracts from HA–bZIP63 OE plants and Col–0. A yeast cell extract containing a HA tagged protein (~ 72kDa) was used as a positive control. Left: blot with the rat anti HA probe. Middle: blot with the mouse anti HA probe. Right: blot of plant number 17 and 23 with the αbZIP63–C1 probe. The arrow indicates a potential signal from HA–bZIP63.

The same blot with a mouse anti HA antibody showed two background bands at 70 and 85kDa in all protein extracts and a unique signal at about 50kDa in the strong overexpressing plant number 23. The αbZIP63–C1 probe recognizes a protein in this plant at about the same size, but not in plant number 17, which had no such band with the mouse anti HA probe (not shown). From this it can be concluded that at mRNA level several of the plants are over–expressor lines, but at protein level expression is not higher in most cases.

Subtraction of the mouse anti HA antibody with Col–0 protein extract could not remove the background signal (not shown). Thus this antibody could not be used for Immunprecipitation of bZIP63 and bZIP63–GFP plants were used instead of the HA–bZIP63 plants.

3.2.3.2. GFP tagged lines

Expression ob bZIP63–GFP in the over–expressor line was not determined on mRNA level with sqRT–PCR, but leaves from young and old leaves were analysed for GFP fluorescence from the tagged protein using a fluorescence microscope. All plants analysed showed transgene expression in both young and old leaves. The transgene was detected in the nucleus exclusively.

To test whether the anti GFP probe would give a backround signal, a Western blot was done with wt and bZIP63–GFP OE plants. The probe detected the transgene specifically and did not give any background in wt plant extracts (see fig 3.14 left).
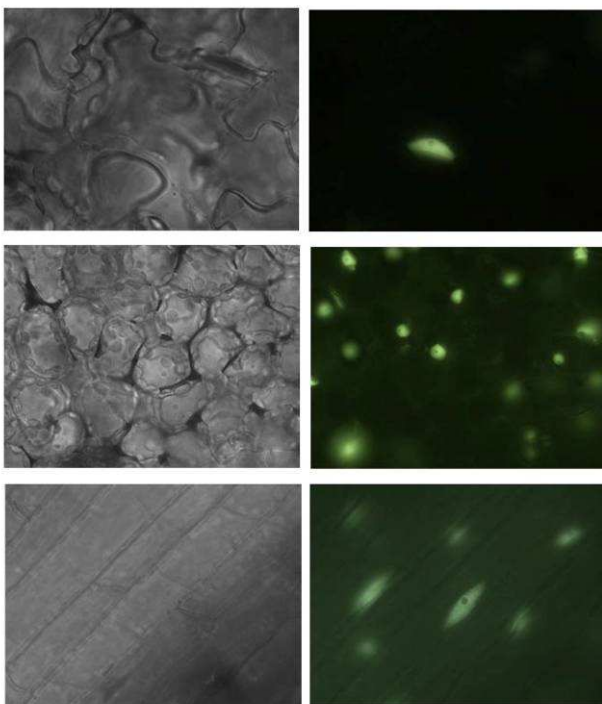

Fig 3.16 bZip63–GFP fluorescence in 35S::*bZIP63–GFP* plants
Left: bright light, Right: GFP fluorescence
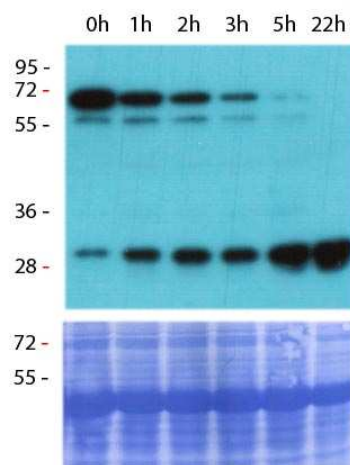Top: epidermal cells, Middle: parenchyma cells, Bottom: leaf vein cells

Fig 3.17 Western blot with anti GFP probe. Samples: protein extracts from 35S::*bZIP63–GFP* plants collected in the light; incubated on ice for up to 22h hours.

3.2.4. BZIP63 stability

BZIP63 is degraded very fast in protein extracts. To determine the rate of degradation, protein extracts were prepared from 35S::*bZIP63–GFP* plants with protein extraction buffer. The extract was incubated on ice and samples were taken after 0h, 1h, 2h, 3h, 5h and 22h and used in a Western blot. The full length protein (~ 70kDa) is completely degraded within 5 hours. Several bands for degradation products can be seen at 58kDa, 45kDa, 36kDa and 30kDa. The 30kDa product, which accumulates, presumably is the GFP part of the protein, which seems to be more stable.

3.2.5. Immunprecipitation of bZIP63 and Identification of in vivo phosphorylation sites

BZIP63–GFP was isolated from leaves of soil grown 35S::*bZIP63–GFP* plants by Immunprecipitation with an anti GFP probe (see fig 3.18). Samples were taken after 4.5 or 16h darkness. Due to the low stability of bZIP63 in protein extracts it was crucial to limit the incubation time of antibody and protein extract. Western blot with an anti GFP probe confirmed the identity of the isolated protein.
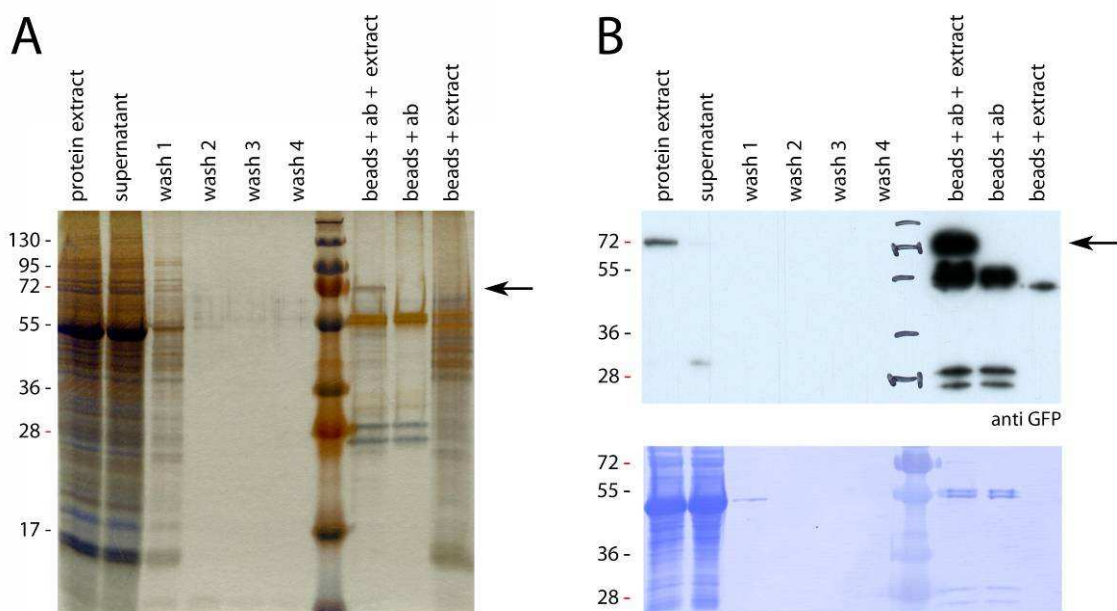
Fig 3.18 Immunprecipitation (IP) of bZIP63.2–GFP with an anti GFP probe (ab).
A: Gel showing the progress of the IP of bZIP63.2–GFP. The arrow indicates the position of the precipitated protein in lane 7. Additional bands at 55kDa, 26kDa and 30kDa in lane 7 and 8 represent the heavy and light chain of the anti GFP probe.
B: Western blot of the IP progress with an anti GFP probe. Signals can be detected for full length bZIP63.2–GFP (lane 1 and 7; arrow) as well as for the anti GFP probe used in the IP (lane 7 and 8).

gel and digested over night with Trypsin or 0.5 – 2h with Subtilisin. Mass spectrometry analysis (MS/MS) of one Trypsin and one Subtilisin digest delivered 4 phosphorylation sites (see fig 3.19 and table 3.2). Each site was found only once. Phosphorylated peptides were

also found unphosphorylated. Another two Subtilisin digests resulted in no identified phosphorylation sites. Two of these sites (S29, S300) were previously shown to be phosphorylated in an *in vitro* phosphorylation reaction with recombinant bZIP63.1 and CPK3 (Hofmann Rodrigues, 2007).

Due to the characteristics of the protein sequence, digest with these two enzymes does not allow complete protein coverage. No peptides were found for the N–terminus of the protein as well as the first half of the bZIP domain containing the NLS. Also a potential SnRK1 target at S294 was not covered. For peptide coverage of the individual digests see supplementary figure 1.
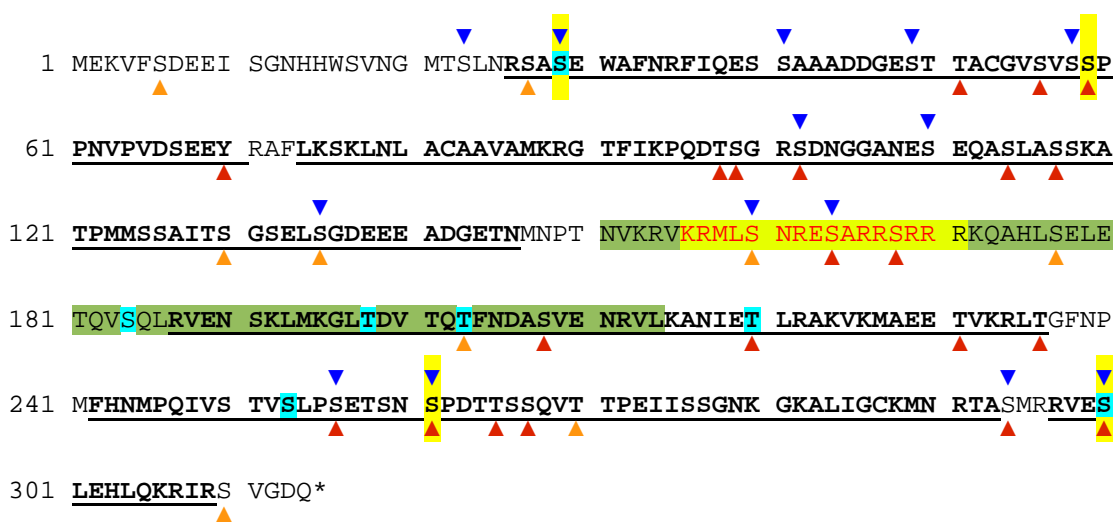
Fig 3.19 Position of the bZIP domain, predicted and identified phosphorylation sites on bZIP63.2.

bZIP domain bZIP-basic domain (DNA binding) bipartate NLS (nuclear import)
PhosPhAt prediction: ▼ general phosphorylation site
NetPhos2.0 prediction: ▲ CKII target site, ▲ general phosphorylation site
in vivo phosphorylation sites identified by IP and MS/MS
sites phosphorylated by CPK3 *in vitro* (Hofmann Rodrigues, 2007)

site	sample	digest
S29	4.5h dark	Trypsin
S59	16h dark	Subtilin (1h)
S261	16h dark	Subtilin (1h)
S300	4.5h dark	Trypsin

Table 3.2 *in vivo* phosphorylation sites

3.2.6. 2D gels as a strategy to determine the in vivo phosphorylation state

Phosphorylation changes the charge of a protein and thus shifts the IP (isoelectric point) to lower pH ranges. Under optimum conditions separation according to the IP would lead to separation of unphosphorylated and phosphorylated protein and each phosphorylation state (0, 1, 2, 3… phosphorylated sites) would give an individual signal (see fig 3.20 A).

To see whether 2D gels can be used to assay the phosphorylation state of bZIP63 *in vivo* in different tissues under different conditions, 2D gels were done on protein extracts from bZIP63–GFP OE plants. The signal from the protein was determined by Western blot with an anti GFP probe.

In the first step Immobiline^TM DryStrips with a pH range from 3 to 11 were used to narrow down the pH range of the signal. Samples from leafs collected after light or dark phase were compared. In both cases a single elongated spot was detected in the area between pH 5 and 6 (see fig 3.20 B).

To improve separation of the signal, Immobiline^TM DryStrips with a pH range from 4 to 7 were chosen. Protein extracts from seedlings collected after light or dark phase were compared. The signal is weaker in the light sample, but overall it seems to shift more to a lower pH indicating stronger phosphorylation (see fig 3.20 C). In the range between pH = 4 and 5 the protein extract shows a horizontal smear, which can be seen both in the Western signal and on the Coomassie stained membrane. This was probably caused by a small gap between the strip and the gel at this position when running the second dimension, but seems not to affect the right half of the blot.

To show that a difference in the phosphorylation state would really lead to a visible shift of the signal, a protein extract from leafs collected after the light phase was treated with λ– Phosphatase for 15min. 2D gels with dephosphorylated and untreated protein extract showed a clear shift of the signal to higher pH due to dephosphorylation (see fig 3.20 D – F). This clearly indicates that bZIP63–GFP is phosphorylated *in vivo*. Due to the long incubation time at 30°C degradation product of bZIP63–GFP can be seen in the Western blot. The lines of spots at 25kDa and 30kDa do not represent different phosphorylation states of the degradation products, because no shift can be observed. Only in the spot–line at 33kDa a shift can be seen, indicating that this C–terminal peptide could contain a phosphorylation site. Most probably the individual spots arise when a charged amino acid is cleaved off in the degradation process changing the IP of the peptide.
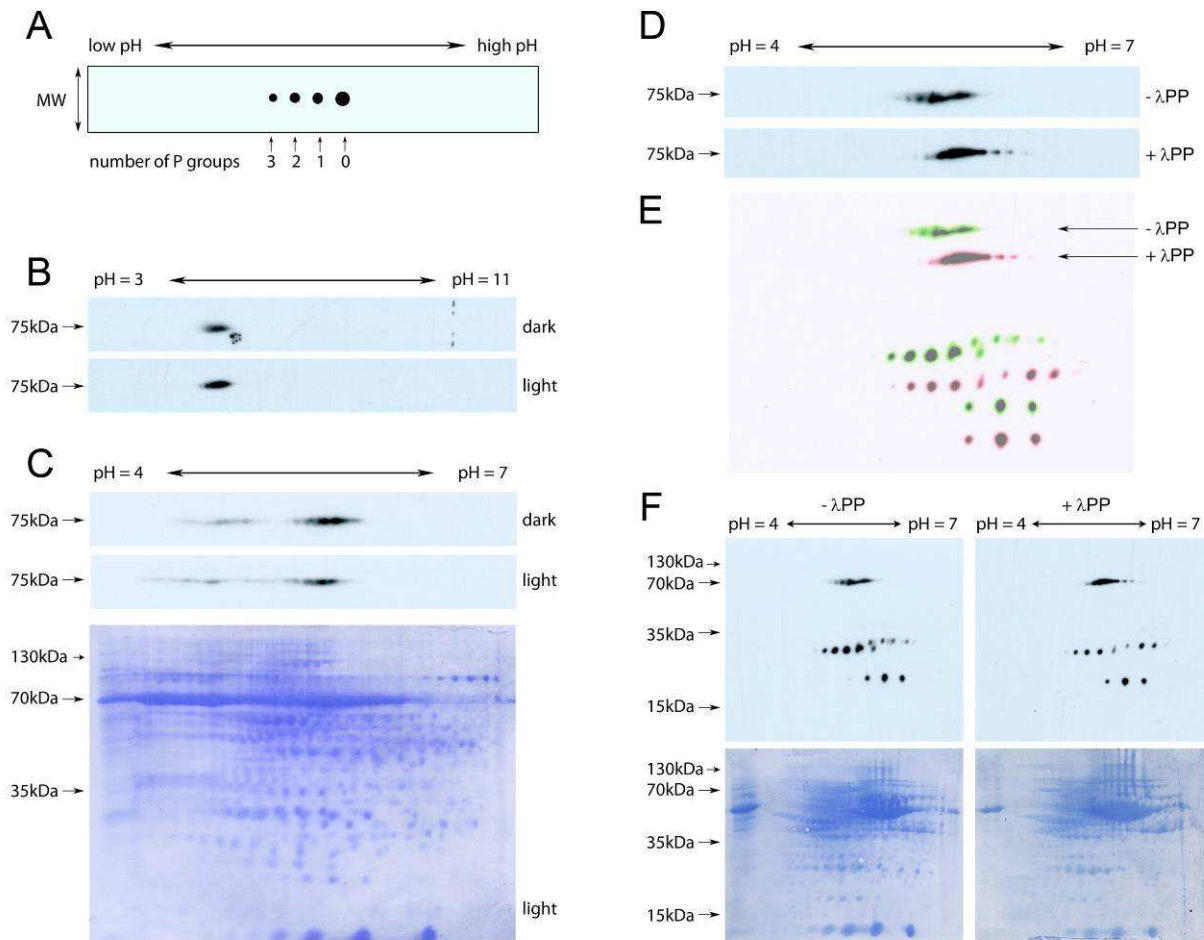
Fig 3.20 2D gels with protein extracts from bZIP63–GFP OE plants. Western blots were done with a mouse anti GFP probe.

A: Scheme showing separation of a protein with 4 different phosphorylation states on a 2D gel. Phosphorylation of the protein leads to a shift of the signal to lower pH ranges.

B: Western blot of 2D gels (pH = 3 – 11) with leaf protein extracts collected after dark or light phase.

C: Above: Western blot of 2D gels (pH = 4 – 7) with protein extracts from 3–week old seedlings grown on ½ MS agar plates, collected after dark or light phase. Below: Coomassie stain of the PVDF membrane of the light sample after blotting.

D–F: Western blots of 2D gels (pH = 4 – 7) with leaf protein extract collected after light phase, treated with or without λ–Phosphatase (λPP).

D: Signal from full length protein.

E: Overlay of the Western blots. Untreated protein extract is shown in green, protein extract treated with λPP is shown in pink and shifted down in vertical orientation for better comparison.

F: Whole Western blots and Coomassie stained membranes.

4. Discussion

4.1.   *In vivo* phosphorylation of bZIP63

Initially the transcription factor bZIP63 was identified as a potential target for the calcium dependent protein kinase CPK3 in a Y2H screen. The kinase could phosphorylate recombinant bZIP63 *in vitro* and several *in vitro* phosphorylation sites were identified (Hofmann Rodrigues, 2007; Kolowrat, 2005).

Now, 2D gels and Immunprecipitation of bZIP63 provided first data about the *in vivo* phosphorylation state of the transcription factor. By dephosphorylation of a protein extract with λ–Phosphatase and Western blot analysis on a 2D gel it could be shown that bZIP63–GFP is indeed phosphorylated *in vivo*. A clear shift of the signal to higher pH ranges was observed in the dephosphorylated compared to the untreated sample, indicating change in the phosphorylation state of the protein.

In total 4 *in vivo* phosphorylation sites could be identified on bZIP63.2–GFP by IP and MS/MS analysis from leaf material of over–expressor plants harvested in the dark. Each site (S29, S59, S261 and S300) was found phosphorylated once, but also unphosphorylated several times. This is not surprising, as not all proteins in the cell are phosphorylated and phosphorylated peptides might be harder to detect by MS due to altered properties. Assuming that phosphorylation of the transcription factor acts as a signal, regulating its activity in one of several possible ways, phosphorylation might only be strong under certain conditions or in certain tissues. In this case choice of the harvesting conditions would strongly influence the probability to find phosphorylated peptides. Two of the phosphorylation sites are identical with the sites phosphorylated by CPK3 *in vitro* (S29 and S300). In case these sites are phosphorylated by CPK3 *in vivo* too, a strong signal would only be expected when CPK3 is activated, like during salt stress (Mehlmer and Teige, unpublished). It is thus possible and probable that bZIP63 is phosphorylated at more than these 4 sites *in vivo*.

A major limiting factor for the identification of phosphorylation sites is the sequence coverage of the protein in the MS analysis, which depends on the protein sequence. The N–terminus as well as the basic part of the bZIP domain could not be accessed by MS analysis. Also, S294, which is a potential SnRK1 target site, could not be covered. Peptides need a certain weight/charge ratio to be detected and neither Trypsin nor Subtilisin digest produced suitable peptides for these protein regions. Especially the basic region harbouring the NLS would be interesting as it contains 3 predicted phosphorylation sites at positions 160, 164 and 168. Phosphorylation in this region could have a strong effect on localisation and DNA binding property of the protein.

For understanding the role of phosphorylation in regulating the activity of the bZIP transcription factor it is essential to know in which tissue and under which conditions the protein is phosphorylated. This also applies to the identification of the *in vivo* phosphorylation sites as it is beneficial to analyse strongly phosphorylated samples.

On 2D gels, phosphorylated and dephosphorylated bZIP63–GFP showed a clear difference in the position of the signal along the pH gradient. This confirmed the potential of 2D gels to assay the *in vivo* phosphorylation state of bZIP63–GFP from different samples.

BZIP63 is proposed to work downstream of the SnRK1 kinases in regulating sugar and amino acid metabolism in response to energy deprivation (Baena-Gonzalez et al., 2007; Baena-Gonzalez and Sheen, 2008). Thus, initially samples taken during day or night were compared. In a light sample from seedlings the signal showed a shift to lower pH compared to a dark sample. The spot representing the weakest phosphorylation state decreased in intensity, while the other spots had the same intensity as in the dark sample. This was in contrast to our expectation and indicates that bZIP63 phosphorylation is stronger during the day. Most probably stronger phosphorylation during the day results from action of other kinases than SnRK1s, like CKII or from increased action of phosphatases during the night. The bZIP transcription factor HY5 exists in 2 isoforms in the cell, an active unphosphorylated and an inactive phosphorylated form. During day both forms are present, but during night the unphosphorylated form is degraded (Hardtke et al., 2000). A similar regulation process could be possible for bZIP63.

However, this assay has only been done once and has to be repeated before conclusions about different phosphorylation conditions during day and night can be made. Also, comparison of samples from different tissues and under different stress conditions will be of interest.

All data on *in vivo* phosphorylation of bZIP63 were obtained from bZIP63–GFP over–expressor plants. This was necessary because the endogenous protein was inaccessible. Peptide antibodies designed against the N– or C–terminus of bZIP63 and bZIP09 did not specifically detect the endogenous proteins in a Western blot, but gave a strong background signal (see also (Hofmann Rodrigues, 2007)). The αbZIP63–C1 probe recognizes bZIP63–GFP in the over–expressor lines, but also gives a strong background signal. This probe has weak specificity for bZIP63 and can be used to detect big amounts of the protein, but is unsuitable to detect low amounts and cannot be used for Immunprecipitation.

4.2.   Identification of kinases phosphorylating bZIP63

With the help of in–gel kinase assays and Mass spectrometry analysis it was possible to identify 3 groups of potential bZIP63–specific kinases: Casein kinase II, SnRK1s and CDPKs.

Two catalytic subunits of CKII were identified, CKA1 and CKA2. These are responsible for the signal at ~35kDa in the in–gel kinase assay as could be confirmed by the use of GTP as a phosphodonor. Also, two regulatory subunits CKB1 and CKB4 were found in the affinity purified samples. In its active form CKII forms a tetramer of 2 catalytic and two regulatory subunits (Riera et al., 2001; Salinas et al., 2006). Either the catalytic or the regulatory subunits could interact with bZIP63, as both variants have been observed for other transcription factors. The high affinity of the kinase to bZIP63 would rather indicate that interaction occurs via the alpha subunits, but this will have to be confirmed by interaction assays. It is also unclear whether only one kind of tetramer binds to bZIP63, as several complex constitutions are possible.

CKII is a very important protein kinase, involved in many different processes and absolutely crucial for a plant's life (Moreno-Romero et al., 2008). Among its various substrates several bZIP transcription factors have been reported. There, phosphorylation leads to altered DNA binding properties, or protein stability (Hardtke et al., 2000; Klimczak et al., 1992). BZIP63 contains a number of predicted CKII target sites, one of them in the strongly conserved basic domain (S160). None of these sites was found phosphorylated *in vivo* yet, but the strong affinity of the kinase to the transcription factor and the clear signal in the *in vitro* kinase assay supports a role for the kinase in bZIP63 phosphorylation.

Two SnRK1 group members, AKIN10 and AKIN11 were identified, representing either one or both of the signals at 50 – 60kDa in the in–gel kinase assay. This finding was not completely unsuspected as these kinases had previously been proposed to target C/S1 group bZIP transcription factors (Baena-Gonzalez et al., 2007; Baena-Gonzalez and Sheen, 2008). Again a regulatory subunit, SNF4, was co–purified by affinity purification. One of the potential SnRK1 phosphorylation sites, S300, was found phosphorylated *in vivo*, S250 was not found phosphorylated and S294 could not be accessed by Trypsin and Subtilisin digest.

AKIN10 and AKIN11 are proposed to be regulators in energy deprivation signalling, regulating enzyme activity and expression of metabolic enzymes (Baena-Gonzalez and Sheen, 2008). The expression pattern of bZIP63 would also be consistent with a role in this process. mRNA levels increase in the dark, when energy is limited and decrease during day and when sugars are provided in the growth medium. Overall our findings support the idea that bZIP63 is regulated by SnRK1 group members.

At least one calcium dependent protein kinase could also play a role in bZIP63 phosphorylation. CPK3 was found in the range of the signals at 50 – 60kDa, again supporting a role for this kinase in bZIP63 regulation. In contrast to this finding, in–gel kinase assays with protein extracts form *cpk3* KO plants showed no difference to extracts from wt

plants. This means that either none of the signals comes from CPK3 or that CPK3 runs at the same position as one of the AKINs or other CDPKs, which could mask the lack of signal from CPK3. Other CDPKs, CPK11 and CPK5, were also found, but peptide coverage for these proteins was unsatisfactory. From these data it is impossible to conclude which, if any, CDPKs regulate bZIP63. However, when calcium was omitted in an in–gel kinase assay the bands at 50 – 60kDa weakened significantly, supporting involvement of calcium dependent protein kinases. To improve identification for the CDPK group it might be necessary to include calcium in the affinity purification, because CDPKs need calcium for activation and substrate binding.

Involvement of other kinases cannot be excluded. When C–terminally tagged bZIP63 was used for affinity purification an additional band at 27kDa could be seen. This band sometimes also appeared in crude protein extracts.

In affinity purification with splicing form 2 and 1 an additional band appeared at 40kDa when splicing form 2 was used. It is tempting to suggest that activity of the different splicing forms is differentially regulated by a kinase. Controversial to this idea this band was not detected in the crude protein extract. The experiment had only been done once and will have to be repeated to see whether the signal is a very low abundant kinase or an artefact from the affinity purification.

For neither of these signals a kinase could be identified by Mass spectrometry.

4.3.   Regulation of bZIP63 activity by phosphorylation

Several *in vivo* phosphorylation sites and kinases have been identified for bZIP63, but at this point it is still unclear what the function of each kinase and phosphorylation site is.

Phosphorylation of the predicted phospho–sites in the basic domain could have an effect on localisation and DNA binding. First DNA binding assays hint that phosphorylation of S164 and S168 would reduce bZIP63 activity (data from Christina Chaban, University of Tuebingen). Whether this is relevant *in vivo* cannot be answered yet, as it was not possible to determine the phosphorylation state of this region. Regulation of bZIP63 activity by changing of subcellular localisation is rather unlikely, as bZIP63–GFP was always observed inside the nucleus.

In vitro target sites for CPK3 were found in the N– and C–terminal part of the protein and especially in the zipper domain. AKIN target sites are in the C–terminal region of bZIP63. Phosphorylation in the leucine zipper, but also in the terminal regions of the protein might affect transcription factor activity by altering dimerization properties. Phosphorylation could

prevent or support dimer formation or lead to interaction with different dimerization partners, which would result in differential gene expression patterns.

Also interaction with other proteins might be affected by phosphorylation. This could have consequences on protein stability like it is the case for HY5 (Hardtke et al., 2000) or on transcription factor activity. It is likely that bZIP activity regulation happens on the level of interaction with other transcriptional activators or repressors or with parts of the transcriptional machinery.

BZIP63 has 3 splicing forms, 2 of which are significantly expressed *in vivo*, as could be shown by sqRT–PCR with splicing form specific primers (data from Bernhard Wurzinger). MRNA levels of splicing forms 1 and 2 are about equal, but splicing form 3 is much lower expressed. Relevancy of this splicing form is further challenged by the fact that in the *Arabidopsis* ecotype Wassilewskija the protein would be 22 amino acids shorter due to a frameshift. One interesting question is whether both splicing form 1 and 2 are relevant *in vivo* and if their function is overlapping or diverse. The same question arises for most *Arabidopsis* proteins, because the presence of more than one splicing form is quite common. Affinity purification with splicing form 2, but not with the 7aa shorter splicing form 1 accumulated a kinase activity at ~ 40kDa. Generally, differential regulation of splicing forms via splicing form specific phosphorylation would be a neat mechanism to quickly change balance between two different forms of a protein. Whether this really happens in the case of bZIP63 is rather questionable. These two splicing forms are very similar and most probably have overlapping properties. At the moment I have too little data to make any conclusion.

4.4.   Conservation of *bZIP63* in different ecotypes

The genomic sequence of Col–0 and Ws–2 *bZIP63* features a number of differences. Many base changes can be observed throughout the DNA sequence, but most of them do not result in amino acid changes. Drastic changes like insertions and deletions are only found in intron sequences and do not affect the protein sequence with the exception of splicing form 3, which is shorter in Ws–2. Overall the protein sequence is quite conserved between these two ecotypes, indicating relevancy of bZIP63 for the plant. Importantly, all of the serines, which were found to be phosphorylated *in vivo* are conserved, but the kinase recognition motif may be disturbed in 2 cases. Two amino acids C–terminal to S29 a tryptophan is exchanged for a glycine and one amino acid C–terminal to S261 a proline is exchanged for a leucine.

5. Outlook

At this point I could identify several protein kinases with the potential to phosphorylate bZIP63 specifically and moreover, I was able to identify 4 *in vivo* phosphorylation sites on bZIP63 *in planta*.

One big aim in the near future is to compare the phosphorylation state of bZIP63–GFP in different tissues and under different conditions with the help of 2D gels. The knowledge under which conditions bZIP63 is phosphorylated strongest will also help to confirm and further identify *in vivo* phosphorylation sites. Knowledge of the *in vivo* sites may then allow to quantify phosphorylation of each individual site in the cell with a mass Western approach (Lehmann et al., 2008).

Apart from that the focus will shift more into the direction of the function of phosphorylation. Relevancy of the identified kinases will be tested in *in vitro* kinase assays and by co–localisation and interaction studies. A big question will be which kinase targets which site. The question of the *in vivo* function of each phosphorylation event on localisation, dimerization, DNA binding and expression of target genes will be addressed together with our collaboration partners within the bZIP network (http://www.zmbp.uni-tuebingen.de/PlantPhysiology/bzip/index.html).

Another effort will be the identification of interaction partners for bZIP63. Interaction with other bZIP transcription factors of the C/S1 group will be addressed with a multi colour BiFC approach to answer the question which heterodimers form preferentially in the cell. One interesting candidate for dimerization is bZIP10, which was found several times in the affinity purified proteins extracts. To address the question of interaction partners more broadly, affinity purification and Co–IP will be used to find candidate proteins.

6. Supplementary figures

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    MEKVFSDEEI SGNHHWSVNG MTSLNRSASE WAFNRFIQES SAAADDGEST TACGVSVSSP
IP2 MEKVFSDEEI SGNHHWSVNG MTSLNRSASE WAFNRFIQES SAAADDGEST TACGVSVSSP
IP4 MEKVFSDEEI SGNHHWSVNG MTSLNRSASE WAFNRFIQES SAAADDGEST TACGVSVSSP
IP5 MEKVFSDEEI SGNHHWSVNG MTSLNRSASE WAFNRFIQES SAAADDGEST TACGVSVSSP
IP6 MEKVFSDEEI SGNHHWSVNG MTSLNRSASE WAFNRFIQES SAAADDGEST TACGVSVSSP

 61 PNVPVDSEEY RAFLKSKLNL ACAAVAMKRG TFIKPQDTSG RSDNGGANES EQASLASSKA
 61 PNVPVDSEEY RAFLKSKLNL ACAAVAMKRG TFIKPQDTSG RSDNGGANES EQASLASSKA
 61 PNVPVDSEEY RAFLKSKLNL ACAAVAMKRG TFIKPQDTSG RSDNGGANES EQASLASSKA
 61 PNVPVDSEEY RAFLKSKLNL ACAAVAMKRG TFIKPQDTSG RSDNGGANES EQASLASSKA
 61 PNVPVDSEEY RAFLKSKLNL ACAAVAMKRG TFIKPQDTSG RSDNGGANES EQASLASSKA

121 TPMMSSAITS GSELSGDEEE ADGETNMNPT NVKRVKRMLS NRESARRSRR RKQAHLSELE
121 TPMMSSAITS GSELSGDEEE ADGETNMNPT NVKRVKRMLS NRESARRSRR RKQAHLSELE
121 TPMMSSAITS GSELSGDEEE ADGETNMNPT NVKRVKRMLS NRESARRSRR RKQAHLSELE
121 TPMMSSAITS GSELSGDEEE ADGETNMNPT NVKRVKRMLS NRESARRSRR RKQAHLSELE
121 TPMMSSAITS GSELSGDEEE ADGETNMNPT NVKRVKRMLS NRESARRSRR RKQAHLSELE

181 TQVSQLRVEN SKLMKGLTDV TQTFNDASVE NRVLKANIET LRAKVKMAEE TVKRLTGFNP
181 TQVSQLRVEN SKLMKGLTDV TQTFNDASVE NRVLKANIET LRAKVKMAEE TVKRLTGFNP
181 TQVSQLRVEN SKLMKGLTDV TQTFNDASVE NRVLKANIET LRAKVKMAEE TVKRLTGFNP
181 TQVSQLRVEN SKLMKGLTDV TQTFNDASVE NRVLKANIET LRAKVKMAEE TVKRLTGFNP
181 TQVSQLRVEN SKLMKGLTDV TQTFNDASVE NRVLKANIET LRAKVKMAEE TVKRLTGFNP

241 MFHNMPQIVS TVSLPSETSN SPDTTSSQVT TPEIISSGNK GKALIGCKMN RTASMRRVES
241 MFHNMPQIVS TVSLPSETSN SPDTTSSQVT TPEIISSGNK GKALIGCKMN RTASMRRVES
241 MFHNMPQIVS TVSLPSETSN SPDTTSSQVT TPEIISSGNK GKALIGCKMN RTASMRRVES
241 MFHNMPQIVS TVSLPSETSN SPDTTSSQVT TPEIISSGNK GKALIGCKMN RTASMRRVES
241 MFHNMPQIVS TVSLPSETSN SPDTTSSQVT TPEIISSGNK GKALIGCKMN RTASMRRVES

301 LEHLQKRIRS VGDQ*
301 LEHLQKRIRS VGDQ*
301 LEHLQKRIRS VGDQ*
301 LEHLQKRIRS VGDQ*
301 LEHLQKRIRS VGDQ*
```

Supplementary figure 1: Sequence coverage of all 4 digests (IP2, IP4, IP5, IP6) and MS/MS runs after Immunprecipitation of bZIP63–GFP. The sequence that was covered by at least one peptide is shown in orange. Underlined and blue amino acids were found phosphorylated.
Lane 1 shows overall peptide coverage. IP2: 16h dark, 1h Subtilisin digest; IP4: 4.5h dark, Trypsin digest; IP5: 4.5h dark, 0.5 Subtilin digest; IP6: 4.5 dark, 2h Subtilisin digest

```
Col-0 ATGGAAAAAG TTTTCTCCGA CGAAGAAATC TCCGGTAACC ATCACTGGTC GGTTAATGGA
Ws-2  ATGGAAAAAG TTTTCTCCGA CGAAGAAATC TCCGGTAACC ATCACTGGTC GTTTAATGGA

  61 ATGACGTCGT TGAATC GCAG TGCTTCCGAA TGGGCATTCA ATCGTTTCAT ACAAGAATCC
     ACGACGTCGT TGAATCXGTAG TGCATCCGAA GGGGCATTCA ATCGCTTCAT ACAAGAATCC

 121 TCCGCCGCTG CAGACGACGG AGAATCTACG ACGGCGTGTG GTGTTTCCGT CTCCTCTCCT
     TCCGCCGCTG CAGACGACGG AGAATCTGCG ACGGCGTGTG GTGTTTCCGT CTCTTCTCCT

 181 CCTAATGTTC CTGTAGATTC AGAGGAATAC AGAGCATTTC TCAAGAGTAA ACTTAATCTT
     CCTAATGTTC CTGTAGATTC AGAGGAATAC AGAGCATTTC TCAAGAGTAA ACTTAATCTT
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 241 GCTTGTGCTG CTGTCGCCAT GAAAAGGgta ctcactaCtc tgctcaagtt tgAgtttttt
     GCTTGTGCTG CTGTCGCCAT GAAAAGGgta ctcactaTtc tgctcaagtt tgGatttttt

 301 t-----ttttttcat agaaa-aagaa aaacAaaacc tttttatctg ttAgtttgat
gagttcactt
     tAtatttttttcat agaaGaagaa aaacCaaacc -----a---- --a----gat
gagttcactt

 361 gaagctaaga aactaatctt gttTtgttat gtttgagtgt taagatctta ttggaCcttt
     gaa------- ------tctt gttGtgttat gtttgagtgt taagatctta ttggaTcttt

 421 tgtatatgat ttgccattat cttccttgag attataattt gcattagtta attgatTttg
     ---------- ---------- ---------- ---------- ---------- attga-----

 481 tggtgttaat attgaaaaTt tGatagGGAA CTTTCATCAA ACCTCAGGAT ACTTCTGGTA
     ---------- ------aaCt tCatagGGAA CTTTCATCAA ACCTCAGGAT ACTTCTGGTA

 541 GATCTGACAA TGGTGGAGCC AATGAATCAG AACAAGCCTC TCTTGCTTCT TCCAAAGgtt
     GATCTGACAA TGGTGGAGCC AATGCATCAG AACAAGGCTC TCTTGCTTCT TCCAAAGgtt

 601 ttacaatAc----a tCtat--------------gata--------a gccttt-----taag
     ttacaatGctctca tGtttctttgaatttggttagatagattgtgga gccttTacctcTatg

 631 atg-----ttaTtga tagttgttgt gTttgttttc aagatttctt gtaaatgcta atggaCCaat
     atgAgccttTtaaga tagttgttgt gAttgttttc aagatctc-- ----atgctG atggaTTaat

 691 ctGtatgcag CTACACCAAT GATGAGCAGT GCTATAACAA GTGGATCTGA GCTCTCTGGT
     ctTtatgcag CTACACCAAT GATGAGCAGT GCTATAACAA GTGGTTCTGA GCTCTCTGGT

 751 GATGAAGAAG AAGCTGATGG TGAAACTAAT ATGAACCCTA CTAATGTTAA ACGCGTTAAA
     GATGAAGAAG AAGCTGATGG TGAAACTAAT ATGAACCCTA CTAATGTTAA ACGCGTGAAA

 811 AGgtaacaaa tttacatcct ttcttGgact tgaacAtgaa catgaacGtg aa--tttgtttg
     AGgtaacaaa tttacatcct ttcttTgact tgaacGtgaa catgaacAtg aacatttgtttg

 871 ttatagGATG CTCTCTAATA GAGAATCAGC TAGACGGTCC AGAAGAAGAA AGCAAGCACA
     ttatagGATG CTCTCTAATA GAGAATCAGC TAGACGATCC AGAAGAAGAA AGCAAGCACA

 931 CTTGAGTGAG CTAGAGACAC AAgtatgtta ctctcgcagc gttcttattc tttctccttt
     CTTGAGTGAG CTAGAGACAC AAgtatgtta ctctcgcagc gttcttattc tttctccttt

 991 aactttgttt agctcActtg t-----gCgacaatc tccatttcgt gtagGTTTCA
CAGCTTCGTG
     aactttgttt agctcCcttg tcagttgtgacaatc tccatttcgt gtagGTTTCG
CAGCTTCGTG

1051 TAGAGAATTC AAAACTCATG AAGGGTCTCA CTGATGTAAC TCAAACATTC AATGATGCAT
     TAGAGAATTC AAAACTCATG AAGGGTCTCA CTGATGTAAC TCAAACATTC AATGATGCAT

1111 CTGTAGAAAA CAGAGTTTTA AAAGCCAATA TTGAGACACT ACGAGCAAAG GT-aaaatact
     CTGTAGAAAA CAGAGTTTTA AAAGCCAATA TTGAGACACT ACGAGCAAAG GTTaaaatact

1171 ttCttttAtc tctctgtcta ccaaaactcg ctattgaaGc atgtttgttt ttggcaggtG
     ttGttttCtc tctctgtcta ccaaaactcg ctattgaaAc atgtttgttt ttggcaggtG

1231 AAAATGGCTG AAGAGACAGT GAAGAGACTC ACTGGCTTTA ACCCAATGTT CCACAATATG
     AAAATGGCTG AAGAGACAGT GAAGAGACTC ACTGGCTTTA ACCCAATGTT CCACACTATG

1291 CCTCAGATTG TTTCAACTGT CTCTCTTCCT TCAGAGACAT CAAATTCTCC AGACACTACA
     CCTCAGATTG TTTCAACTGT CTCTCTTCCT TCAGAGACAT CAAATTCTCT AGACACTACA

1351 AGCAGCCAAG TGACTACACC AGAGATCATT AGCTCGGGGA ACAAAGGCAA GGCCTTGATA
     AGCAGCCAAG TGACTACACC AGAGATCATC AGCTCGGGGA ACAAAAGCAA GGCCTTGATA
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90

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1411 GGGTGCAAGA TGAACAGAAC AGCTTCGATG CGTAGAGTTG AGAGCTTGGA ACATCTGCAG
     GGGTGCAAGA TGAACAGAAC AGCTTCGATG CGTAGAGTTG AGAGCTTGGA AC????????

1471 AAACGTATTC GAAGCGTTGG GGATCAGTAG
     ?????????? ?????????? ??????????
```

Supplementary figure 2: Genomic sequence comparison of *bZIP63* from ecotype Columbia 0 (Col–0) and Wassilewskija 2 (Ws–2)
splicing form 2: EXON, intron
splicing form 1: like 2, but 507 – 527 = intron
splicing form 3: like 2, but 1163 – 1241 = exon, stop at 1241
sequence differences: base changed, base lacking
X position of the T–DNA insertion in the *bzip63* KO line
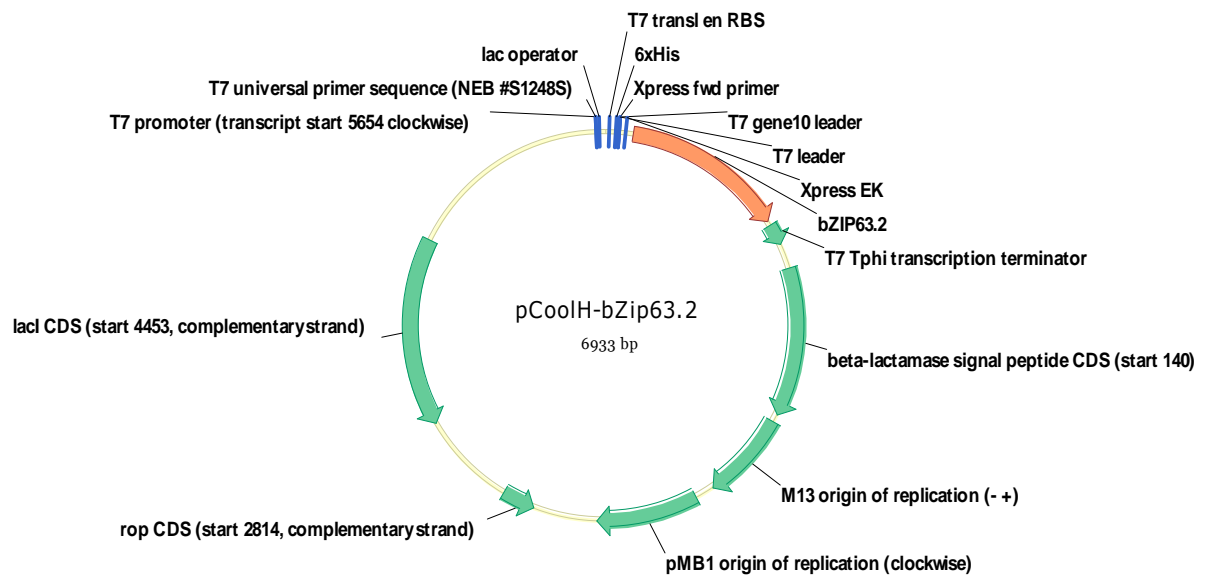
Supplementary figure 3: Vector map of pCoolH–bZIP63.2

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