Analysis of a novel kelch domain-containing protein from maize

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“What is known for certain is dull.”

Max Ferdinand Perutz

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Abbreviations

aa	amino acid	**HEPES**	4-(2-hydroxyethyl)-1-piperazinethane
ABA	abscisic acid		sulfonic acid
ACC	1-amino-1-cyclopropane carboxylic acid	**IAA**	indole-3-acetic acid
AD	activation domain	**kb**	kilo base(s)
APS	ammonium persulfate	**MES**	2-(N-morpholino)ethane sulfonic acid
3-AT	3-aminotriazole	**MOPS**	3-(N-morpholino)propane sulfonic acid
BAP	benzylaminopurine	**mRNA**	messenger ribonucleic acid
BD	binding domain	**NAA**	naphthalene acetic acid
bp	base pair(s)	**OD_x**	optical density at the wavelength x
BSA	bovine serum albumin	**PAGE**	polyacrylamide gel electrophoresis
cDNA	complementary deoxyribonucleic acid	**PCR**	polymerase chain reaction
co-IP	co-immunoprecipitation	**PEG**	polyethylene glycol
2,4-D	2,4-dichlorphenoxy acidic acid	**RNA**	ribonucleic acid
dap	day(s) after pollination	**RT-PCR**	reverse transcription polymerase chain
DEPC	diethylpyrocarbonate		reaction
dH_2O	deionised water	**SDS**	sodium dodecyl sulfate
DNA	deoxyribonucleic acid	**TCA**	trichloracetic acid
DTT	dithiothreitol	**TEMED**	N,N,N',N'-tetramethylethylenediamine
EDTA	ethylenediamine tetraacetic acid	**TNT**	*in vitro* transcription/translation reaction
GA_3	gibberellin A_3	**Tris**	Tris(hydroxymethyl)aminomethane
GST	glutathione-S-transferase	**YFP**	yellow fluorescent protein

1. Introduction

1.1 Domains are conserved building blocks of proteins

1.2 Molecular propellers: neu, RCC, WD-40 and kelch repeats

1.3 Kelch domain proteins are multifunctional all-rounder

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1.5 Object of this thesis

1. Introduction

"*[Macromolecules] carry their own names in the form of the dispositions of nucleotides and amino acids in chemical space, either as linear sequences or on the surfaces of three-dimensional structures. The objects have their own names: they are chemical names written in the language of DNA sequences and the arrangements of amino acids on protein surfaces. It is the interactions between these objects that create the processes that produce outcomes for cells, organs and organism.*"

This sentence from Sydney Brenner clearly reveals the most challenging task of today's science: not isolated proteins, but only their interactions with each other lead to a real understanding of their function.

Proteins, the fundamentals of life, have a multitude of functions: they are catalytically active nano-machines producing complex compounds and storable forms of energy, they build stabilizing structures and regulating gates where boundaries have to be overcome; they are involved in the perception of internal and external signals and provide tools for their implementation – but all of these tasks cannot be accomplished by a single protein, but require the interactions with others.

To completely understand a protein's function, it is thus essential to look at the context in which it acts. This is not an easy task, but often the presence of certain conserved domains with known functions proves to be a very helpful tool.

1.1 Domains are conserved building blocks of proteins

Domains are spatially distinct regions of the protein that can fold and function in isolation (DOOLITTLE, 1995; JANIN and CLOTHIA, 1985).

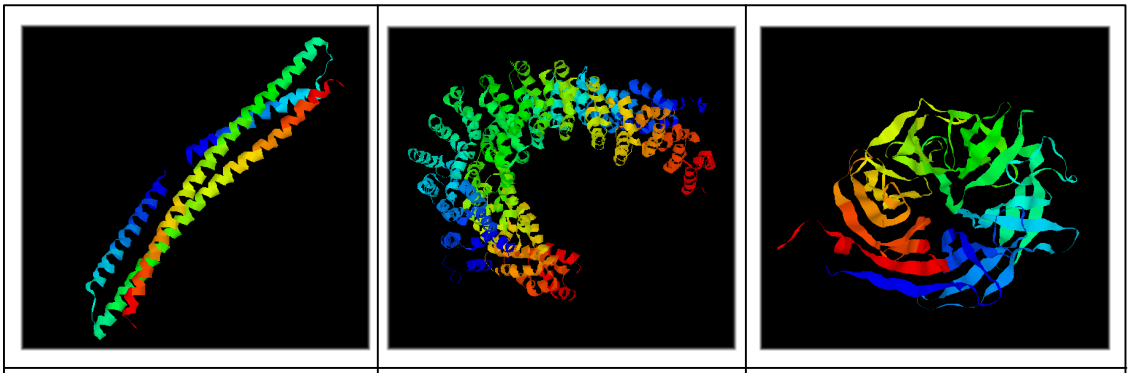
Interestingly, three-dimensional structures are often more conserved than sequences. Two sequences can diverge beyond the limits of sequence similarity detection methods, but can still have a similar structure and function. Good examples for this phenomenon are adenylate cyclase and DNA polymerase: though they lack obvious sequence simila-

rity, both enzymes contain certain conserved residues at their active sites and catalyse similar reactions (ARTYMIUK et al., 1997).

Both essential and hyper-adaptable domains that are suited to many beneficial functional niches are of ancient origin and can be found in all three forms of cellular life, that is in archaea, bacteria and eukarya. On the other hand, there are domains that are unique to eukaryotes, like chromatin-associated (e.g., bromodomains) or actin-binding cytoskeletal domains (like the cofilin-domain) that have not been found in prokaryotic proteomes (PONTING *et al.*, 2000).

Domains are often formed by a number of repeated elements, created through several steps of duplication and recombination within a single gene (OHNO, 1999).

These repeats can be classified into three groups, namely those forming linear rods like in spectrin, superhelices (e.g., in HEAT repeats) or those forming closed structures like β-trefoils or β-propellers (PONTING and RUSSELL, 2002). Examples for members from each of these groups are shown in figure 1.1.



A	***spectrin***	***B***	***protein phosphatase***	***C***	***sialidase***

Fig. 1.1: *Domains formed by repeats can be categorised into three groups:* ***A:*** *linear rods as in spectrin from Drosophila melanogaster (chain A, PDB id: 2spc; YAN et al., 1993),* ***B:*** *superhelices like in the regulatory domain of human protein phosphatase 2a, pr65 alpha formed by HEAT-repeats (chain A, PDB id: 1b3u; GROVES et al., 1999) or* ***C:*** *closed structures as the sialidases/neuraminidases repeats forming a b-propeller found for example in the N-terminal domain of Micromonospora sialidase (PDB id: 1eur; TAYLOR et al., 1992; GASKELL et al., 1995). All structures are viewed with RasMol.*

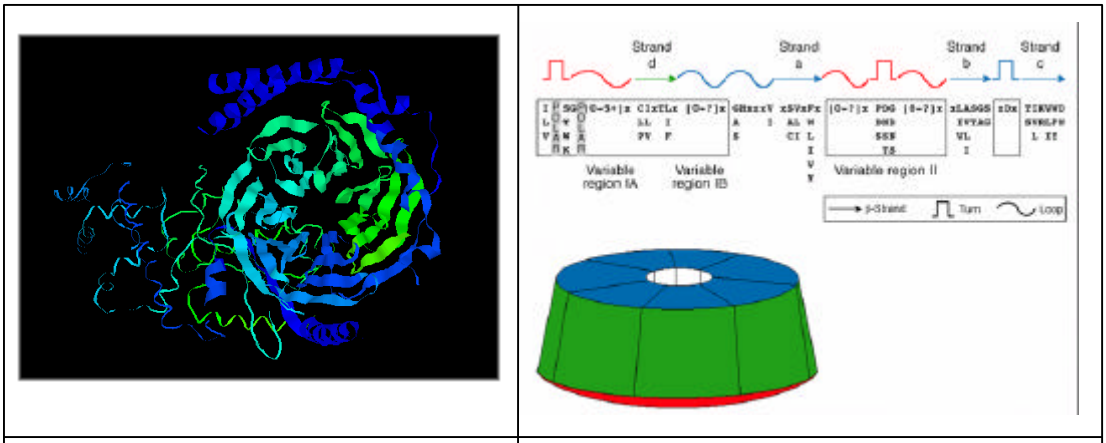
1.2 Molecular propellers: neu, RCC, WD-40 and kelch repeats

To date, four classes of repetitive elements are known to form β-propellers: neu, RCC, kelch and WD-40 repeats. RCC and neu have only a very limited distribution and are present in just one class of proteins, from which they got their names: neu-repeats were discovered in neuraminidases/sialidases that cleave sialic acid from glycoproteins, and RCC repeats have merely be found in the regulator of chromosome condensation (RCC1). Neu repeats form propellers with six blades (CRENNELL *et al.*, 1993 and 1996), RCC those with seven blades (RENAULT *et al.*, 1998).

These highly restricted repetitive elements stand in contrast to the WD-40 repeats that are found in many different classes of proteins. The best characterized WD-40 protein and the one, in which the WD-40 repeats have originally been found, is the β-subunit of the heterotrimeric guanine nucleotide-binding (G)-protein (FONG *et al.*, 1986).
This protein-complex is comprised of three subunits: the α-, β- and γ-subunit (Gα, Gβ and Gγ). The trimer is associated with membrane-bound G-protein-coupled receptors through Gβ. Upon activation of the receptor, the GDP bound by Gα is exchanged for GTP and subsequently Gα and the Gβ/Gγ-dimer dissociate. Both components will then elicit the cellular response.
The crystal-structure of the heterotrimeric complex has been solved (WALL *et al.*, 1995; LAMBRIGHT *et al.*, 1996) and is shown in figure 1.2.

Other prominent members of the WD-40 repeat family are the RACKs (receptor for activated C kinase) that are highly homologous to Gβ.
RACKs are anchor proteins that participate in protein kinase C (PKC) signalling. They do not only bind to PKC, but also to other proteins like phospholipase Cγ (DISATNIK *et al.*, 1994), dynamin (RODRIGUEZ *et al.*, 1999), integrins (LILIENTAL and CHANG, 1998), Src (CHANG *et al.*, 1998) and phosphodiesterase 4D5 (YARWOOD *et al.*, 1999). They connect different signalling pathways and different organelles or sites within the cell (SCHECHTMAN and MOCHLY-ROSEN, 2001) and changes in the expression pattern of

mammalian RACK proteins are related to different forms of cancer (BERNS *et al.*, 2000; CASTAGNA *et al.*, 1982).



A ***trimeric G-protein*** ***B*** ***structure of WD-40 repeats***

Fig. 1.2: *The trimeric G-protein as an example for WD-40 proteins* ***A:*** *Three-dimensional structure of the human trimeric G-protein (PDB id: 1got; viewed with RasMol). The WD-40 repeat domain forms the b-propeller in the centre of the protein, while Ga and the small Gg are at the sides (WALL et al., 1995; LAMBRIGHT et al., 1996).* ***B:*** *Consensus sequence of WD-40 repeats and schematic illustration of the three-dimensional structure. Colours used in the sequence indicate the position of the strand in the schematic three-dimensional structure. Taken from SMITH et al. (1999).*

Not much is known about the function of plant RACKs. RACK-like proteins have been found in many plants like *Arabidopsis* (accession U77381 and AB020749), alfalfa (MCKHANN *et al.*, 1997) and rice. The best characterised plant RACK is an auxin-inducible gene from tobacco, *arcA* (auxin-responsive gene in cultured cells; ISHIDA *et al.*, 1993 and 1996). Besides its hormone-responsiveness, it is thought to be involved in the cell cycle arrest caused by salicylic acid and UV irradiation (PERENNES *et al.*, 1999).

Most WD-40 proteins contain seven repeats, but the number varies from three (as in coronin from *Trichomonas vaginalis*; BRICHEUX *et al.*, 2000) to sixteen (like in Q55563 from *Synechocystis spec.*; KANEKO *et al.*, 1995). They are only weakly conserved and consist of about forty to sixty amino acids that are initiated by a glycine-histidine dipeptide and stop with the tryptophan-aspartic acid dipeptide that gave rise to the name (WD; SMITH *et al.*, 1999).

Although they do not share any conserved residues, both the WD-40 and the kelch repeats, another propeller-forming group of repetitive elements, form the same tertiary

structure: each repeat builds a four-stranded β-sheet resulting in a blade-like structure that lies tilted around a central axis forming a β-propeller (ITO *et al.*, 1999; illustrated in figure 1.3). The β-strands are linked via loops that can project above, below or at the sides of the circular structure. The result is a flattened shape with three accessible surfaces: the top, the bottom and the rim.

Although galactose oxidase from the fungus *Hypomyces rosellus* is the only kelch domain protein with a known three-dimensional structure, it is proposed that this propeller-like arrangement is conserved among all kelch domain proteins, because of the invariant ordering of primary sequence in all other known β-propellers and the sequence identities within the kelch motifs (MURZIN, 1992).

Kelch repeats are typically between 44 and 56 amino acids in length and have a very low sequence identity. Only eight conserved key residues are necessary: four hydrophobic amino acids that are followed by a double glycine and two separated aromatic residues, typically tyrosine and tryptophan (ADAMS *et al.*, 2000). An alignment of one kelch repeat from different kelch domain proteins together with the consensus sequence is shown in figure 1.3. It also indicates the characteristic spacing of the conserved residues.

Kelch domains typically consist of six or seven kelch repeats (ADAMS *et al.*, 2000), but sequences containing fewer repeats are also deposited in the databases (like FbfB from *Stigmatella aurantiaca* with only three repeats; SILAKOWSKI *et al.*, 1998). However, it is not clear, whether or not these proteins are able to form β-propellers as the most advantageous number of repeats for the formation of such a structure is seven like in galactose oxidase, although six repeat-propellers are sterically possible, if they contain small residues on the inner strands (MURZIN, 1992).

Kelch repeats are found in many different protein types, including enzymes, as opposed to WD-40 repeats that have so far only been reported from regulatory proteins.

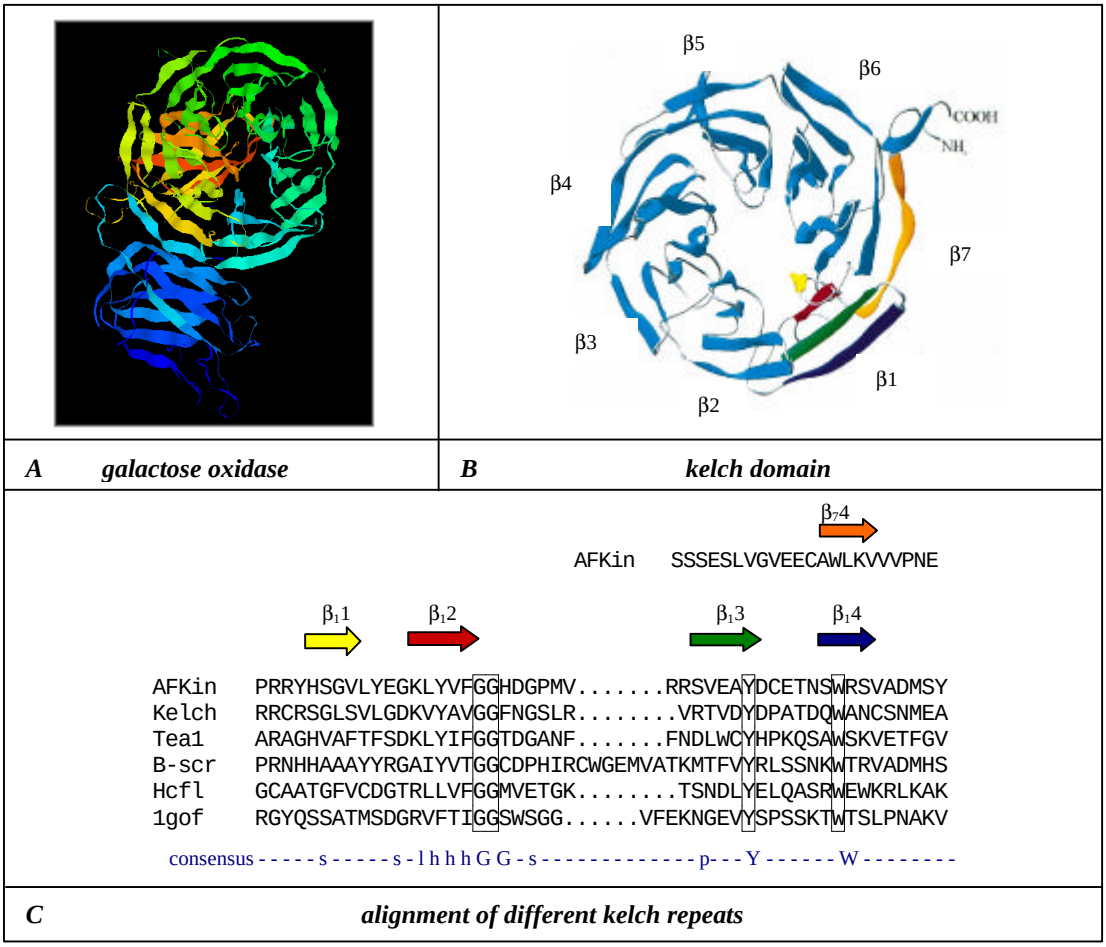


Fig. 1.3: *Kelch repeats form b-propellers.* ***A:*** *Three-dimensional structure of galactose oxidase from Hypomyces rosellus at pH 4.5 (PDB id: 1gof; viewed with RasMol).* ***B:*** *similar to A, but only the kelch domain with seven kelch repeats (named b1-b7) is shown (taken from* ADAMS *et al., 2000).* ***C:*** *Alignment of one kelch repeat from six different kelch domain proteins. The consensus sequence is given below: only the diglycine, tyrosine and tryptophan residues are conserved in over 90% of all kelch repeats. Arrows mark individual b-strands (colour coded as in B; according to* ADAMS *et al., 2000).* ***Abbreviations: h*** *hydrophobic residue,* ***l*** *large residue,* ***p*** *polar residue,* ***s*** *small residue.*

1.3 Kelch domain proteins are multifunctional all-rounder

Kelch domains, like the WD-40 repeats, usually represent only one part of a protein. They are often combined with other domains that allow these proteins to function in many different contexts. Therefore, the presence of kelch repeats does not reveal a protein's function, although many of the so far characterized kelch domain proteins are

associated with the cytoskeleton. However, most kelch repeat-containing proteins share the property of acting via protein/protein interactions.

The repeats were first found in the *Drosophila* kelch gene, which, when mutant, confers female sterility. The resulting incomplete egg-shell is goblet-shaped, kelch being the German word for goblet (SCHÜPBACH and WIESCHHAUS, 1991).
In *Drosophila*, the egg cell is connected with 15 nurse cells via intracellular bridges (the so called ring canals) that result from incomplete cytokinesis (for review see SPRADLING, 1993; COOLEY and THEURKAUF, 1994). During maturation of the egg, different proteins are added to these arrested cleavage furrows. Among them are F-actin (KOCH and KING, 1969), a phosphotyrosine containing protein (ROBINSON *et al.*, 1994), the Hu-li tai shao ring canal protein (YUE and SPRADLING, 1992; ROBINSON *et al.*, 1994) and the kelch protein (XUE and COOLEY, 1993; ROBINSON and COOLEY, 1997).
Kelch is the last protein to be located in the ring canal and does not arrive until the maximum number of actin filaments has been recruited to it (TILNEY *et al.*, 1996). Upon the arrival of kelch, the ring canal expands from a diameter of 3-4 µm to 10 µm. The kelch mutant shows normal morphology of the ring canal before the time of this expansion, but becomes disorganised later on and results in a female sterile phenotype due to insufficient plasma transfer into the oocyte.
It is assumed that kelch is required to maintain the organisation of actin filaments rather than for their assembly (ROBINSON *et al.*, 1994; TILNEY *et al.*, 1996).

Besides the *Drosophila* kelch protein, many other proteins containing kelch repeats have been found to be associated with the cytoskeleton. The human protein mayven shares 63% identity with the *Drosophila* kelch and has also been shown to bind actin. It is predominantly expressed in brain tissue and changes its distribution within the cell upon depolarisation of primary neurons (SOLTYSIK-ESPANOLA *et al.*, 1999).
The human kelch domain protein Keap1 interacts not only with actin, but also with myosin. Keap1 suppresses the transcription factor Nrf2 and thus prevents the induction of detoxifying and oxidative stress proteins in the presence of electrophiles and reactive oxygen species (VELICHKOVA *et al.*, 2002).

Another interesting example of kelch domain proteins are α- and β-scruin. Both proteins are composed of two kelch domains, one at the N- and one at the C-terminus. They share 67% identity with each other, but α-scruin is involved in cross-linking the actin filaments in the acrosomal process of *Limulus* sperm (SCHMID *et al.*, 1991 and 1994; SHERMAN *et al.*, 1999; WAY *et al.*, 1995a), while β-scruin is located within the acrosomal vesicle, where no actin is found (WAY *et al.*, 1995b).

Although most of the above mentioned kelch domain proteins interact with the cytoskeleton, their function and mode of action remains unknown. In contrast, the actin-fragmin kinase of the slime mold *Physarium polycephalum* that contains six kelch repeats in its C-terminal half has been shown to phosphorylate actin (EICHINGER *et al.*, 1996) and therefore has kinase activity.
Another kelch domain protein with enzymatic activity is galactose oxidase that oxidises primary alcohols to aldehydes (MCPHERSON *et al.*, 1992). It is also different from most other proteins in that it is extracellular. Another extracellular protein containing kelch repeats is attractin that promotes monocyte spreading and T-cell clusters in the human immune response (DUKE-COHAN *et al.*, 1998).
There is even a kelch domain protein among the class of transmembrane proteins, encoded by the mouse mahogany locus that suppresses agouty-lethal-yellow pigmentation and obesity (GUNN *et al.*, 1999; NAGLE *et al.*, 1999)

1.4 Plant kelch domain proteins

Kelch domain proteins are also found in the plant kingdom. The first two genes encoding such proteins, *ZEITLUPE* (*ZTL*) and *FKF1* (for flavin-binding, kelch repeat, F box) from *Arabidopsis thaliana* were published simultaneously; both are clock-associated proteins involved in the determination of the flowering-time (NELSON *et al.*, 2000; SOMERS *et al.*, 2000). *ZTL* was also published as *LKP1* (LOV kelch protein 1) by KIYOSUE and WADA (2000). A third member of the ZTL-like kelch proteins, LKP2, has been characterised by SCHULTZ *et al.* (2001) and is also involved in the circadian clock. Overexpression of these genes leads to an early flowering phenotype and the lack of functional *ZTL* causes late flowering.

Besides the kelch domain, all three proteins also contain the F-box motif. They belong to the large group of F-box/kelch domain proteins, a family with at least 48 members in *Arabidopsis* (ANDRADE *et al.*, 2001). Studies on mammalian F-box proteins indicate that they are involved in the controlled degradation of cellular regulator proteins through ubiquitination and proteolysis by the 26S proteasome (BAI *et al*., 1996; SKOWYRA *et al*., 1997).

The second group of plant kelch domain proteins is the family of protein phosphatases with kelch-like repeat domains (PPKLs; KUTUZOV and ANDREEVA, 2002). In the *Arabidopsis* genome there are four such PPKLs of which three have been found in EST-databases and are therefore expressed. They all contain five complete kelch repeats and an additional one lacking the C-terminal half. So far nothing is known about their target proteins or the context in which they act.

1.5 Object of this thesis

As mentioned above, there are currently only two groups of kelch domain proteins described in plants – the ZTL-family of clock-associated genes and the protein phosphatases with kelch-like repeats. Nevertheless, by the end of 2002 there were already 126 proteins containing a kelch domain listed in the Pfam database (http://pfam.wustl.edu/index.html; BATEMAN *et al*., 2002) for *Viridiplantae* and 155 for *Metazoa* (see table 1).

Taxon		**Number of kelch domain proteins found**
Eukaryota	Viridiplantae	126
	Euglenozoa	1
	Mycetozoa	2
	Fungi	12
	Metazoa	155
	Alveolata	1
Bacteria	Proteobacteria	6
	Firmicutes	1
Archaea	Crenachaeota	1
	Euryarchaeota	1

Tab. 1: *List of all proteins found in the Pfam-database containing a kelch domain*

Out of these 126 plant kelch domain proteins, 123 are from *Arabidopsis* and three are from rice. It can be assumed that kelch repeats are not only versatile and important components of metazoan proteins, but are of equal importance in plants. It shows that there is a backlog in research on plant kelch domain proteins and therefore, it seemed reasonable to work on a novel type of kelch domain protein that was found in maize (HUEROS, unpublished).

This protein, preliminary called ZmKEL1, was furthermore of special interest, because it also contains a conserved sequence stretch in the N-terminus that can be found in several other plant proteins and is for that reason thought to be a novel, plant-specific domain. However, none of these proteins has a precisely defined function, and thus no prediction regarding the function of this domain was possible.

The subject of this thesis was to analyse the function of ZmKEL1 including the role of this novel N-terminal domain. As the presence of a kelch domain does not indicate a defined function, nothing about the cellular context in which it acts was known.
Proteins showing homology to ZmKEL1 are present in *Arabidopsis* and rice. The presence of an *Arabidopsis*-homologue was thought to be particularly helpful as it facilitates working with transgenic RNAi-, antisense- or overexpression-lines and it was hoped to find knock-out mutants in the huge collection of T-DNA- and transposon-insertion lines available for *Arabidopsis*.
An important tool in this thesis was the yeast two-hybrid system that provided access to putative interacting partners and was used to understand the context in which the novel kelch domain protein acts.

2. Material and Methods

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2. Material and Methods

2.1 Material

2.1.1 Chemicals, radioisotopes, enzymes and oligonucleotides

Chemicals were obtained from Biomol (Hamburg), Duchefa (Haarlem, NL), Fluka (Neu-Ulm), Invitrogen (Karlsruhe), Merck (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg) and Sigma (Deisenhofen).

Enzymes were purchased from Invitrogen, New England Biolabs (Frankfurt/Main), Promega (Mannheim) and Roche (Mannheim).

Radioisotopes were ordered from Amersham Buchler (Braunschweig). Custom synthesized oligonucleotides were prepared by Invitrogen and Operon (Köln).

2.1.2 Antibodies

All readily available antibodies (both primary and secondary) used in this thesis are listed in table 2.1.

Tab. 2.1: *List of readily available antibodies used for this work.*

antigen	species of origin	conjugate	working dilution	supplier/reference
actin	mouse	-	1:500	Amersham Phamacia Biotech (Freiburg)
glutathione-S-transferase	rabbit	-	1:2000	Sigma (Saint Louis, Missouri, USA)
immuno-phillin	rabbit	-	1:2000	HUEROS *et al*., 1998
mouse IgG	goat	peroxidase	1:5000	Sigma
rabbit IgG	goat	peroxidase	1:5000	Sigma

Antisera raised in the course of this thesis are listed in table 2.2. Polyclonal immune sera against ZmRIK and ZmRACK were prepared by Biogenes Gesellschaft für Biopolymere mbH (Berlin). Preimmune sera were checked prior to immunisation to find suitable animals. Antigens were provided as purified GST- or $(His)_6$-tag-fusions. Immunisations were performed according to the company's standard protocol.

antigen	name of antiserum
GST-ZmRACK(aa 1-334)	59
denatured	60
$(His)_6$-ZmRik (aa 1-284)	57
native	58
GST-ZmRik (aa 1-284)	79
native	80

Tab. 2.2: *List of all antisera made in the course of this work. Two rabbits were immunised with each antigen and the numbers given to the animals were used for the identification of the antisera.*

Standard working dilution for all six antisera was 1:1000 if not otherwise stated.

2.1.3 Vectors

The following cloning and expression vectors were used in this thesis:
pAD-GAL4-2.1, pBD-GAL4 Cam and pBluescript KS (+) from Stratagene (La Jolla, California, USA); pGEM-T and pGEM-T easy from Promega (Mannheim); pGEX-3X and pGEX-4-2T from Amersham Pharmacia (Little Chalfont, Buckinghamshire, GB); pQE60 from Qiagen (Hilden); pMON999 YFP (SHAH *et al*., 2001).
Modification of pMON999 YFP necessary for C-terminal fusion constructs: the YFP-sequence was amplified via PCR without the stop-codon and the original YFP was exchanged for this PCR-product using the *Nco*I and *Bam*HI sites.

2.1.4 Microorganisms and plant material

All microorganisms used in this thesis are listed in table 2.3.

Tab. 2.3: *List of microorganisms.*

name	genotype	reference
Escherichia coli BL21-CodonPlus(DE3)-RP	F^-, *ompT*, *hsdS*($r_B^-m_B^-$), *dcm*$^+$, Tet^r, *gal*λ(DE3), *endA*, Hte, [*argU*, *proL*, Cam^r]	Stratagene (La Jolla, California, USA)
E. coli M15[pREP4]	Nal^S, Str^S, Rif^S, Thi^-, Lac^-, Ara^+, Gal^+, Mtl^-, F^-, $RecA^+$, Uvr^+, Lon^+	Qiagen (Hilden)
E. coli XL1-blue	*rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17(r_K^-, m_K^+), *sup*E44, *rel*A1, *lac*, [F', *pro*AB$^+$, *lac*I^qZΔM15, ::Tn10(Tet^r)]	BULLOCK *et al.* (1987)
Saccharomyces cerevisiae YRG-2	Matα, *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *trp1-901*, *leu2-3 112*, *gal4-542*, *gal80-538*, LYS2::UAS_{GAL1}-$TATA_{GAL1}$-HIS3, URA3::$UAS_{GAL4\ 17mers(x3)}$-$TATA_{CYC1}$-*lacZ*	Stratagene (La Jolla, California, USA)

Plant material:

Nicotiana tabacum Bright Yellow 2 (BY-2)

Zea mays L. cv A69Y

Zea mays suspension cell line HE/89

2.2 Methods

2.2.1 Standard molecular biology methods

Standard molecular biology methods, including the culture of microorganisms, nucleic acid manipulations and cloning, were performed as described in MANIATIS *et al.* (1989).

Isolation of plasmid DNA from *E. coli* was made using the QIAprep Spin Miniprep kit and the QIAGEN Plasmid Midi/Maxi kit (all from Qiagen, Hilden) according to the manufacturer's protocol.

The preparation of plasmids from yeast was done as described by HOFFMAN and WINSTON (1987). Plant genomic DNA was isolated according to SHARP *et al.* (1988), and poly(A)$^+$-mRNA was isolated following the protocol of BARTELS and THOMPSON (1983).

Nucleic acids were quantified via spectrophotometry at 260/280 nm.

The amplification of specific DNA-sequences was done via polymerase chain reaction (PCR, SAIKI *et al*., 1985), either with *Taq*- or *Pfu*-polymerase (Promega, Mannheim), following the manufacturer's protocol using the provided buffers.

PCR-products were purified using the QIAquick Purification kit from Qiagen as described by the manufacturer.

Cloning of PCR-products was performed by inserting restriction enzyme sites into the oligonucleotides. Alternatively, the pGEM-T (Easy) Vector kit from Promega was used for *Taq* PCR-products.

DNA-fragments were isolated from agarose gels using the QIAex II Gel Extraction kit (Qiagen).

2.2.2 High-efficiency transformation of yeast

- 10x amino acid stock solution: 0.2g/l of adenine hemisulfate salt, L-arginine, (L-histidine HCl monohydrate), L-methionine, (L-tryptophan), uracil; 0.3 g/l of L-isoleucine, L-lysine, L-tyrosine; 0.4 g/l of L-serine; 0.5 g/l of L-phenylalanine, 1 g/l of (L-leucine), L-glutamate, L-aspartate; 1.5 g/l of L-valine, 2 g/l of L-threonine; amino acids written in brackets have to be left out for certain selective media
- PEG/LiAC: 10 mM Tris/HCl pH 7.5, 1 mM EDTA, 100 mM lithium acetate, 40% (w/v) PEG 3350
- SD: 0.67 (w/v) yeast nitrogen base (w/o amino acids), 2% (w/v) glucose, 1x amino acid stock solution; 1.5% (w/v) agar (where necessary)
- TE/LiAc: 10 mM Tris/HCl pH 7.5, 1 mM EDTA, 100 mM lithium acetate
- YPDA: 2% (w/v) peptone, 1% (w/v) yeast extract, 2% (w/v) glucose; 2% (w/v) agar (where necessary)

High-efficiency yeast-transformation was performed according to GIETZ *et al*. (1997). An over-night culture of the corresponding yeast-strain was used to inoculate 300 ml of YPDA to give an OD_{600} of 0.1-0.2. After three hours of shaking at 30° C, the cells were washed with sterile dH_2O and resuspended in 1 ml of TE/LiAc. To 1 ml of cell suspension, 100 µg of plasmid-DNA, 2 mg of salmon sperm DNA and 6 ml of PEG/LiAC were added and then incubated at 30° C for 30 minutes. After the addition of 700 µl DMSO, the cells were kept at 42° C for 15 minutes with occasional swirling. After this heat shock, they were allowed to recover in YPDA for two hours, then washed thoroughly with dH_2O and plated on selective SD plates.

2.2.3 Sequencing reactions

DNA sequences were determined by the MPIZ DNA core facility (ADIS) on Applied Biosystems (Weiterstadt) Abi Prism 377 and 3700 sequencers using BigDye-terminator chemistry. Premixed reagents were purchased from Applied Biosystems.

2.2.4 Transfer of nucleic acids onto nylon membranes and hybridisation with radioactive probes

- 20x SSC: 3 M NaCl; 0.3 M trisodium citrate; pH 7
- 100x Denhardts: 2% (w/v) Ficoll 400, 2% (w/v) polyvinylpyrrolidone, 2% (w/v) BSA
- prehybridisation/hybridisation buffer (PHB): 5x SSC, 5x Denhardts, 50% (v/v) formamide, 1% (w/v) SDS, 100µg/ml salmon sperm DNA

Electrophoretically separated nucleic acids were transferred onto nylon membranes via capillary blot, according to SOUTHERN (1975), using 10x (RNA) or 20x (DNA) SSC as transfer buffer. The transfer was done over night and the nucleic acids were immobilized by baking the membrane under vacuum for two hours at 80°C.
Prehybridisations were done in PHB for two to three hours at 42°C and hybridisations were carried out over night in fresh buffer at the same temperature. Filters were washed for 2x 5 minutes with 2x SSC/0.1% SDS, 2x 5 minutes with 0.2x SSC/0.1% SDS (at room temperature) and 2x 15 minutes with 0.2 x SSC/0.1% SDS at 42° C.
Membranes were sealed in plastic foil and exposed to X-ray films (Kodak X-Omat) or phosphoimager screens.

2.2.5 Preparation of ^{32}P-labelled DNA-probes

Radioactively labelled probes were generated from 25-50 ng of purified PCR-product using the Rediprime II kit from Amersham Pharmacia basing on random primed labelling (FEINBERG and VOGELSTEIN, 1983 and 1984) according to the manufacturer's protocol. Unincorporated nucleotides were removed with the QIAquick Purification kit from Qiagen (Hilden).

2.2.6 Protein extraction from plant material and quantification

- Extraction buffer: 0.5 M NaCl, 50 mM Tris/HCl pH 7, 1 mM EDTA, 1 mM DTT, 100 µl/10 ml proteinase inhibitor cocktail (for use with plant cell extract; Sigma, St. Louis, Missouri, USA)

The plant material was ground to a fine powder in liquid nitrogen, mixed with two times the amount of extraction buffer and centrifuged for 15 minutes at 9,000x g. The protein concentration of the supernatant was determined using the BioRad protein assay system based on the method described by BRADFORD (1976).

2.2.7 Purification of GST- and $(His)_6$-fusion proteins

- glutathione-agarose: 70 mg glutathione-agarose/5 ml MTBS
- GST-elution buffer: 50 mM Tris/HCl pH 8.0, 10 mM glutathione (reduced)
- MTBS: 150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4
- His-lysis buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole; pH 8.0
- His-wash buffer: lysis buffer, 20 mM imidazole
- His-elution buffer: lysis buffer, 250 mM imidazole

The purification of GST-fusion proteins was done according to the protocol of SMITH and JOHNSON (1988). An over-night culture of the corresponding *E. coli* strain was inoculated 1:10 in 500 ml LB + antibiotics, grown until the OD_{600} reached 0.6 and then induced with a final IPTG-concentration of 1 mM.
After four hours, the cells were harvested and resuspended in 9 ml of MTBS. Lysozyme was added to a final concentration of 1 mg/ml. After incubating on ice for 15 minutes, the cell suspension was sonicated five times each for 15 seconds. The lysate was centrifuged at 10,000x g for 15 minutes and the supernatant was incubated with 1 ml of glutathione-agarose on a shaker for one hour at 4° C. After washing five times with 1 ml MTBS, the fusion proteins were eluted with 2x 200 µl GST-elution buffer.
The purification of $(His)_6$-tagged recombinant protein is based on the technique described by PORATH (1992) and was performed as described above with the following changes: The cell pellet was resuspended in 3 ml lysis buffer per g of cells and 1 ml of Ni-NTA-agarose (Qiagen) per ml was added to the cleared lysate. Washing and elution were done in the same way, but using the His-washing and -elution buffer.
Quality and quantity of the purified protein were assayed via SDS-PAGE and aliquots were stored at –70° C.

2.2.8 Protein separation by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis

- Coomassie: 50% (v/v) methanol, 10% (v/v) acetic acid, 0.05% (w/v) Coomassie Brilliant Blue R-250
- Coomassie destain: 7% (v/v) acetic acid, 50% (v/v) methanol
- Laemmli buffer: 0.25 M Tris/HCl pH 8.2, 0.4% (w/v) SDS, 767 mM glycine
- Ponceau: 3% (v/v) trichloracetic acid, 0.2% (w/v) Ponceau S
- SDS sample buffer (6x): 0.28 M Tris/HCl pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 0.3 M 2-mercaptoethanol, 0.001% (w/v) bromphenol blue
- TBS: 1.37 M NaCl, 0.1 M Tris, 5 mM KCl; pH 7.4
- TBST: TBS + 0.05% (v/v) Tween®20
- transfer buffer: 25 mM Tris, 192 mM glycine, 20% (v/v) methanol

Proteins were separated on 10-15% SDS polyacrylamide gels according to LAEMMLI (1970). The gels were either stained with Coomassie (one hour at room temperature, shaking) and destained (for more than two hours) or were subject to Western blotting and immunodetection.

Transfer onto nylon membranes was done by electroblotting (four hours, 300 mA or over night at 150 mM) and transfer efficiency was controlled with Ponceau staining. Membranes were blocked in TBS + 5% (w/v) skimmed milk powder for one hour, incubated with the first antibody for one to eight hours, washed (2x with TBST, 1x with TBS, 15 minutes each) and incubated with the second antibody for one hour. After washing as before, bands were detected using the ECL™ Western Blotting Detection System on Hyperfilm™ ECL™ (both from Amersham Pharmacia).

2.2.9 Subcellular fractionation

- Buffer I: 0.5 M sucrose, 0.05 M Tris/HCl pH 7.5, 1 mM EDTA, 0.1% (w/v) BSA, 0.05% cysteine (w/v), 100 µl/10 ml proteinase inhibitor cocktail (Sigma, St. Louis, Missouri, USA)
- Buffer II: 0.5 M sucrose in 0.05 M Tris/HCl pH 7.5
- Buffer III: 0.4 M sucrose in 0.05 M Tris/HCl pH 7.5

Subcellular fractionations were done as described by MASLOWSKI *et al*. (1977).
Approximately 20g of maize leaf material was homogenised in a Waring blendor with 50 ml of buffer I and filtered through four layers of cheesecloth. An aliquot of the filtrate was kept for further analysis and the supernatant was subsequently centrifuged at 200x g (15 minutes; pellet washed once with 10% sucrose), 1,000x g (10 minutes, pellet washed twice with buffer II), 10,000x g (15 minutes), 14,000x g (15 minutes) and 100,000x g (one hour, pellet resuspended in buffer II and centrifuged again at 80,000x g for one hour). The 14,000x g pellet was discarded.
The remaining pellets comprised the following crude fractions: nuclei, chloroplasts, mitochondria and microsomes. The supernatant after the 100,000x g centrifugation contained the soluble, cytosolic proteins.

2.2.10 Preparation of antibody-sepharose

- CNBr/acetonitrile: 62.5% (w/v) cyanogen bromide in acetonitrile
- Tris/saline/azide (TSA) solution: 10 mM Tris/HCl pH 8.0, 0.14 M NaCl, 0.025% (w/v) sodium acide

Antibodies were coupled to sepharose using the cyanogen bromide activation method described by CUATRECASAS (1970). First, the immune serum was dialysed against 500x volumes of 0.1 M $NaHCO_3$/0.5 M NaCl for twenty-four hours at 4° C. Aggregates were removed by centrifuging for one hour at 100,000x g. The protein concentration was adjusted to 5mg/ml by measuring the optical absorption at 280 nm, assuming that 1 mg IgG/ml = A_{280}/1.44.
Meanwhile, the amount of Sepharose CL-4B equal to the volume of antibody was washed with 10x the volume of water and resuspended in the same amount of 0.2 M Na_2CO_3. Dropwise, 3.6 ml of CNBr/acetonitrile per ml slurry were added to the slowly stirring sepharose. The sepharose was filtered and aspirated to semi-dryness. After washing with the same amount of 0.1 M HCl, it was wetted with 0.1 M HCl and immediately mixed with the prepared antibody solution. After stirring over night at 4° C, an excess of 50 mM glycine was added to saturate all remaining reactive groups. The antibody-sepharose was stored in TSA solution at 4° C.

2.2.11 Co-immunoprecipitation

- binding-buffer: 150 mM NaCl, 20 mM Tris/HCl pH 7.5, 0.1% (v/v) Igepal

A crude protein extract was prepared as described in 2.2.6, but using binding-buffer instead of the stated extraction buffer. The antibody-sepharose was washed with binding-buffer and approximately 1 ml antibody-sepharose/10 ml protein extract were incubated over-night on an over-head shaker. The beads were washed 5x with binding-buffer and eluted with 1 ml of 0.1 M glycine, pH 3. The eluate was precipitated with 2.5 volumes of methanol and incubating over-night at –20°C.

2.2.12 Coupled *in vitro* transcription/translation reaction

Radioactively labelled protein was obtained in a coupled *in vitro* transcription/translation (TNT) reaction using the TNT Rabbit Reticulocyte Lysate kit from Promega according to the manufacturer's instructions.

2.2.13 *In vitro* pull-down assay

- binding buffer: 150 mM NaCl, 20 mM Tris/HCl pH 7.5, 0.1% (v/v) Igepal

In vitro pull-down assays were performed with modifications according to GOLDSTEIN *et al.* (1999). Approximately 20 µg of purified GST-fusion protein were bound to 200 µl of glutathione agarose and free binding capacities were blocked with 300 µl binding buffer containing 5 mg/ml skimmed milk powder for one hour. The agarose was washed with binding buffer, resuspended in 200 µl of binding buffer plus 1 mg/ml milk powder and 5-10 µl of ^{35}S-labelled protein were added and incubated for two hours. All steps were performed at 4° C and samples were incubated on an over-head-shaker.
After washing with 5x 1 ml binding buffer, the samples were subsequently analysed via SDS-PAGE (see 2.2.8) and the dried gel was exposed to an X-ray film over night.

2.2.14 Culture of the maize suspension cell line HE/89

The maize suspension cell line HE/89 was cultivated as recommended by MOROCZ *et al.* (1990) in modified N6 maize culture medium according to CHU *et al.* (1975), in

which the original micro-elements were exchanged for MS-microelements (MURASHIGE and SKOOG, 1962).

2.2.15 Culture and transient transformation of BY-2 protoplasts

- K3: 4.3 g/l MS-salts (Duchefa, Harlem, NL), 0.4 M sucrose, 100 mg/l inositol, 230 mg/l xylose, 1ml/l MS-vitamins (Duchefa, Harlem, NL); pH 5.6
- MaMg: 450 mM mannitol, 15 mM $MgCl_2$, 0.1% (w/v) MES; pH 5.6
- Enzyme solution: 1% (w/v) cellulase, 0.1% (w/v) pectolyase, 0.4 M mannitol; pH 5.5
- W5: 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM saccharose; pH 5.6-6.0
- PEG-solution: 25% (w/v) PEG 1500, 0.1 M $MgCl_2$, 0.45 M mannitol, 20 mM HEPES; pH 6.0

The culture of the tobacco Bright Yellow 2 (BY-2) cell suspension line was done according to NAGATA *et al.* (1992). Protoplasts were prepared from cells harvested three days after subculture as described by OKADA *et al.* (1986). Cells were resuspended in 50 ml of enzyme solution and incubated on a rotary shaker (120 rpm) for approximately three hours at 28° C until the digestion of the cell walls was complete. The protoplasts were washed with MaMg, counted and the cell density was adjusted to 2x 10^6/300 µl with MaMg.

For the transient transformation, a modified version of the protocol by NEGRUTIU *et al.* (1987) was followed by mixing 300 µl of protoplast solution with 10-20 µg of DNA and 600 µl of PEG-solution. After incubating for 20 minutes at room temperature, 10 ml of K3 were added. Expression analysis was done after an over night incubation at 28° C.

2.2.16 Transient transformation of maize leaf cells with particle bombardment

Maize leaves, of approximately two weeks old maize plants, were cut and put on petri dishes containing 1% agar and 85 µM benzimmidazole. The coating of the gold particles was done as described by SCHENK *et al.* (1998). The bombardment was performed

with the particle delivery system Biolistic® PDS-1000/He from Bio-Rad (Hercules, California, USA) using rupture disks bursting at 900 psi.

2.2.17 Microscopy

Fluorescence microscopy was done using a Zeiss Axiophot light microscope equipped with epiflourescence optics. For visualizing GFP-fluorescence, a HQ GFP LP filter with an excitation wavelength of 450-490 nm and an emission of LP 520 nm was used.
Confocal laser scanning microscopy was performed on the Carl Zeiss LSM 510 META laser scanning microscope using suitable filter-sets for YFP and chlorophyll-autofluorescence. For excitation, the 514 nm band of the argon-laser was used. Between thirty and forty optical sections in z-direction were taken and the three-dimensional reconstruction was made with the software provided by Carl Zeiss, Jena.

2.2.18 Computer software

Sequence analysis was done using the indicated World Wide Web based programmes and the GCG-package version 9.0 from the Genetic Computer Group (Madison, Wisconsin, USA).
X-ray films were scanned using Photo-Paint 8.0 (Coral Corporation). Image processing was performed using Photo-Paint 8.0, CoralDRAW 8.0 (Coral Corporation) and Adobe Photoshop 4.0 (Adobe Systems).

3. Results

3. Results

3.1 *In silico* analysis of ZmKEL1

By the end of 2002, the GOLD Genomes Online Database (http://igweb.integrated genomics.com/GOLD/) lists already 117 completely sequenced genomes: 16 from archaea, 86 from bacteria and 15 from eukaryotes, including *Arabidopsis thaliana* (THE *ARABIDOPSIS* GENOME INITIATIVE, 2000), *Oryza sativa* L. ssp. *indica* (YU *et al*., 2002) and ssp. *japonica* (GOFF *et al*., 2002). Most of this data is publicly available through web-based databases and provides a valuable tool for a variety of applications.

Besides these enormous collections of mere sequence data, more and more genes and proteins are analysed in detail and their functions are revealed. These results often elucidate general molecular mechanisms that can be converted into algorithms to predict certain properties of novel genes or proteins. Although the results of such computational approaches are only of theoretical nature and have to be confirmed experimentally, they often provide a good starting-point for further analysis. Therefore, it was reasonable to first analyse ZmKEL1 *in silico*.

A BLAST-search (http://www.ncbi.nlm.nih.gov:80/BLAST/; ALTSCHUL *et al*., 1997) was made to find homologues of ZmKEL1, ideally with assigned functions. One homologue from *Arabidopsis* and two from rice were found and were designated AtKEL1 and OsKEL1/OsKEL2, respectively. Unfortunately, the sequences from these three kelch domain proteins resulted only from sequencing projects and therefore do not provide a hint as to the function of ZmKEL1. An alignment of these three together with ZmKEL1 is shown in figure 3.1.

Only OsKEL1 shows high homology over the whole length of ZmKEL1 (72% identity on the amino acid level). The other two kelch domain proteins are relatively high conserved in the C-terminal kelch domain (58 % identity on the amino acid level) and the N-terminus (53% identity), but not in the central region (14% identity).

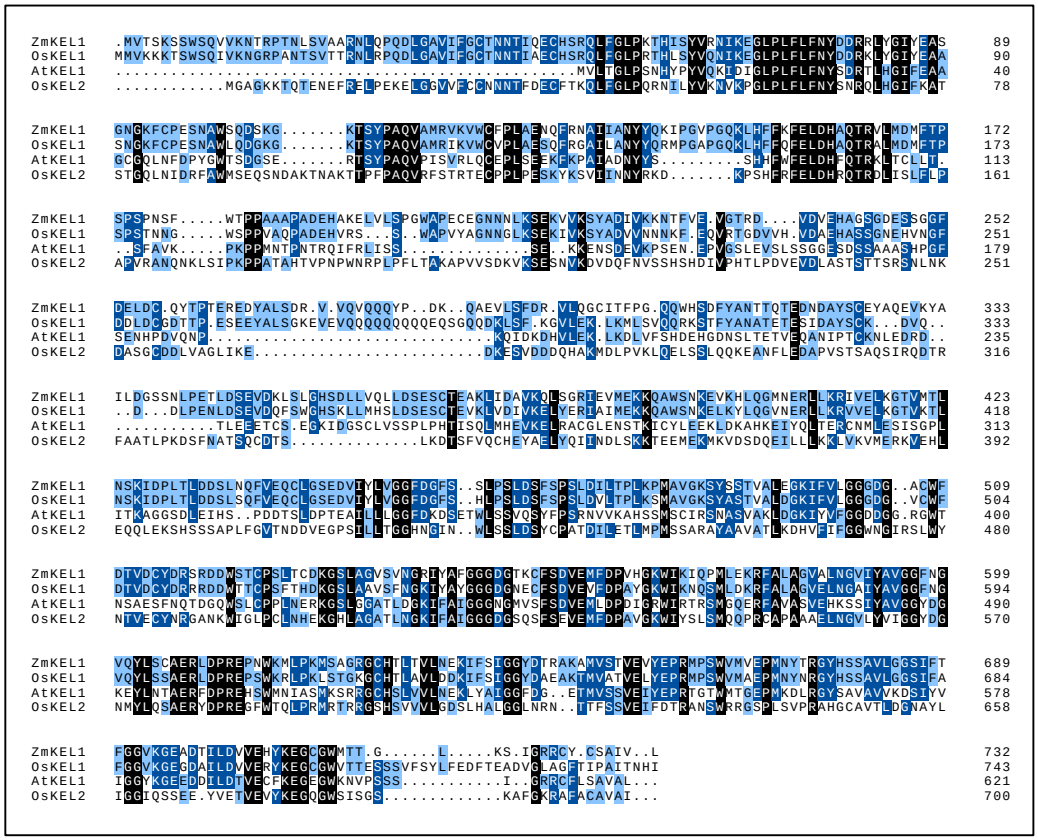


Fig. 3.1: *Alignment of all four kelch domain proteins from Arabidopsis (AtKEL1), rice (OsKEL1 and OsKEL2) and maize (ZmKEL1). The numbers on the right indicate the position of the last amino acid of the row within each protein.* ***Shading: black:*** *100% conserved,* ***dark blue:*** *at least 75%,* ***bright blue:*** *at least 50%.*

The alignment with OsKEL1 indicates that the coding sequence of ZmKEL is already the full-length and that no essential parts are missing.

No proteins, other than OsKEL1, were found with significant homology to the central region of ZmKEL1, but the BLAST-algorithm identified several proteins that share homology with the N-terminus of the kelch domain proteins. The homology applies only to this region. The remainder of these proteins is not conserved, and they greatly vary in length. The shortest consists of 204 amino acids, while the longest is composed of 1615 residues. Most of these proteins are from *Arabidopsis* and rice, but homologues for this N-terminal region of ZmKEL1

have also been found in carrot, garden pea and grapefruit. Figure 3.2 shows an alignment of some of these proteins together with the four kelch domain proteins.

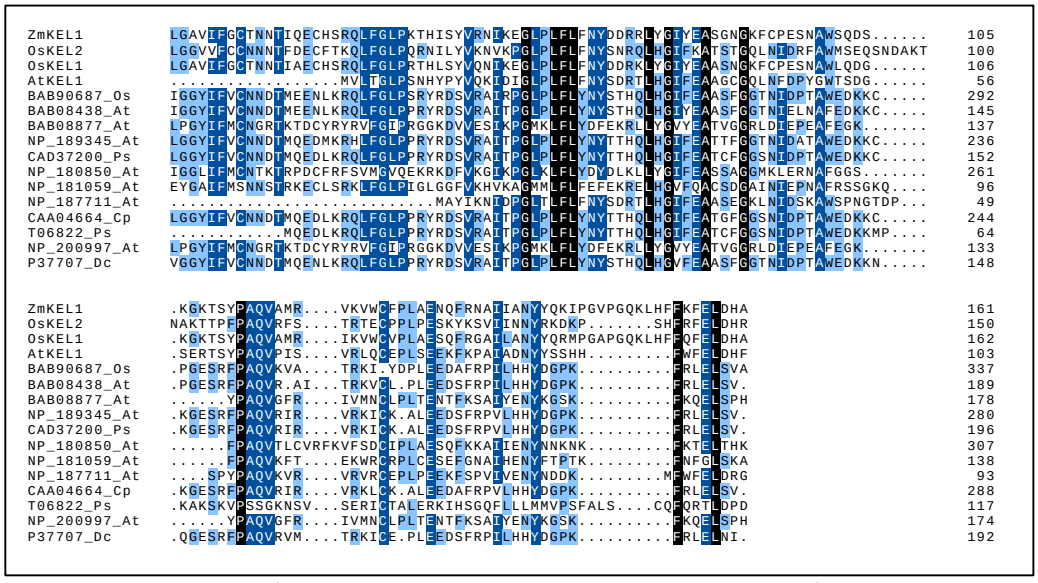


Fig. 3.2: *Alignment of the proteins containing the N-terminal domain found in ZmKEL1. Numbers on the right give the position of the last amino acid of the row within each protein.* ***Shading: black:*** *100% conserved,* ***dark blue:*** *at least 75%,* ***bright blue:*** *at least 50%.* ***Abbreviations: AT*** *Arabidopsis thaliana,* ***CP*** *Citrus x paradisi,* ***DC*** *Daucus carota,* ***PS*** *Pisum sativum*

The alignment suggests that the N-terminus of ZmKEL1 contains a novel, so far undescribed domain. Unfortunately, none of the proteins containing this domain has an assigned function and, consequently, does not imply a role for this domain. As long as no function for this domain has been revealed, it will be designated LFL-domain in accordance to the highly conserved tripeptide leucine-phenylalanine-leucine.

Interestingly, this novel domain is always at the N-terminus of a protein and proteins with the LFL-domain have only been found in plants. It can thus be assumed that this novel domain is plant-specific.

Pfam (BATEMAN *et al.*, 2002; http://pfam.wustl.edu/index.html) identified six kelch repeats in the C-terminus of each of the four kelch domain proteins, consisting of 34 to 47 amino acids each. A graphical overview of the domain structure of all four kelch domain proteins is given in figure 3.3.

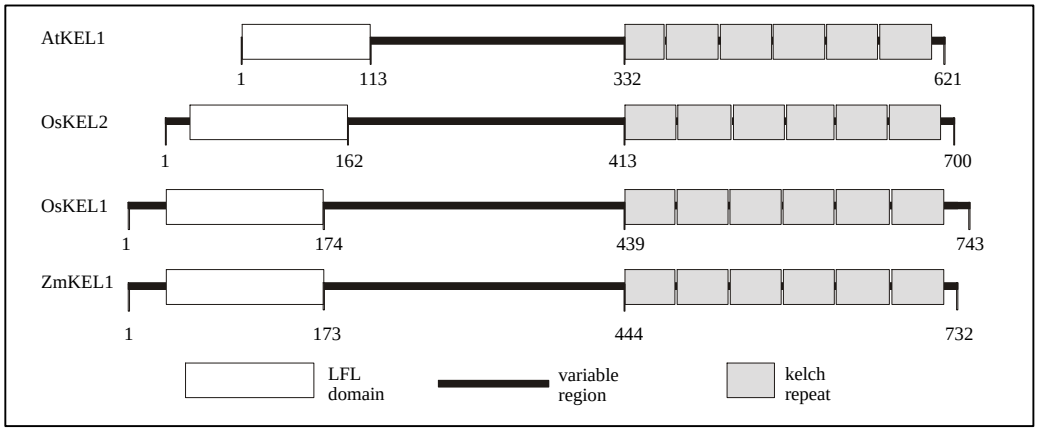


Fig. 3.3: *Graphical overview of the domain structure of AtKEL1, OsKEL1, OsKEL2 and ZmKEL1. White boxes display the LFL-domain, grey boxes represent kelch repeats and variable regions are indicated with black bars. Numbers indicate the position of selected amino acids. Drawn to scale.*

Furthermore, analyses with the PSORT-algorythm (http://psort.nibb.ac.jp/form.html) did not reveal any convincing targeting signals.

3.2 The two-hybrid system: Finding interacting partners of ZmKEL1

Most kelch repeat proteins described so far function via protein-protein interactions (reviewed by ADAMS *et al.*, 2000). For this reason, it can be assumed that ZmKEL1 also acts in a protein complex and knowing the interacting partner(s) will provide an inside into the cellular context in which it acts.

A very suitable tool for this attempt provides the yeast two-hybrid system (FIELDS and SONG, 1989), because it allows the screening of complete cDNA libraries for putative interacting partners (CHIEN *et al.*, 1991). It further makes it possible to identify the domains or even single amino acids involved in an interaction between two proteins (LI and FIELDS, 1993).

The system is based on the properties of the Gal4 transcription factor that consists of two physically separable domains: the DNA binding domain (BD) and the transcription activation domain (AD). Both domains on their own are transcriptionally inactive, but when fused to interacting proteins, and by these brought into close

vicinity, they constitute a functional transcription factor and drive the expression of a reporter gene like, for example, *HIS*3.
For library screens, the protein of interest is transcriptionally fused to the BD of Gal4 ("bait"), while the cDNA library is fused to the AD ("prey"). Cotransformation of both bait and prey plasmid into a histidine-auxotroph yeast strain should only result in colony growth in the absence of histidine, if the hybrid proteins interact with each other and thus reconstitute a functional transcription factor that then drives the transcription of *HIS*3.

For a two-hybrid screen with ZmKEL1, the full-length cDNA was amplified via PCR with added *Sma*I/*Sal*I-sites. The PCR-product was cloned into the corresponding sites of the vector pBD-GAL4 Cam and was used as bait in a screen with a maize endosperm cDNA library made seven days after pollination (dap; custom-made by Stratagene, La Jolla, CA, USA). It was the same library from which the kelch domain protein was isolated initially and was thus likely to contain the cDNAs from interacting partners.
Some proteins cannot be used as baits because of their autoactivating properties. This is frequently a problem with transcription factors. In this case, 3-amino-1,2,4-triazol (3-AT), an inhibitor of the *HIS3*-encoded IGP-dehydrogenase, can be used to reduce background growth. To test for the autoactivating properties of ZmKEL1, the bait together with the empty AD vector were cotransformed into the yeast strain YRG-2 and plated onto selective medium with different concentrations of 3-AT. The cotransformants showed only very weak growth on plates without 3-AT and this background was completely suppressed with 1 mM 3-AT. To minimise unspecific interactions, the screen was performed with 5 mM 3-AT.
In total, 5.2×10^6 cotransformants were analysed and sixty-six colonies grew on selective media. After rescuing the plasmids and transforming them back into yeast, fourteen candidates still showed histidine-auxotrophy and were sequenced.
Seven of these clones did not show significant homology to any other protein or were homologues of proteins with unknown functions. Four clones were known false-positives frequently found in two-hybrid screens (protease, proteasome sub-

unit, heat-shock protein, ribosomal binding protein; for reference see list of frequently found false-positives on http://www.fccc.edu/research/labs/golemis/main_false.html).

Furthermore, β-tubulin was found, and twice a gene that showed highest homology (89% identity on the amino acid level) to a gene from rice called *rwd* (rice protein containing the WD-40 repeat; IWASAKI *et al*., 1995).

The putative interaction with β-tubulin was very interesting, because kelch domain proteins are known to interact with the cytoskeleton, but the interaction was only very weak in the two-hybrid system, and it was not possible to purify tagged recombinant β-tubulin under native conditions for pull-down assays.

3.2.1 Analysis of the two-hybrid clones 15 and 20

The *rwd*-like clones (clones 15 and 20) were the most promising ones as they were found twice independently. Figure 3.4 shows the alignment of these two clones together with the rice homologue RWD.

Fig. 3.4: *Sequence alignment of the two clones 15 and 20 found in the two-hybrid screen with RWD from rice.* ***Shading: black:*** *100% identity,* ***blue:*** *66% identity. The numbers on the right give the position of the last amino acid of the row within the protein.*

The alignment suggests that clone number 15 contains already the full-length coding sequence, while clone number 20 is only a partial length. It cannot be determined, if both two-hybrid clones represent the same or different genes: Although the amino acid sequences differ in four residues only (with two different amino acids directly at the 5'-end of clone number 20), there are already 28 exchanges on the nucleotide level (data not shown).

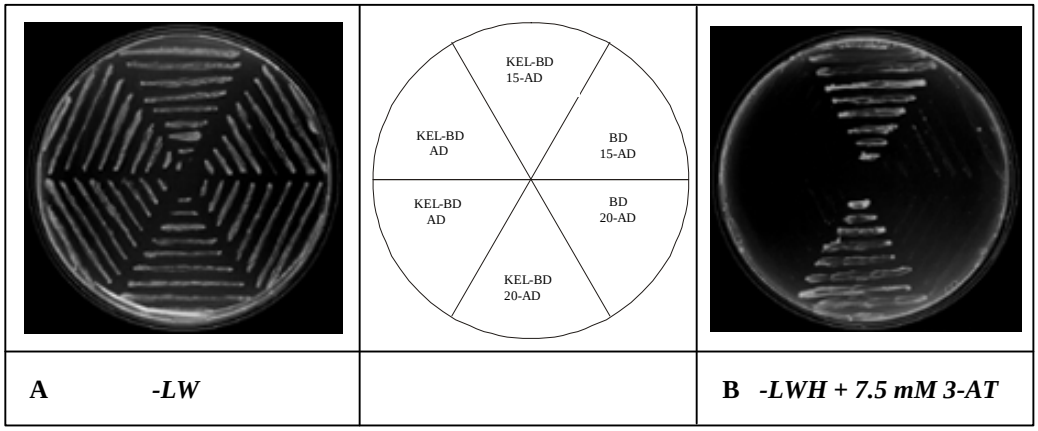


Fig. 3.5: *Control plating for clones 15 and 20.* ***A:*** *Selecting for the presence of both bait and prey plasmid on media without leucine and tryptophan (–LW).* ***B:*** *Testing for the ability to interact on medium additionally lacking histidine (-LWH) +7.5 mM 3-AT. The way of plating is indicated in the middle.*

Control platings after a new cotransformation revealed that the *rwd*-like clones 15 and 20 only conferred histidine-auxotrophy in the presence of *Zmkel1* fused to the BD, but not when cotransformed with the empty BD-vector (see figure 3.5). This indicates that the interaction between the kelch domain protein and the RWD-like protein is specific in the two-hybrid system.

As mentioned above, the two-hybrid clones share highest homology to RWD from rice. RWD consists of seven WD-40 repeats that are assumed to form a β-propeller like the kelch repeats (WALL *et al.*, 1995; LAMBRIGHT *et al.*, 1996; see also figure 1.2). This is the typical characteristic for both the β-subunits of the trimeric G-protein (Gβ) and the receptors for activated C kinase (RACK; BUBIS and KHORANA, 1990). Both proteins are highly homologous - a fact that made it difficult to classify the two-hybrid clone into one of the two groups.

To solve this problem, a phylogenetic tree with characterized Gβs and RACKs was made (see figure 3.6). The tree has two branches that clearly separate the Gβs and the RACK proteins. The two-hybrid clone can be found on the RACK-branch and is even closer related to mammalian RACK proteins than to plant Gβs. Thus the two-hybrid clone was named ZmRACK.

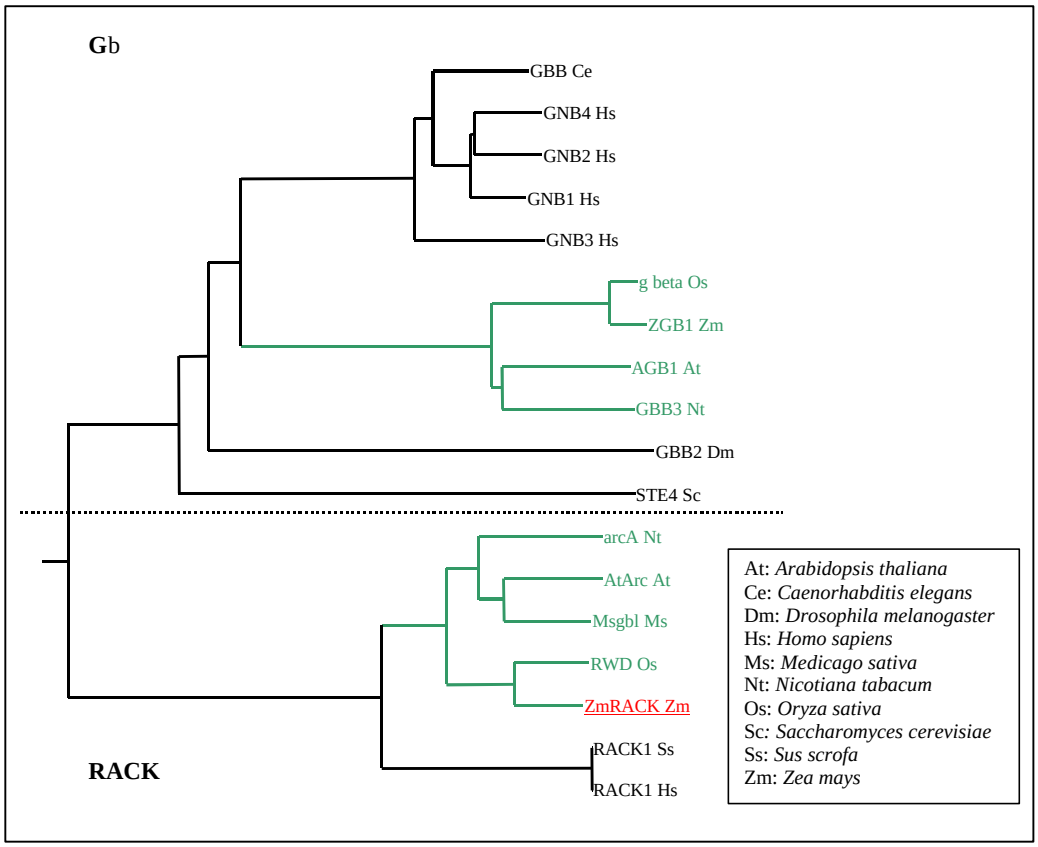
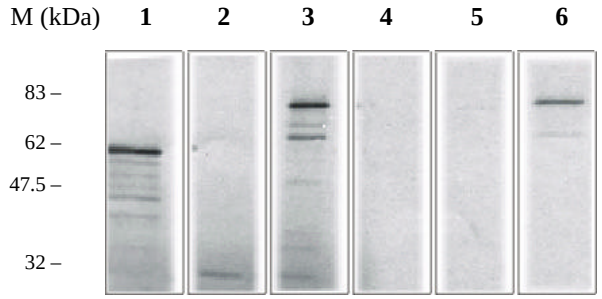


Fig. 3.6: *Phylogenetic tree displaying the relationship between the b-subunit of the trimeric G-protein (Gb) and the receptors for activated C kinase (RACKs). ZmRACK is highlighted in red and plant proteins in green.*

3.2.2 Pull-down assays with ZmRACK

To repeat the interaction between the kelch domain protein and ZmRACK with another technique than the two-hybrid system, pull-down assays with ^{35}S-labelled ZmKEL and GST-ZmRACK were made.

To obtain GST-tagged ZmRACK protein, the coding sequence of *Zmrack* was amplified with added restriction sites for *Bam*HI and *Eco*RI and cloned into the same sites of the vector pGEX-4T-2. *Zmrik* was amplified via PCR with added *Xho*I and *Bam*HI sites and cloned into the vector pBS-KS(+). ^{35}S-labelled ZmRIK was obtained in a coupled *in vitro* transcription/translation (TNT) reaction. Negative controls included GST (from the empty vector pGEX-4T-2) and the unrelated protein luciferase (included in the TNT kit from Promega).



1: TNT product luciferase
2: TNT product empty vector
3: TNT product ZmKEL1
4: GST-ZmRACK + ^{35}S-luciferase
5: GST + ^{35}S-ZmKEL1
6: GST-ZmRACK + ^{35}S-ZmKEL1

Fig. 3.7: *Pull-down assays with ^{35}S-labelled ZmRIK and GST-ZmRACK. Samples were separated on a 10% SDS-PAGE and the size of maker bands in kDa is given on the left.*

As shown in figure 3.7, no interactions were obtained with GST alone or with ^{35}S-labelled luciferase, but ^{35}S-ZmKEL was able to specifically bind to the GST-fusion of ZmRACK. For this reason, the kelch domain protein was named ZmRIK for *Zea mays* RACK-interacting kelch domain protein.

3.3 Determination of the domain(s) of ZmRIK involved in the interaction with ZmRACK

The fact that ZmRIK consists of three domains (the N-terminal LFL-domain, the central region and the kelch domain) raises the question as to which of these regions is required for the interaction between ZmRIK and ZmRACK.

3.3.1 Pull-down assays with different partial lengths of ZmRIK

To identify the region of ZmRIK responsible for the interaction with ZmRACK, more pull-down assays were made. For this purpose, it was necessary to clone five more constructs as shown in figure 3.8 A. The corresponding regions of *Zmrik* were amplified via PCR with added restriction sites for *Xho*I and *Bam*HI and cloned into the vector pBS-KS(+). The TNT-products of these clones together with the full-length of ZmRIK were then used for pull-down assays with GST-ZmRACK. The results are shown in figure 3.8 B.

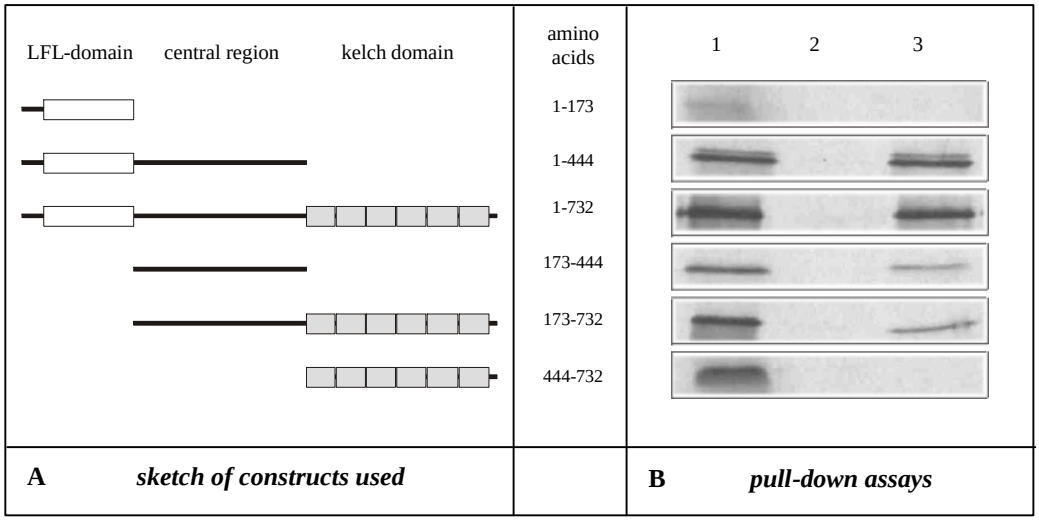


Fig. 3.8: *Determination of the domain(s) responsible for the interaction of ZmRIK with ZmRACK.* ***A:*** *Schematic drawing of the constructs used.* ***B:*** *Pull-down assays with different partial lengths of ZmRIK.* ***Explanations: 1:*** *TNT-product,* ***2:*** *GST + TNT-product,* ***3:*** *GST-ZmRACK +* ^{35}*S-labelled ZmRIK*

The TNT-reaction of ZmRIK(aa 1-174) was not very efficient and was thus not very informative. However, it is evident that only those constructs were able to interact with ZmRACK that included the central region of ZmRIK and this region on its own was already sufficient for binding.

3.3.2 Two-hybrid tests with each of the three domains of ZmRIK

To avoid the problem with the insufficient amount of ^{35}S-labelled ZmRIK(aa 1-173), the two-hybrid system was used to support the results from the pull-down assays.

The same constructs as shown in figure 3.8A were amplified with added *Sal*I sites and cloned into the vector pBD-GAL4 Cam. The right orientation of the inserts was verified through PCR and sequencing. These constructs, together with pAD-*Zmrack*, were cotransformed into the histidine-auxotroph yeast strain YRG-2. Selection for the presence of both plasmids was performed by plating the cotransformants on medium without leucine and tryptophan (-LW) and the ability to interact was tested on medium additionally lacking histidine (-LWH). To reduce unspecific background growth, 15 mM 3-AT were added.

The drawback of the two-hybrid system was that ZmRIK(aa 173-732) showed very strong autoactivating (intrinsic transcription activation) properties (data not shown). Weak autoactivation was also observed with ZmRIK(aa 173-444). For this reason, only the results of the platings with each of the three domains and the full-length of ZmRIK are shown in figure 3.9.

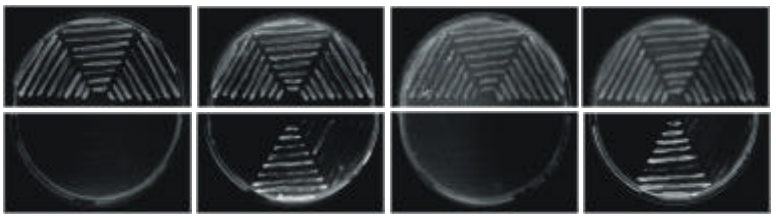


Fig. 3.9: *Two-hybrid plating with ZmRACK and different partial lengths of ZmRIK. The way of plating is indicated on the right. The plates were incubated for two days at 30°C.* ***Explanation****: 1: ZmRIK(X)-BD + pAD, 2: ZmRIK(X)-BD + ZmRACK-AD, 3: pBD + ZmRACK-AD (plated on - LW). Bold numbers: plated on –LWH + 15 mM 3-AT. X: indicated partial length of ZmRIK.*

Growth was only observed with the full-length of ZmRIK and ZmRIK(aa 173-444), representing the central region of ZmRIK. The LFL-domain and the kelch domain were not able to confer histidine-auxotrophy, indicating that they did not interact with ZmRACK.

These data coincide very well with the results obtained from the pull-down assays and clearly indicate that the central region of ZmRIK with its amino acids 173 to 444 is necessary and sufficient for the interaction with ZmRACK. The central region of ZmRIK was therefore designated the RACK-interacting (RI) region.

3.4 Testing the ability of the RIK-homologues to interact with different RACK proteins

The results from chapter 3.3 clearly indicate that the central region is necessary and sufficient for the interaction between ZmRIK and ZmRACK. But the alignment from figure 3.1 shows that only OsKEL1 shares significant homology to

ZmRIK in this region and that AtKEL1 and OsKEL2 do not. This suggests that only OsKEL1 might be able to interact with RACK-like proteins, but not AtKEL1 and OsKEL2.

3.4.1 Pull-down assays with all RACKs and all kelch domain proteins

To test this hypothesis, the coding sequence of *Atkel1* was amplified via RT-PCR from polyA$^+$-mRNA prepared from different *Arabidopsis* var. Columbia tissues and cloned into the pGEM-T system from Promega (in an orientation that sense-RNA was obtained from the T7-promoter).

For unknown reasons, the coding sequence of *Oskel1* was recalcitrant to amplification. It was only possible to amplify the central region of *Oskel1* from total RNA prepared from rice leaves and stems. This RT-PCR product, containing an artificial start-ATG, was subsequently cloned into the *Bam*HI/*Xho*I sites of the vector pBS-KS(+). The same was made with *Oskel2*, but with added recognition sites for *Eco*RI and *Xho*I.

Sequencing of the two full-length kelch domain proteins showed that *AtKEL1* had the same sequence as published in the databases, but *Oskel1* had a 9 bp insertion adding the amino acids tyrosine, leucine and glutamine at position 572. Whether or not this is cultivar-specific was not further analysed.

Besides *Zmrack* and *rwd* from rice, there are two RACK-like genes in *Arabidopsis*: *AtARC* with the accession number U77381 and *AtRACK* with the accession number AB020749. Figure 3.10 shows an alignment of all four RACK proteins that share an over-all identity of 82% on the amino acid level.

The two RACK proteins from *Arabidopsis* together with the rice *rwd* were cloned via RT-PCR using the same polyA$^+$-mRNA/total RNA as for the cloning of the kelch domain proteins. The RT-PCR products were cloned into the *Bam*HI and *Eco*RI sites of the vector pGEX-4T-2. The four GST-tagged RACK proteins were purified and used for pull-down assays together with the ^{35}S-labelled kelch domain proteins.

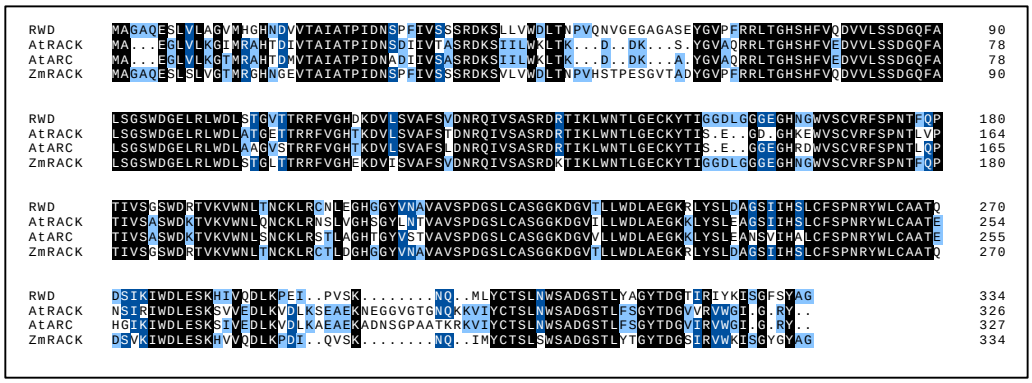
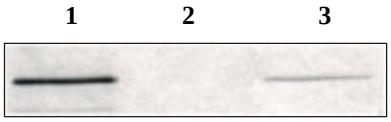


Fig. 3.10: *Alignment of the four RACK-like proteins AtARC and AtRACK from Arabidopsis, RWD from rice and ZmRACK from maize.* ***Shading: black:*** *100% conserved,* ***dark blue:*** *75% conserved,* ***bright blue:*** *50% conserved.*

First, the ability of OsKEL1(aa 174-439) to interact with GST-RWD, the rice-homologue of ZmRACK, was tested and the results are shown in figure 3.11. GST was used as a negative control.

As already anticipated from the alignment of the four kelch domain proteins shown in figure 3.1, the central region of OsKEL1 was also able to specifically interact with its corresponding RACK protein, RWD. For this reason, this kelch domain protein from rice was named OsRIK.



1: ^{35}S-OsRIK(aa 174-439)

2: GST + ^{35}S-OsRIK(aa 174-439)

3: GST-RWD + ^{35}S-OsRIK(aa 174-439)

Fig 3.11: *Pull-down assay with GST-RWD and ^{35}S-labelled OsRIK(aa 174-439).*

In a next step, the ability of the other two kelch domain proteins to interact with RACK-like proteins was tested. ZmRIK was used as a positive control. The results of the pull-down assays are shown in figure 3.12.

Interestingly, ZmRIK was not only able to bind to ZmRACK, but also to the two RACK proteins from *Arabidopsis* and to RWD from rice. The kelch domain proteins from *Arabidopsis* and rice showed no interaction with any of the four RACK proteins.

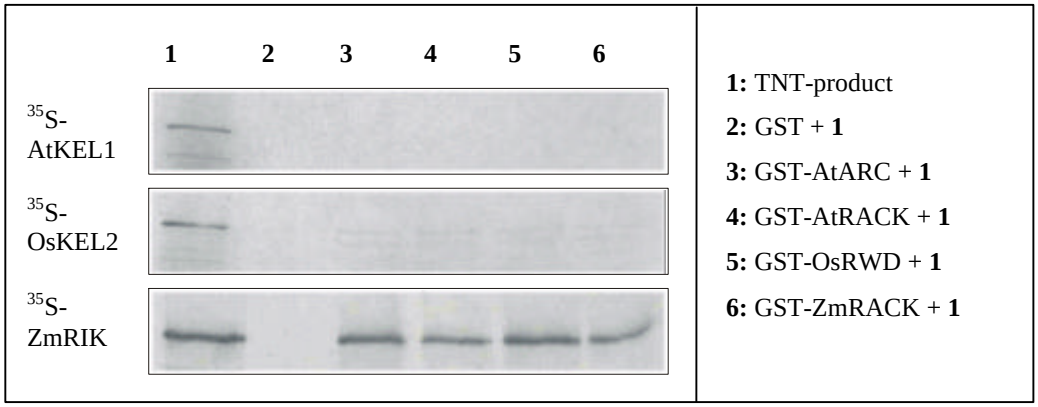


Fig. 3.12: *Pull-down assays with all four RACK proteins from Arabidopsis (AtARC and AtRACK), rice (OsRWD) and maize (ZmRACK) and the three kelch domain proteins (AtKEL1 from Arabidopsis, OsKEL2 from rice and ZmRIK from maize).*

These findings coincide with the results from chapter 3.3. As these two kelch domain proteins from *Arabidopsis* and rice are homologous to the N- and C-terminus of ZmRIK, but do not share the RI region that gave rise to the name of ZmRIK, they were named RLP for RIK-like proteins.

3.4.2 The central region of ZmRIK confers RACK-binding on AtRLP

As the central region of ZmRIK has been shown to mediate the interaction with ZmRACK, it should be possible to get an interaction of AtRLP with a RACK protein, if its central region is exchanged for the corresponding maize region.

To test this, each of the three domains of AtRLP were amplified separately via PCR and cloned into the vector pBS-KS(+) as shown in figure 3.13.

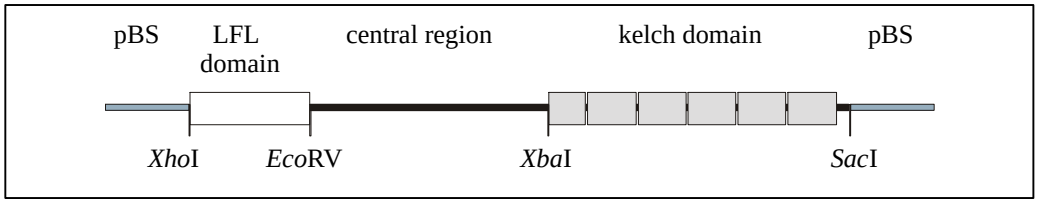


Fig. 3.13: *Schematic drawing of the cloning strategy required for exchanging the central region of AtRLP for the RI region from maize.*

This construct was designated RLP(AAA), indicating that all three domains are from the *Arabidopsis* kelch domain protein. RLP(AZA) was cloned by exchanging the central region of *RLP(AAA)* for the RI region of *Zmrik.*

Pull-down assays were made with ^{35}S-labelled ZmRIK (positive control), RLP (AAA) and RLP(AZA) together with GST-ZmRACK. As can be seen in figure 3.14, there was no interaction between RLP(AAA) and the RACK protein, but RLP(AZA) was able to specifically bind to GST-ZmRACK.

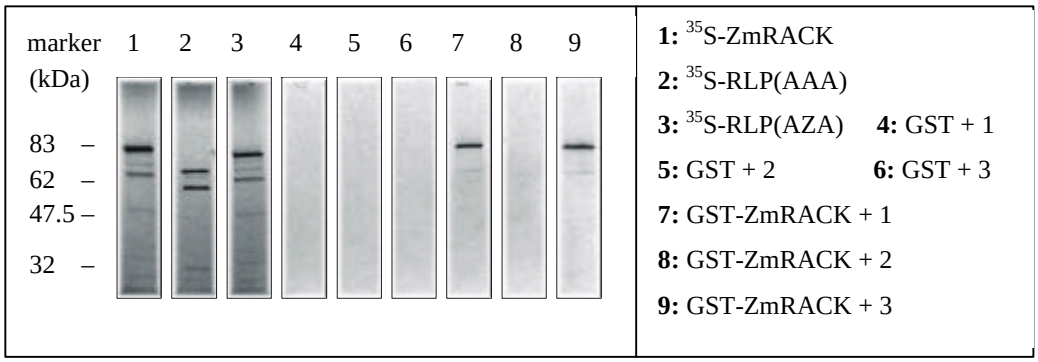


Fig. 3.14: *Pull-down assays with ZmRACK, RLP(AAA) and RLP(AZA) with GST-ZmRACK. Samples were separated on a 10% SDS-PAGE, 1µl of each TNT product and the total volume of each pull-down reaction was loaded.*

The central region of ZmRIK thus conferred the ability to interact with RACK proteins and this confirmed the finding that the central region is sufficient for the interaction with RACK proteins.

3.5 Southern blot analysis of *Zmrik* and *Zmrack*

Since the genomes of *Arabidopsis thaliana* and rice have been sequenced (THE *ARABIDOPSIS* GENOME INITIATIVE, 2000; YU *et al*., 2002; GOFF *et al*., 2002), the copy number of genes in these plants can easily be determined. The *Arabidopsis* database reveals that *AtRLP* (accession NM_120244) is a single copy gene and that the genome contains two *RACK* genes (*AtARC* and *AtRACK* with the accession numbers U77381 and AB020749, respectively).

The situation in rice is just the opposite. While there is only one *rack* (*rwd*), there are two *rik* or *rik*-like genes: *Osrik* resembling *Zmrik,* and *Osrlp* that is only con-

served in the N- and C-terminal domains. To get an idea about the copy number of ZmRIK and ZmRACK in maize, Southern blot analyses was performed.
Genomic DNA of the maize line A69Y+ was hydrolysed with different restriction enzymes, electrophoretically separated and blotted onto a Hybond-N nylon membrane. The filters were successively hybridised with a ^{32}P-labelled PCR-fragment of the full-length of *Zmrack* (bp 1-1005) and a partial length of *Zmrik* (bp 282 to 1831 = 1549 bp). The hybridisations were performed at 42°C and washing steps were done at medium stringency. The results are shown in figure 3.15.

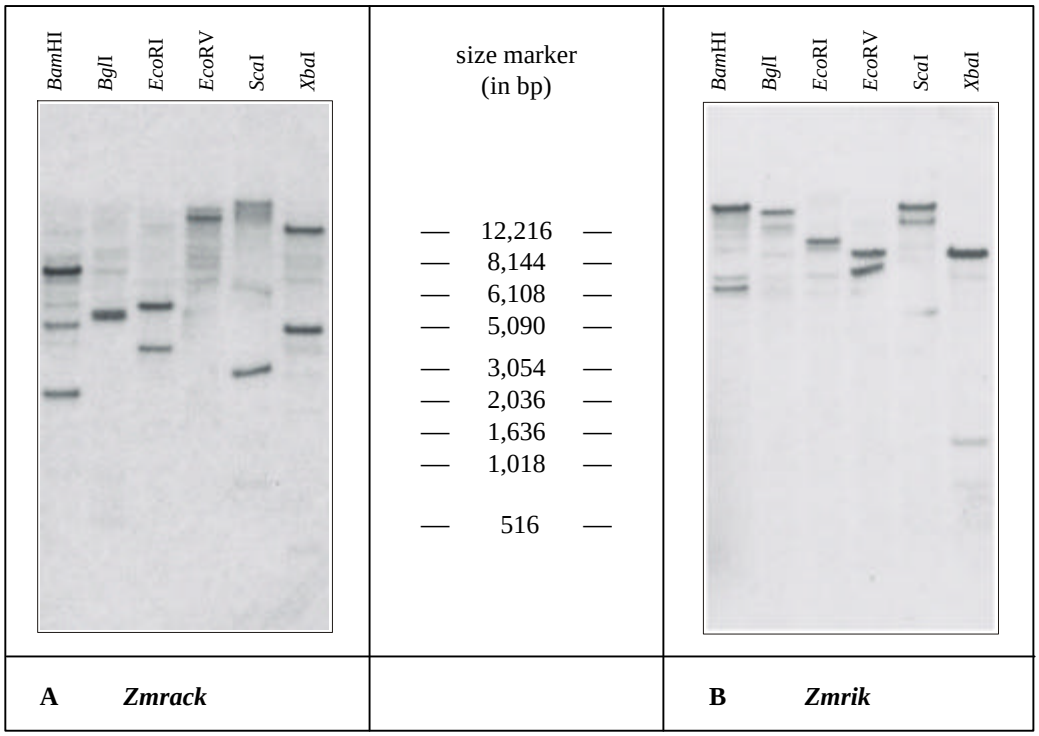


Fig. 3.15: *Southern blot analysis of Zmrack* ***(A)*** *and Zmrik* ***(B).*** *Each lane contained 10 µg of genomic maize DNA cut with the restriction enzymes indicated above. The filter was stripped and used for both hybridisations. The position of marker bands (in bp) is indicated in the middle.*

The *Zmrack*-specific probe detected between one and three main bands. As the genomic sequence of *Zmrack* is not known, these results do not reveal, if there are one or two copies in the maize genome and it can only be stated that *Zmrack* is a low copy gene. The fact that the two-hybrid clones number 15 and 20 contained

twenty-eight differences on the nucleotide level suggests there are two copies of *Zmrack* in the maize genome.

The hybridisation with a probe specific for *Zmrik* showed similar results. One or two main bands were detected together with several weaker ones, but it is not possible to determine, if *Zmrik* is a single copy gene or if there are two copies present in maize.

3.6 Analysis of the expression patterns of ZmRIK and ZmRACK

The expression patterns of ZmRIK and ZmRACK were analysed both on the transcript (Northern blot) and on the protein level (Western blot).

The results should give an insight into the impact of the two proteins. Expression in only one/few tissue(s) would indicate a specialised function, while a ubiquitous expression would point towards a more general function.

3.6.1 Northern blot analysis

The first Northern blots were made with 25 µg of total RNA, but no bands could be detected with a *Zmrik*-specific probe. This indicated that the expression level was very low and thus poly-A^+mRNA was isolated from different maize tissues (shoots, leaves, husks, kernels harvested at different dap, silks, anthers and pollen). Approximately 2 µg of each sample was loaded onto a 0.7% denaturing agarose gel and blotted onto HybondN nylon membranes (Amersham). ^{32}P-labelled probes were made from the full-length coding region of *Zmrack* (bp 1-1005) or from bp 282-1831 of *Zmrik*. β-tubulin was used as loading control.

As the expression of *Zmrik* was extremely low, it was necessary to expose the filter to a phosphoimager screen for two weeks to obtain at least a weak signal, while *Zmrack*-specific probes produced a suitable signal already after a one-day exposure to a normal X-ray film.

Figure 3.16 A shows that both probes detected only one band of approximately 3 kb (in the case of *Zmrik*) or 1.6 kb (*Zmrack*), and this coincided with the expected sizes estimated from the corresponding coding regions.

Zmrik showed the highest expression in leaves, husks, kernels (mixture of 12 to 24 dap), unpollinated cobs and silks and a weaker expression in shoots. It was below the detection limit in anthers and pollen.

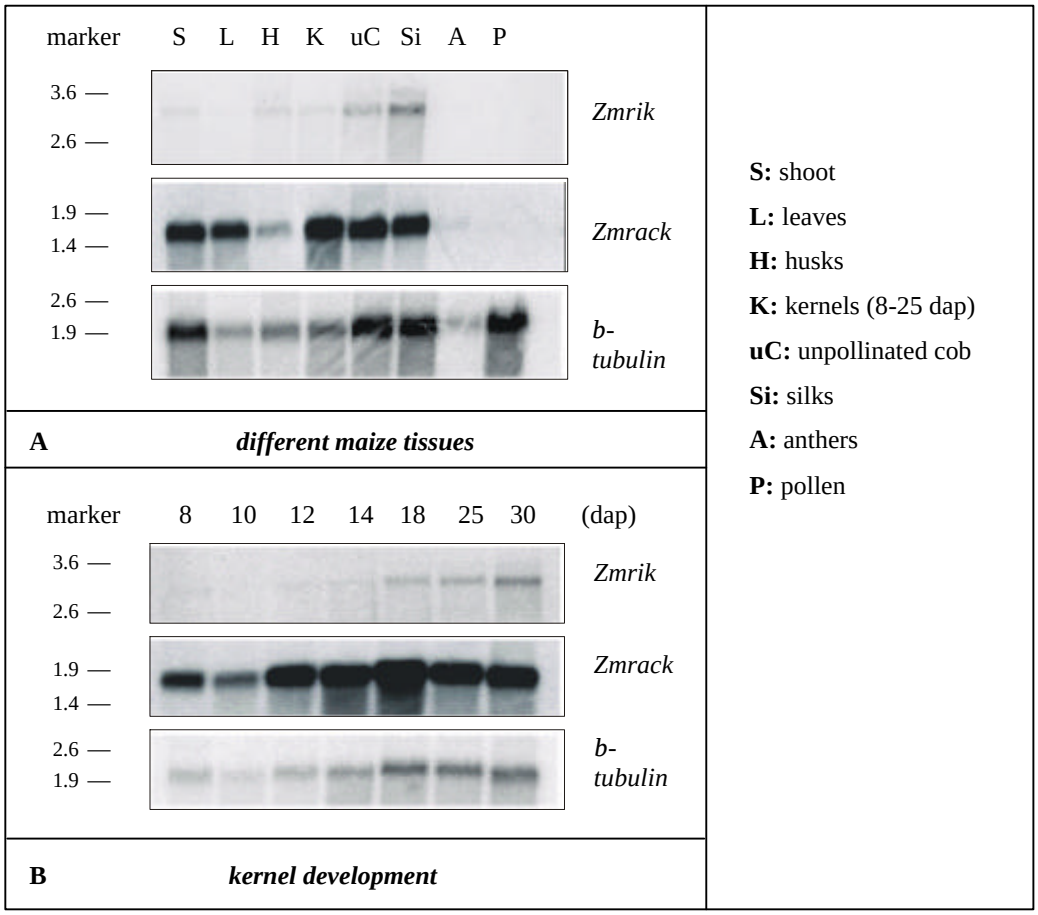


Fig. 3.16: *Northern blot analysis of the expression of Zmrik and Zmrack in* ***A:*** *different maize tissues and* ***B:*** *kernels at different developmental stages. The positions of marker bands and their sizes in kb are given on the left.*

The relative levels of *Zmrack* transcript were similar to those from *Zmrik*: the highest amounts of transcript were found in leaves and kernels, followed by unpollinated cob, silks, shoots and husks. Only a very weak signal was obtained from anthers and the amount of *Zmrack*-transcript in pollen was also below the detection limit.

A difference was observed during kernel development: while the amount of *Zmrik*-transcript increased during the maturation of the kernels, it remained constant in the case of *Zmrack*.

3.6.2 Western blot analysis

For an expression analysis of ZmRACK on the protein level, polyclonal antibodies were raised against the whole protein. Prior to immunisation, preimmune sera of several rabbits were tested on crude extracts of different maize tissues and two rabbits without cross-reactivity were chosen. Immunisations were carried out with native GST-ZmRACK that was also used for the pull-down assays (see chapter 3.2.2).
Both antisera (named 59 and 60) were then used for Western blot analysis with crude protein extracts from different maize tissues. Equal loading was controlled by running an extra gel until all proteins have just migrated into the separating gel. The gel was stained with Coomassie Brilliant Blue and the amount of protein was adjusted where necessary (data not shown).

Of each protein sample, 20 µg were loaded onto a 12% SDS-PAGE and blotted onto nylon membranes (Schleicher and Schuell, Dassel). The blots were incubated for one hour at room temperature (antiserum 59) or over night at 4° C (antiserum 60).
As can be seen in figure 3.17, both antisera detected a protein with an apparent molecular weight of 36 kDa. This is exactly the size calculated from the cDNA and this indicates that both antisera detect the correct protein.
Both antisera detected a very similar expression pattern. ZmRACK was found in every tissue tested, showing the strongest expression in roots, shoots, husks, cob axis, kernels (collected between twelve and twenty-four dap) and anthers. The expression was much weaker in leaves and silks, but was still detectable. The amount of protein did not change during seed development.

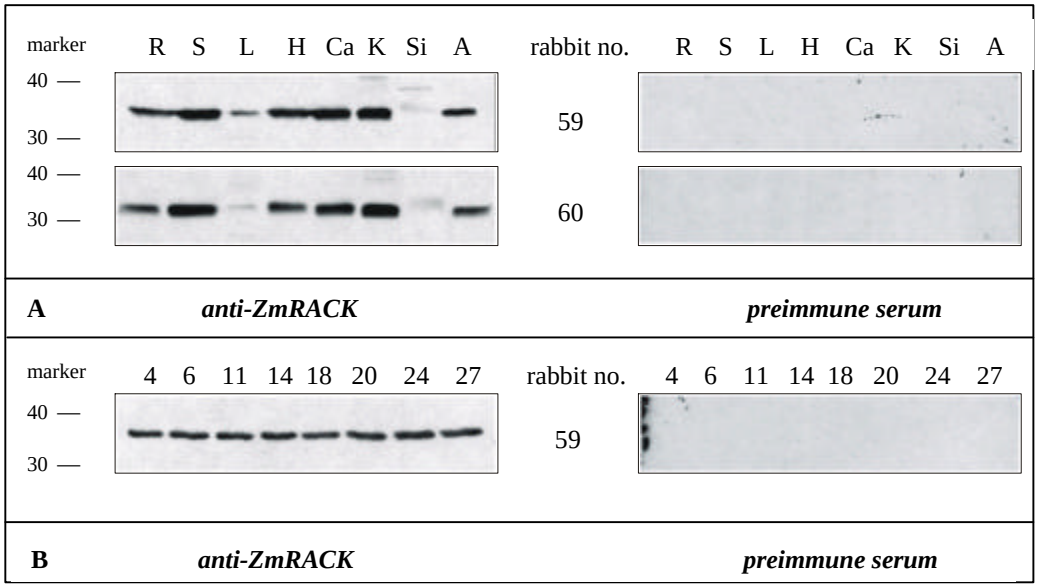


Fig. 3.17: *Western blot analysis of ZmRACK expression* ***A:*** *in different maize tissues,* ***B:*** *during kernel development. Numbers in B indicate the age of the kernels in dap, numbers on the left give the size of marker bands in kDa.* ***Abbreviations: R:*** *roots,* ***S:*** *shoots,* ***L:*** *leaves,* ***H:*** *husks,* ***Ca:*** *cob axis,* ***K:*** *kernels (twelve to twenty-four dap),* ***Si:*** *silks,* ***A:*** *anthers.*

Antisera were also raised against ZmRIK. For this purpose, four rabbits were immunised: two with denatured GST-fusion protein and two with native $(His)_6$-fusion protein.

For the GST-fusion, the coding region of ZmRIK(aa 1-284), including the LFL-domain and about half of the RI region, was amplified via PCR and cloned into the *Bam*HI and *Eco*RI sites of pGEX-3X. The construct was transformed into the *E. coli* strain BL21-CodonPlus(DE3)-RP, but no native protein could be purified. Instead, a huge amount of protein was found in inclusion bodies. Therefore, the protein was eluted from a 10% SDS-PAA-gel, run on a 9-18% gradient gel and was again eluted from the gel. The purity of this protein was more than 99% as judged from Coomassie staining. Two rabbits (numbers 79 and 80), with no cross-reactivity to maize proteins, were immunised with this antigen.

As an antiserum against the native protein might prove more useful in co-immunoprecipitations, it was tried to purify $(His)_6$-tagged protein under native conditions.

The same region of *zmrik* as for the construction of the GST-fusion was cloned into the *Bgl*II and *Nco*I sites of the vector pQE60. This construct was transformed into the *E. coli* strain M15[pREP4] and it was possible to purify this fuion protein under native conditions. This antigen was used to immunise the rabbits with the numbers 57 and 58.

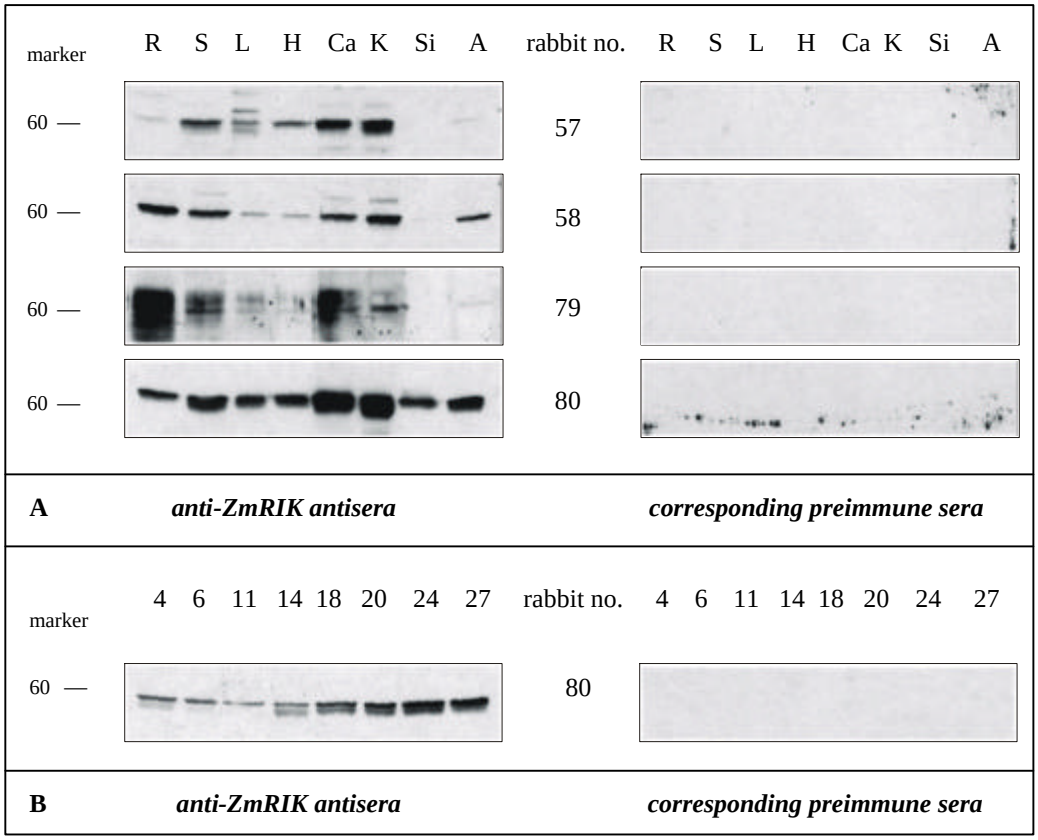


Fig. 3.18: *Western blot analysis of ZmRIK expression* ***A:*** *in different maize tissues,* ***B:*** *during kernel development. Numbers in B give the age of the kernels in dap, numbers on the left give the size of marker bands in kDa.* ***Abbreviations: R:*** *roots,* ***S:*** *shoots,* ***L:*** *leaves,* ***H:*** *husks,* ***Ca:*** *cob axis,* ***K:*** *kernels (twelve to twenty-four dap),* ***Si:*** *silks,* ***A:*** *anthers.*

All four antisera were tested on crude extracts from different maize tissues and kernels at different developmental stages. The results are shown in figure 3.18.
For unknown reasons, the antiserum 79 always showed a high background and was not used for further experiments. The antisera 57 and 58 showed some weak cross-reactivity with other proteins, but number 80 detected only one clear band.

It also had the highest titer and was used in most of the following experiments.
The expression pattern of ZmRIK was very similar to that of ZmRACK. ZmRIK was expressed in all tissues analysed, with the highest expression in shoots, cob axis, kernels and anthers and with a very low expression in silks. As opposed to ZmRACK, the amount of ZmRIK protein increased during seed maturation. This coincided with the results form Northern blot analyses
The expression patterns detected with all antisera were similar and the only differences were seen in roots. While the amount of protein in roots detected with the antisera 57 and 80 was lower than compared to the expression in leaves, it is the opposite with 58. These differences might result from posttranslational modifications of the protein in roots that mask the epitopes for antiserum 57 and 80 and, therefore, inhibit proper binding of the antibody.

Surprisingly, all three (and even number 79) antisera detected a band of 60 to 62 kDa, but the predicted molecular weight of ZmRIK (as deduced from the cDNA) is 81 kDa. Such a big difference in the apparent molecular weight can sometimes be explained with running-abnormalities during gel electrophoresis, but this usually results in a higher apparent molecular weight (MATAGNE *et al.*, 1991; CASAREGOLA *et al.*, 1994). As the TNT-product of ZmRIK runs at its expected size of 81 kDa, this can be excluded.
The fact that all four preimmune sera did not show any cross-reactivity with maize proteins prior to immunisation, and that all antisera detected only one main band, led to the conclusion that ZmRIK must be co- or posttranslationally modified, resulting in the deletion of a peptide of approximately 20 kDa. Another explanation could be alternative splicing, but this can be excluded from the Northern blot results (see chapter 3.6.1).
It is unlikely that an internal region of the protein is deleted, and it is more likely that this modification takes place at either the N- or at the C-terminus of ZmRIK. Kelch domains are one structural unit making it improbable that only parts of it are removed. Deleting the comlete kelch domain would result in a protein of approximately 50 kDa, making it too small for the detected protein. But if the

LFL-domain was deleted, the remaining protein would have a molecular weight of 61 kDa.

It can therefore be hypothesised that the N-terminal LFL-region is not present in the mature protein and that this region contains a recognition site for this modification rather than a functional domain.

3.7 Co-immunoprecipitation with anti-ZmRACK antiserum

The availability of specific antisera made it possible to verify the interaction between ZmRIK and ZmRACK also with co-immunoprecipitations (co-IP).
For a negative control, unspecific rabbit serum was coupled to sepharose using the cyanogen bromide method and was incubated with a crude protein extract from maize shoots. The eluate was loaded onto a 12% SDS-PAGE and blotted onto a nitrocellulose membrane, which was cut into stripes and incubated with different antisera. The results are shown in figure 3.19A. Neither ZmRACK nor ZmRIK can be detected in the eluate using the antisera 59 and 80. This indicates that there is no unspecific binding of ZmRACK or ZmRIK to the sepharose beads.

Many kelch domain proteins are known to interact with actin and this has also been shown with Co-IPs (HERNANDEZ *et al*., 1997). For this reason, it was also tested if there was unspecific binding of actin to the sepharose-beads. Unfortunately, it was indeed detected in the eluate, making it impossible to use this method for the detection of an interaction between ZmRIK and the actin cytoskeleton. It was not possible to optimise the procedure.

In a next step, the anti-ZmRACK antibody 59 was coupled to sepharose and used for a co-IP with a crude extract from maize shoots. The results are shown in figure 3.19B. A band of approximately 55 kDa was detected in all lanes and is caused by co-eluted antibody, because it is detected by the secondary anti-rabbit antibody, even when no first antibody was used.

The anti-ZmRACK 59 antiserum detected a band with an apparent molecular weight of 36 kDa that could not be detected with its preimmune serum. This showed that coupling the antiserum to the sepharose beads did not inhibit its binding capacity.

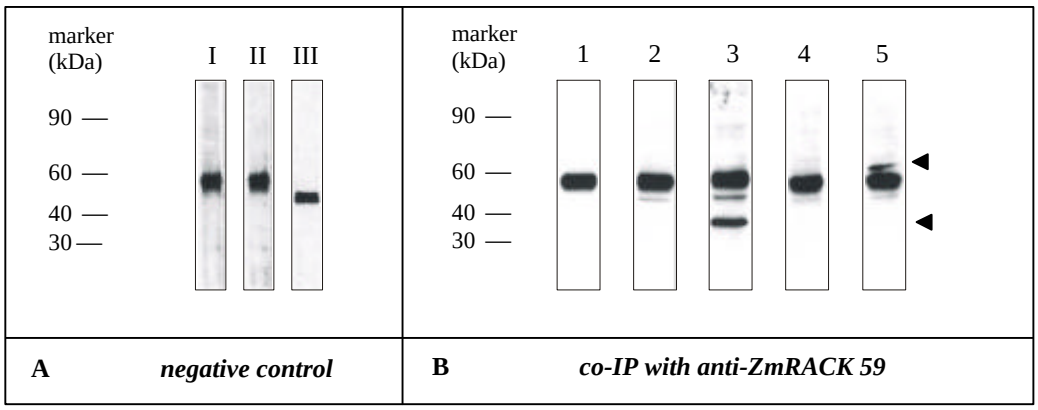


Fig. 3.19: *Co-IP with a crude protein extract from maize shoots.* ***A:*** *negative control with rabbit serum coupled to sepharose.* ***B:*** *co-IP with anti-ZmRACK 59 coupled to sepharose.* *Arrowheads indicate the position of ZmRACK and ZmRIK, respectively.* ***Explanation:*** *first antibody* ***I:*** *anti-ZmRACK 59,* ***II:*** *anti-ZmRIK 80,* ***III:*** *anti-actin;* ***1:*** *no first antibody,* ***2:*** *preimmune serum 59,* ***3:*** *anti-ZmRACK 59,* ***4:*** *preimmune serum 80,* ***5:*** *anti-ZmRIK 80.*

While no band of 61 kDa was detected with the preimmune serum of ZmRIK 80, the corresponding immune serum clearly detected a band at the size of ZmRIK. Therefore, it was also possible to show an interaction between ZmRIK and ZmRACK with a co-IP.

This also indicated that the band detected with the anti-ZmRIK antisera was indeed ZmRIK: if the 61 kDa bands detected in the Western blots resulted from unspecific cross-reactions, it is unlikely that this protein also specifically interacts with ZmRACK.

3.8 Subcellular localization of ZmRIK and ZmRACK

Knowing the subcellular localisation of a protein often provides a hint to its function. This can be analysed in different ways, but the easiest is fusing the protein of interest to a fluorescent protein and to transiently transform protoplasts with this construct. This was done with both ZmRACK and ZmRIK using yellow fluores-

cent protein (YFP) fusions and their localisation was analysed in tobacco protoplasts and in maize leaf cells. The availability of specific antisera also allowed the analysis of the subcellular localisation of the endogenous proteins with subcellular fractionations.

3.8.1 Expression analysis of ZmRACK-YFP and YFP-ZmRIK(aa 173-732) in BY-2 protoplasts

To analyse the subcellular localisation of ZmRACK and ZmRIK in tobacco BY-2 protoplasts, YFP-fusions with both proteins were prepared. Unfortunately, the N-terminal fusion of ZmRIK to YFP (or to any other fluorescent protein) inhibited the fluorescence of YFP and could not be used. Therefore, only the C-terminal fusion was analysed.

As already described in chapter 3.6.2, it can be assumed that the N-terminal LFL-domain is not present in the mature ZmRIK protein. The fusion was therefore only made with ZmRIK(aa 173-732), containing the RI region and the kelch domain. This excluded the risk that the fluorescent protein will be separated from the rest. The coding region of *Zmrack* was PCR-amplified with added *Nco*I sites, *Zmrik*(aa 173-732) was amplified with added *Bam*HI sites and both PCR-products were cloned into the corresponding sites of the vector pMON999 YFP. Both constructs, together with the empty vector (YFP-control), were transiently transformed into BY-2 protoplasts. The expression was analysed twenty-four hours after the transformation and the results are shown in figure 3. 20.

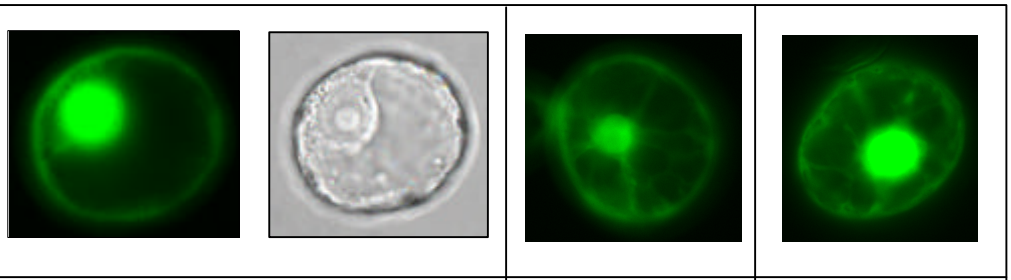
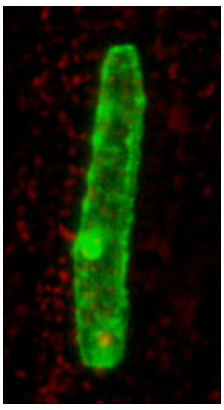


Fig. 3.20: *Localisation-studies of YFP-tagged ZmRACK and ZmRIK in BY-2 protoplasts.* ***A:*** *YFP-control, fluorescence (left) and bright-field image (right).* ***B:*** *ZmRACK-YFP and* ***C:*** *YFP-ZmRIK(aa 173-732).*

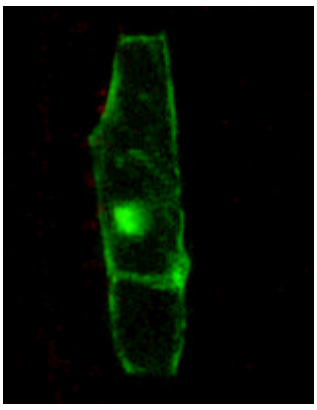
YFP is a small protein without a targeting sequence, leading to a ubiquitous localisation of the protein. But also the YFP-fusions of ZmRACK and ZmRIK did not show any particular subcellular localisation: the fluorescence was observed in all cellular compartments.

3.8.2 Localisation of ZmRACK-YFP and YFP-ZmRIK(aa 173-732) in maize leaf cells

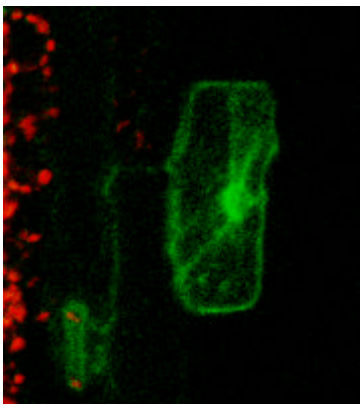
Protoplasts provide a convenient system for studying the localisation of proteins tagged with a fluorescent protein, but their morphology is not always comparable to intact cells within a multi-cellular context. For this reason, the above mentioned YFP-constructs were also transiently transformed into young maize leaf cells using particle-bombardment. The expression analysis was done with a confocal laser scanning microscope, making twenty-five to forty sections in x-direction for each cell. The pictures shown in figure 3.21 are overlays of all individual images and show not only the YFP-fluorescence (displayed in green), but also the chlorophyll autofluorescence (red).



A ***YFP***



B ***ZmRACK-YFP***



C ***YFP-ZmRIK(aa 173-732)***

Fig. 3.21: *Confocal laser scanning images of particle-bombarded maize-leaves viewed with filters specific for YFP and chlorophyll fluorescence.* ***A:*** *YFP control,* ***B:*** *ZmRACK-YFP,* ***C:*** *YFP-ZmRIK(aa 173-732).*

The results are similar to those obtained with the BY-2 protoplasts: both YFP-fusion proteins do not show any particular subcellular localisation and have a similar distribution within the cell.

3.8.3 Subcellular fractionations

The results obtained with the recombinant YFP-fusion proteins must not necessarily represent the subcellular distribution of the endogenous protein. On the one hand, adding the YFP-fusion might inhibit the proper function of the protein resulting in an artificial localisation. On the other hand, the fusion proteins are overexpressed and this massive amount of protein might also lead to mislocalisations. Further, it cannot be completely excluded that the LFL-domain of ZmRIK contains some kind of a targeting signal, which, when not present in the fusion protein, might also lead to an artificial localisation. To circumvent these problems, subcellular fractionations were made.

This was done with two different tissues, namely maize leaves and kernels, harvested between sixteen and twenty-five dap. Differential centrifugation was used to obtain fractions enriched in nuclei, chloroplasts, mitochondria, microsomes and cytosolic proteins. Approximately 20 µg of each fraction were loaded onto a 10 or 12% SDS-PAA gel and blotted onto nitrocellulose membranes.

As can be seen in figure 3.22, ZmRIK could be detected in all fractions and showed a similar distribution in the two tissues analysed. Most of protein was found in the cytoplasmatic and the crude organell fractions, but parts were also detected in the crude nuclear fraction.

The subcellular distribution of ZmRACK was very similar to that of ZmRIK. Remarkably, it differed in the two tissues analysed: while there was no signal in the crude nuclear fraction of leaves, there was a clear band in this fraction from kernels. This stands in contrast to the localisation of the ZmRACK-YFP fusion in maize leaf cells, where it has been shown to be clearly nuclear-localised.

It is currently not possible to decide, if the ZmRACK-YFP fusion protein is not functional and thus localises improperly, of if the RACK protein present in the

nuclei of leaves (but not from kernels) has been modified, masking the epitope, so that it cannot be detected by the antiserum.

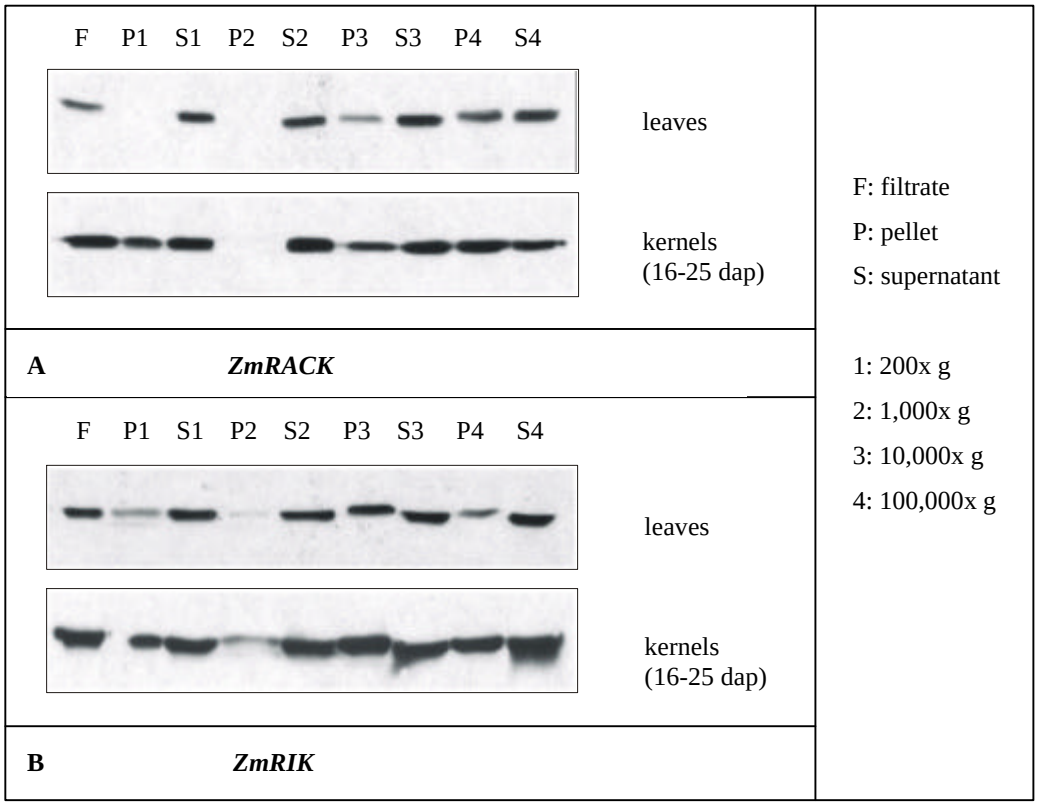


Fig. 3.22: *Subcellular fractionations of maize leaves and kernels. Western blots were incubated with anti-ZmRACK antiserum 59 (**A**) or anti-ZmRIK antiserum 80 (**B**).*

The results from all three experiments showed that ZmRIK and ZmRACK are present in the same cellular compartments and that an interaction between these two proteins is sterically possible.

3.9 Testing the hormone inducibility of ZmRACK and ZmRIK

The best-characterised plant RACK is *arcA* (auxin-responsive gene from cultured cells) from tobacco. ISHIDA *et al*. (1993 and 1996) have shown that the level of *arcA* transcript increases upon the addition of both natural and synthetic auxins, but not through the addition of abscisic acid (ABA), 1-amino-1-cyclopropane carboxylic acid (ACC), gibberellin A_3 (GA_3) or benzylaminopurine (BAP).

MsGBL, a RACK-like protein from *Medicago sativa*, was found to be regulated by hormones, although not by auxins but cytokinins (MCKHANN *et al*., 1997). Because of these properties of the two plant RACK proteins, it was of interest to see if the expression of ZmRACK, or maybe even ZmRIK, is also regulated by hormones. ISHIDA *et al.* (1996) and MCKHANN *et al.* (1997) have obtained their data through Northern blot analyses, but the availability of specific antibodies for ZmRACK and ZmRIK suggested the analysis on the protein level.

According to the procedure described by ISHIDA *et al*. (1996) for the tobacco BY-2 system, the maize suspension line HE/89 was cultivated in hormone-free medium for three days before the hormones listed below were added. The cells were harvested after seven hours, and crude protein extracts were prepared. The hormones used included ABA (5µM final concentration), ACC (1 mM), BAP (4.4 µM), GA_3 (5 µM), indole-3-acetic acid (IAA, 11.4 µM), naphthalene acetic acid (NAA, 5.4 µM) and 2,4-dichlorphenoxy acidic acid (2,4-D, 0.9 µM). Included was also a sample without hormone-starvation and one without added hormones. An immunophillin-antiserum (HUEROS *et al*., 1998) was used as a loading control.

As can be seen in figure 3.23, neither the expression of ZmRACK nor ZmRIK changed during hormone-starvation and no effects were observed upon the addition of any of the hormones added to the cell suspension culture.

These findings lead to the conclusion that ZmRACK and ZmRIK are not induced (or suppressed) by any of the tested plant hormones and that the hormone-responsiveness of the tobacco and alfalfa RACK proteins is not a common property shared by all plant RACK proteins.

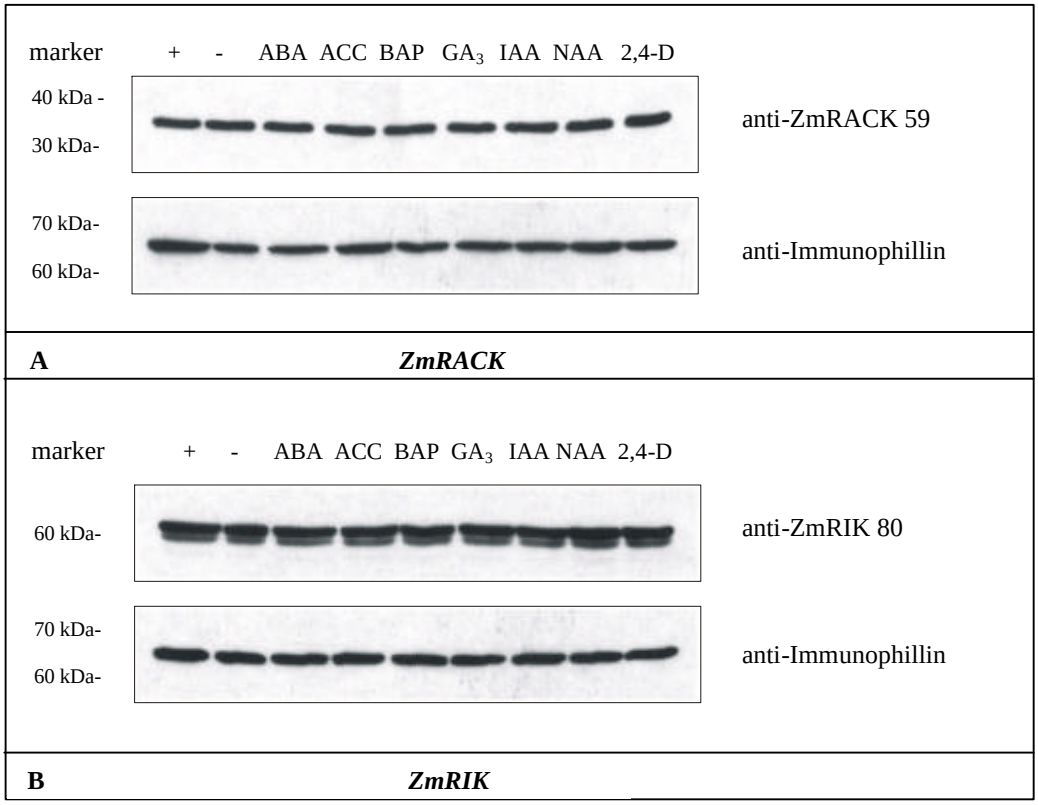


Fig. 3.23: *Testing the hormone-inducibility of ZmRACK and ZmRIK. Immunophillin was used as a loading control.* ***Explanation: +:*** *no hormone-starvation;* ***-:*** *no hormones added;* ***ABA, ACC, BAP, GA_3, IAA, NAA, 2,4-D:*** *indicated hormones added.*

3.10 Two-hybrid screen with ZmRACK as bait

RACK proteins are molecular adaptors that are known to interact with many different proteins. The human RACK1 has been shown to interact not only with βII protein kinase c (RON *et al.*, 1994), but also with phospholipase C_γ (DISATNIK *et al.*, 1994), ras-GAP, a GTPase activating protein (VAN DER GEER *et al.*, 1997) and Src, a tyrosine kinase (MOASSER *et al.*, 1999).

This suggests that ZmRACK might also interact with other proteins than ZmRIK. For this reason, ZmRACK was used as bait in another two-hybrid screen.

For this purpose, the coding sequence of *Zmrack* was PCR-amplified with added restriction sites for *Eco*RI and *Sal*I and cloned into the same sites of the vector pBD-GAL4 Cam. This construct, together with the seven dap endosperm library, was then cotransformed into the yeast strain YRG-2.

A total of 2 x 10^6 cotransformants were analysed. Six colonies grew on selective media containing 7.5 mM 3-AT. Sequencing revealed that clone number 4 was a RNA-binding protein. It was not analysed further as this class of proteins frequently causes unspecific interactions in the two-hybrid system. Clones number 1 and 3 only showed homology to proteins with unknown functions. They were named *Zmuri1* and *Zmuri2* for *Zea mays* unknown RACK-interacting protein.

Clone number 2 was the maize homologue of *arf1* from rice (designated *Zmarf1*) and number 5 was a homeobox leucine zipper protein that showed highest homology to *Oshox1* from rice (MEIJER *et al*., 1997). This clone was named *Zmhox3*, because *Zmhox1* and *Zmhox2a/b* have already been described by BELLMANN and WERR (1992) and KLINGE *et al.* (1996), respectively.
The last clone was highly homologous to AfVIP, the VIVIPAROUS-interacting protein from wild oat (JONES *et al*., 2000). The control platings after retransformation into yeast are shown in figure 3.24. All five clones found in the two-hybrid screen specifically conferred histidine-auxotrophy in the presence of ZmRACK-BD. The interactions were relatively strong, because growth on –LWH + 15 mM 3-AT was not weaker than on –LW.

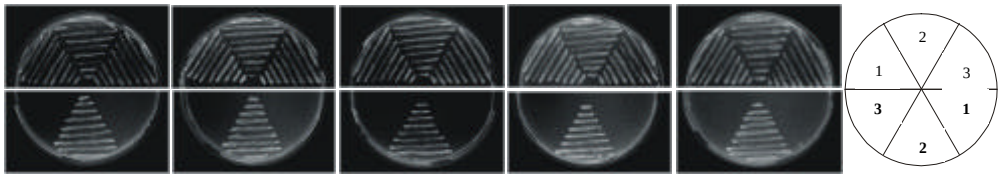


Fig. 3.24: *Two-hybrid platings with ZmRACK-BD and the five putative interacting partners ZmURI1, ZmURI2, ZmARF1, ZmHOX3 and ZmVIP as AD-fusion proteins. Plates were incubated at 30° C for two days.* ***Explanation 1:*** *ZmRACK-BD + pAD,* ***2:*** *ZmRACK-BD + X-AD,* ***3:*** *pBD + X-AD (on –LW medium), bold numbers on –LWH + 15 mM 3-AT;* ***X:*** *indicated prey-plasmid.*

4. Discussion

4. Discussion

4.1 Isolation of ZmRACK as an interacting partner of ZmRIK, a novel kelch domain protein from maize

Unlike other domains (e.g. the protein kinase domain), the presence of a kelch domain does not implicate a certain function for a given protein. The indications relating to function at the outset of this project was the fact that most kelch domain proteins act via protein/protein interactions and that they are part of protein complexes.

Different techniques can be used for the identification of interacting partners. Cross-linking experiments or, if specific antibodies are available, co-immunoprecipitations might prove useful, but subsequently require sequencing of the proteins of interest and cloning of corresponding genes.

Genetic approaches like the split-ubiquitin or the yeast two-hybrid system circumvent this problem as they directly provide the cDNAs of putative interacting partners. Currently, the two-hybrid system is the most commonly used technique and has been succesfully applied to identify interacting partners of many kelch domain proteins. For example, the interaction between Kel1p and Kel2p from yeast (PHILIPS and HERSCHKOWITZ, 1998), the inhibitory interaction of mammalian Keap1 with the transcription factor Nrf2 (ITOH *et al*., 1999), and its binding to myosin-VIIa (VELICHKOVA *et al*., 2002), and the interaction of the microtubule-associated protein 1B with gigaxonin (DING *et al*., 2002), were all identified with the two-hybrid system.

For this reason, the yeast two-hybrid system was also used to identify putative interacting partners for the kelch domain protein of unknown function from maize.

The screens made using a cDNA library prepared from seven dap endosperm successfully identified two independent clones for a receptor of activated C kinase that was designated ZmRACK. A close homologue of ZmRACK, RWD, has been described from rice (IWASAKI *et al*., 1995). RWD shares 89% identity on the ami-

no acid level with ZmRACK. An alignment of the two-hybrid clones together with RWD showed that one of the clones found was already the full-length coding sequence, while the other one encoded only for the amino acids 183 to 334. From the latter it can be concluded, that the C-terminal half of ZmRACK is already sufficient for the interaction with the kelch domain protein.
This interaction could be repeated with pull-down assays and a co-immunoprecipitation. Therefore, it can be assumed that the interaction is specific, and thus the novel kelch domain protein was named ZmRIK for *Zea mays* RACK-interacting kelch domain protein.

All RACKs are comprised of seven WD-40 repeats (SCHECHTMAN and MOCHLEY-ROSEN, 2001). These repeats form a β-propeller, like the kelch domain, and they too are known to facilitate protein/protein interactions (compare figure 1.2; NEER *et al*., 1994). RACKs are highly homologous to the β-subunits of the trimeric G-protein (Gβ), but lack the N-terminal α-helix necessary for the correct folding of the subunit (SCHMIDT and NEER, 1991), and for its interaction with the γ-subunit (BUBIS and KHORANA, 1990). The presence of a RACK in *Chlamydomonas reinhardtii* (although originally designated Gβ; SCHLOSS, 1990), prompted NEER *et al.* (1994) to suggest that the biological function of RACK proteins was established before the separation of the animal and the plant kingdoms occurred. Mammalian RACK1 is expressed in all tissues, suggesting that it has an important function in most, if not all, cells (CHOU *et al*., 1999).
Northern and Western blot analysis have shown that also ZmRIK and ZmRACK are expressed in all tissues analysed (although at different levels), and that the relative levels of the two transcripts/proteins are similar. This points towards a basic function of ZmRIK and ZmRACK.
Both ZmRIK and ZmRACK were detected in nearly all subcellular compartments. This indicates that an interaction between RIK and RACK can indeed occur, and that it is not artificially induced by bringing two proteins together (in yeast) that would not get in contact *in vivo*.

4.2 RACKs and protein kinase C signalling in plants: virtually virgin territory

Several plant RACK sequences are deposited in the databases, but only few of these proteins have been analysed in detail. In contrast to the situation in plants, mammalian RACK proteins are very well characterised. They are known to be molecular adaptors that bind to many different components of diverse signal transduction pathways and that are responsible for the correct temporal and spacial localisation of their interacting partners (reviewed by SCHECHTMAN and MOCHLY-ROSEN, 2001).

One such component binding to RACK is protein kinase C (PKC) that gave rise to the name of this group of adapter proteins. PKC-signalling is part of a complex signal transduction network that requires the activation of the kinase. External signals lead to the hydrolysis of membrane-bound phospholipids by phospholipase C (PLC) to generate diacylglycerol and inositol triphosphate. These two, together with Ca^{2+}, bind to PKC and cause its activation, resulting in its relocation from the cytosolic to the membrane fraction (KRAFT and ANDERSON, 1983). Some isoforms of PKC have been shown to colocalise with cytoskeletal structures (ZALEWSKI *et al*., 1988; KILEY and JAKEN, 1990; MOCHLEY-ROSEN *et al*., 1990) suggesting the involvement of some kind of anchor or adaptor. The first such adaptor was isolated from rat and was called RACK1 (receptor for activated C kinase, RON *et al.*, 1994). The binding of PKC to its RACK brings the kinase in contact with some of its substrates, but also away from others and therefore confers the functional specificity on the different isozymes (MOCHLY-ROSEN, 1995).

Little is known about plant RACK proteins. The best characterised one is ARC from tobacco. It was found to be induced by the auxin 2,4-D upon hormone-starvation (ISHIDA *et al*., 1993) and it is hypothesised that it is involved in UV light and salicylic acid-induced cell cycle arrest (PERENNES *et al*., 1999). MsGBL from alfalfa has been shown to be involved in cytokinin-mediated cell division (MCKHANN *et al*., 1997). The rice RACK, RWD, is only characterised in terms of its expression pattern (IWASAKI *et al*., 1995). Other RACK-like proteins such as

ARC and RACK from *Arabidopsis* have not yet been analysed and only their sequences are deposited in the databases. Furthermore, no interactions with other proteins have been so far described, and thus the binding of ZmRIK to ZmRACK is the first contribution towards understanding of the network in which plant RACK proteins act.

The fact that both *arcA* and *Msgbl* are hormone-induced on the transcript level (MCKHANN *et al*., 1997) led to the suggestion that plant RACKs could have a general function in hormone-controlled cell division. To test the generality of this hypothesis, the hormone inducibility of ZmRACK in the maize suspension cell line HE/89 was tested. The availability of specific antibodies made it possible to analyse hormone-induced changes on the protein- and not on the transcript-level. However, in contrast to the other two studied plant RACK proteins, ZmRACK (like ZmRIK) showed no hormone-responsiveness.
Changes on the transcript level do not necessarily result in changes in the amount of protein. It thus remains unclear whether *arcA* and *Msgbl* also show changes at the protein level or, *vice versa*, if the amount of transcript of ZmRACK changes upon the addition of hormones. However, it can be stated that hormone-responsiveness is not a general feature of plant RACK proteins.

The question of whether or not plant RACK proteins could accomplish the same function as mammalian RACK proteins has still to be answered. One matter of debate is also the issue of whether or not plants have a protein kinase C, no such plant protein sequence has been identified so far. With all the sequence information available including the complete genomes of *Arabidopsis* and rice, no protein with significant homology to mammalian PKCs could be identified. Early attempts to detect plant PKCs by using antibodies raised against PKC from bovine brain (ELLIOTT and KOKKE, 1987a and 1987b; MORELLO *et al.*, 1993) did not produce convincing data, although ELLIOTT and KOKKE (1987) and MORELLO *et al*., (1993) were able to biochemically detect PKC-activity in plants.

The first convincing evidence for the existence of a plant PKC was presented by CHANDOK and SOPORY (1998), who biochemically purified a maize protein with the typical characteristics of a PKC: the kinase was activated by phorbol myristate acetate (PMA), the activation was Ca^{2+}-dependent, and the addition of Ca^{2+} in the presence of phosphatidyl serine and PMA lowered its K_m. It was further able to phosphorylate histone H1, and its activity was inhibited by two general PKC-inhibitors, namely staurosporine and H-7. Although the sequence identity of this PKC remains still unknown, there is persuasive evidence for the presence of a plant protein with the properties of PKC.

The fact that RACK proteins are conserved in unicellular algae, plants and animals suggests that they may also fulfil the same, conserved, function. The data available for plant RACK proteins also supports this hypothesis: all so far analysed plant RACKs are ubiquitously expressed in all tissues like their mammalian counterparts, and evidence has been presented that ARCA and MsGBL are involved in signal transduction (MCKHANN *et al.*, 1997; PERENNES *et al.*, 1999). So at the moment, nothing would contradict there being a similar function for mammalian and plant RACK proteins.

4.3 Finding functions for each of the three domains of ZmRIK

The fact that RACKs bind to different components of signal transduction pathways raises the question as to what the function of ZmRIK could be. To address this issue, the (putative) function of each of the three domains will be discussed below.

The central region of ZmRIK was shown to be necessary and sufficient for the interaction with ZmRACK and was for that reason called the RACK-interacting (RI) region.

Although AtRLP and OsRLP show relatively high homology to the N- and C-terminus of the RIK proteins from rice and maize, the central regions of these proteins bear no homology to their RI region. As a consequence, the RLP proteins are not able to interact with RACK proteins. They might still be present in one com-

plex with the corresponding RACK, and might carry out a similar function to that of ZmRIK or OsRIK, but with the difference that the interaction between the kelch domain protein and the rest of the complex is mediated by a protein other than the RACK itself. Another possible explanation for the function of RLP might be that the RLP proteins are the result of a combination of three domains with different properties that form a protein with a new function. Unfortunately, the function of RLP from *Arabidopsis* and rice was not further analysed.
The fact that RIK proteins have been found in maize and rice, but not in *Arabidopsis* or members of plant families other than the *Poaceae*, leads to the suggestion that RIKs might be specific for this monocotyledonous plant family.

The N-terminal LFL-domain has been identified by its relatively high level of conservation between different plant proteins and got its name from the invariant tripeptide leucine-phenylalanine-leucine. Proteins containing the LFL-domain have been found in such diverse plant families as the *Apiaceae*, *Brassicaceae*, *Fabaceae*, *Poaceae* and *Rutaceae*, but not outside the plant kingdom. It can thus be assumed that LFL-domain containing proteins are widespread among many (if not all) plant families, and that this domain is plant-specific.
Unfortunately, no function is yet known for any protein containing the LFL-domain. Interestingly, the LFL-domain is always at the N-terminus of these proteins.
The results from Western blot analyses with anti-ZmRIK antibodies together with the findings from the co-immunoprecipitations showed that the apparent molecular weight of ZmRIK was smaller than that predicted from the coding sequence (approximately 61 kDa versus 81 kDa). As running abnormalities during gel electrophoresis and alternative splicing can be excluded, this suggests that ZmRIK is translated as a pre-protein and that part of it is removed during a so far unknown maturation process.
As the 61 kDa-protein can still interact with ZmRACK, it is unlikely that the RI region is deleted. Furthermore, the kelch domain is one functional unit, making it doubtful that a cleavage takes place within this domain. Removal of the complete kelch domain would result in a protein of about 50 kDa – too small for the detec-

ted protein with an apparent molecular weight of 61 kDa. However, the LFL-domain is roughly 20 kDa in size and its deletion would result in a protein with a molecular weight of 61 kDa - matching exactly the size detected with the antisera.

It remains unknown how and where this processing of ZmRIK happens. The presence of inteins (the so called "protein introns") can be excluded for two reasons. On the one hand, inteins can splice out without the requirement of supporting factors. According to this, the splicing reaction would also take place in an *in vitro* transcription/translation (TNT) reaction, but TNT-reactions with the full-length coding sequence of ZmRIK produced a protein with the expected molecular weight of 81 kDa. On the other hand, inteins require certain conserved residues for the splicing mechanism (PERLER *et al*., 1997; PIETROKOVSKI, 1998) that are not present in the sequence of ZmRIK or any of the LFL-domain proteins.
Further, the cleavage of a targeting leader peptide appears to be unlikely as no specific subcellular localisation has been found. The fact that no 81 kDa protein has ever been detected, suggests that the modification occurs with a very high affinity during or immediately after translation. The mechanism and the function of this modification remain unclear.

Unfortunately, no interacting partners for the kelch domain of ZmRIK have been found and thus its function remains still unknown. Mammalian RACKs bind transcription factors like the Epstein-Barr virus activator protein BZLF1 (SMITH *et al*., 2000), kinases like members of the src kinase family (CHANG *et al*., 1998 and 2001), and other components of signal transduction pathways like phosphodiesterase 4D5 (YARWOOD *et al*., 1999) and phospholipase Cγ (DISATNIK *et al*., 1994; RON *et al*., 1995), but it is unlikely that ZmRIK could perform one of these functions. In fact, there are kinases among the big super-family of kelch domain proteins, like AFKin (EICHINGER *et al*., 1996) and the group of plant protein serine/threonine phosphatases with kelch-like repeat domains (PPKL; KUTUZOV and ANDREEVA, 2002), but their kinase activity is not the property of the kelch domain.

The kelch domains of all so far analysed proteins have not displayed any enzymatic properties and their functions may lie exclusively in mediating protein/protein interactions. Many kelch domain proteins are interacting directly with the cytoskeleton, like actinfilin (CHEN *et al*., 2002), mayven (SOLTYSIK-ESPANOLA *et al.*, 1999) or IPP (KIM *et al*., 1999). This interaction is always mediated through the kelch domain, and this leads to the hypothesis that the kelch domain of ZmRIK could also be associated with the cytoskeleton.

For technical reasons, it is not possible to use the two-hybrid system for the identification of direct interactions with the cytoskeleton: the filamentous nature of its components causes steric hindrance and prevents the required nuclear-localisation. The co-immunoprecipitations shown in chapter 3.7 also could not be used to demonstrate an interaction with actin-filaments, as actin has been shown to bind unspecifically to the beads. Further, co-sedimentations with ^{35}S-labelled ZmRIK (aa 173-732) were not successful, because the high salt concentrations required for the maintenance of the actin-filaments caused the aggregation of ZmRIK (data not shown), resulting in an actin-independent sedimentation of ZmRIK. Thus, because of technical problems, it was so far not possible to test the hypothesis that ZmRIK is associated with the cytoskeleton.

Currently, co-localisation-studies with anti-actin and anti-ZmRIK antibodies are under-way.

4.4 RIK and RACK: A shuttle and its load?

RACK proteins have been shown to colocalise with the cytoskeleton (MOCHLEY-ROSEN *et al*., 1990; ZALEWSKI *et al*., 1988) and it is assumed that the RACK-complex moves through the cell along cytoskeletal elements. But so far it was not possible to show that RACK proteins bind directly to the cytoskeleton, and it remains unknown, as to how the RACK-complex is actually moving through the cell.

The fact that many kelch domain proteins are associated with the cytoskeleton leads to the hypothesis that RIK (and maybe even RLP) could be the missing link

between the RACK-complex and the cytoskeleton. RIK would then act as a shuttle for the RACK-complex, using the cytoskeleton like train tracks.

The fact that ZmRIK can be found in all cellular compartments excludes the possibility that it is permanently bound to the cytoskeleton. Both proteins, ZmRACK and ZmRIK, are partly cytoplasmic, leading to the assumption that the contact between RIK and RACK might occur in the cytoplasm. ZmRIK could then bind to cytoskeletal filaments and carry the RACK-complex along the filaments to the different subcellular compartments.

This movement has to be coordinated and directed, and it is likely that it requires the presence of other proteins bound to the complex. These could either interact with ZmRIK or ZmRACK. Two-hybrid screens with ZmRIK(aa 173-732) did not identify any convincing binding partners, but different putative interacting partners for ZmRACK were found. Besides two proteins with no assigned functions, one auxin-responsive factor (ARF) with high homology to OsARF1 from rice, one homeobox leucine zipper protein homologous to the transcription repressor OsHOX1 from rice, (MEIJER *et al.*, 1997), and the maize homologue of AfVIP, the VIVIPAROUS1-interacting protein from wild oat (*Avena fatua*; JONES *et al.*, 2000), have been found to specifically interact with ZmRACK in the two-hybrid system. Unfortunately, it was not possible to obtain sufficient amounts of *in vitro* transcribed/translated proteins for pull-down assays to repeat the interaction with a technique other than the two-hybrid system. But the fact that the interaction in the two-hybrid system was specific and very strong makes it likely that the detected interactions are indeed real.

The interaction of ZmRACK with ZmARF1 and ZmVIP shows that also plant RACK proteins could also be connecting different signal transduction pathways: ARFs are transcription factors involved in auxin-induced growth response (ULMASOV *et al.*, 1997 and 1999), while VIP and its interaction with VIVIPAROUS1 is part of an abscisic acid-regulated signal transduction pathway (JONES *et al.*, 2000).

4.5 Mutants of OsRIK: a starting point for further investigations

"Man kann nach wie vor Hypothesen aufstellen und versuchen, sie zu widerlegen oder zu… - nun ja, wirklich beweisen kann man nie, aber man kann mehr und mehr zeigen." (Günther Blobel)

["One can still set up hypotheses and can try to disprove them or to…- well, you will never be able to prove, but you can reveal more and more."]

As said by Günter Blobel, it is not possible to find the ultimate proof for a given hypothesis. Frequently, mutants provide useful tools to identify the function of a protein and make it possible to analyse its impact in the context of the living organism.

Unfortunately, no *Arabidopsis*-mutants for AtRLP were found and they would have not necessarily provided a hint as to the function of RIK proteins. Only recently, the collection of the *Tos17* mutant lines of the Functional Genomics Laboratory, National Institute of Agrobiological Sciences (NIAS) in Japan was made publicly available. BLAST search with a database of flanking regions of the transposon insertion lines identified seven different mutants with insertions evenly distributed over the whole length of *Osrik*. Two of them, lines NF9873 and NE3539, are located in an intron and the other five are inserted in different exons. The positions of the *Tos17* insertions in the different lines are shown in figure 4.1 together with some of the pictures that are available from the NIAS World Wide Web page (http://tos.nias.affrc.go.jp/~miyao/pub/tos17/) for four of the lines (NF7037, NE 3539, ND7004 and ND1004).

Tos17 is a rice retrotransposon that is highly active during tissue culture phases, but remains stably integrated during normal growth (HIROCHIKA, 1997 and 2001). The average insertion number in the rice cultivar Nipponbare is two per haploid genome. The mutant lines were regenerated after five month of tissue culture and have an average of eight insertions (HIROCHIKA, 1997 and 1999). The NIAS homepage provides also some information on the phenotypes of the mutants, but they have not yet been correlated to their genotypes. However, the fact that lines

with insertions in similar regions show similar phenotypes can be taken as an indication that the observed phenotypes indeed result from insertions in *Osrik*.

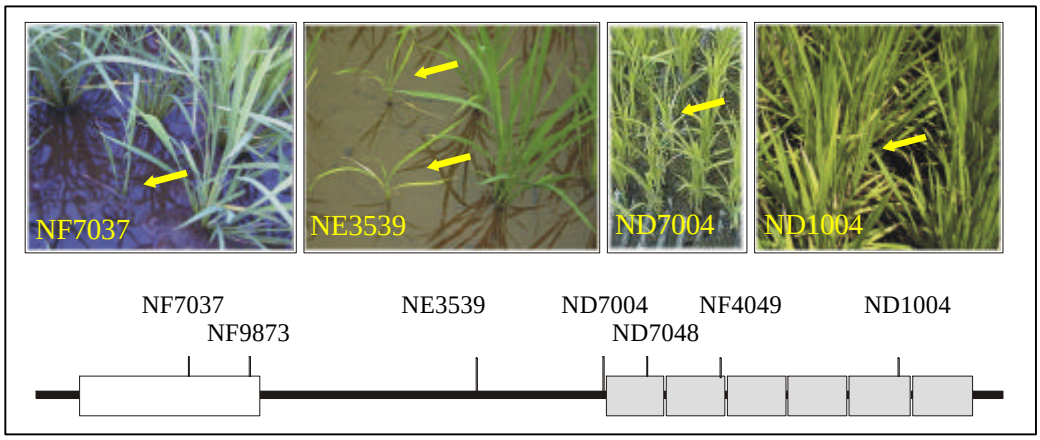


Fig. 4.1: *Sketch of the Tos17 insertions in respect to their corresponding position within OsRIK. Pictures of some of the mutants from the NIAS homepage are shown in the top panel. Arrows mark individual mutant plants or a whole row of mutants*

The lines NF7037 and NE3539 with insertions in the LFL- and the RI region both show a dwarf phenotype. Additionally, NE3539 was described to produce less tillering and yellowish leaves. The mutant line ND7004 (inserted at the transition RI region/ kelch domain) also shows a dwarf and albino phenotype. The more C-terminal the insertions are, the less severe the phenotypes are: ND1004 with a *Tos17* insertion in the fifth kelch repeat only shows yellowish leaf-tips and is sterile.

At the moment, not much can be said about the phenotypes of the mutant lines as at the time of submission of the thesis, the plants were too small to analyse their genotypes. Several seedlings already show a dwarf phenotype and have a defective pigmentation.

Once they are big enough they will be subject to Southern blot analysis. *Tos17*- and gene-specific probes will be used to show whether or not the observed phenotypes cosegregate with the insertion of *Tos17* in *Osrik*.

The available pictures of the mutant lines show that the knock-out lines have very serious defects. This indicates that RIK proteins are involved in essential develop-

mental processes, and that also this novel class of kelch domain proteins plays an important role for the coordinated growth and development of the organism.

5. Summary

Kelch domain proteins are multifunctional all-rounders involved in many cellular processes. In mammals, kelch domain proteins have been found to be involved in many important aspects of cellular life, but in plants only the *ZEITLUPE*-family of kelch domain proteins has been analysed in detail. The fact that there are 126 plant proteins with a kelch domain listed in the Pfam-database in the end of 2002, shows that this group of proteins is of equal importance in plants and that there is a clear backlog in this field.

In this thesis, ZmRIK, a novel kelch domain protein from maize, was analysed. The protein consists of three domains: the C-terminal kelch domain with six kelch kelch repeats, a novel conserved region in the N-terminus called LFL-domain and a central region that is only conserved in the RIK homologue from rice.

Evidence has been presented that ZmRIK is translated as a pre-protein, of which a 20-kDa peptide is removed during a currently unknown maturation process. It can be hypothesised that the LFL-domain functions as a recognition site for this modification, and that this region is not present in the mature protein. The central region of ZmRIK has been shown to mediate the interaction with a receptor of activated C kinase (ZmRACK), but no function could be assigned to the kelch domain.

The fact that kelch domains are often associated with the cytoskeleton led to the assumption that the kelch domain of ZmRIK could also mediate an interaction with the cytoskeleton.

RACK proteins are known to be molecular adaptors that bind to components of different signal transduction pathways, and that are responsible for their correct spatial and temporal localisation. They have been shown to be associated with the cytoskeleton and it is hypothesised that they use it for their movement through the cell. The results from the work presented here suggest that ZmRIK could act like a shuttle, using the cytoskeletal filaments like train tracks for this movement and thus being the missing link between the RACK-complex and the cytoskeleton.

6. Zusammenfassung

Kelch-Domänenproteine sind multifunktionelle Proteine, die an den verschiedensten zellulären Prozessen beteiligt sind. Während diese Familie von Proteinen in Säugern bereits sehr eingehend untersucht worden ist, ist über pflanzliche Kelch-Domäneneproteine kaum etwas bekannt. Bis Ende 2002 waren in der Pfam-Datenbank bereits 126 pflanzliche Proteine mit einer Kelch-Domäne aufgelistet, was darauf hindeutet, dass diese Proteinsuperfamilie auch in Pflanzen eine wichtige Rolle spielt.

Gegenstand dieser Arbeit war die Analyse von ZmRIK, eines neuartigen Kelch-Domänenproteins aus Mais. Das Protein besteht aus drei verschiedenen Abschnitten. Im C-Terminus besitzt es die Kelch-Domäne mit sechs Kelch-Wiederholungen. N-terminal befindet sich eine bisher noch nicht beschriebene, pflanzenspezifische Domäne, die aufgrund eines hochkonservierten Tripeptides aus Leucin-Phenylalanin-Leucin als LFL-Domäne bezeichnet wird. Die Ergebnisse dieser Arbeit legen nahe, dass ZmRIK als Pre-Protein gebildet wird, von dem im Verlauf einer bisher noch unbekannte Prozessierung ein Peptid von etwa 20 kDa entfernt wird. Es wird vermutet, dass die LFL-Domäne im reifen Protein nicht mehr vorhanden ist und keine funktionelle Domäne, sondern vielmehr eine Erkennungssequenz für diese Modifikation enthält.

Die LFL- und die Kelch-Domäne werden durch eine Region getrennt, die zusätzlich nur noch in einem homologen Protein aus Reis konserviert ist. Diese Region interagiert mit einem Rezeptor für aktivierte C Kinase, ZmRACK, was auch zur Namensgebung des Proteins führte (*Zea mays* RACK-interagierendes Kelch- Domänenprotein). Die Interaktion wurde zunächst im Hefe-Zwei-Hybrid-System gefunden, konnte aber auch mit *in vitro*-Bindungstests und Co-Immunprezipitationen nachgewiesen werden. ZmRACK und ZmRIK weisen ein ähnliches Expressionsmuster auf und sind in fast allen subzellulären Kompartimenten vorhanden. Das zeigt, dass die Interaktion zwischen den beiden Proteinen auch tatsächlich stattfinden kann und nicht auf einer artifiziellen Zusammenführung zweier *in vivo* räumlich getrennter Proteine beruht.

RACK-Proteine sind in Säugern bereits eingehend untersucht worden (nicht zuletzt auch wegen Ihrer Beteiligung an verschiedenen Krebsarten), allerdings ist über die pflanzlichen RACKs kaum etwas bekannt. RACK-Proteine sind molekulare Adaptoren, die an Elemente verschiedener Signaltransduktionswege binden und die für deren zeitlich und räumlich korrekte Lokalisierung verantwortlich sind. Es gibt Hinweise, dass die Bewegung des RACK-Komplexes entlang des Zellskeletts erfolgt, jedoch konnte bisher keine direkte Bindung der RACK-Proteine an das Zytoskelett nachgewiesen werden.

Was könnte nun die Funktion von ZmRIK sein? Die Überlegung, dass sehr viele Kelch-Domänenproteine direkt mit Elementen des Zytoskeletts interagieren, führte zu der Hypothese, dass auch ZmRIK über die Kelch-Domäne diese Funktion ausüben könnte. ZmRIK wäre somit das noch fehlende Bindeglied zwischen dem RACK-Komplex und den Filamenten des Zytoskeletts. Die Koordination des Transportes zu den verschiedenen Kompartimenten könnte durch andere, sich in dem RACK-Komplex befindende, Proteine erfolgen. Als mögliche weitere Interaktionspartner von ZmRACK konnten ein Homöobox-Leucin-Zipper Protein, ein ARF-Transkriptionsfaktor und das Mais-Homolog von AfVIP, dem VIVIPAROUS-interagierendem Protein aus Flughafer, im Hefe-Zwei-Hybrid-System identifiziert werden.

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8. Appendix

I. Oligonucleotides

Construct	Restriction sites	Sequence (5' ® 3'); restriction sites are underlined
pBD-ZmRACK	*Eco*RI	GATCGAATTCATGGCCGGCGCGCAG
	*Sal*I	GATCGTCGACTTCTAGCCTGCGTAGCCGT
pBD-ZmRACK	*Eco*RI	GATCGAATTCATGGCCGGCGCGCAG
	*Sal*I	GATCGTCGACTTCTAGCCTGCGTAGCCGT
pBD-ZmRIK	*Sma*I	GATCCCCGGGCCATGGTGACTAGCAAATCC
	*Sal*I	GACTGTCGACTCAGAGAACAATCGCTGAG
pBS-OsRIK(aa 174-439)	*Xho*I	GATCCTCGAGATGCCTTCTACCAACAATGGC
	*Bam*HI	GATCGGATCCTTAGACATCTTCTGAACCCAA
pBS-OsRLP	*Xho*I	GATCCTCGAGATGGGTGCTGGAAAGAAG
	*Eco*RI	GATCGAATTCTCAAATGGCAACGGCGCA
pBS-RLP(AAA)	*Xho*I	GATCCTCGAGATGGTTTTGACAGGT
LFL-domain	*Eco*RV	GATCGATATCACATGTCAACTTCCT
pBS-RLP(AAA)	*Eco*RV	GATCGATATCCTGCTAACATCTTTTG
central region	*Xba*I	GATCTCTAGAATCTAAGCTGGTATC
pBS-RLP(AAA)	*Xba*I	GATCTCTAGACCAACTGAGGCAATT
kelch domain	*Sac*I	GATCCCGCGGTTACAAAGCCACCGC
pBS-RLP(AZA)	*Eco*RV	GATCGATATCATGTTTACTCCTTCT
central region	*Xba*I	GATCTCTAGAACCCAAACATTGTTC
pBS-ZmRIK	*Xho*I	GATCCTCGAGATGGTGACTAGC
	*Bam*HI	GATCGGATCCTCAGAGAACAATC
pBS-ZmRIK(aa 1-173)	*Xho*I	GATCCTCGAGATGGTGACTAGC
	*Bam*HI	GATCGGATCCTCAAGGAGAAGGAGTAAAC
pBS-ZmRIK(aa 1-444)	*Xho*I	GATCCTCGAGATGGTGACTAGC
	*Bam*HI	GATCGGATCCTCAATCTTCTGAACCCAAA
pBS-ZmRIK(aa 173-444)	*Xho*I	GATCCTCGAGATGTCTCCCAACAGTTTCT
	*Bam*HI	GATCGGATCCTCAATCTTCTGAACCCAAA
pBS-ZmRIK(aa 173-732)	*Xho*I	GATCCTCGAGATGTCTCCCAACAGTTTCT
	*Bam*HI	GATCGGATCCTCAGAGAACAATC
pBS-ZmRIK(aa 444-732)	*Xho*I	GATCCTCGAGATGGTCATTTATTTAGTTG
	*Bam*HI	GATCGGATCCTCAGAGAACAATC
pGEMT-AtRLP	*Eco*RI	GATCGAATTCATGGTTTTGACAGGTTTACCCTCTAAC
	*Xho*I	GATCCTCGAGTTACAAAGCCACCGCAGATAGAAAGCA
pGEX-AtARC	*Bam*HI	GATCGGATCCATGGCGGAAGGACTCGTTTTG
	*Eco*RI	GATCGAATTCCTAGTAACGACCAATACCCCA
pGEX-AtRACK	*Bam*HI	GATCGGATCCATGGCCGAGGGACTCGTATTG
	*Eco*RI	GATCGAATTCCTAGTAACGACCAATACCCCA
pGEX-OsRWD	*Bam*HI	GATCGGATCCATGGCCGGCGCGCAGGAGTCT
	*Eco*RI	GATCGAATTCCTAGCCGGCGTAGCTGAAACC
pGEX-ZmRACK	*Not*I	GATCGCGGCCGCCTAGCCTGCGTAGCCGT
	*Eco*RI	GATCGAATTCATATGGCCGGCGCGCAG
pGEX-ZmRIK(aa 1-284)	*Bam*HI	GATCGGATCCCCATGGTGACTAGCAATCC
	*Eco*RI	GATCGATTCTCACTGCTTATCAGGG
PQE60-ZmRIK(aa 1-284)	*Nco*I	GATCCCATGGTGACTAGCAATCC
	*Bgl*II	GTCAAGATCTCTGCTTATCAGGG
pYFP-ZmRACK	*Cla*I	GATCATCGATATGGCCGGCGCGCAGGAGT
	*Cla*I	GATCATCGATGCCTGCGTAGCCGTA
pYFP-ZmRIK(aa 173-732)	*Bam*HI	GATCGGATCCCCTTCTCCCAACAG
	*Bam*HI	GATCGGATCCTCAGAGAACAATC

II. Sequences

ZmRIK

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M V T S K S S W S Q V V K N T R P T N L S V A A R N L Q P Q D L G A V I F G C T N N T I
ATGGTGACTAGCAAATCCTCGTGGAGCCAGGTTGTAAAAAATACCAGGCCGACAAACCTGTCCGTTGCTGCAAGAAACCTCCAACCACAGGACCTTGGAGCCGTGATATTTGGTTGCACAAACAATACCA

  Q E C H S R Q L F G L P K T H I S Y V R N I K E G L P L F L F N Y D D R R L Y G I Y E
TTCAAGAGTGTCACTCACGGCAACTTTTTGGCTTGCCAAAAACACATATTTCGTATGTACGAAACATTAAGGAAGGACTACCTCTCTTCCTCTTCAATTATGATGATCGAAGATTGTATGGTATTTACGA

 A S G N G K F C P E S N A W S Q D S K G K T S Y P A Q V A M R V K V W C F P L A E N Q
AGCTTCAGGGAATGGAAAATTCTGTCCTGAATCAAATGCATGGTCACAAGATAGCAAGGGCAAAACAAGCTATCCTGCCCAGGTTGCAATGCGGGTAAAGGTGTGGTGTTTTCCGCTAGCAGAGAATCAG

F R N A I I A N Y Y Q K I P G V P G Q K L H F F K F E L D H A Q T R V L M D M F T P S P
TTCAGAAATGCAATTATAGCCAATTACTACCAGAAAATTCCCGGTGTCCCTGGCCAGAAGCTTCATTTTTTCAAGTTTGAATTGGATCATGCTCAAACACGTGTTTTGATGGATATGTTTACTCCTTCTC

  S P N S F W T P P A A A P A D E H A K E L V L S P G W A P E C E G N N N L K S E K V V
CTTCTCCCAACAGTTTCTGGACGCCCCCTGCTGCAGCACCAGCTGACGAGCATGCGAAAGAATTAGTGTTGTCACCTGGATGGGCACCAGAGTGTGAAGGGAACAATAACCTCAAATCAGAAAAGGTTGT

 K S Y A D I V K K N T F V E V G T R D V D V E H A G S G D E S S G G F D E L D C Q Y T
CAAGTCATATGCAGACATAGTGAAGAAGAACACATTTGTGGAAGTTGGGACAAGAGATGTGGATGTGGAACATGCAGGCTCAGGTGATGAATCTTCTGGTGGTTTCGATGAACTGGATTGTCAGTACACA

P T E R E D Y A L S D R V V Q V Q Q Q Y P D K Q A E V L S F D R V L Q G C I T F P G Q Q
CCAACAGAGAGGGAGGACTACGCTTTATCAGATAGGGTGGTTCAAGTGCAACAACAGTACCCTGATAAGCAGGCAGAAGTGCTTAGCTTTGATCGGGTTTTACAAGGGTGCATCACTTTTCCTGGTCAGC

  W H S D F Y A N T T Q T E D N D A Y S C E Y A Q E V K Y A I L D G S S N L P E T L D S
AGTGGCATTCTGATTTTTATGCCAATACTACCCAAACTGAGGATAACGATGCATATAGCTGCGAGTATGCCCAAGAGGTCAAATATGCAATTCTGGATGGCAGCTCTAATTTGCCAGAAACCTTAGATTC

 E V D K L S L G H S D L L V Q L L D S E S C T E A K L I D A V K Q L S G R I E V M E K
 TGAAGTTGATAAACTGTCCCTGGGGCATTCTGATTTGCTGGTGCAGTTGTTGGACTCTGAATCATGCACAGAAGCTAAGCTGATTGATGCAGTGAAACAGTTATCTGGACGAATAGAGGTGATGGAGAAG

K Q A W S N K E V K H L Q G M N E R L L K R I V E L K G T V M T L N S K I D P L T L D D
AAGCAGGCTTGGTCCAACAAAGAAGTCAAACATCTACAAGGGATGAATGAGAGGTTACTGAAAAGAATTGTTGAACTAAAGGGAACCGTTATGACATTGAATTCTAAAATAGATCCTTTAACCCTAGATG

  S L N Q F V E Q C L G S E D V I Y L V G G F D G F S S L P S L D S F S P S L D I L T P
ATTCACTTAACCAGTTTGTAGAACAATGTTTGGGTTCAGAAGATGTCATTTATTTAGTTGGTGGTTTTGATGGCTTCTCATCTTTGCCATCACTAGACTCTTTTTCCCCTTCATTGGACATACTAACACC

 L K P M A V G K S Y S S T V A L E G K I F V L G G G D G A C W F D T V D C Y D R S R D
CCTTAAACCAATGGCTGTTGGCAAGTCATATTCCTCAACTGTTGCACTAGAGGGCAAGATATTTGTTCTTGGTGGTGGTGATGGTGCTTGCTGGTTTGATACAGTTGACTGTTATGACCGAAGCCGTGAT

D W S T C P S L T C D K G S L A G V S V N G R I Y A F G G G D G T K C F S D V E M F D P
GATTGGTCCACATGCCCATCGTTGACTTGTGACAAGGGGAGCCTTGCTGGGGTTAGTGTCAACGGGAGAATCTATGCATTTGGTGGTGGAGATGGAACCAAGTGTTTCTCTGATGTTGAAATGTTTGATC

  V H G K W I K I Q P M L E K R F A L A G V A L N G V I Y A V G G F N G V Q Y L S C A E
CTGTGCATGGAAAGTGGATAAAGATTCAACCTATGCTGGAAAAGCGCTTTGCTCTAGCTGGTGTAGCACTAAATGGTGTGATTTATGCTGTTGGTGGCTTCAATGGTGTTCAATATCTGAGCTGTGCTGA

 R L D P R E P N W K M L P K M S A G R G C H T L T V L N E K I F S I G G Y D T R A K A
AAGACTTGATCCTCGGGAGCCTAACTGGAAAATGCTGCCAAAGATGAGTGCAGGAAGGGGCTGTCATACACTTACAGTTCTTAATGAGAAGATATTCTCGATTGGTGGATATGACACTAGGGCTAAAGCA

M V S T V E V Y E P R M P S W V M V E P M N Y T R G Y H S S A V L G G S I F T F G G V K
ATGGTGTCTACTGTTGAGGTGTATGAGCCAAGGATGCCATCGTGGGTGATGGTTGAACCCATGAACTACACCAGAGGATACCATTCTTCAGCTGTGCTCGGTGGCTCAATATTTACCTTTGGTGGGGTGA

  G E A D T I L D V V E H Y K E G C G W M T T G L K S I G R R C Y C S A I V L *
AAGGTGAAGCAGACACAATCTTGGATGTGGTGGAACACTACAAGGAAGGGTGTGGCTGGATGACAACGGGATTGAAGTCGATTGGCAGAAGATGCTACTGCTCAGCGATTGTTCTCTGA
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ZmRACK

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M A G A Q E S L S L V G T M R G H N G E V T A I A T P I D N S P F I V S S S R D K S V L
ATGGCCGGCGCGCAGGAGTCCCTCTCCCTGGTGGGCACGATGCGCGGCCATAACGGCGAGGTGACGGCGATCGCGACCCCGATCGACAACTCGCCTTTCATCGTCTCCTCCTCCCGGGACAAGTCCGTGC

  V W D L T N P V H S T P E S G V T A D Y G V P F R R L T G H S H F V Q D V V L S S D G
TGGTGTGGGACCTGACCAACCCGGTCCACTCCACCCCGGAATCCGGCGTCACCGCCGACTACGGCGTCCCCTTCCGCCGCCTCACCGGCCACTCCCACTTCGTCCAGGACGTCGTCCTTAGTTCCGACGG

 Q F A L S G S W D G E L R L W D L S T G L T T R R F V G H E K D V I S V A F S V D N R
CCAGTTCGCACTCTCTGGCTCCTGGGACGGCGAGCTCCGCCTCTGGGACCTCTCTACCGGCCTCACCACCCGCCGCTTCGTCGGCCACGAGAAGGACGTCATCTCCGTCGCCTTCTCCGTCGACAACCGC

Q I V S A S R D K T I K L W N T L G E C K Y T I G G D L G G G E G H N G W V S C V R F S
CAGATCGTCTCCGCGTCCCGCGACAAGACCATCAAGCTCTGGAACACTCTCGGCGAGTGCAAGTACACCATCGGCGGCGACCTCGGCGGCGGCGAGGGCCACAACGGGTGGGTCTCCTGCGTTAGGTTCT

  P N T F Q P T I V S G S W D R T V K V W N L T N C K L R C T L D G H G G Y V N A V A V
CCCCAAACACCTTCCAGCCCACCATCGTCTCCGGATCCTGGGACCGCACCGTCAAGGTCTGGAACCTTACCAACTGCAAGCTGCGCTGCACTCTGGATGGGCACGGTGGCTATGTTAACGCCGTCGCTGT

 S P D G S L C A S G G K D G V T L L W D L A E G K R L Y S L D A G S I I H S L C F S P
GAGCCCCGACGGGTCGCTGTGCGCCTCCGGCGGGAAGGACGGTGTTACTCTGCTGTGGGATTTGGCTGAGGGGAAGAGGCTGTACTCGCTGGACGCGGGCTCCATCATCCACTCCCTCTGCTTCTCACCC

N R Y W L C A A T Q D S V K I W D L E S K H V V Q D L K P D I Q V S K N Q I M Y C T S L
AACCGCTACTGGCTCTGCGCTGCGACGCAGGACTCTGTCAAGATCTGGGACCTCGAGTCGAAGCACGTTGTGCAGGACCTCAAGCCCGACATCCAGGTCTCCAAGAACCAGATCATGTACTGTACAAGCT

  S W S A D G S T L Y T G Y T D G S I R V W K I S G Y G Y A G *
TGAGCTGGAGTGCGGACGGAAGCACCCTCTACACTGGCTACACCGATGGGTCTATCAGGGTTTGGAAGATCTCCGGGTACGGCTACGCAGGCTAG
```

ZmURI1 (partial)

GGCACGAGGTCAGATTTCAGGGGTTCTTCTGGAAAAACAATTCTAGATAGGCCTGAGCCATCAAAGTCTAATAACTCAGATGATGAGGCACACCAG
TTTTGGCATAAGCAGCTTCCGGATGACATTATTCCAGACTTCACTTCTTTTGAGAAAGTTGAACAAGGGCCTGAACTGTCCCTTGCTGGATTATCCTT
GAATGCACCCCCCTTTTATGGGACAACTTCCAGTCGCTTCTCAAGAGAATATCACGAATTATCTTCTCCAGTTACCAAGGGCCTGGAACTGGAACTA
GAACATACTAATATGCTATATGAAGATAAAAGCAACTGGGAGCAAAATTATATTGGTGATCTTCATATTGCTAATGGAAACCAGGACCTTCATTAT
GATTCTGAATCTGGTGTAAGCTTTTCTGATAGCTTTGCTAGTGAGTATGTTGCCCCATCAGATGGCCTTTTTGCTCCCCTGGAGTACTTGGCATCTCA
GTTCCCTGGATTTTCAGCAGAGAGCCTTGCAGAGCTTTACTACGCAAATGGGTGTGATTTCAACCATACTATTGAAATCCTCACCCAGCTAGAGATG
CAAGTTGATGCTACACCCAATCACACACTGAATCTGCCCCACAGCACACCGAACTTCAGCACTGGGGATTTCCCTGCACTTCCAACAGCTGAGGAC
CAAAATGGTTTCAATCAGGGTAATGTGGATGTACTTGGCATGTTCAATGGGGCGCAGGTTCATCTGCGATGCCTACTGGGAGCTNGGTGATTTGTTT

ZmURI2 (partial)

GGCACCAGGGGGAGCGCCCTGGCGCTGGAGGATGACGAGGAGGAGCCGGGCGCGGCGGCACTGTCGTCGTCGCCCAACGACAGCGCGGGCTCCTT
CCCGCTGGACCTGGGAGGCCCACGCGCCCACGCCGAGGGCGCCGCGGCGCGGGCCGGCGGCGAGCGGTCCTCGTCTCGCGCCAGCGATGAGGACG
AGGGCGCGTCCGCGCGCAAGAAGCTGCGCCTCTCCAAGGAGCAGTCTGCGTTCCTGGAGGAGAGCTTCAAGGAGCACAGCACCCTCAACCCTAAG
CAGAAGGCGGCGCTGGCGAAGCAGCTCAACCTCCGGCCGCGACAGGTAGAAGTCTGGTTCCAGAACCGCCGAGCCAGGACGAAGCTGAAGCAGA
CGGAGGTGGACTGCGAGTACCTGAAGCGCTGCTGCGAGACGCTGACGGAGGAGAACCGGCGGCTGCACAAGGAGCTCGCGGAGCTGCGCGCGCTC
AAGACGGCGCCGCCCTTCTTCATGCGCCTCCCGGCCACCACCCTCTCCATGTGCCCCTCCTGCGAGCGCGTCGCCTCCGGCCCCAGCCCTGCCTCCA
CCTCGGCACCTGCGTCGTCCACGCCGCCTGCCACAGCCGCCACCACCGCCATCTCGTACGCTG

ZmARF1 (partial)

Parts of the sequence of the mRNA have been deposited in the databases with the accession number AY104269, that codes for a protein with the sequence given below. The two-hybrid clone encoded the last 380 amino acids.

TRPRTRGGMQRKLGSDIWMRMNRPDGYSEMLSGYQPPNEDVRNSQGFCSLPDQIAAGRPNFWHTVNAHYQDQQGNHNLFPGSWSMMPSSTGFGMNRQ
SYPMIQEVGGMSQSCTNTKFGNGVYAALPGRGIDRYPSGWFGHTTPGGRVDDAQPRVIKPQPLVLAHGEALKMKGNSCKLFGIHLDSPAKSEPLKSPPSV
ATPAAEKWMADGIDADKSPEPHKTPKQLGATQVDPVPERCPQASRGTQCKSQGGSTRSCKKVHKQGMALGRSVDLTKFNGYTELVAELDEMFDFNGEL
KGCSKEWMVVYTDYEGDMMLVGDDPWNEFCSMVHKIFVYTREEVQRMNPGALNSRPEDSGLANSTERGSASTAAAREAPGYQSASSLNSDNC

ZmHOX3 (partial)

GGCACCAGGGGGAGCGCCCTGGCGCTGGAGGATGACGAGGAGGAGCCGGGCGCGGCGGCACTGTCGTCGTCGCCCAACGACAGCGCGGGCTCCTT
CCCGCTGGACCTGGGAGGCCCACGCGCCCACGCCGAGGGCGCCGCGGCGCGGGCCGGCGGCGAGCGGTCCTCGTCTCGCGCCAGCGATGAGGACG
AGGGCGCGTCCGCGCGCAAGAAGCTGCGCCTCTCCAAGGAGCAGTCTGCGTTCCTGGAGGAGAGCTTCAAGGAGCACAGCACCCTCAACCCTAAG
CAGAAGGCGGCGCTGGCGAAGCAGCTCAACCTCCGGCCGCGACAGGTAGAAGTCTGGTTCCAGAACCGCCGAGCCAGGACGAAGCTGAAGCAGA
CGGAGGTGGACTGCGAGTACCTGAAGCGCTGCTGCGAGACGCTGACGGAGGAGAACCGGCGGCTGCACAAGGAGCTCGCGGAGCTGCGCGCGCTC
AAGACGGCGCCGCCCTTCTTCATGCGCCTCCCGGCCACCACCCTCTCCATGTGCCCCTCCTGCGAGCGCGTCGCCTCCGGCCCCAGCCCTGCCTCCA
CCTCGGCACCTGCGTCGTCCACGCCGCCTGCCACAGCCGCCACCACCGCCATCTCGTACGCTGCAGCAGCCGC

ZmVIP

The clone found in the two-hybrid screen coded for the last 199 amino acids. The sequence of the complete mRNA has been deposited in the databases with the accession number AY107616, encoding the following protein:

MENGDETLASPTAAAETDALNGGVAEEEQVPITHPAKSYVTLADDNPAPNGGVVKEEEGGAHTTAKSYAAVAAQAEIEDLRAAK
LDLEEKLAEARRENKSLAEETHRSEGIFTQAREEVTIAEFAATSAEKEVASLRAVERLDAVLRIEKGEHELDKQRHEKVAKEVDAV
RQEKLKLEEEIRALKASATAAATTKEREAAPASEAPKEGEVAWLGMAVAAAGAAGTAAIMLVYLRLKR*

Erklärung

Ich versichere, dass die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschliesslich Tabellen, Karten und Abbildungen - , die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist, sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Herrn Priv-Doz. Dr. Richard D. Thompson betreut worden.

Köln, im März 2003

Sonja Vorwerk

Poster:
Sonja Vorwerk, Gregorio Hueros, Heinz-Albert Becker, Richard Thompson:
Untersuchungen zu ZmKEL1, einem Kelch-Domänen-Protein aus Mais
15. Tagung Molekularbiologie der Pflanzen, Dabringhausen

Sonja Vorwerk, Richard D. Thompson:
ZmRACK: Per RIK-Shuttle durch die Zelle?
16. Tagung Molekularbiologie der Pflanzen, Dabringhausen

Publikation:
Sonja Vorwerk, Franscesco Salamini, Richard D. Thompson: Analysis of ZmRIK, a putative mediator between the RACK complex and the cytoskeleton. In Vorbereitung.

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"The fundamental thing is the opportunity to be creative."
Martin Rodbell

Lebenslauf

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Stipendien

01/98-05/00	*Stipendiatin der Studienstiftung des Deutschen Volkes, Bonn*
06/00-05/03	*Wechselnd assoziiertes/volles Mitglied des Graduiertenkollegs „Molekulare Analyse von Entwicklungsprozessen“, einer Kooperation zwischen der Universität zu Köln und dem MPIZ*
01/01-03/03	*Promotionsstipendiatin (wechselnd finanzielle/ideelle Förderung) der Studienstiftung des Deutschen Volkes, Bonn*