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Contents

Scope

Receptor-like protein kinases (RLKs) are central components of many signal transduction chains. RLKs perceive an extracellular signal and transduce it to its effectors inside the cell. In this way they are mediating processes like cell division, cell growth, cell differentiation and cell death. RLKs consist of an extracellular domain, a transmembrane domain and an intracellular catalytic kinase domain. Plant RLKs can be divided into different subfamilies based on their extracellular domain. A large family among the plant RLKs are the leucine-rich repeat (LRR) containing transmembrane receptors. LRRs are found in many different proteins and are believed to be involved in mediating protein-protein interactions. In general, protein kinases catalyse the transfer of the γ-phosphate of ATP to an amino acid side chain resulting in the phosphorylation of that residue. In plants, most protein kinases autophosphorylate on serine and threonine residues. As a result of kinase activity, the 3D-organisation of the phosphorylated protein may be rearranged resulting in a change of activity. In addition new protein-interaction sites can be created that may or may not include the phosphorylated residues themselves.

The *Arabidopsis* Somatic Embryogenesis Receptor-like Kinase 1 (*At*SERK1) was identified as an RLK containing a leucine zipper, five leucine-rich repeats, a single transmembrane domain and a functional serine-threonine kinase. The *At*SERK1 gene is expressed during ovule development and early embryogenesis. Ectopic expression of *At*SERK1 increases somatic embryo formation in culture. Thus *At*SERK1 was proposed to participate in an uncharacterised signal transduction cascade involved in ovule and embryo development. Previous work showed that *At*SERK1 interacts *in vitro* and in plant cells with the kinase-associated protein phosphatase (KAPP).

The major goal of the research presented in this thesis was to find additional proteins that interact with the kinase domain of *At*SERK1 and that may function in the signal transduction cascade mediated by the *At*SERK1 receptor kinase. To achieve this goal, we employed the yeast two-hybrid screen using the *At*SERK1 kinase domain as bait.

In Chapter 1, an overview of the regulatory levels of kinase signalling is presented. Besides the activation and inactivation of protein kinases, the role of protein binding

domains, adaptor, scaffold and anchoring proteins is discussed with an emphasis on the regulatory roles of 14-3-3 proteins.

In Chapter 2, the yeast two-hybrid screen for proteins that interact with the *At*SERK1 kinase domain is described. The details of the screening procedure are described as well as the procedures to verify their interaction in yeast and *in vitro*. In addition, it is shown whether these proteins can be transphosphorylated *in vitro* by *At*SERK1.

In Chapter 3, the analysis of the interaction between the putative *At*SERK1 interacting 14-3-3 protein GF14λ and *At*SERK1 is presented. The interaction between *At*SERK1 and GF14λ was verified *in vitro* using binding assays, in plant cells based on the occurrence of FRET between fluorescently labelled *At*SERK1 and GF14λ proteins and *in vivo* using co-immunoprecipitation. Finally, GF14λ was shown to be transphosphorylated by *At*SERK1.

In Chapter 4, the analysis of the interaction between the putative *At*SERK1 interacting AAA-ATPase protein *At*CDC48 and *At*SERK1 is presented. The analysis of *At*CDC48 phosphorylation by *At*SERK1 is described, as well as the verification of the interaction between *At*SERK1 and *At*CDC48 in yeast, *in vitro* using binding assays and in *Arabidopsis* cells using co-immunoprecipitation. The results of a pairwise interaction test between the *At*SERK1 interacting proteins in yeast are also presented.

In Chapter 5, an overview is presented of the presence of putative 14-3-3 binding domains in *Arabidopsis* protein kinase domains. The potential biological significance of the presence of this domain is discussed.

In Chapter 6, the results of this research are compiled in a summarising discussion.

Chapter 1

Regulation of signal transduction via protein kinases

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Sumbitted for publication

Abstract

Receptor kinases are very important for intracellular and intercellular signal transduction. The activity of protein kinases can be regulated by various mechanisms mainly involving multiple phosphorylation and dephosphorylation events. Another level of regulation involves the recruitment of substrate proteins to the receptor kinase. Kinases may bind their substrate proteins through protein-binding domains present on the receptor kinase itself or on adaptor or scaffold proteins mediating the interaction. One such adaptor or scaffold protein is the 14-3-3 protein that binds to proteins containing a phosphoserine-containing motif. Besides regulating the binding of substrate proteins, receptor kinase activity may be modulated by the localisation of the receptor kinase itself, which involves endocytosis and subsequent recycling or degradation.

Protein kinases

Serine/threonine and tyrosine kinases are widely employed as tools for signal transduction. Sequencing of eukaryotic genomes, including the human and the *Arabidopsis* genome, indicates that 2-4% of all genes encode protein kinases (AGI, 2000; IHGS, 2001). Protein kinases catalyse the transfer of the γ-phosphate of ATP to an amino acid side chain. Most eukaryotic protein kinases are thought to be specific either for Ser and/or Thr residues or for Tyr residues, but some phosphorylate residues from both of these classes. Phosphorylation can cause significant changes in proteins. A residue with an added phosphate group (pK_a ~6.7) is likely to be dianionic at physiological pH. While the property of a double-negative charge is not available among naturally occurring non-phosphorylated amino acids, phosphorylation of a residue will influence the 3D-organisation of its local peptide region. As a result, a new protein-interaction site can be created that may or may not include the phosphorylated residue. More long-range conformational changes can also occur, thereby rearranging the protein and enabling (new) protein-protein interactions or changing the catalytic activity.

The catalytic domains of protein kinases range from 250 to 300 amino acid residues corresponding to about 30 kD. Crystal structures of several kinases have been determined, showing that the kinase fold is extremely well conserved among serine/threonine and tyrosine kinases (Johnson et al., 1998). Although the catalytic domains of serine/threonine-specific and tyrosine-specific kinases are very similar, it is possible to differentiate between them based on sequence (Hanks and Hunter, 1995). The kinase domain is divided into two subdomains, or lobes (Fig. 1; Johnson et al., 1998). The smaller N-terminal lobe is composed of a five-stranded β-sheet (β1-β5) and one α-helix called helix αC. The larger C-terminal lobe is predominantly helical. ATP is bound in a deep cleft between the two lobes, located beneath a highly conserved loop connecting strands β1 and β2. This phosphate-binding loop (P-loop) contains a glycine-rich sequence motif and is important for fixing the orientation of the phosphates of ATP. A centrally located loop, the activation loop, provides a protein-binding platform for the peptide substrate (Huse and Kuriyan, 2002). The bound peptide substrate is then located across the ATP-binding cleft, close to the γ-phosphate of ATP.

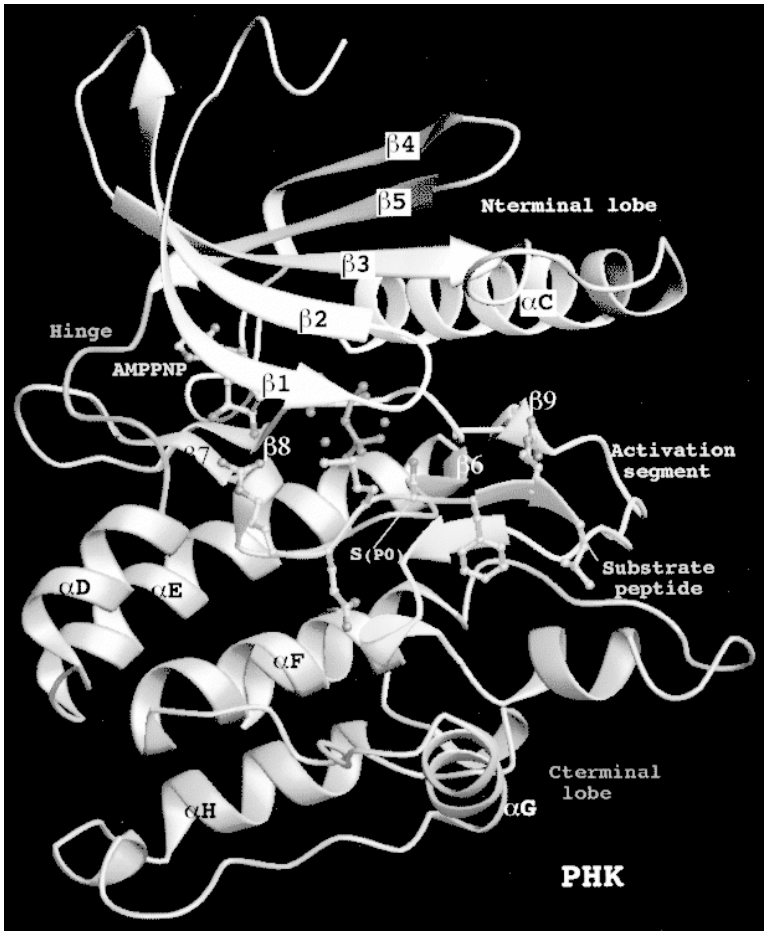

Figure 1: Schematic diagram of the fold of phosphorylase kinase (PhK) taken from Johnson et al. (1998), illustrating a typical protein kinase fold. α-Helices are labelled from αC to αI (αI is obscured by αF). β-Strands are labelled β1–β9.

Regulation of protein kinase activity through phosphorylation

All kinases perform the same biochemical reaction. Therefore, it is not surprising that the conformation of their active catalytic regions is very similar. However, their inactive conformations show large differences indicating that different regulatory mechanisms have evolved for kinase activation. Protein kinase activity can be regulated by inhibitory elements of the kinase itself or by inhibitor proteins that in most cases directly or indirectly block the active centre (Kemp et al., 1994). During the process of activation the inhibiting structural element vacates the active centre or the inhibitory protein dissociates.

Activation of a kinase domain can be promoted by changes in the oligomerisation state, thereby facilitating phosphorylation events necessary for activation (Huse and

Kuriyan, 2002). In most kinases the activation loop is phosphorylated when the kinase is active. Due to this phosphorylation, the activation loop can undergo large conformational changes when the kinase switches between the inactive and the active state. In the inactive state the activation loop can act as an auto-inhibitory element by blocking the active site (intrasteric inhibition). For example, the activation loop of the inactive insulin receptor kinase (IRK) blocks the active site by occupying both the protein substrate and the ATP binding sites (Hubbard et al., 1997; Till et al., 2001). Upon phosphorylation, the activation loop moves away, thereby reorganising the catalytic centre in such a way that substrate binding and catalysis can take place. The C-terminal region of the plasma membrane Ca^{2+} pump contains two autoinhibitory regions (Enyedi et al., 1996). In the presence of diacylglycerol (DAG), protein kinase C phosphorylates a residue in the second inhibitory domain, resulting in partial activation of the pump. Full activation is obtained when calmodulin binds to the first inhibitory domain in the presence of Ca^{2+}, resulting in complete neutralisation of autoinhibition. Eph tyrosine kinase receptors contain an N-terminal autoinhibition mechanism (Wybenga-Groot et al., 2001). The juxtamembrane segment of the inactive Eph receptor kinase adopts a helical conformation that distorts the small lobe of the kinase domain and thereby blocks the activation loop from attaining an activated conformation. Phosphorylation of conserved juxtamembrane tyrosines relieves this autoinhibition by disturbing the association of the juxtamembrane segment with the kinase domain, while liberating phosphotyrosine sites for the binding of target proteins. The inhibitory mechanisms of the tyrosine kinase Src involve the SH2 and SH3 modular binding domains present N-terminal to the kinase domain (Hubbard, 1999). When Tyr^{527} within the Src C-terminal tail is phosphorylated by the non-receptor kinase Csk, repression of the Src kinase activity occurs. The Src SH2 domain will bind to the phosphorylated Tyr^{527} residue, enabling the SH3 domain to bind to the linker segment separating the SH2 domain and the kinase domain. These interactions result in the inactivation of the Src kinase by repositioning of the αC helix in the small N-terminal lobe of the kinase domain, preventing the formation of a salt bridge that is crucial for kinase activity as it is involved in positioning the phosphates of ATP. The interactions of the SH2 and SH3 domains with Src are suboptimal. Therefore, phosphotyrosine- or polyproline-containing sequences of activating proteins can favourably compete with the Src sequences and release the kinase

from its repressed state. Autophosphorylation of the kinase activation loop will then rapidly occur, leading to a fully active Src kinase.

Regulation of protein kinase activity through dephosphorylation

Phosphorylation is a reversible process, with protein phosphatases opposing the action of protein kinases. Protein phosphorylation may be used to switch enzyme activities on and off, but the same is true for the dephosphorylation of proteins. Phosphatases can be divided into two major families: the protein Ser/Thr phosphatases and the protein Tyr phosphatases. Both catalyse the removal of the phosphoryl group from a phosphorylated residue. However, in contrast to kinases, phosphatases represent a structurally and evolutionary more diverse group of proteins. Ser/Thr phosphatases and Tyr phosphatases have completely different structures and distinct catalytic mechanisms (Denu et al., 1996).

In the *Arabidopsis* genome, 112 putative phosphatases can be found (Kerk et al., 2002). These are mainly Ser/Thr phosphatases with the major part (69) being phosphatases that belong to the PP2C group of phosphatases that require Mg^{2+} or Mn^{2+} for their activity. The kinase-associated protein phosphatase (KAPP) is a PP2C phosphatase that is homologous to two other plant phosphatases, one from *Oryza sativa* and one from *Zea mays*, but is not closely related to any other *Arabidopsis* protein. KAPP has been shown to be a modulator of receptor-like kinase signalling pathways. It negatively regulates the CLAVATA signalling pathway (Trotochaud et al., 1999) and it is involved in internalisation of the *At*SERK1 receptor kinase (Shah et al., 2002).

Besides the regulation of kinase activity, there is also a strict control on the dephosphorylation rate in a cell. The expression patterns of the phosphatase-encoding genes and the subcellular localisation of the corresponding proteins ensure that protein phosphatases are only active in a subset of cells at defined subcellular sites (Pujol et al., 2000). PP2C phosphatases contain, besides the catalytic core, N-terminal domains of variable length that might play an important role in targeting the PP2C activity (Rodriguez, 1998). The N-terminal domain of KAPP contains an amino-terminal signal anchor and a kinase interaction (KI) domain. The KI domain targets the PP2C activity of KAPP to phosphorylated kinases (Stone et al., 1998; Shah et al., 2002). Protein phosphatases may also be phosphorylated themselves. Phosphorylation of phosphatases

may influence their catalytic activity, create docking sites for substrate proteins or control their subcellular localisation. Thus the combined activities of kinases and phosphatases are regulated by coordinated phosphorylation and dephosphorylation events.

The CLAVATA1 (CLV1) kinase domain undergoes autophosphorylation upon ligand binding. KAPP binds to the phosphorylated form of CLV1 and negatively regulates CLV1 activity through dephosphorylation (Williams et al., 1997). The KAPP protein is phosphorylated in the presence of CLV1, suggesting that the activity of KAPP may be modulated by the activated CLV1 receptor. Like CLV1, *At*SERK1 is also able to bind and phosphorylate KAPP and KAPP in return is able to dephosphorylate *At*SERK1 (Shah et al., 2002). Thus, receptor kinase signalling may be mediated through a negative feedback loop involving phosphatases.

Localisation and modulation of protein kinase activity

Once the receptor kinase is activated, its phosphorylation activity can be used for amplification and transduction of the signal. Verveer et al. (2002) showed that upon ligand binding, activation of the mammalian Erb1 receptor spreads laterally independent from further ligand binding. In this way, the signal propagates along the plasma membrane providing an early amplification mechanism. In order to transduce the signal, the phosphorylation activity of the kinase is in most cases used for regulating protein-protein interactions. Signals can be transduced via protein-protein interactions by inducing a conformational change or stabilising an existing conformation in the substrate protein thereby causing a change of activity at another site of that protein. The mechanism of ‘induced protein proximity’ can be used to propagate signalling via receptor kinases and to confer specificity of protein-protein interactions by recruiting only those proteins that are necessary to transduce the signal (Kholodenko et al., 2000). This mechanism elevates the local concentration of proteins at a certain site, thereby increasing the number of productive signalling complexes that can be formed and hence increasing the extent of activation of downstream processes. Mechanistically, anchoring a receptor and its substrate proteins to the membrane causes them to be at a higher local concentration, and association between the proteins should be favoured.

In order to recruit substrate proteins to a membrane located receptor kinase, several mechanisms have evolved. Lipid anchors are commonly used to anchor a protein to a membrane in the vicinity of the receptor kinase. Substrate proteins can also be recruited to the receptor kinase via protein-protein interactions, mediated by protein binding domains. Through these domains, substrates can either be directly attached to the receptor kinase or to adaptor or scaffold proteins that mediate the interaction with the receptor kinase. Apart from regulating the subcellular localisation of kinase substrates, kinase signalling can also be controlled by regulating the subcellular localisation of the kinase itself. Below, the mechanisms mentioned above will be discussed in more detail.

Membrane localisation using lipid anchors

Posttranslationally attached lipid anchors composed of hydrophobic residues such as fatty acids, isoprenoids or complex glycolipids can target a protein to the plasma membrane, as well as to other subcellular compartments (Yalovsky et al., 1999). Lipid modifications, like palmitoylation, are reversible and can therefore have regulatory functions in signal transduction through regulation of the subcellular localisation of signalling proteins. Myristylation and prenylation are generally constitutive processes and permanent modifications. However, the hydrophobicity of a myristylated or a prenylated peptide alone is not always sufficient to anchor a protein stably in the plasma membrane, suggesting that additional mechanisms are necessary (Bhatnagar et al., 1997). Methylation of the prenylated peptide can already increase the membrane affinity tenfold. In addition, a common mechanism by which N-myristoylated proteins, but also prenylated proteins, create a stable association with membranes is to have positively charged residues that can cooperate with the myristoyl moiety. Most biological membranes have a very acidic surface, due to the acidic phospholipid head groups. Therefore, the surface charge of a lipidated protein can have a large influence on the membrane affinity. For example family members of the mammalian non-receptor kinase Src use myristylation to associate with the plasma membrane (Resh et al., 1994). Phosphorylation of serine residues near the cytoplasmic surface produces negative charges that reduce the net positive charge, thereby weakening the electrostatic interactions and releasing the myristoylated protein from the membrane. Addition of a second lipid group is another method to create extra membrane affinity (Bhatnagar et al., 1997).

Protein-protein interactions mediated by protein binding domains

Another important mechanism to localise or anchor a protein to a membrane located receptor kinase is through direct protein-protein interactions using protein interaction domains (Pawson et al., 2002). Many kinases have one or several 'docking sites' that allow a substrate to bind directly to the kinase in a sequence specific manner, thereby increasing the efficiency of phosphorylation. The formation of a kinase-substrate complex may also involve adaptor or scaffold proteins that bridge the interaction or anchor a protein to a subcellular structure close to the receptor kinase (Burack and Shaw, 2000). Interaction domains recognise exposed features of their binding partners, for example sequences that contain phosphoserine or -threonine residues. A growing family of interaction domains that bind phosphoserine/threonine-containing motifs has been identified in plants, including FHA, WW and WD domains (Yaffe and Elia, 2001). In addition, 14-3-3 proteins were also found to bind phosphoserine/threonine-containing motifs. In contrast to domains like FHA, WW and WD, which are usually part of signalling proteins, 14-3-3 proteins are complete functional proteins that can function as adaptor or scaffold.

The Forkhead-associated (FHA) domain is a modular binding domain of 130-140 amino acids (Durocher et al., 2002). The FHA domain has been found in more than 200 different proteins in species ranging from prokaryotes to higher eukaryotes. FHA domains recognise phosphorylated residues, preferentially phosphothreonine. Different FHA domains recognise distinct binding motifs, specificity being determined by the sequence motifs flanking the central phosphothreonine residue on both sides. The *Arabidopsis* protein phosphatase KAPP binds to the receptor-like kinases (RLKs) *At*SERK1 and CLV1 with the FHA domain present in its kinase interaction (KI) domain (Stone et al., 1998; Shah et al., 2002). The conserved residues in the FHA domain of KAPP are critical for KI domain function. Interactions between the KI domain and RLKs have a very slow association rate (~10^3 M^{-1} s^{-1}) and an even slower dissociation rate (~10^4 M^{-1} s^{-1}) (Li et al., 1999). These kinetics indicate that the KI domain binds slowly, but once bound the interaction is very stable. This may reflect the physiological function of KAPP: KAPP may remain bound to phosphorylated RLKs until the dephosphorylation process is (nearly) complete.

WW domains are small protein-protein interaction modules of 38-40 amino acids (Sudol et al., 2000). The name refers to two signature tryptophan (W) residues that are spaced 20-22 amino acids apart and play an important role in the structure and function of the domain. WW domains bind to short proline-rich motifs in proteins and based on their ligand preference they can be divided into four groups. WW domains of Group IV have preference for pSer-Pro or pThr-Pro containing proteins. The best-characterised pSer/Thr-binding WW domain is one in the human peptidyl-prolyl isomerase (PPIase) Pin1, which catalyses the cis-trans isomerisation of pSer/pThr-proline bonds (Lu et al., 2002). Pin1 is essential for mitotic progression and is required for the DNA replication checkpoint. Pin1 binds a defined subset of mitosis specific phosphoproteins through interaction with pSer or pThr. The *Arabidopsis* Pin1 protein lacks the WW-domain, but it does specifically bind pSer-Pro or pThr-Pro containing proteins and it is able to catalyse the same reaction as human Pin1 (Landrieu et al., 2002). While *At*Pin1 does not contain a WW-domain there are at least 14 *Arabidopsis* proteins that do contain such a domain including some RNA helicases and the flowering control protein FCA (accession nr. At4g16280). These proteins were found using the SMART tool (Letunic et al., 2002), to find modular domains.

WD repeat and leucine-rich repeat (LRR) domains may presumably function as pSer/Thr binding modules in F-box proteins (Yaffe and Elia, 2001). In contrast to WW and FHA domains, structural proof for direct binding of WD repeats or LRR domains to pSer-containing sequences is lacking. F-box proteins recognise substrates of the SCF (Skp1-Cdc53/Cullin-F-box) ubiquitin ligase complex, targeting them for phosphorylation-dependent ubiquitination and proteasome degradation (Andrade et al., 2001). In *Arabidopsis*, more than 300 putative F-box proteins were identified, many of them containing WD repeat and LRR domains (AGI, 2000). Besides being present in F-box proteins, WD domains can be found in many other proteins, for example in the Gβ subunit of heterotrimeric G proteins. The WD repeat contains 44-60 residues that typically contain a WD dipeptide at the C-terminus (Smith et al., 1999). A WD repeat domain comprises 4-16 copies of the WD repeat and adopts a β-propellor fold. WD repeats can be found in proteins involved in diverse processes, but their function is poorly understood. So far no catalytic activity has been attributed to the domain. LRR domains are present as tandem arrays of 20-29 a.a. with conserved leucine residues and are commonly involved in

protein-protein interactions and interactions with other molecules (Kobe and Diesenhower, 1994). In plants, many LRR domains can be found in the extracellular region of receptor-like kinases (RLKs) that share a common function of signal transduction across membranes (Zhang et al., 1998). For example, the *Arabidopsis* CLV1 receptor binds the small CLV3 protein and is involved in shoot meristem maintenance (Trotochaud et al., 1999). Upon binding of brassinosteroids the BRI1 receptor initiates a signal transduction cascade that controls growth and development (Wang et al., 2001).

Adaptor, scaffold and anchoring proteins

As mentioned before, the formation of a kinase-substrate complex may also involve adaptor, scaffold or anchoring proteins (Burack and Shaw, 2000). These proteins do not have enzymatic activity themselves, but function in mediating protein-protein interactions. The binding of adaptor, scaffold or anchoring proteins may bring about binding specificity and/or induce conformational changes that alter the catalytic activity of the interacting proteins.

Anchoring proteins are attached to a subcellular structure like the plasma membrane and serve to localise enzymes close to their site of action (Pawson and Scott, 1997). They contain at least two functional domains: a protein binding domain and a targeting site responsible for anchoring to the subcellular structure.

Adaptor proteins function to provide additional docking sites for modular signalling proteins (Pawson and Scott, 1997). Once an adaptor protein is associated with its appropriate activated receptor, it becomes phosphorylated at multiple sites. These sites can in turn interact with specific binding domains of signalling proteins that recognise phosphoserine or -threonine residues. In this way a single receptor may bind multiple substrates, thereby amplifying the signal received by the receptor.

Scaffold proteins function to co-localise a group of molecules that participate in the same signalling process to a specific area of a cell (Burack and Shaw, 2000). The co-localised proteins do not necessarily directly act on each other, but are rather all involved in the same signalling pathway. In this way scaffolds can enhance the efficiency of signal propagation. A good example of a scaffold protein is Ste5p, which is involved in the formation of mitogen-activated protein kinase (MAPK) cascade assemblies (Elion, 2001). MAPKs are members of large families of serine threonine protein kinases and are found in

all eukaryotes (Bögre et al., 2000). In eukaryotes, MAPKs are known to play an important role in transducing signals from receptors to effector proteins resulting in responses such as target gene expression or changes in cellular organisation. During the formation of MAPK cascades, Ste5p does not function as a passive scaffold (Elion, 2001). A major function of Ste5p is to recruit MAP kinases to the plasma membrane at the right time for their activation. Nuclear shuttling regulates Ste5p localisation and controls the access of Ste5p to the plasma membrane. Signal cross-talk can normally occur between pathways activated by different receptors, but also between pathways activated by a single receptor. While MAPKs can function in multiple MAPK cascades, Ste5p may be used to provide pathway specificity. This occurs at the level of selective binding of Ste5p to the MAPK Fus3p. Ste5p is suggested to positively regulate the activity of Fus3p at the plasma membrane by interacting with multiple signal transduction components required for Fus3p activation (Elion, 2001). In this way scaffold proteins can provide pathway insulation to prevent cross-talk.

14-3-3 proteins can function as adaptor or scaffold molecules. They bind in a sequence specific manner to phosphoserine or –threonine residues of a diverse range of proteins. They generally recognise proteins containing the sequence motif [RK]$X_{(2,3)}$pSXP, where X stands for any amino acid and pS denotes both phosphoserine and phosphothreonine residues; Muslin et al., 1996; Yaffe et al., 1997; Sehnke et al., 2002). However, some 14-3-3 interacting proteins have been identified whose binding sequences deviate significantly from the motif mentioned above, or do not require phosphorylation of the 14-3-3 binding sequence for interaction.

The name 14-3-3 was given to an abundant mammalian brain protein family due to its particular migration pattern on two-dimensional DEAE-cellulose chromatography and starch gel electrophoresis (Moore and Perez, 1967). 14-3-3 proteins exist as protein families in many species (Rosenquist et al., 2000). They exist as dimers in a native situation and also *in vitro* they readily form heterodimers (Wu et al., 1997). The crystal structure that was determined for two human 14-3-3 proteins (Xiao et al., 1995; Liu et al., 1995) shows that each molecule of the 14-3-3 dimer consists of nine antiparallel α-helices, together resulting in an U-shaped structure (Fig. 2). The N-terminal domains form the dimerisation interface and the floor of the cleft; the C-terminal domains form the sides of the channel. The residues that line the interior of the protein-binding channel, including

many of the residues that form direct ligand contacts, are highly conserved between the different isoforms. More variable residues are distributed over the outer surface of the protein. Each monomer produces a cleft that is sufficient in size and shape to accommodate the interaction with a phosphorylated peptide from a binding partner (Yaffe et al., 1997). Thus, the 14-3-3 dimer is able to interact with two different phosphorylated peptides.

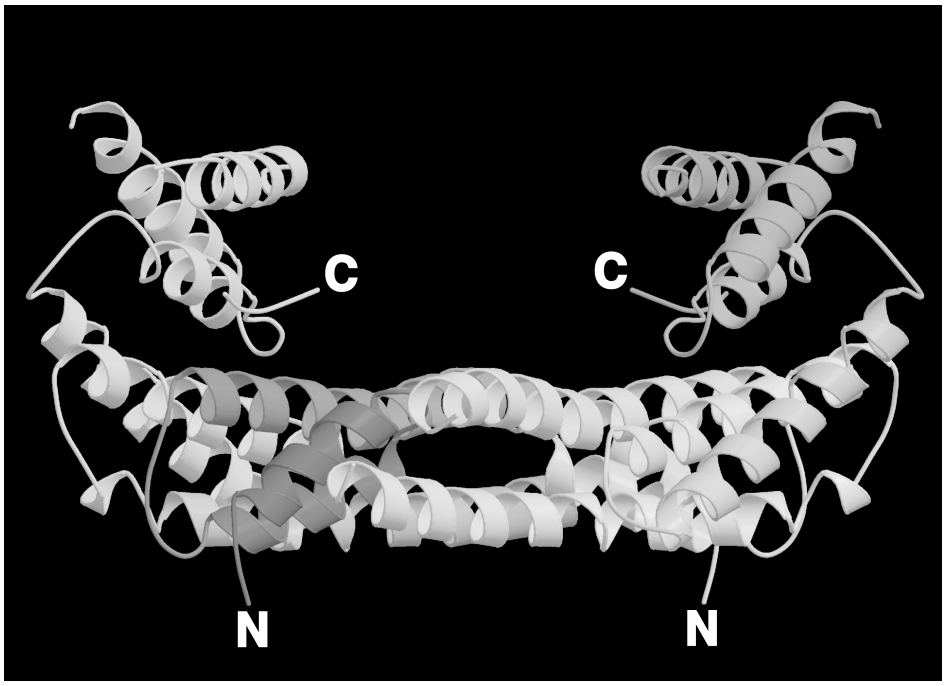

Figure 2: Schematic diagram of the fold of the mammalian 14-3-3ζ homodimer taken from Liu et al. (1995). Each molecule of the 14-3-3 dimer consists of nine antiparallel α-helices, together resulting in an U-shaped structure. The N-terminal domains of the two 14-3-3 proteins form the dimerisation interface and the floor of the cleft; the C-terminal domains form the sides of the channel.

Although 14-3-3 isoforms are very homologous to each other and the residues involved in ligand binding are highly conserved, there are differences in specificity towards target proteins. It was shown by surface plasmon resonance that there are large differences in affinity between nine *Arabidopsis* 14-3-3 isoforms for a target peptide representing the binding motif present in the C-terminus of the plant plasma membrane H^+-ATPase (Rosenquist et al., 2000). In addition, 14-3-3 proteins can be phosphorylated, which can influence the specificity of interaction with a target protein (van Zeijl et al.,

2000). Besides differences in binding specificity, there are also differences in the localisation of 14-3-3 proteins. Different 14-3-3 isoforms are expressed during different developmental stages and/or in different cell types or tissues (Testerink et al., 1999). Some 14-3-3 isoforms are localised to specific subcellular compartments. For example, the *Arabidopsis* GF14κ isoform is found in the nucleus and the plasma membrane region, and the GF14υ isoform is found in the cytoplasm and in the chloroplast stroma (Sehnke et al., 2002).

14-3-3 proteins are dimers and each monomer can bind a target protein. In this way the 14-3-3 dimer can function as adaptor or scaffold protein bridging the interaction of two binding partners and promoting the assembly of signalling complexes. This has been demonstrated for the interaction between Raf-1 and protein kinase C (PKC) (van der Hoeven et al., 2000), where a 14-3-3 dimer facilitates the coupling between these proteins as a response of the cell to mitogenic stimuli. PKC activates Raf-1 by phosphorylation and subsequent phosphorylation of 14-3-3 by PKC will weaken the interaction. Thus, 14-3-3 proteins can also attenuate signalling when 14-3-3 phosphorylation leads to complex dissociation. In addition, binding of a 14-3-3 protein can change the conformation of an interacting protein resulting in a change in activity of that protein. Raf-1 is maintained in an inactive state by the binding of a 14-3-3 dimer to a single Raf-1 polypeptide at two phosphorylated serine residues (Ser^{259} and Ser^{621}; Light et al., 2002). Activation of the Raf-1 kinase domain occurs after displacement of the 14-3-3 protein from Ser^{259} by Ras-GTP. This stabilises Raf-1 association with the plasma membrane and allows its phosphorylation and activation. 14-3-3 and Ras-GTP have been suggested to compete with each other for binding to a cysteine rich domain in the N-terminal domain of Raf. This suggests that 14-3-3 antagonises Raf-1 recruitment to the plasma membrane to ensure that Raf-1 is not activated in resting cells and cannot be activated by all Ras-related small G proteins (Light et al., 2002). Thus 14-3-3 proteins may also function as competitive inhibitor.

Besides their function as adaptor/scaffold proteins, 14-3-3 proteins are also involved in the translocation of their interacting proteins. Nuclear or cytoplasmic transport of proteins involves localisation signals that may be present in the protein itself or attached to the protein in the form of an anchoring protein that targets the interacting protein to a certain location in the cell (Quimby and Corbett, 2001). The exposure of

localisation signals can be regulated by phosphorylation or by masking mechanisms in which a change in the 3D-conformation of the protein is needed for exposure of the signal or in which a binding protein is involved that hides the localisation signal. Nuclear export of 14-3-3 proteins is dependent on nuclear export signals (NES) and the nuclear exclusion factor Crm1 (Brunet et al., 2002). However, there are two theories regarding how nuclear shuttling occurs via 14-3-3 binding. It has been postulated that a NES is present within the 14-3-3 protein (Lopez-Girona et al., 1999). 14-3-3 proteins might then function as attachable NES, resulting in nuclear exclusion of the interacting protein. The other theory is that the mere binding of 14-3-3 proteins to proteins that contain nuclear targeting or exclusion signals masks these intrinsic signals and precludes nuclear import or export of these proteins (Muslin and Xing, 2000).

In *S. pombe*, entry into M-phase is controlled by the B-type mitotic cyclin-dependent kinase Cdc2. 14-3-3 proteins inhibit progression through the cell cycle by keeping Cdc2 in an inactive form, thereby preventing the G_2-to-M transition (van Hemert et al., 2001). During interphase and after DNA damage, the activity of Cdc2 is suppressed through phosphorylation by the Wee1 and Myt/Mik1 kinases. The nuclear Cdc25 protein phosphatase can dephosphorylate Cdc2, resulting in the activation of Cdc2 that leads to entry into mitosis. After DNA damage, the activity of the Cdc25 phosphatase is repressed through phosphorylation by the checkpoint protein kinase Chk1, thereby creating a binding site for 14-3-3 proteins. As a consequence of 14-3-3 binding, Cdc25 is rapidly exported from the nucleus away from its substrate Cdc2, and the cell cycle is blocked. Thus, one function of these 14-3-3 proteins is to keep Cdc25 out of the nucleus.

Although many 14-3-3 proteins are predominantly localised within the cytoplasm, a large number of their interacting proteins are localised in the nucleus (Brunet et al., 2002). Lopez-Girona et al. (2000) argued that 14-3-3 proteins can function as an attachable NES for nuclear export of Cdc25. They proposed that the 14-3-3-dimer interacts with Cdc25 and Crm1 simultaneously to facilitate nuclear export. The 14-3-3 NES is present in helix α1, which plays an essential role in binding to phosphorylated proteins (Rittinger et al., 1999). The primary and general function of this α-helix is to participate in binding of interacting proteins. Moreover, Brunet et al. (2002) showed that a 14-3-3 mutant (K49E) that was deficient in binding phosphopeptide ligands while preserving its NES-like sequence, was found predominantly in the nucleus. Thus, in the

absence of bound proteins, the NES-like leucine rich sequence within 14-3-3 proteins seems not to function as a NES. Nucleo-cytoplasmic transport of the human Forkhead transcription factor FKHRL1 was shown to need both FKHRL1 NESs and interaction with 14-3-3 proteins in the nucleus (Brunet et al., 2002). Therefore, NES sequences in the interacting protein may drive the export of 14-3-3-interactor complexes out of the nucleus and 14-3-3 binding may facilitate this process. *Xenopus* Cdc25C was found to contain two putative NES sequences (Kumagai and Dunphy 1999). These NESs are necessary for its exclusion from the nucleus, while binding of 14-3-3 proteins is not sufficient for export of Cdc25. Thus, analogous to FKRHL1, 14-3-3 proteins may only play a facilitating role in the regulation of the cellular localisation of Cdc25.

14-3-3 proteins may have an additional role in the cytoplasm in preventing the nuclear re-import of proteins by masking nuclear localisation signals (Dalal et al., 1999; Kumagai and Dunphy, 1999; Brunet et al., 2002). 14-3-3 proteins may thus function as "molecular chauffeurs", where the destination of the 14-3-3-bound complex is determined by instructions contained within the sequence and structure of the bound cargo rather than through intrinsic properties of the 14-3-3 protein (Brunet et al., 2002). The ultimate fate of the cargo is then determined by 14-3-3 protein-mediated alterations in the kinetics of nuclear-cytoplasmic transport.

Although it is clear that 14-3-3 proteins can function as adaptor or scaffold proteins, the exact function of 14-3-3 proteins is still a question of debate. However, it is becoming clear that 14-3-3 proteins have a range of regulatory functions and that the binding of 14-3-3 proteins often serves more than one function.

Kinase signal attenuation and receptor endocytosis

During the signal transduction process, signal attenuation is as important as signal initiation. The activity of kinases must be tightly regulated and properly balanced in order to mediate their normal cellular tasks and their many physiological responses. Several mechanisms exist for signal attenuation. Most important is the deactivation of kinases by the action of phosphatases. A more permanent way of signal attenuation is receptor degradation, which irreversibly terminates the life span of the receptor (Strous and Gent, 2002). In order for protein degradation to occur, receptor-mediated endocytosis is an

essential process whereby the receptor is internalised and processed. This process further involves ubiquitination of the receptor before endocytosis.

Many eukaryotic plasma membrane proteins undergo regulated ubiquitination, which triggers their internalisation (Hicke, 1999). Ubiquitination of proteins is a posttranslational modification in which a 76 a.a. polypeptide, ubiquitin, or a multiubiquitin chain is attached to proteins. A polyubiquitin chain of at least four subunits long is efficiently recognised by the proteasome, where the ubiquitinated protein will be degraded. A shorter ubiquitin tail is sufficient for rapid endocytosis and entry in the endocytic pathway. Internalised receptors are sorted to the endosomal compartment. Along the endocytic pathway endosomes are modified in protein composition and pH, and their contents are sorted for shipment to the appropriate cellular destination (Bevan et al., 1996). Among these fates are retention in the endosomal compartment, recycling back to the plasma membrane and delivery toward a lysosomal degradation pathway.

Insulin binding activates and induces rapid internalisation of the insulin receptor kinase (IRK) (Wiley and Burke, 2001). After insulin-induced endocytosis, insulin and the IRK promptly dissociate because their association is sensitive to the endosomal acidic pH. Dissociation of insulin from the IRK allows receptor recycling and rapid insulin degradation. Thus, the timely termination of insulin action is facilitated by rapid breakdown of the ligand. In many cases, the internalised receptor is still active. Potentially, the internalised receptor is then exposed to substrates that are not accessible at the membrane. Signalling can then occur in each of these locations either initiating or extending the signal (Leof, 2000). For example, the animal epidermal growth factor (EGF) receptor kinase generates some signals at the cell surface while others appear to be generated from within endosomes (Wiley and Burke, 2001). The EGF receptor functions in cell fate determination, proliferation, cell migration and apoptosis. Following ligand binding, the EGF receptor is rapidly internalised by a mechanism that requires intrinsic receptor kinase activity and specific motifs in the C-terminal domain of the receptor. The EGF receptor is subsequently sorted into endosomes and then either targeted to lysosomes or recycled back to the cell surface. Since the internalised receptor remains active until it reaches the late endosomes, EGF receptor signalling can occur from the endosomes. In this way, endocytosis may play a role in placing the activated cell surface receptor in the appropriate cellular location to interact with downstream signalling molecules. In addition

to its presence in the endocytic pathway, the presence of the EGF receptor was also reported in the nuclei of cells in highly proliferating tissues (Lin et al., 2001). Following ligand binding EGF receptors are proposed to migrate to the nucleus where they associate with the promoter region of cyclin D1, a protein involved in mitogenesis. In this way, receptors can regulate their specific target genes directly.

Conclusions

Kinase signalling involves a network of phosphorylation and dephosphorylation reactions. Multiple phosphorylation sites present in a substrate protein may be phosphorylated by different kinases and each individual phosphorylation site may have a distinct function so that specific regulation of protein activity is possible. Phosphorylation of receptor kinases may create protein-interaction domains for direct binding of substrate proteins. Adaptor and scaffold proteins may also bind to receptors and play a role in the formation of protein complexes thereby interconnecting signalling pathways or preventing cross-talk by pathway insulation. Regulated protein localisation and spatial separation of proteins is a commonly used mechanism to prevent spontaneous signal activation. The exact location of the ligand-activated receptor and the presence of specific substrates may dictate which pathways can be activated. Not only the subcellular localisation of substrates may be regulated, but also the subcellular localisation of the receptor via endocytosis followed by recycling or degradation. Because almost all members of signalling networks belong to multigene families, an additional level of complexity involves the assembly and disruption of the right signalling complexes in a cell-specific and subcellular localisation-specific way. Combination of all these possible regulatory levels makes kinase signalling pathways and their role in cellular function an intriguing research area that is wide open for future research.

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Chapter 2

A search for proteins interacting with the kinase domain of *At*SERK1 by yeast two-hybrid screening

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Abstract

Receptor-like protein kinases (RLKs) are key elements in many signal transduction chains. The Somatic Embryogenesis Receptor-like Kinase 1 (*At*SERK1) is an RLK containing five leucine-rich repeats. *At*SERK1 is proposed to participate in a signal transduction cascade involved in ovule and embryo development. The downstream components of the *At*SERK1 signal transduction cascade are mostly unknown. Therefore we used the yeast two-hybrid system to screen for proteins interacting with the kinase domain of *At*SERK1. As a result of the screen we found six different putative *At*SERK1 interacting proteins. These proteins were predicted to encode the 14-3-3 protein GF14λ, the AAA-ATPase *At*CDC48, a putative RNA binding protein (RBP), the potassium transporter KEA1, the aquaporin *At*SIP1 and a racemase-like protein. GF14λ, *At*CDC48 and RBP can be phosphorylated by *At*SERK1 *in vitro*.

Introduction

Protein interactions play important roles in regulating cellular functions. In 1993 Gyuris et al. described the yeast (*Saccharomyces cerevisae*) two-hybrid interaction trap to screen for protein interactors. Subsequently two-hybrid systems have been extensively used for identifying components of plant signalling pathways. For example Ikeda et al. (2000) performed a two-hybrid screening with the wheat protein kinase WPK4, which plays a role in catabolite repression. They isolated two cDNA clones both encoding 14-3-3 proteins that bind to the C-terminus of WPK4. Nam and Li (2002) searched for interactors of the *Arabidopsis* brassinosteroid receptor BRI1 and found the leucine-rich repeat receptor-like protein kinase BAK1, which is identical to *At*SERK3, to be involved in brassinosteroid signalling.

Receptor-like protein kinases (RLKs) are key elements in many signal transduction chains. RLKs consist of an extracellular domain, a transmembrane domain and an intracellular catalytic kinase domain. The Somatic Embryogenesis Receptor-like Kinase 1 (*At*SERK1) is an RLK containing a leucine zipper, five leucine-rich repeats, a single transmembrane domain and a functional serine-threonine kinase (Hecht et al., 2001; Shah et al., 2001b). *At*SERK1 is proposed to participate in an uncharacterised signal transduction cascade involved in ovule and embryo development (Hecht et al., 2001). Previous work has shown that *At*SERK1 interacts *in vitro* and *in vivo* with the kinase-associated protein phosphatase (KAPP; Shah et al., 2002).

To identify additional components of the *At*SERK1 signalling complex, we used the yeast two-hybrid system to identify proteins interacting with the kinase domain of *At*SERK1. Six different putative *At*SERK1 interacting proteins were found encoding the 14-3-3 protein GF14λ, the AAA-ATPase *At*CDC48, a putative RNA binding protein (RBP), the potassium transporter KEA1, the aquaporin *At*SIP1 and a racemase-like protein. We will present the analysis of these proteins and the verification of their interaction in yeast. In addition we will test whether these proteins can be transphosphorylated by *At*SERK1. A more detailed analysis of GF14λ and *At*CDC48 will be presented elsewhere.

Materials and methods

Yeast two-hybrid screening

The DuplexA yeast two-hybrid system (Origene Technologies Inc.) was used to screen for proteins interacting with *At*SERK1 (Genbank accession nr. A67827). The *At*SERK1 kinase domain (*At*SERK1^{kinase}, a.a. 266-625), the LRR domain (*At*SERK1^{LRR}, a.a. 26-234) and the mature *At*SERK1 protein minus the signal sequence (*At*SERK1$^{\Delta SS}$, a.a. 31-625) were cloned in the bait vector pEG202 and in the prey vector pJG4-5 (Origene Technologies Inc.) as translational fusions to LexA and B42 respectively (Shah et al., 2001a). A repression assay was performed to verify proper entry of LexA fusion proteins into the yeast nucleus. Plasmid pJK101 (Origene Technologies Inc.) contains two LexA operators that are placed between the yeast GAL1 promoter and the lacZ reporter gene. LexA fusion proteins will bind to these operators and decrease the level of GAL1-driven lacZ. As a positive control the plasmid pRFHM1 (Origene Technologies Inc.) was used encoding a LexA fusion protein that enters the nucleus. For the assay, yeast strain EGY48 (MATα, trp1, his3, ura3, leu2::6 LexAop-*LEU2*) was cotransformed with either pEG202-*At*SERK1^{kinase} and pJK101, with pRFHM1 and pJK101, or with pJK101 alone. Autoactivation of the lacZ gene or the LEU2 reporter by pEG202-*At*SERK1^{kinase} was tested by cotransforming EGY48 with pEG202-*At*SERK1^{kinase} and plasmid pSH18-34 (Origene Technologies Inc.). pSH18-34 contains the lacZ reporter gene with four LexA operators. Transformants were grown on selection medium (gal/raf, ura$^-$, trp$^-$) containing 80 mg/mL Xgal for 48 hrs at 30°C. The repression assay and autoactivation test were also performed for pEG202-*At*SERK1^{LRR} and pEG202-*At*SERK1$^{\Delta SS}$.

An *Arabidopsis thaliana* ecotype Landsberg cDNA library was prepared as a fusion to the activation domain B42 in vector pJG4-5 using mRNA from young silique tissue (Grebe et al., 2000). The primary library contained approximately $2 \cdot 10^6$ cDNA clones. The library was amplified once in *E. coli* and used to screen for proteins interacting with *At*SERK1^{kinase}. For the screening, yeast EGY48 was first cotransformed with pEG202-*At*SERK1^{kinase} and pSH18-34 and then transformed with the pJG4-5 cDNA library. This resulted in $1.8 \cdot 10^6$ primary transformants. After amplification, $30 \cdot 10^6$ colonies were screened for reporter gene activation on selective induction medium (his$^-$, ura$^-$, trp$^-$, leu$^-$) containing 80 mg/L Xgal. Colonies were judged positive when they grew in

the absence of leucine at 30ºC and showed visible β-galactosidase activity within three days. Positive colonies were transferred twice to new selection plates (his^-, ura^-, trp^-) containing 20 g/L glucose as carbon source, in order to eliminate the presence of additional pJG4-5 library plasmids encoding non-interacting proteins.

pEG202-*At*SERK1^{kinase} was removed from each positive clone by taking away the selection pressure for the pEG202 plasmid via growth of yeast on medium containing histidine. Loss of pEG202-*At*SERK1^{kinase} from yeast was verified by comparing growth on medium with or without histidine. Yeast clones without pEG202-*At*SERK1^{kinase} that did not grow on medium without histidine were plated on selective induction medium (ura^-, trp^-) containing Xgal to test for reporter gene activity. Library clones that autoactivated the reporter genes were eliminated. A mating assay was performed to test for *At*SERK1^{kinase}-specific interaction. Yeast EGY48 clones without pEG202-*At*SERK1^{kinase} were mated with yeast strain RFY206 containing either pEG202-*At*SERK1^{kinase} or pEG202-Lamin C. Diploid yeast were transferred to selective induction plates (his^-, ura^-, trp^-) containing Xgal to test for reporter gene activity. Library clones that show interaction with *At*SERK1^{kinase} and with Lamin C were eliminated. To obtain the pJG4-5 plasmids encoding the resulting positive library clones, total yeast DNA was isolated and electroporated into *E. coli* strain Kc8 ($trpC^-$). *E. coli* colonies containing a pJG4-5 library plasmid were obtained by tryptophan and ampicillin selection. pJG4-5 plasmid DNA was purified from *E. coli* and the inserts were sequenced.

In order to verify the interaction with *At*SERK1, the pJG4-5 plasmids with inserts encoding putative *At*SERK1^{kinase} interactors (Table. 1) were cotransformed with either pEG202-*At*SERK1^{kinase}, pEG202-*At*SERK1^{LRR} or pEG202-*At*SERK1$^{\Delta SS}$ into EGY48, plated on selective induction plates (his^-, ura^-, trp^-) containing Xgal and grown for two days at 30°C. A comparable experiment was done with a longer part of the putative *At*SERK1 interactors as encoded by EST cDNA clones (Table1; Fig. 1). EST cDNA clones were obtained from the *Arabidopsis* Biological Resource Centre. Interaction of the EST encoded peptides with *At*SERK1 was also tested using *At*SERK1 cloned in pJG4-5 as prey, and the EST encoded peptides cloned in pEG202 as bait. To make in frame fusions a 921 bp cDNA fragment of EST 99M10 and a 495 bp cDNA fragment of EST 124L19, encoding RBP$^{118-357}$ and KEA1$^{528-618}$ respectively, were amplified by PCR from the corresponding λZiplox plasmid using a gene-specific forward primer containing an *EcoRI*

site (5'-CGG AAT TCG GCG GTG GTC GTG AAG GAC-3', 5'-CGG AAT TCG GAT TTG GTC GAG TTG GTC-3') and a reverse primer on the λZiplox plasmid containing a *XhoI* site (5'-CCG CTC GAG GGC CAG TGA ATT GAA TTT AGG-3'). The fragments were digested with *EcoRI* and *XhoI* and inserted in the corresponding sites of pEG202 and pJG4-5.

Protein expression and affinity purification

To express *At*SERK1^{kinase}, the peptides encoded by the pJG4-5 plasmids isolated from the two-hybrid library and the peptides encoded by the EST cDNA clones, translational fusions were made to Glutathione-S-transferase (GST) and to the maltose binding protein (MBP). The cDNA sequence encoding complete GF14λ was amplified from EST cDNA clone 190N19 in plasmid λZiplox with a gene specific primer on the transcription start of GF14λ containing an *EcoRI* site (5'-GG GAA TTC ATG GCG GCG ACA TTA GGC AG-3') and a λZiplox specific primer containing an *XhoI* site (5'-CCG CTC GAG GGC CAG TGA ATT GAA TTT AGG-3'). The PCR fragment was digested with *EcoRI* and *XhoI* and cloned in the corresponding sites of vector pGEX-4T1 (Pharmacia Biotech, Inc). A 290 bp cDNA fragment encoding *At*CDC48$^{747-809}$ and a 735 bp cDNA fragment encoding *At*SIP1$^{188-240}$ were amplified by PCR from the corresponding pJG4-5 plasmids using pJG4-5 derived primers containing a *BamHI* restriction site in the forward primer (5'-CGC GGA TCC TAC CCT TAT GAT GTG CC-3') and a *SalI* site in the reverse primer (5'-ACG CGT CGA CTC TGG CGA AGA AGT CC-3'. The PCR fragment was digested with *BamHI* and *SalI* and inserted in the corresponding sites of pGEX-4T1 and pMal-c2x (New England Biolabs). pJG4-5 plasmids encoding racemase$^{248-330}$, RBP$^{213-357}$ and KEA1$^{592-618}$ were digested with *EcoRI* and *XhoI*. The obtained fragments of 470 bp, 657 bp and 360 bp respectively, were purified from agarose gel and cloned into pGEX-4T1 and pEG202. In addition, pJG4-5-KEA1$^{592-618}$ was digested with *EcoRI* and *BamHI* releasing a 244 bp fragment that was purified from agarose gel and cloned into pMal-c2x. pEG202-racemase$^{248-330}$ and pEG202-RBP$^{213-357}$ were digested with *EcoRI* and *SalI* whereupon the obtained fragments of 475 bp and 660 bp respectively, were inserted in pMal-c2x. The cDNA fragments encoding RBP$^{118-357}$ and KEA1$^{528-618}$ that were used for making the two-hybrid constructs were also inserted in pGEX4T1. For cloning in pMal-c2x a 925 bp and a 500 bp *EcoRI-SalI* fragment were

digested from pEG202-RBP$^{118-357}$ and pEG202-KEA1$^{528-618}$ respectively, and inserted in the corresponding sites of pMal-c2x. All plasmid constructs were transformed to *E. coli* BL21 for expression of fusion proteins.

For expression of GST-fusion proteins, 3 mL of an overnight culture was transferred to 300 ml of 2xYT medium and the cells were allowed to grow to an OD_{600} of 0.6 at 30°C. Production of fusion proteins was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a concentration of 0.1 mM and cultures were grown for a further 4 hours at 30°C. The cells were collected by centrifugation, resuspended in phosphate-buffered saline, sonicated and cleared by centrifugation at 12,000 g. Soluble GST-fusion proteins were purified from the supernatant by affinity chromatography using glutathione sepharose 4B (Pharmacia Biotech, Inc) according to the manufacturer's instructions. Proteins were eluted from the resin with 15 mM glutathione in 50 mM Tris pH 8.0, containing 150 mM NaCl. The GST-GF14λ fusion protein was digested for seven hours with thrombin, releasing GF14λ from glutathione sepharose 4B bound GST. For expression of MBP-fusion proteins, cells were grown as above but in LB medium containing 2 g/L glucose. Production of fusion proteins was induced by adding IPTG to a concentration of 0.3 mM. The cells were collected by centrifugation, resuspended in column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA and 1 mM DTT), sonicated and cleared by centrifugation at 12,000 g. Soluble MBP-fusion proteins were purified by affinity chromatography using amylose resin (New England Biolabs). Proteins were eluted from the resin with 15 mM maltose in column buffer. Protein concentrations were determined by Bradford micro-assay (Bio-Rad) using BSA as a standard.

Phosphorylation assays

Transphosphorylation of the peptides encoded by the two-hybrid cDNA clones and EST clones was tested by incubating 500 ng GST- or MBP-fusion protein in the presence of 500 ng GST-*At*SERK1^{kinase} in kinase buffer (20 mM HEPES, 150 mM KCl, 10 mM $MgCl_2$), containing 50 μM ATP and $3.7 \cdot 10^5$ Bq [γ-^{32}P] ATP in a final volume of 30 μL. After incubation for 30-60 minutes at 30°C the reaction was stopped by adding SDS-PAGE sample buffer, incubated for five minutes at 100°C and separated by 12% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue to verify equal loading. After

drying the gel radioactivity was detected with a PhosphoImager using the Image Quant program (Molecular Dynamics). *At*SERK1^{kinase}-specific phosphorylation was tested by incubating the GST- and MBP-fusion proteins with ATP in the absence of GST-*At*SERK1^{kinase}.

Results

Yeast two-hybrid screening

A LexA version of the yeast two-hybrid system was used to identify proteins that interact with the kinase domain of *At*SERK1. The *At*SERK1 kinase domain (*At*SERK1^{kinase}, a.a. 266-625) was therefore cloned in the bait vector pEG202 as a translational fusion to LexA. A series of control experiments were performed to ensure that pEG202-*At*SERK1^{kinase} could indeed be used as bait. When transformed into yeast pEG202-*At*SERK1^{kinase} does not autonomously activate transcription of the reporter genes. A repression assay indicated that the fusion protein pEG202-*At*SERK1^{kinase} enters the yeast nucleus and binds to the LexA operators. Therefore, we conclude that the pEG202-*At*SERK1^{kinase} construct is useful for performing interaction screens to identify downstream binding partners of the *At*SERK1 receptor. The LRR domain (*At*SERK1^{LRR}, a.a. 26-234) was also cloned into pEG202. The resulting construct pEG202-*At*SERK1^{LRR} was less efficient in binding to the LexA operators in the repression assay. The mature *At*SERK1 protein minus the signal sequence (*At*SERK1$^{\Delta ss}$, a.a. 31-625) was cloned in pEG202 to give the construct pEG202-*At*SERK1$^{\Delta SS}$. pEG202-*At*SERK1$^{\Delta SS}$ behaved similarly as pEG202-*At*SERK1^{LRR}, suggesting that the presence of the LRR domain interfered with efficient screening.

The *Arabidopsis* cDNA library from young silique tissue (Grebe et al., 2000) was transformed into yeast containing pEG202-*At*SERK1^{kinase} and pSH18-34 resulting in $1.8 \cdot 10^6$ transformants. After amplification $30 \cdot 10^6$ colonies were plated on selection plates containing Xgal to screen for reporter gene activity. After growing at 30°C for three days 198 positive colonies were selected for further analysis. Positive colonies can contain more than one pJG4-5 library plasmid. Therefore all positives were transferred to new selection plates twice to eliminate the presence of additional pJG4-5 plasmids.

False positives were discarded via several experiments. Upon elimination of the bait plasmid pEG202-*At*SERK1^{kinase} via growth without plasmid selection pressure it was possible to test for activation of the reporter genes by the pJG4-5-library clone alone. Autoactivation of the reporter genes by the library clone was observed 13 times. These clones were discarded. To exclude aspecific binding, occurrence of interaction was tested between the library clone and the unrelated human protein Lamin C in comparison with *At*SERK1^{kinase}. For this, yeast clones containing only the pJG4-5-library plasmid and the reporter plasmid were mated with yeast containing either pEG202-*At*SERK1^{kinase} or pEG202-Lamin C. 62 library clones were discarded that showed interaction with *At*SERK1^{kinase} as well as with Lamin C. This leaves 123 clones that passed all tests. pJG4-5-plasmid DNA of these clones was then isolated and sequenced.

Sequence analysis of the 123 positive clones showed that 65 clones contained a cDNA insert cloned in the opposite orientation and 17 clones contained an insert cloned in an incorrect reading frame. Another 11 clones contained inserts encoding known false positives, such as elongation factors or ribosomal proteins (Serebriiskii et al., 2000; www.fccc.edu/research/labs/Golemis/ InteractionTrapInWork.html), and 14 clones did not contain an insert at all. In total, 16 clones were left encoding seven different putative *At*SERK1 interacting proteins. To confirm binding to the kinase domain of *At*SERK1, one representative clone of each putative interacting protein was tested for interaction with *At*SERK1^{kinase} in comparison with *At*SERK1^{LRR} and *At*SERK1$^{\Delta SS}$. All seven putative interacting proteins showed interaction with the kinase domain. However, one protein showed additional interaction with the LRR domain of *At*SERK1. The four clones encoding this protein were eliminated. Not all putative interactors show interaction with *At*SERK1$^{\Delta SS}$. This can be explained by the less efficient entry of the complete receptor into the yeast nucleus and/or the aberrant folding of the *At*SERK1 protein in the nucleus of yeast.

In conclusion, the yeast two-hybrid screening using the kinase domain of *At*SERK1 as bait resulted in the isolation of twelve cDNA clones encoding six different putative *At*SERK1 interacting proteins.

Analysis of putative *At*SERK1 interacting proteins

Sequence analysis of the isolated yeast two-hybrid clones showed that they represent the 14-3-3 protein GF14λ, the AAA-ATPase *At*CDC48, a putative RNA binding protein (RBP), the potassium transporter KEA1, the aquaporin *At*SIP1 and a racemase-like protein. In Table 1 an overview is presented of the isolated two-hybrid clones and the peptides they represent. Except for GF14λ, all isolated two-hybrid cDNA clones encoded only a C-terminal part of the putative *At*SERK1 interacting proteins. All three clones encoding GF14λ are identical, suggesting that they were derived from the same primary clone after library amplification. The same is true for the four clones that encode the racemase-like protein. For comparison, in Table 1 the predicted protein-lengths and the peptides encoded by the corresponding two-hybrid clones and ESTs are also included. Sequence analysis of the putative interacting proteins will provide insight into the organisation of the proteins and their putative *At*SERK1 interacting domain. Therefore, further details of Table 1 will be discussed below. The analysis of the two-hybrid clones that are identical to GF14λ (3 clones) or to *At*CDC48 (2 clones) and their putative interaction with *At*SERK1 will be described in more detail in Chapters 3 and 4, so only a brief summary is given here. Figure 1 gives an overview of the organisation of putative interacting proteins. For each protein, the regions that are encoded by the representative two-hybrid clone and the corresponding ESTs are indicated.

IR127, IR141 and IR183 encode GF14λ

Three two-hybrid clones were isolated, IR127, IR141 and IR 183, all encoding the complete coding region of the 14-3-3 protein GF14λ (Figure 1A). IR141 is taken as a representative clone. The gene encoding GF14λ is located on BAC F12B17 on chromosome 5. Many *Arabidopsis* EST cDNA clones can be found that encode GF14λ. EST 190N19 (accession nr. R90640) is an example.

IR55 and IR146 encode *At*CDC48

Two two-hybrid clones were isolated, IR55 and IR146, both encoding the C-terminus of AAA-ATPase *At*CDC48 (Fig. 1B). IR55 is taken as a representative clone. The gene encoding *At*CDC48 is located on BAC F8A24 on chromosome 3. A large

Table 1: Overview of the isolated two-hybrid clones and the proteins they encode. Compared are predicted protein lengths with the peptides encoded by the two-hybrid clones. ESTs that code for (part of) the proteins are also mentioned, together with their length and the peptide they encode.

Yeast two-hybrid clone			**Protein**			**EST clone**			
Clone number	Clone length (bp)	Residues encoded by clone	Identity	Accession number	Length (aa)	Clone name	Accession number	Clone length (bp)	Residues encoded by clone
IR 55	290	62	*At*CDC48	S60112	809	104O24	T22005	893	260
IR 146	299	65				180L9	H36923	1406	423
IR 72	471	82	Racemase-like protein	AAD39669	330	701671290	AI99701	542?	91?
IR 117	471	82							
IR 172	471	82				RAFL9-58N9	AV809263	433?	70?
IR 196	471	82							
IR 136	735	52	*At*SIP1	AAF26804	240	AFZL43b02F	AV523893	623	160
						701554115	AI997457	553	79
IR 174	657	144	RBP	BAA97154	357	99M10	T22589	942	239
IR 190	244	26	KEA1	AAD01191	618	124L18	T44799	1829	547
IR 127	1015	248	GF14λ	AAD51781	248	190N19	R90640	1015	248
IR 141	1015	248							
IR 183	1015	248							

number of *Arabidopsis* ESTs can be found that encode *At*CDC48. EST 180L1 (accession nr. H36923) and EST 104O24 (accession nr. T22005) were chosen for further analysis.

IR174 encodes a putative RNA binding protein

Two-hybrid clone IR174 encodes the C-terminal 144 residues of an unknown *Arabidopsis* protein of 357 residues (Fig. 1C). The gene encoding the protein is located on BAC MQL5 on chromosome 5. A large number of ESTs were found that encode the protein. EST 99M10 (accession nr. T22589), encoding the C-terminal 239 residues was chosen for further analysis (Table 1).

Sequence analysis, using a BLASTp search, revealed that the unknown protein is homologous to RNA binding proteins with the *Arabidopsis* nuclear RNA binding protein A-like protein (accession nr. NP_193485) as its most related homologue. Therefore, we designated the unknown protein as a putative RNA binding protein (RBP). The protein shows very low homology to the heat shock protein family HSP70 based on a match with the PFAM protein family HMM PF00012. Hsp70 chaperones help folding many proteins in an ATP-dependent manner. Hsp70 proteins are made up of two regions: the amino terminus is the ATPase domain and the carboxyl terminus is the substrate-binding region (Bukau and Horwich, 1998). The putative RNA binding protein shows homology only with the substrate-binding domain.

No signal sequence or transmembrane domain was found in the putative RNA binding protein RBP using several membrane and/or signal-peptide localising programs, implying that it is a soluble protein that is putatively transported to the nucleus where it could bind RNA. Yeast two-hybrid clone IR174 encodes the C-terminal 144 residues of the putative RNA binding protein ($RBP^{213-357}$) that shows homology with the substrate-binding domain of Hsp70 proteins. *At*SERK1 binding should take place within these 144 residues.

IR190 encodes the K^+-efflux antiporter KEA1

The two-hybrid clone IR190 encodes the C-terminal 26 residues of the *Arabidopsis* K^+-efflux antiporter KEA1 (Fig. 1D). The gene encoding KEA1 is located on BAC T1N6 on chromosome 1. KEA1 is a protein of 618 residues that is encoded by at least three

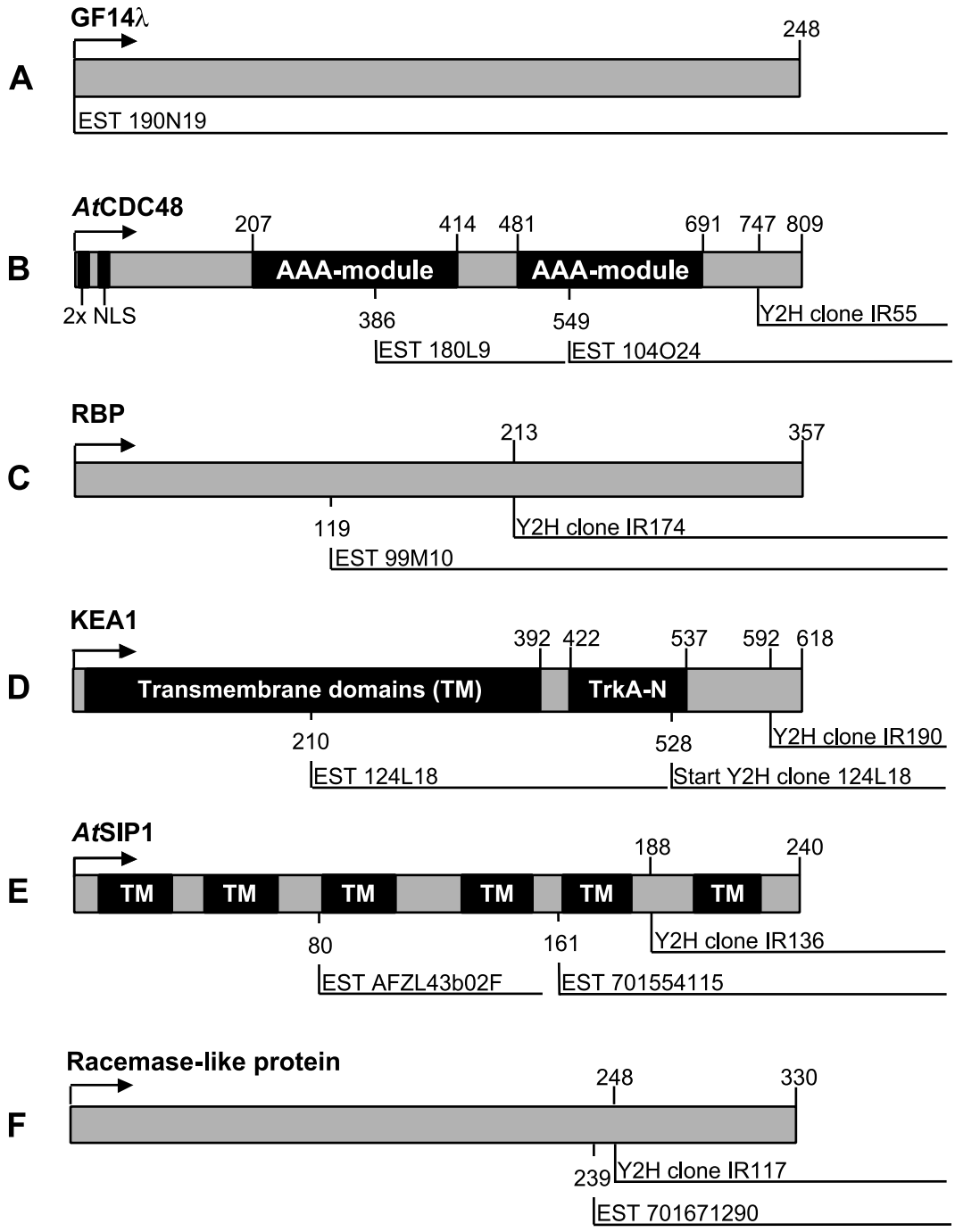

Figure 1: Schematic overview of the putative *At*SERK1 interacting proteins: (**A**) GF14λ, (**B**) *At*CDC48, (**C**) RBP, (**D**) KEA1, (**E**) *At*SIP1, (**F**) racemase-like protein. Drawn are the protein organisation and the localisation of the two-hybrid clones (Y2H) and ESTs. TM = transmembrane domain, TrkA-N = potassium transporter NAD-binding domain.

ESTs. EST 124L18 (accession nr. T44799) encoding residues 71-618 of KEA1 (KEA1$^{71-618}$) is used for further analysis.

Sequence analysis revealed that KEA1 is a member of the Na^+/H^+-exchanger family based on a match with PFAM protein family HMM PF00999. KEA1 is 81% identical to a putative K^+/H^+ antiporter (*At*KEA2; *At*4g00630) which is like KEA1 a monovalent cation:proton antiporter family 2 (CPA2 family) member (Saier et al., 1999), and similar to the SWISS-PROT:SPP03819 *E. coli* glutathione-regulated potassium-efflux system protein kefC (K^+/H^+ antiporter (Miller et al., 1997). Both the Na^+/H^+ exchanger and K^+/H^+ antiporter belong to the superfamily of P-type ion pumps. The family of P-type ion pumps includes primary transporters energised by hydrolysis of ATP with a wide range of specificities for small cations and perhaps also phospholipids (Axelsen and Palmgren, 2001). P-type ion pumps undergo covalent phosphorylation during the transport cycle hence the name P-type. Plant P-type ion pumps are characterised structurally by having a single subunit, eight to twelve transmembrane domains, N and C termini exposed to the cytoplasm and a large central cytoplasmic domain including the phosphorylation and ATP binding sites. Like in P-type ion pumps, KEA1 contains a putative ATP binding site (P-loop) at residues 317-324 and multiple transmembrane domains. The cytoplasmic C-terminal tail of KEA1 contains a TrkA-N domain (PFAM PF02254) spanning residues 422 to 537, which binds nicotinamide adenine dinucleotide (NAD). NAD binding domains are found in a wide variety of proteins, including potassium channels, phosphoesterases and various other transporters (Schlosser et al., 1993).

Two-hybrid clone IR190 encodes only the C-terminal 26 residues of KEA1 (KEA1$^{592-618}$) implying that *At*SERK1 interaction should take place at the end of the cytoplasmic tail within these 26 residues.

IR136 encodes the putative aquaporin *At*SIP1

Two-hybrid clone IR136 encodes the C-terminal 52 residues of a hypothetical *Arabidopsis* protein (accession nr. AAF26804) of 240 residues (Fig. 1E). The gene encoding the protein is located on BAC T6K12 on chromosome 3. EST APZL43b02F (accession nr. AV523893) and EST 701554115 (accession nr. AI997475) were found to encode the protein (Table 1). The two-hybrid and EST cDNA clones all have a different polyadenylation site.

Sequence analysis revealed that the hypothetical protein shows homology with MIP (Major Intrinsic Protein) domain proteins like aquaporins, based on a match with the PFAM protein family HMM PF00230. MIP-domain proteins exhibit essentially two distinct types of transport properties: specific water transport, such as carried out by aquaporins and transport of small neutral solutes, such as glycerol by the glycerol facilitators. The MIP family proteins have no signal sequence and seem to contain six transmembrane segments separated by loops. Both types of MIP-domain proteins differ from each other by their amino acid content and the length of predicted loop regions (Froger et al., 1998).

Like in MIP-domain proteins, the hypothetical protein has no signal sequence and contains six membrane-spanning regions. It contains the characteristic Asn-Pro-Ala (NPA) motif in the second and fifth loop, and it contains almost all residues characteristic for aquaporins (Froger et al., 1998). The hypothetical protein is very homologous to the maize aquaporin family member *Zm*SIP1-2 (small basic membrane integral protein 1-2; Chaumont et al., 2001). Therefore we designated the hypothetical protein AAF26804 as *At*SIP1. The C-terminal 52 residues of the protein, encoded by IR136 (*At*SIP1$^{188-240}$), span the last loop, the sixth transmembrane domain and the C-terminal cytoplasmic tail. This implies that interaction with the *At*SERK1 kinase domain most likely took place within the C-terminal cytoplasmic 13 residues.

IR72, IR117, IR172 and IR196 encode a racemase-like protein

Four two-hybrid clones were isolated, IR72, IR117, IR 172 and IR196, all encoding the C-terminal 82 residues of an asp-glu racemase-like protein (Figure 1F). IR117 is taken as a representative clone and designated racemase$^{248-330}$. The gene encoding the racemase-like protein is located on BAC F9L1 on chromosome 1 (bp 115107..115823 (exon 1), bp 115936..116211 (exon 2). Two ESTs were found that encode the racemase-like protein: EST 701671290 (accession nr. AI997901) is identical to the second exon starting just before the end of the intron (F9L1: bp 115893-116460) and EST RAFL9-58N9 (accession nr. AV809263) encodes at least the second exon (Table 1). The question marks in Table 1 are given because we did not sequence these clones and therefore do not know their exact length. The racemase-like protein is annotated as a protein of 330 residues being a member of the aspartate-glutamate racemase family

(accession nr. AAD39669; *Arabidopsis* Genome Initiative, 2000). It was described as a member of this family based on a match with the PFAM protein family HMM PF0177. Aspartate (EC 5.1.1.13) and Glutamate racemase (EC 5.1.1.3) are two evolutionary related bacterial enzymes that convert aspartate and glutamate residues respectively, from their L-configuration into the D-configuration. Glutamate racemase provides bacteria with a source of D-glutamate, which is an important building block of the peptidoglycan layer in bacterial cell walls (Glavas and Tanner, 2001).

Verification of the interaction in yeast with EST derived peptides corresponding to the putative *At*SERK1 interactors

Protein-protein interactions depend on the conformation of the proteins involved. Several of the putative *At*SERK1 interactors as found in the yeast two-hybrid screen were represented only by a small C-terminal part of the protein. When this part of the protein would normally not be exposed due to the conformation of the complete protein, the observed interaction with *At*SERK1 can be artefactual. To rule out this possibility, longer versions of the putative *At*SERK1 interactors were tested for interaction with *At*SERK1^{kinase} in yeast. This was not necessary for GF14λ as the complete protein was found as *At*SERK1 interactor in the yeast two-hybrid screen. All interacting proteins are represented by at least one EST. However, the cytoplasmic tail of the putative aquaporin *At*SIP1 is already coded for by the yeast clone and the presence of several transmembrane domains in the corresponding EST will most likely prevent proper interaction in the yeast nucleus. Therefore the *At*SIP1 EST was not tested for interaction with *At*SERK1. The racemase-like protein is represented by an EST that encodes approximately the same C-terminal part of the protein as the two-hybrid clones and was therefore not tested for interaction with *At*SERK1. Longer versions of the proteins were only obtained for *At*CDC48 (see Chapter 4), RBP and KEA1. EST 99M10 encodes a substantial part of RBP (RBP$^{118-357}$; Fig. 1C). The EST was cloned into the yeast bait and prey vectors. EST 124L18 encodes almost the complete KEA1 protein (KEA1$^{71-618}$; Fig. 1D). However only the cytoplasmic tail was cloned into the yeast vectors to avoid difficulties due to the presence of the predicted membrane domains (KEA1$^{528-618}$, Fig. 1D).

Table 2 shows that interaction of RBP and KEA1 with the *At*SERK1 kinase domain was observed in all cases. However, when RBP$^{118-357}$ is cloned in the bait vector

pEG202 it seems to interact with all prey-samples tested. It is likely, that RBP$^{118-357}$-pEG202 is able to autoactivate the reporter genes. This was confirmed by an autoactivation test (results not shown). When RBP$^{118-357}$ is cloned in the prey vector pJG4-5, it does interact with *At*SERK1^{kinase}. We conclude that GF14λ, *At*CDC48, RBP and KEA1 are most likely valid interactors with the *At*SERK1 kinase domain. The racemase-like protein and the putative aquaporin *At*SIP1 remain candidates that would require other approaches for verification of their interaction.

Table 2: Interaction of RBP and KEA1 with the kinase domain of *At*SERK1 is confirmed with RBP$^{125-357}$ (EST 99M10), and KEA1$^{528-618}$ (EST 124L18) using the yeast two-hybrid system. When possible, interaction was determined using *At*SERK1 either as bait or as prey. pEG202 = bait vector (AD), pJG4-5 = prey vector (AD). Compared are the interactions of *At*SERK1 with the peptides encoded by the two-hybrid clones and with the ESTs. ++ strong interaction, + interaction, -/+ poor interaction, - no interaction.

	Putative RNA binding protein (RBP)			**KEA1**		
	AD-RBP (213-357)	AD-RBP (118-357)	BD-RBP (118-357)	AD-KEA1 (592-618)	AD-KEA1 (528-618)	BD-KEA1 (528-618)
*At*SERK1$^{\Delta SS}$	-	-	-/+	-	-	-
*At*SERK1^{kinase}	+	++	+	+	++	++
*At*SERK1^{LRR}	-	-	-/+	-	-	-
Lamin C	-	-	-/+	-	-	-

*At*SERK1^{kinase} transphosphorylates putative *At*SERK1 interactors *in vitro*

*At*SERK1^{kinase} is able to autophosphorylate, but can also transphosphorylate other proteins (Shah et al., 2001b). Therefore, phosphorylation assays were performed to further substantiate the interaction between *At*SERK1 and the putative interacting partners. The *At*SERK1 kinase domain was expressed as fusion protein with glutathione-S-transferase (GST). Because GST is known to form homodimers by itself we expressed GF14λ, *At*CDC48$^{747-809}$, RBP$^{213-357}$, KEA1$^{592-618}$, *At*SIP1$^{188-240}$ and racemase$^{248-330}$ encoded by the

isolated two-hybrid clones as fusion proteins with maltose binding protein (MBP) to be able to perform future *in vitro* binding assays with *At*SERK1^{kinase}.

For the phosphorylation assays GST-*At*SERK1^{kinase} together with one of the two-hybrid MBP-fusion proteins was incubated in the presence of labelled ATP (Fig. 2). Only GF14λ, *At*CDC48$^{747-809}$ and RBP$^{213-357}$ are transphosphorylated by *At*SERK1^{kinase} (Fig. 2b lanes 1, 4 and 6). *At*SERK1 does not transphosphorylate GST nor MBP (Fig. 2 lanes 7-8).

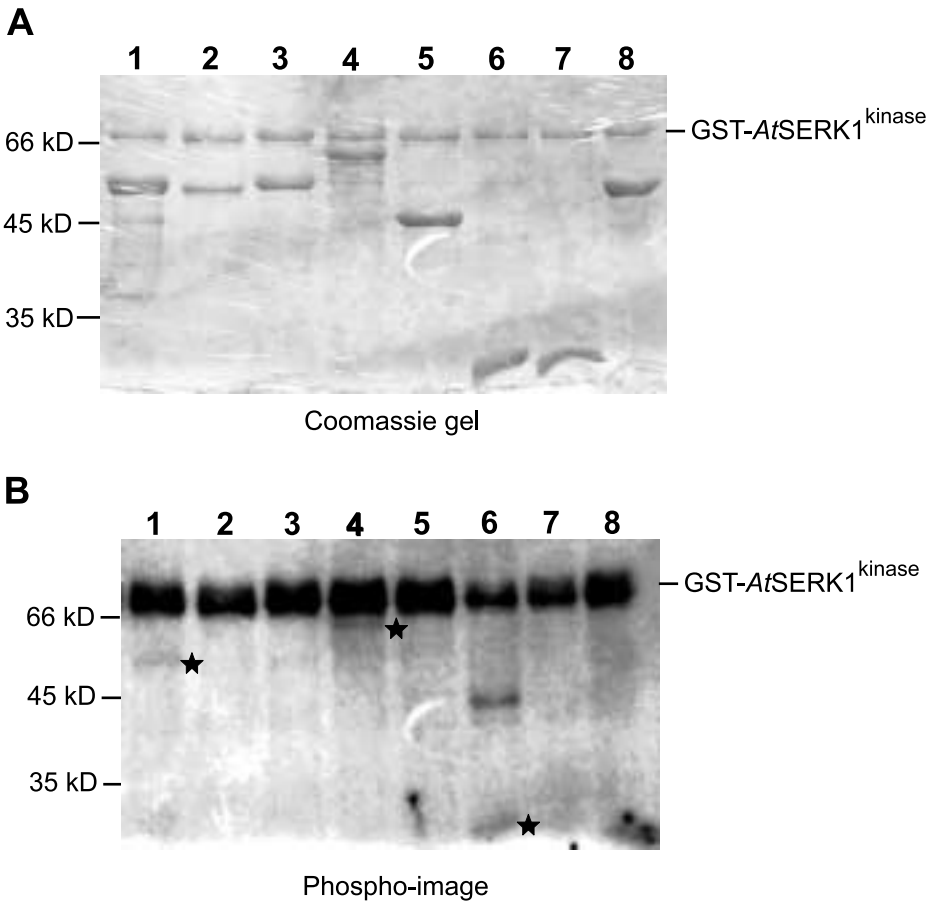

Figure 2: Transphosphorylation assays with *At*SERK1^{kinase} of the peptides encoded by the two-hybrid clones. After electrophoresis, gels were stained with Coomassie Brilliant Blue (**A**) to verify equal loading. After drying the gels, radioactivity was detected with a PhosphoImager (**B**). **A** and **B**: GST-*At*SERK1^{kinase} + MBP-*At*CDC48$^{747-809}$ (**Lane 1**), + MBP-racemase$^{248-330}$ (**Lane 2**), + MBP-*At*SIP$^{188-240}$ (**Lane 3**), + MBP-RBP$^{213-357}$ (**Lane 4**), + MBP-KEA1$^{592-618}$ (**Lane 5**), + GF14λ (**Lane 6**), + GST (**Lane 7**), + MBP (**Lane 8**). Transphosphorylated *At*SERK1-interacting peptides are indicated by an asterisk.

In comparison, *At*SERK1^{kinase} autophosphorylation is much more efficient than transphosphorylation of the putative *At*SERK1 interactors. In lane 6 besides autophosphorylated GST-*At*SERK1^{kinase} and transphosphorylated GF14λ an additional band is present. This band represents autophosphorylated *At*SERK1^{kinase} without GST due to the presence of thrombin in the GF14λ protein sample.

Transphosphorylation of GF14λ, *At*CDC48$^{747-809}$ and RBP$^{213-357}$ was confirmed with GST-fusion proteins. *At*SERK1^{kinase}-dependent phosphorylation was confirmed by incubating the two-hybrid MBP-fusion proteins with labelled ATP in the absence of *At*SERK1^{kinase}. More detailed analysis of GF14λ and *At*CDC48 are given in Chapters 3 and 4. The results for RBP are shown in Figure 3. *At*SERK1^{kinase} transphosphorylated RBP$^{213-357}$ and the observed phosphorylation was *At*SERK1-dependent (Fig. 3b), as RBP$^{213-357}$ is not phosphorylated in the absence of *At*SERK1^{kinase}. Transphosphorylation was also observed for the longer RBP$^{119-358}$ peptide fused to GST- or MBP (Fig. 3d). In comparison, the longer KEA1$^{528-618}$ peptide fused to GST- or MBP was not transphosphorylated (results not shown). We conclude that *At*SERK1 transphosphorylates GF14λ, *At*CDC48 and RBP *in vitro*. KEA1, *At*SIP1 and the racemase-like protein were not transphosphorylated by *At*SERK1 and were therefore excluded from further analysis.

Discussion

Short description of results

We used the yeast two-hybrid system to screen for proteins interacting with the intracellular domain of *At*SERK1. Six different putative *At*SERK1 interacting proteins were found encoding the 14-3-3 protein GF14λ, the AAA-ATPase *At*CDC48, a putative RNA binding protein (RBP), the potassium transporter KEA1, the putative aquaporin *At*SIP1 and a racemase-like protein. GF14λ was found as a full-length protein. The interaction of *At*SERK1^{kinase} with *At*CDC48, RBP and KEA1 was confirmed in yeast by using longer EST-derived parts of the proteins. Under *in vitro* conditions, GF14λ, *At*CDC48 and RBP can be transphosphorylated by the active kinase of *At*SERK1. Based on these results we conclude that GF14λ, *At*CDC48 and RBP have a potential role in *At*SERK1 signalling.

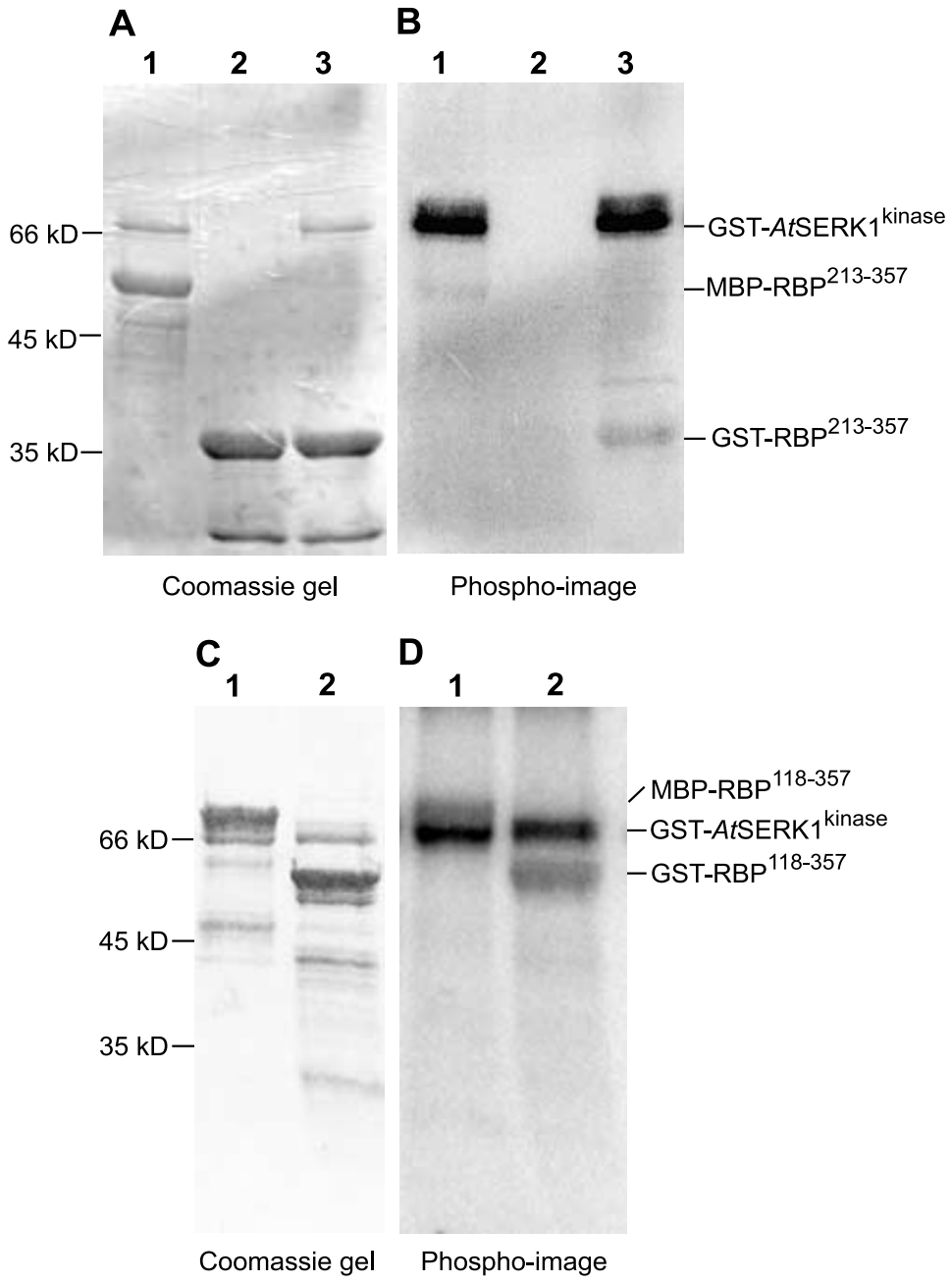

Figure 3: Transphosphorylation assays with *At*SERK1^{kinase} of the RBP peptides encoded by the two-hybrid clone and the EST cDNA 99M10. After electrophoresis, gels were stained with Coomassie Brilliant Blue (**A** and **C**) to verify equal loading. After drying the gels, radioactivity was detected with a PhosphoImager (**B** and **D**). **A** and **B**. **Lane 1**: GST-*At*SERK1^{kinase} + MBP-RBP$^{213-357}$, **Lane 2**: MBP-RBP$^{213-357}$, **Lane 3**: GST-*At*SERK1^{kinase} + GST-RBP$^{213-357}$. **C** and **D**. **Lane 1**: GST-*At*SERK1^{kinase} + MBP-RBP$^{118-357}$, **Lane 2**: GST-*At*SERK1^{kinase} + GST-RBP$^{118-357}$.

Two-hybrid screening; detection of false positives

The yeast two-hybrid interaction trap (Gyuris et al., 1993) consists of three critical components, a vector for expression of the bait protein fused to the DNA binding domain protein LexA (BD), a vector for expression of the prey (library) protein fused to the transcription activation domain B42 (AD) and two reporter genes. One of the reporter genes used is the LEU2 gene fitted with six LexA binding sites that is integrated in the yeast genome (yeast strain EGY48). The second reporter gene is lacZ, with eight LexA binding sites, that is located on a plasmid. The bait protein is constitutively expressed. The prey protein is only expressed in the absence of glucose, in order to allow reproductive growth of yeast when the library plasmid encodes a toxic protein. The LEU2 reporter of EGY48 is very sensitive; it is even activated by AD-fusion proteins that interact weakly with LexA BD-fusions (Estojak et al., 1995). The lacZ reporter is less sensitive. Therefore, proteins that interact strongly with *At*SERK1^{kinase} will give a Leu^{+} and dark blue phenotype. Proteins that interact weakly with *At*SERK1^{kinase} will give a Leu^{+} phenotype, but the lacZ phenotype can range between light blue and white. Yeast that is Leu^{+} and LacZ^{-} can still contain biologically relevant interactors (Estojak et al., 1995). However, in this study yeast that were Leu^{+} but LacZ^{-} were not selected for further research, due to the large amount of positive clones obtained.

In our library screen, we isolated one or more proteins that are presumed to be false positive. False positives are defined either as proteins that impossibly or not likely interact with the bait used in the screen, or as proteins that are subsequently shown to interact with multiple unrelated baits (Serebriiskii et al., 2000). Therefore several experiments were performed to detect and eliminate false positives. An autoactivation test of BD-library fusion proteins and a mating assay were performed to confirm *At*SERK1^{kinase} binding, which resulted in the elimination of 75 out of 198 clones that were determined to be false positives. A disadvantage of the mating assay is that the sensitivity of the reporters is generally less in diploid cells relative to haploid cells (Finley and Brent, 1997). Therefore, putative *At*SERK1^{kinase} interacting proteins that interact only very weakly could have been accidentally eliminated. Sequencing of the remaining 123 clones revealed that many clones contained library inserts cloned in the opposite orientation or in an incorrect reading frame or had no insert at all. Because the LEU2 reporter of EGY48 is very sensitive, even weak transcription activators fused to LexA can activate it. In

addition, some library proteins can enhance the intrinsic transcriptional activity of the LexA-fused bait with which it is expressed. Therefore, almost every LexA fusion protein will activate the LEU2 reporter in EGY48 to some extent by itself (Estojak et al., 1995). Although the *At*SERK1^{kinase} bait we used passes the autoactivation test, this could explain the presence of some false positives. Out of the 123 clones, 12 clones were identified encoding putative *At*SERK1^{kinase} interacting proteins. Although these clones passed all tests, they still could be false positives. Some proteins are hypothesised to possess their false positive character because they are intrinsically 'sticky' because their biological function involves binding a large number of different proteins or because of non-specific charge or coiled-coil interactions (Serebriiskii et al., 2000). However, not all baits bind to the same 'sticky' proteins. Therefore, interaction of the positive library clones was tested with the unrelated bait protein Lamin C and with *At*SERK1^{LRR} as an alternative bait protein. Only *At*SERK1^{kinase} interactors that did not interact with Lamin C and *At*SERK1^{LRR} were subsequently analysed.

β-Galactosidase activity can be described as absent, low or high based on colonies being white, light blue or dark blue. A number of considerations must be taken into account when describing interactions between proteins in yeast based on β-galactosidase activity (Serebriiskii and Golemis, 2000). In the two-hybrid screening three plasmids are used, that are partitioned into daughter cells such that copy numbers of the plasmids may vary between 10 and 40 copies per cell in total. The ratio of bait, prey and reporter plasmids may vary between separately transformed colonies. Relative ratios of β-galactosidase activity observed in yeast grown for short periods can differ from those obtained from yeast grown for longer periods. In addition, in the case of toxic or otherwise disfavoured transcriptional activators, yeast cells are efficient at genetically or epigenetically adapting to limit the strength of the activator, either by modifying the core transcriptional apparatus or by eliminating expression of individual disfavoured proteins (which could be either bait, prey, or both) (Serebriiskii et al., 2000). Yeast cells grow optimally on media of pH 5.5 and continue to acidify their media as they grow. The optimal pH for enzymatic activity of β-galactosidase is 7.0. While X-gal plates are buffered to approach pH 7.0 to enhance enzyme function, yeast grow sub-optimally under these conditions potentially predisposing them to toxicity effects. If yeast cells grow over longer periods to high density the growth medium is eventually acidified impairing the

effectiveness of the assay (Serebriiskii and Golemis, 2000). In order to avoid elimination of clones due to a variation in β-galactosidase activity, in our experiments all interaction tests were done with five different individual transformants. Relative β-galactosidase activity was determined at multiple points after commencement of the assay and finally yeast cells were grown not longer than three days.

It is possible that one or more of the interactions detected involves an individual domain of a protein that is normally masked in the full-length protein. In a previous two-hybrid screening of the same cDNA library, using pEG202-*At*SERK1$^{\Delta SS}$ as bait (Schmidt and de Vries, unpublished results) a clone was isolated encoding the C-terminal residues (a.a. 373-449) of an unknown protein (accession nr. AAL32518) and a clone encoding the C-terminal residues (a.a. 555-964) of a putative aminopeptidase (accession nr. AAG52429). When a longer part of the unknown protein or the putative aminopeptidase was used in a yeast interaction test either as prey or as bait, interaction with *At*SERK1$^{\Delta SS}$, *At*SERK1^{kinase} or *At*SERK1^{LRR} was no longer observed. This makes it less likely that these positives represent biologically relevant interactors.

The interaction with *At*SERK1 was confirmed for a longer EST encoding part of *At*CDC48, RBP and KEA1. The C-terminal tail of KEA1 (KEA1$^{528-618}$) autoactivates the reporter genes when expressed as a fusion to LexA. pJG4-5-KEA1$^{528-618}$ does not autoactivate the reporter genes, but does show interaction with *At*SERK1^{kinase}. Therefore, KEA1 was not eliminated as a false positive. The racemase-like protein and the putative aquaporin *At*SIP1 were not included in this analysis. *At*SIP1 contains multiple transmembrane domains with only a very small intracellular C-terminal tail. Due to expected folding problems in the absence of a membrane, interaction of the full-length protein with *At*SERK1^{kinase} was not further investigated. For the racemase-like protein some uncertainties arose when the coding sequence of the ESTs was compared with the annotated protein. Besides coding for exon two of the putative racemase protein, EST 701671290 encodes several residues of the putative intron. Because of this, and the fact that all 4 isolated two-hybrid clones encode only the second exon of the putative racemase protein, it is doubtful that the annotation is correct. In addition, a racemase is an enzyme that converts amino acid residues from the L-configuration into the D-configuration. Amino acid residues with a D-configuration cannot be found in plants. Therefore, interaction of the putative racemase protein with *At*SERK1 was not further investigated. In

conclusion, GF14λ, *At*CDC48, RBP and KEA1 passed all tests to detect false positives and could therefore be considered as potential *At*SERK1^{kinase} interacting proteins.

Phosphorylation assays were performed to further substantiate the interaction between *At*SERK1 and its putative interacting partners. The phosphorylation assays showed that *At*SERK1 only phosphorylates GF14λ, *At*CDC48 and RBP. The KEA1 protein, either the short version encoded by the two-hybrid clone or the C-terminal cytoplasmic tail (KEA1$^{528-618}$), fails to be phosphorylated by *At*SERK1^{kinase}. Therefore we discontinued further analysis of the interactions between *At*SERK1^{kinase} and KEA1.

Biological significance of the potential *At*SERK1^{kinase} interactors

The homology with nuclear RNA binding protein A as found for one of the potential *At*SERK1^{kinase} interactors suggests a direct role for *At*SERK1 in post-transcriptional gene expression by regulation of RNA processing at the level of splicing, stability or translation. The AAPK-interacting protein (APK1), with sequence homology to heterogeneous nuclear RNA binding protein A/B is a substrate of the fava bean abscisic acid-activated protein kinase (AAPK) (Li et al., 2002). AAPK-dependent phosphorylation is required for the interaction of APK1 with mRNA. Both APK1 and AAPK are nuclear proteins. *At*SERK1 is a transmembrane receptor protein, which makes it difficult to envisage how *At*SERK1 can interact and phosphorylate a nuclear protein. However, RBP does not have an obvious nuclear localisation signal. Therefore, the subcellular localisation of RBP and verification of the *in vivo* interaction with *At*SERK1 are necessary to determine whether RBP is a valid *At*SERK1 interactor.

For the *At*SERK1 interactors GF14λ and *At*CDC48 there is an indication that these proteins are present in a complex together with the phosphatase KAPP that was recently shown to interact with *At*SERK1 (Shah et al., 2002). It has been shown that the human protein tyrosine phosphatase PTPH1 interacts with 14-3-3β and uses valosin-containing protein VCP (the mammalian CDC48 homologue) as a substrate (Zhang et al., 1999). It is of great interest to determine whether such a complex is also present in *Arabidopsis*. This led us to continue our studies with the GF14λ (Chapter 3) and the *At*CDC48 proteins (Chapter 4).

Conclusion

While two-hybrid screens can give artifactual results, such false positives can be frequently eliminated through either comparison against a database of common false positives or by simple secondary characterisation. However, when a putative interactor is found it is still necessary to verify the validity of the interaction in a different experimental system. For example the interaction can be verified *in vitro* by pull-down assays and *in vivo* by an overlap in expression pattern in combination with co-immunoprecipitation or a molecular interaction in protoplasts between fluorescently labelled proteins. When a kinase is used as bait, a good biochemical test to verify the interaction is a phosphorylation assay to determine whether the kinase can use its interactors as substrate. Nevertheless, for the majority of intracellular proteins, the two-hybrid system is capable of detecting biologically significant protein-protein interactions (Serebriiskii et al., 2000). However, a yeast two-hybrid screening is never saturating. Most probably not all *At*SERK1^{kinase} interacting proteins have been found in our screen. Some weakly interacting proteins could have been missed because colonies that were Leu^+ but $LacZ^-$ were not isolated from the screening. Many proteins of higher eukaryotic organisms undergo extensive post-translational modifications that are essential for their function. In yeast, the relevant modifying enzymes may be absent. In addition, accessory proteins or small components that are not provided by yeast could be necessary for an interaction to occur. Modified and alternative approaches to the original two-hybrid screenings, like three-hybrid systems for ternary complex analysis or the Sos recruitment system for transcriptionally active or membrane proteins, were developed to circumvent these problems (reviewed by Colas and Brent, 1998 and Fashena et al., 2000). In the future a three-hybrid system might be used to search for proteins interacting with *At*SERK1 that need the presence of one of the interactors found in our two-hybrid screen.

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Chapter 3

The *Arabidopsis* Somatic Embryogenesis Receptor-like Kinase 1 interacts with the 14-3-3 protein GF14λ

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Abstract

Leucine-rich repeat containing transmembrane receptor-like kinases are important components of plant signal transduction. The Somatic Embryogenesis Receptor-like Kinase 1 (*At*SERK1) is an RLK, containing five leucine-rich repeats. *At*SERK1 is proposed to participate in a signal transduction cascade involved in ovule and embryo development. As a result of a yeast two-hybrid screening GF14λ, a member of the *Arabidopsis thaliana* family of 14-3-3 proteins, was found to interact with the kinase domain of *At*SERK1. GST pull-down assays with *At*SERK1 fused to GST showed that GF14λ preferentially binds to the phosphorylated *At*SERK1 kinase domain. *In vitro* *At*SERK1 is able to transphosphorylate GF14λ. In plant cells, the interaction between *At*SERK1 and GF14λ was demonstrated by using co-immunoprecipitation and the occurrence of FRET between fluorescently labelled *At*SERK1 and GF14λ proteins.

Introduction

The perception and transduction of signals is essential for the regulation of cellular behaviour. Receptor-like protein kinases (RLKs) are key elements in many signal transduction chains. They consist of an extracellular domain, a transmembrane domain and an intracellular catalytic kinase domain. A large family amongst the plant RLKs consists of the leucine-rich repeat (LRR) containing transmembrane receptors. The LRR motif is believed to be involved in mediating protein-protein interactions or interactions with other molecules (Kobe and Deisenhofer, 1994). Thus, in receptors it may interact directly or indirectly with the ligand. In the *Arabidopsis* genome around 200 LRR-RLKs have been found for which only a few biological functions are known (AGI, 2000). Cf9 (Romeis et al., 1999) and Xa21 (Century et al., 1999) are two examples of LRR-RLKs involved in disease resistance. The LRR receptor kinase FLS2 was found to be the receptor for flagellin, a polypeptide originating from pathogens that induces defence responses is plants (Gomez-Gomez et al., 2001). In addition, it was recently found that the perception of the peptide plant hormone phytosulfokine involves an LRR receptor kinase (Matsubayashi et al., 2002). The same is true for the perception of systemin (Scheer and Ryan, 2002).

We have previously identified the *Arabidopsis* Somatic Embryogenesis Receptor-like Kinase 1 (*At*SERK1), which is an RLK containing a leucine zipper, five LRRs, a single transmembrane domain and a functional serine-threonine kinase (Hecht et al., 2001; Shah et al., 2001b). *At*SERK1 is highly expressed in ovule primordia, the entire female gametophyte while low expression has been detected in adult vascular tissue (Hecht et al., 2001). Ectopic expression of *At*SERK1 does not result in obvious phenotypes in *Arabidopsis* plants but increases somatic embryo formation in culture. Based on its specific expression pattern and its involvement in embryo formation, *At*SERK1 is proposed to participate in an uncharacterised signal transduction cascade involved in ovule and embryo development (Hecht et al., 2001).

In several studies additional components of a signal transduction system have been identified using a variety of techniques such as analysis of additional and non-allelic mutants, co-immunoprecipition experiments or yeast interaction screens. In *Arabidopsis* the signal transduction systems involving the brassinosteroid receptor BRI1 (Wang et al.,

2001) or the CLAVATA genes (Trotochaud et al., 1999) have been extensively investigated.

The *Arabidopsis* brassinosteroid (BR) receptor BRI1 encodes an LRR-RLK that upon sensing of BR initiates a signal transduction cascade that controls growth and development (Wang et al., 2001). Additional elements of BR signalling were found. The putative secreted carboxypeptidase BRS1 was found to negatively regulate the BR response probably at an early step in BRI1 signalling (Li et al., 2001). BR signalling through BRI1 likely inhibits the kinase BIN2 (Brassinosteroid Insensitive 2). This allows accumulation of BZR1 (Brassinazole Resistant 1) and BES (BRI1-EMS-suppressor 1) in the nucleus thereby positively regulating BR signalling by mediating BR induced growth responses (He et al., 2002; Yin et al., 2002). BIN2 negatively regulates BZR1 as phosphorylation of BZR1 increases its degradation by the proteasome. Recently, the LRR-RLK BAK1 (BRI1-Associated receptor Kinase 1) was identified as interaction partner of BRI1 based on a suppressor screen as well as a yeast interaction screen (Li et al., 2002; Nam and Li, 2002). BAK1 has high sequence homology with *At*SERK1 and is identical to the *At*SERK family member *At*SERK3 (accession nr. AF384970). The two LRR-receptor kinases BAK1 and BRI1 bind *in vitro* and *in vivo* and can phosphorylate each other. Most likely they function together through heterodimerisation to mediate BR signalling in *Arabidopsis* (Li et al., 2002)

The *Arabidopsis CLAVATA* (*CLV*) genes are involved in shoot meristem maintenance (Clark et al., 1997; Jeong et al., 1999; Trotochaud et al., 1999). *CLV1* encodes an LRR-RLK forming a complex with CLV2, which is an LRR protein without kinase domain. CLV1 is present in two complexes of 185 kD and 450 kD (Trotochaud et al., 1999). The formation of the bigger complex is dependent on the presence of the small, secreted CLV3 protein, which is the proposed ligand. In addition, the kinase-associated protein phosphatase (KAPP) that functions as negative regulator of CLV1 and a Rho GTPase-related protein were also shown to be part of the 450 kD receptor complex.

The components of the *At*SERK1 signalling complex are mostly unknown. Previous work has shown that *At*SERK1 interacts *in vitro* and *in* vivo with KAPP, which was found to play a role in internalisation of *At*SERK1 (Shah et al., 2002). To identify additional components of the *At*SERK1 signalling complex, we used the yeast two-hybrid system to find proteins interacting with the kinase domain of *At*SERK1. We found that

*At*SERK1 interacts with the 14-3-3 protein GF14λ *in vitro* and *in vivo*. CFP/YFP fusions with GF14λ and *At*SERK1 show that the interaction takes place at the cell membrane. GF14λ association with *At*SERK1 is enhanced when the *At*SERK1 kinase domain is phosphorylated. Besides binding, *At*SERK1 can also transphosphorylate GF14λ *in vitro*.

Materials and methods

Yeast two-hybrid screening

The DuplexA yeast two-hybrid (Origene Technologies Inc.) system was used to screen for proteins interacting with *At*SERK1. An *Arabidopsis thaliana* (ecotype Landsberg erecta) cDNA library was prepared as a fusion to the activation domain in vector pJG4-5 (Origene Technologies Inc.) using mRNA from young silique tissue (Grebe et al., 2000). The activation domain in vector pJG4-5 is an 88-residue acidic *E. coli* peptide (B42) that is able to activate transcription in yeast. The primary library contained approximately 2.10^6 cDNA clones. The library was amplified once in *E. coli.* The nucleotide sequence encoding the kinase domain of *At*SERK1 (Genbank accession nr. A67827; a.a 266-625) was cloned into the bait vector pEG202 (Origene Technologies) to produce a fusion protein with LexA (Shah et al., 2001a). The following control experiments were performed to ensure that LexA-*At*SERK1^{kinase} could be used as bait: 1) a repression assay that indicated that the LexA-*At*SERK1^{kinase} protein is transported correctly into the nucleus and 2) an autoactivation assay that verified that the LexA-*At*SERK1^{kinase} protein does not autonomously activate the *lacZ* or the *LEU2* reporter-genes. The cDNA library was transformed into yeast strain EGY48 (MATα, his3, trp1, ura3, $LexA_{op(x6)}$-*LEU2*) containing pEG202-*At*SERK1^{kinase} and the reporter plasmid pSH18-34. The transformation resulted in $1.8x10^6$ colonies. Approximately 30.10^6 transformants were plated on selection plates containing 80 mg/L Xgal.

Colonies were judged positive when they grew in the absence of leucine at 30°C and showed visible β-galactosidase activity within three days. To exclude *At*SERK1^{kinase}-independent activation of leucine and β-galactosidase expression, EGY48 was grown on selection plates containing only the pJG4-5 plasmid with a library clone and the pSH18-34 plasmid. *At*SERK1^{kinase}-dependent reporter gene expression was verified by comparing the interaction of the positive pJG4-5 library clones with pEG202-*At*SERK1^{kinase} and with

pEG202-lamin C. Library clones that only activated the reporter genes in an *At*SERK1^{kinase} dependent manner were used for further analysis. pJG4-5 plasmid DNA was purified and the inserts were sequenced.

One of the isolated putative interacting proteins was identified as GF14λ (accession nr. NP_568229). GF14λ *At*SERK1 interaction was analysed in yeast with either the *At*SERK1 kinase domain (a.a. 266-625) or the entire mature *At*SERK1 protein without the N-terminal signal sequence (*At*SERK1$^{\Delta SS}$, a.a. 31-625) in comparison with the *At*SERK1 extracellular domain (*At*SERK1^{LRR}; a.a. 26-234).

GST-fusion protein expression

To express the proteins GF14λ, GF14λ$^{39-248}$ and A*t*SERK1^{kinase}, translational fusions were made to glutathione-S-transferase (GST). The cDNA sequence encoding complete GF14λ was amplified from the EST cDNA clone 190N19 (accession nr. R90640; *Arabidopsis* Biological Resource Centre) in plasmid λZiplox with a gene specific primer on the transcription start of GF14λ containing an *EcoRI* site (5'-GG GAA TTC ATG GCG GCG ACA TTA GGC AG-3') and a λZiplox specific primer containing an *XhoI* site (5'-CCG CTC GAG GGC CAG TGA ATT GAA TTT AGG-3'). The PCR fragment was digested with *EcoRI* and *XhoI* and cloned in the corresponding sites of vector pGEX-4T1 (Pharmacia Biotech, Inc.). The pJG4-5-GF14λ$^{39-248}$ plasmid was digested with *EcoRI* and *XhoI*, releasing a 900 bp fragment that was purified from agarose gel and inserted into pGEX-4T1. The plasmids pGEX-GF14λ, pGEX-GF14λ$^{39-248}$ and pGEX-*At*SERK1^{kinase} and the empty vector pGEX-4T1 were transformed into *E. coli* strain BL21 for protein expression.

Protein expression of GST-fusion proteins and GST was induced with 0.1 mM isopropyl β-D-thiogalactopyranoside. Proteins were solubilised by sonification and GST-fused proteins were bound to glutathione sepharose 4B beads following the manufacturer's instructions (Pharmacia Biotech). GST and GST-*At*SERK1^{kinase} proteins were eluted from the beads with 15 mM reduced glutathione in 50 mM Tris pH 8.0 and 150 mM NaCl. They were then dialysed against 50 mM Tris pH 7.4, 1 mM DTT and 0.1 mM EDTA. The GST-GF14λ and GST-GF14λ$^{39-248}$ fusion proteins were digested for

seven hours with thrombin, releasing GF14λ and GF14λ$^{39-248}$ from glutathione sepharose 4B bound GST. Protein concentrations were determined by Bradford micro-assay (Bio-Rad) using BSA as a standard.

Production of αGF14λ and α*At*SERK1^{kinase} antibodies

αGF14λ antibodies were generated by immunising rabbits with the purified full length GF14λ protein without the GST-tag. The rabbit serum was purified from antibodies directed against bacterial proteins by passing the serum over immobilised bacterial protein extract. The αGF14λ antiserum was used at a 1:15,000 dilution for Western analysis. Trace amounts of GST, not detected by SDS-PAGE gel electrophoresis, were present in the purified GF14λ protein sample. Therefore, besides detecting the GF14λ protein on western blot, the antiserum also detects GST and GST fusion proteins. To specifically detect *At*SERK1, peptide antibodies (αSP1) were generated in rabbit. The antibodies are directed against the peptide DLGNAELSGHLVPELGVLKNL (nr 789) corresponding to amino acid residues 75-95 in the LRR domain of *At*SERK1.

***In vitro* protein binding assays**

GST pull-down assays of GF14λ by the *At*SERK1 kinase domain (GST-*At*SERK1^{kinase}) were performed to confirm the observed yeast two-hybrid interactions using both phosphorylated and non-phosphorylated GST-*At*SERK1^{kinase}. GST-*At*SERK1^{kinase} (3 μg) was phosphorylated by adding 100 μM ATP and incubated for 30 minutes at 30ºC in a total volume of 30 μL protein binding buffer (PBB: 20 mM HEPES pH 7.5, 150 mM KCl, 1 mM DTT, 0.1 mM EDTA, 5 mM $MgCl_2$). 3 μg GST or GST-*At*SERK1^{kinase} was incubated with glutathione sepharose 4B for 30 minutes at room temperature. After binding GST or GST-*At*SERK1^{kinase} the beads were first blocked with 1% (w/v) skimmed milk, 0.1% (v/v) Triton X-100 and then with 2% (w/v) BSA, 0.1% (v/v) Triton X-100 both for 30 minutes at 4ºC. For the binding assay 1 μg of GF14λ or GF14λ$^{39-248}$ was added to immobilised GST or GST-*At*SERK1^{kinase} and incubated for two hours at room temperature in 300 μL PBB. The immobilised protein complexes were washed three times with 1 mL PBB. Bound proteins were removed from the beads by adding SDS-PAGE sample buffer to the beads and incubating at 100°C for five minutes.

The proteins were then separated by 12% SDS-PAGE and transferred to nitrocellulose membrane (Schleicher and Schuell) followed by Western analysis using αGF14λ antiserum. Proteins on the Western blots were visualised using an alkaline phosphatase-based detection system with standard conditions.

Phosphorylation assays

Transphosphorylation of GF14λ and GF14λ$^{39-248}$ was tested by incubating 500 ng protein in the presence of 500 ng GST-*At*SERK1^{kinase} for 45 minutes at 30°C in phosphorylation buffer (20 mM Tris, pH 7.5, 150 mM KCl, Triton, 10 mM $MgCl_2$) containing 50 µM unlabeled ATP and $3.7 \cdot 10^5$ Bq of [γ-^{32}P]ATP in a final volume of 30 µL. The reaction was stopped by adding SDS-PAGE sample buffer and incubated for five minutes at 100°C. The proteins were separated by 10% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue to verify equal loading. After drying the gel, radioactivity was detected with a PhosphoImager using the Image Quant program (Molecular Dynamics).

Immunoprecipitation of GF14λ

Protein extracts were obtained from *Arabidopsis thaliana* cell suspension (Menges and Murray, 2002) by grinding the tissue in extraction buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% Glycerol, 5 mM EGTA and 1% Triton X-100) containing protease inhibitors (1mM PMSF, 1% aprotinin and 10 µg/mL leupeptin) and phosphatase inhibitors (5 mM EDTA, 100 mM NaF). For the co-immunoprecipitation 20 µL of αSP1 *At*SERK1 peptide antibodies or αGF14λ antiserum, bound to sepharose-protein A, was incubated with 50 µg of plant protein extract at 4°C for 1.5-2 hours. The immune-complexes were washed once with high salt buffer (50 mM Tris pH 7.5, 0.1% Triton-X100, 0.5 M NaCl), then once with medium salt buffer (50 mM Tris pH 7.5, 0.1% Triton X100, 0.15 M NaCl) and finally twice with low salt buffer (50 mM Tris pH 7.5, 0.1% Triton X100). Immunoprecipitated proteins were eluted from the sepharose by adding sample buffer and incubating at 100°C for five minutes. The proteins were separated on 12% SDS-PAGE and analysed by Western analysis using αGF14λ antiserum. Visualisation of proteins on

the Western blots was performed using a horseradish peroxidase-based detection system (ECL, Amersham) according to standard conditions.

Localisation and interaction of CFP/YFP fusion proteins in protoplasts

The cDNA sequence encoding complete GF14λ was amplified from the EST cDNA clone 190N19 in plasmid λZiplox with a gene specific primer on the transcription start of GF14λ containing an *EcoRI* site (5'-GGG AAT TCG GCG GCG ACA TTA GGC AGA G-3') and a λZiplox specific primer containing a *XhoI* site (5'-CCG CTC GAG GGC CAG TGA ATT GAA TTT AGG-3'). The PCR fragment was digested with *EcoRI* and *XhoI* and cloned in the corresponding sites of vector pMON999 containing the CFP gene, resulting in the CFP-GF14λ construct. In addition, the PCR fragment was also digested with *EcoRI* and *BamHI* and cloned in the corresponding sites of vector pEYFP*-C1 (Clontech). The resulting construct was then digested with *NheI* and *BamHI* and the YFP-14-3-3 fragment was cloned in the *XbaI* and *BamHI* sites of vector pMon999, resulting in the YFP-GF14λ construct. Preparation of the *At*SERK1^{kinase}-YFP construct was described previously (Shah et al., 2001a). Both YFP-GF14λ and CFP-GF14λ fusion proteins contain a linker region of 10 a. a. in between the fluorescent protein and the GF14λ protein. The *At*SERK1-YFP fusion protein does not contain a linker region. All CFP/YFP transgenes are expressed under the control of the 35S promoter. The CFP/YFP fusion proteins were transiently expressed in cowpea (*Vigna unguiculata* L.) mesophyll protoplasts. Protoplasts were isolated and transfected via polyethyene glycol mediated transformation. The intracellular CFP and YFP fluorescence was analysed by Confocal Laser Scanning Microscopy (CLSM) using a Zeiss LSM 510 confocal microscope (Carl-Zeiss, Germany). All measurements and imaging experiments were performed as described previously by Shah et al. (2002). For DAPI staining, protoplasts were fixated for 10 minutes in phosphate-buffered saline with 4% paraformaldehyde and washed three times with phosphate-buffered saline (pH 7.5). Protoplasts were stained with 1 µg/mL DAPI (4, 6-dianidino-2-phenylindole) in Vectashield antifade solution (Vector Laboratories Inc, Burlingane, CA, U.S.A) and analysed with a Zeiss epi-fluorescent microscope equipped with a CCD-camera. Pictures were taken with a DAPI filter and a FITC filter using Genus software (Applied Imaging).

Fluorescence Resonance Energy Transfer (FRET) between the fluorescently labelled *At*SERK1^{kinase} and 14-3-3 proteins was measured by Fluorescence Spectral Imaging Spectroscopy (FSPIM) as described by Shah et al., (2002). Spectral images were acquired using a 60x oil immersion objective with a 150 groove/mm grating on the entrance slit of the spectrograph, set at a central wavelength of 500 nm and a slit width of 100 μm corresponding to 5 μm in the object plane. Typical exposure and CCD integration time was three seconds. The resulting fluorescence spectra were corrected for background fluorescence and camera bias by background subtraction using an extracellular region from the same spectral image. Acceptor photobleaching (APB) (Wouters and Bastiaens, 1999) was performed by bleaching the YFP fluorophore in a previously defined region for two seconds with 50-100% laser power at 514 nm. Images of the protoplast were taken with a 3.25 second time interval.

Results

GF14λ interacts with the *At*SERK1 kinase domain in yeast

To identify proteins that physically interact with *At*SERK1 (Hecht et al., 2001), a yeast two-hybrid cDNA library from *A. thaliana* young silique tissue (Grebe et al., 2000) was screened with the kinase domain of *At*SERK1 (a.a. 266-625, *At*SERK1^{kinase}). Screening of approximately 30.10^6 yeast transformants resulted in 200 clones that showed β-galactosidase activity and leucine auxotrophy. Control experiments were performed to remove false positives. After excluding library clones with *At*SERK1^{kinase} independent activation or autoactivation of the reporter genes, 12 clones of the initial 200 positives encoded putative *At*SERK1 interacting proteins. Among these positives were three clones containing the complete coding sequence of GF14λ, a member of the *Arabidopsis* family of 14-3-3 proteins. All three clones were identical, suggesting that they were derived from the same primary clone after library amplification. In an earlier screening of the same cDNA library, using the mature *At*SERK1 protein minus the signal sequence (*At*SERK1$^{\Delta SS}$) as bait, another clone encoding GF14λ was found (Schmidt and de Vries, unpublished results). This clone lacks the N-terminal 38 residues and was designated GF14λ$^{39-248}$. In none of the two-hybrid interaction screens we performed with *At*SERK1

any of the other 14-3-3 family members were found, suggesting a highly specific interaction between *At*SERK1 and GF14λ.

Control experiments in yeast showed that full length GF14λ, besides interacting with *At*SERK1^{kinase}, weakly interacts with *At*SERK1$^{\Delta SS}$, but does not interact with the *At*SERK1 extracellular domain encoded by *At*SERK1^{LRR} (results not shown). We could not perform the reciprocal interaction experiment, because GF14λ auto-activates reporter gene expression when fused to LexA. This was also observed for other 14-3-3 proteins and is probably due to the acidic nature of 14-3-3 proteins (Wu, 1997; Wang, 1999). The observed weak interaction between GF14λ and *At*SERK1$^{\Delta SS}$, when compared with the interaction between GF14λ and *At*SERK1^{kinase}, may be due to aberrant folding of the complete *At*SERK1 membrane protein in the yeast nucleus.

*At*SERK1 is able to transphosphorylate GF14λ as well as GF14λ$^{39-248}$

It has been reported that some protein kinases can use a 14-3-3 protein as substrate (Lu et al., 1994; van der Hoeven et al., 2000). In order to further substantiate the interaction between *At*SERK1 and GF14λ, phosphorylation assays were performed to determine whether *At*SERK1 can use GF14λ as substrate. According to the prediction of phosphorylation sites by NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos) there are no putative serine or threonine phosphorylation sites within the N-terminal 38 amino acids of GF14λ. However, *At*SERK1 is able to phosphorylate on tyrosine residues of the common substrate myelin basic protein (Shah et al., 2001b). Within the N-terminal 38 amino acids of GF14λ the program identified two putative tyrosine phosphorylation sites. Therefore, we determined whether *At*SERK1 is able to transphosphorylate GF14λ and whether this phosphorylation is restricted to residues 1-38 of GF14λ.

For the phosphorylation assays, *At*SERK1^{kinase}, GF14λ and GF14λ$^{39-248}$ were expressed as C-terminal fusion proteins with glutathione-S-transferase (GST) in *E. coli* and purified by affinity chromatography on glutathione resin. GST was cleaved from GF14λ by thrombin. The purified GST, GST-*At*SERK1^{kinase} and GF14λ proteins show molecular masses of 27 kD, 66 kD and 31 kD respectively (Figure 1). GF14λ$^{39-248}$ with a calculated molecular mass of 24 kD should run faster than GF14λ (28 kD) but on 12%

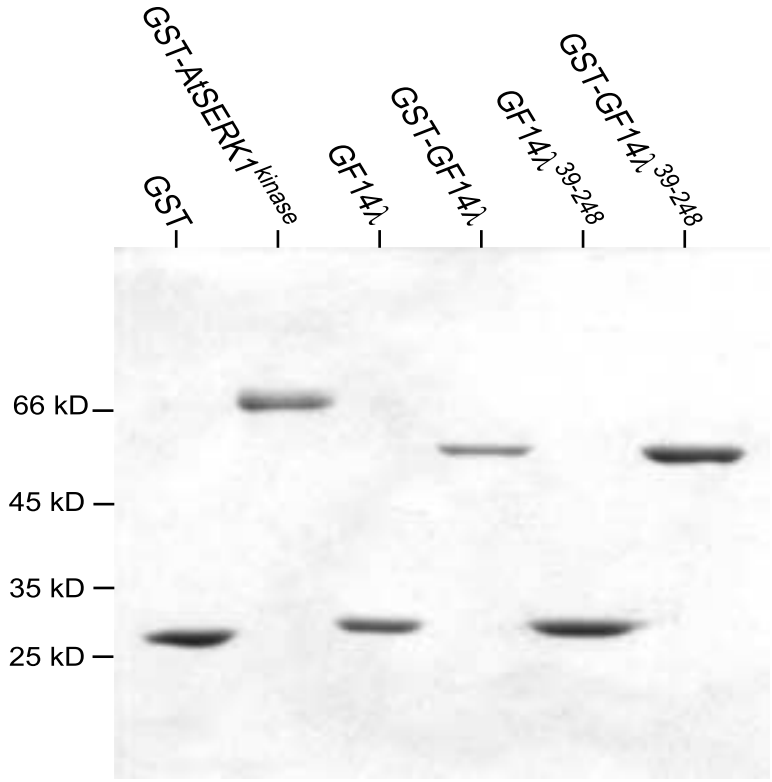

Figure 1: Overview of the proteins used in this study on a 12% SDS-PAGE gel stained with Coomassie Brilliant Blue. Proteins were expressed in *E. coli* as translational fusions to glutathione-S-transferase and purified by affinity chromatography on glutathione resin. GST was cleaved from GF14λ and GF14λ$^{39\text{-}248}$ by thrombin.

SDS-PAGE it appears to run at the same molecular size as the complete GF14λ protein (Figure 1).

Phosphorylation assays were performed by incubating the GF14λ and GF14λ$^{39\text{-}248}$ proteins in the presence of GST-*At*SERK1^{kinase} and [γ-^{32}P]ATP. The reaction was stopped by adding protein sample buffer and the samples were subjected to SDS-PAGE and subsequent autoradiography. The results are shown in Figure 2. Besides autophosphorylation, GST-*At*SERK1^{kinase} can transphosphorylate both GF14λ and GF14λ$^{39\text{-}248}$ with comparable intensity. The kinetics of GF14λ phosphorylation by *At*SERK1^{kinase} was compared to *At*SERK1^{kinase} autophosphorylation in a time course (Fig. 2b). In a standard assay with 10 mM $MgCl_2$ and 500 ng *At*SERK1^{kinase} autophosphorylation is complete within 30 min (Shah et al., 2001b). In the time-interval we used, GF14λ is being transphosphorylated as soon as *At*SERK1 is autophosphorylated.

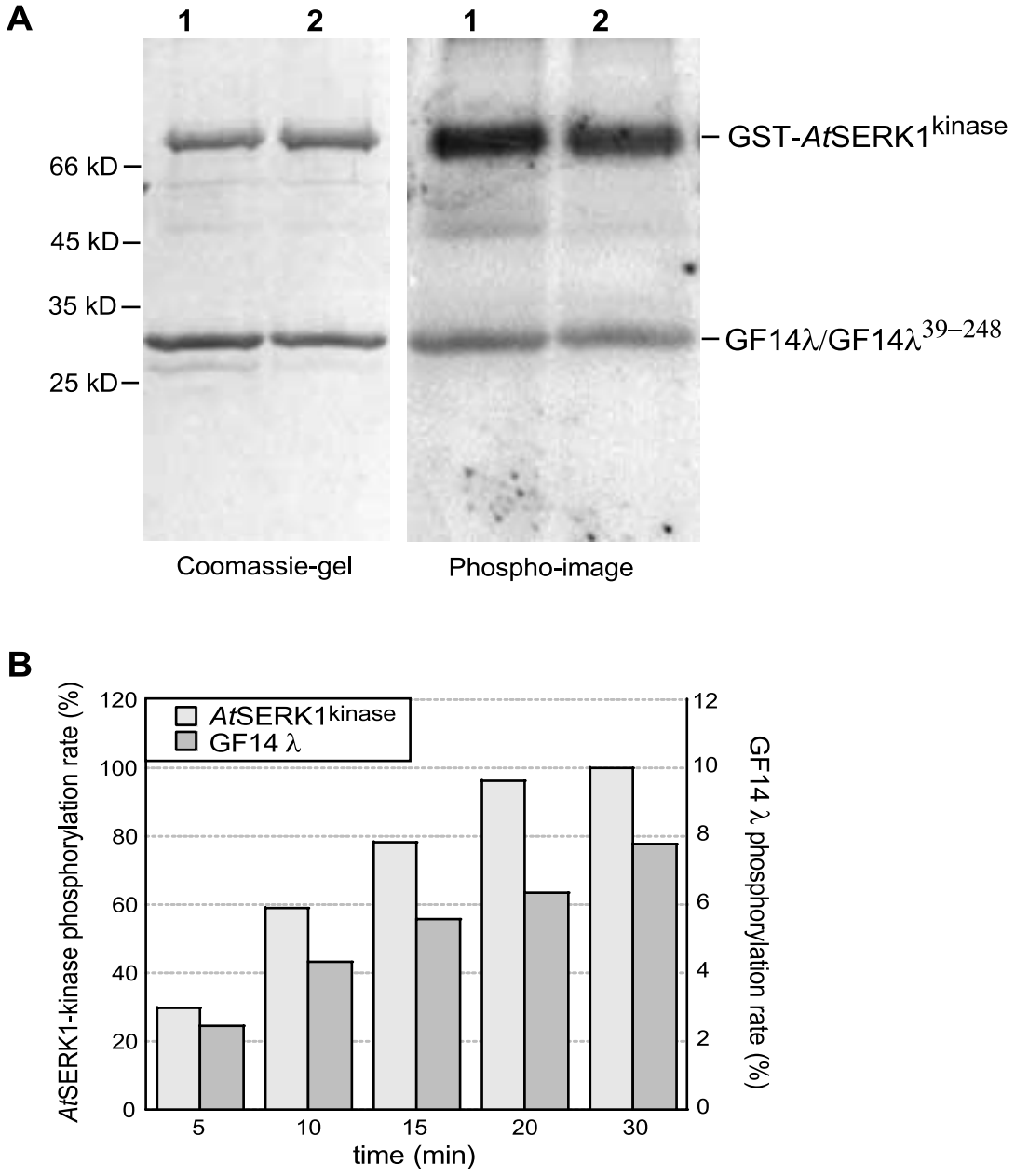

Figure 2: **A**: *In vitro* phosphorylation assay of GF14λ and GF14λ$^{39\text{-}248}$ by GST-*At*SERK1. Both GF14λ and GF14λ$^{39\text{-}248}$ are phosphorylated by *At*SERK1^{kinase} when incubated together at 30°C in the presence of ATP ([γ-^{32}P]ATP). **Lane 1**: GST-*At*SERK1^{kinase} with GF14λ, **Lane 2**: GST-*At*SERK1^{kinase} with GF14λ$^{39\text{-}248}$. **B**: Time series of the GST-*At*SERK1 and GF14λ phosphorylation rate after *in vitro* phosphorylation.

The observed GF14λ phosphorylation is *At*SERK1-dependent as no phosphorylation was observed after incubating GF14λ or GF14λ$^{39-248}$ with [γ-^{32}P]ATP in the absence of *At*SERK1^{kinase} (results not shown). These results show that *At*SERK1 is able to phosphorylate GF14λ and that phosphorylation is not restricted to the N-terminal 38 residues of GF14λ.

GF14λ interacts with the *At*SERK1 kinase domain *in vitro*

In the kinase domain of *At*SERK1 we found a putative 14-3-3 binding motif surrounding Ser-394, with the sequence RPPS^{394}QPP. Many of the proteins that interact with a 14-3-3 protein contain a 14-3-3 binding motif based on the consensus motif [RK]X$_{(2,3)}$pSXP, where X stands for any amino acid and p denotes a phosphorylated amino acid (Muslin et al., 1996; Yaffe et al., 1997; Sehnke et al., 2002). A three dimensional model of the *At*SERK1 kinase domain was made (Fig. 4; Shah et al., 2001b),

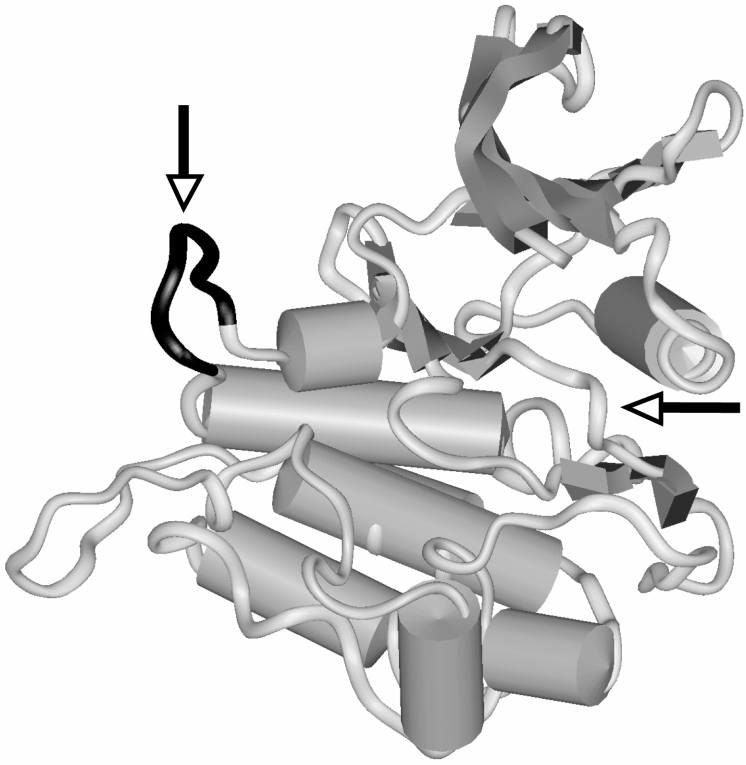

Figure 3: Homology based model of the kinase domain of *At*SERK1. The kinase domain of the human insulin receptor (accession nr. P06213) was used as template (Shah et al., 2001b). The putative 14-3-3 binding sequence is located in a loop extending from the kinase and shown in black. The active site of the kinase and the 14-3-3 binding sequence are both marked by an arrow.

based on homology with the kinase domain of the insulin receptor (accession nr. P06213; Hubbard et al., 1994). According to this model, the putative 14-3-3 binding sequence of *At*SERK1 is present in a loop extending from the kinase domain. The same loop is present in the insulin receptor although it does not contain a 14-3-3 binding motif and does not bind 14-3-3 (Furlanetto et al., 1997).

To confirm the existence of a direct interaction between *At*SERK1 and GF14λ, GST pull-down assays were performed. The results of the pull-down assay employing GST-*At*SERK1^{kinase} and GF14λ are shown in Figure 4. GF14λ interacts with GST-*At*SERK1^{kinase} (Fig. 4, lane 1), but not with GST alone (Fig. 4, lane 5). Phosphorylation of the serine residue in the 14-3-3 binding domain has been shown to be important for the interaction with a 14-3-3 protein (Muslin et al., 1996; Yaffe et al., 1997). Therefore, pull-down experiments with phosphorylated *At*SERK1^{kinase} were performed to investigate whether the phosphorylation status of *At*SERK1 affects the interaction with GF14λ. GST-*At*SERK1^{kinase} was phosphorylated by incubating the protein with ATP at phosphorylating conditions. Autophosphorylation activity of the *At*SERK1 kinase domain was verified by incubating the GST-*At*SERK1^{kinase} protein in the presence of [γ-^{32}P]ATP. When the pull down assay is performed with the full-length GF14λ protein and phosphorylated GST-*At*SERK1^{kinase} protein, more GF14λ was bound to phosphorylated GST-*At*SERK1^{kinase} (Fig. 4, lane 2) compared to non-phosphorylated GST-*At*SERK1^{kinase} (Fig.4, lane 1). Through comparison of the band intensities on the Western blot we estimated that interaction of GF14λ with phosphorylated *At*SERK1 is about five fold higher compared to non-phosphorylated *At*SERK1.

The same experiments were repeated for GF14λ$^{39-248}$. Interaction between non-phosphorylated GST-*At*SERK1^{kinase} and either GF14λ or GF14λ$^{39-248}$ is observed with equal intensities (Fig.4, lane 1 and 3). When the pull down assay is performed using phosphorylated GST-*At*SERK1^{kinase} (Fig. 4, lane 4) no difference was observed in the ability to bind GF14λ$^{39-248}$ compared to non-phosphorylated GST-*At*SERK1^{kinase} (Fig. 4, lane 3). From these results we conclude that there is a phosphorylation-independent interaction between GF14λ and the *At*SERK1 kinase domain as well as a phosphorylation-dependent interaction. This phosphorylation-dependent interaction requires the first 38 amino acids of the GF14λ protein.

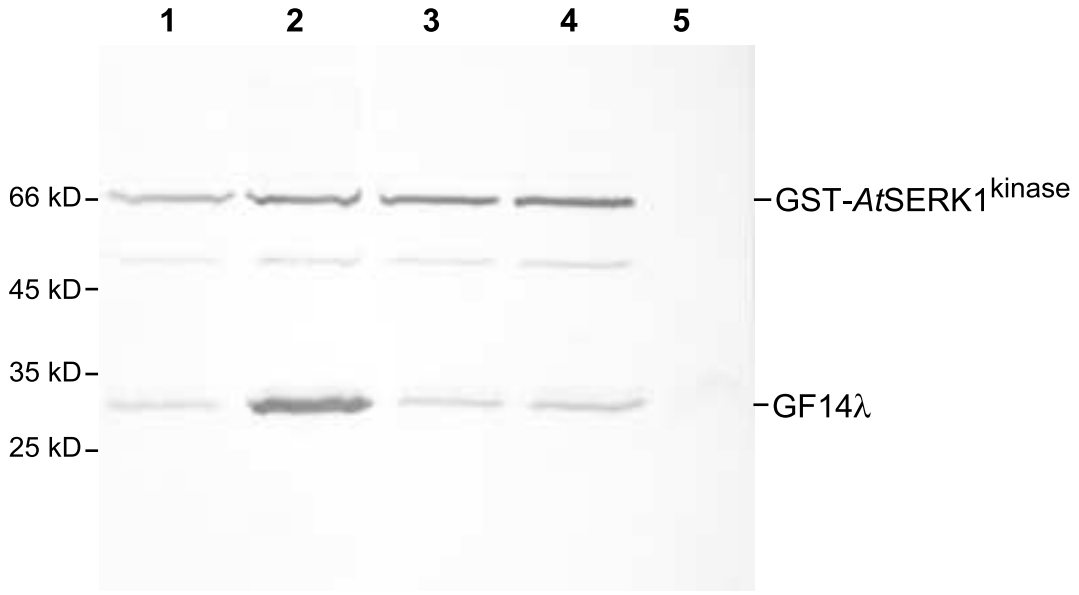

Figure 4: GST pull down assays for the binding of GF14λ and GF14λ$^{39-248}$ to the kinase domain of *At*SERK1. GF14λ was incubated with immobilised GST or GST-*At*SERK1^{kinase}. Proteins bound to the sepharose beads are pelleted, washed, subjected to SDS-PAGE and detected by Western blotting using αGF14λ antiserum. These antibodies also recognise besides GF14λ GST-*At*SERK1^{kinase}. **Lane 1-2**: Pull down assays with GST-*At*SERK1^{kinase} and GF14λ under non-phosphorylating and phosphorylating conditions. **Lane 3-4**: Pull down assays with GST-*At*SERK1^{kinase} and GF14λ$^{39-248}$ under non-phosphorylating and phosphorylating conditions. **Lane 5**: Pull down assay with GF14λ and GST.

GF14λ interacts with *At*SERK1 *in vivo*

*At*SERK1 is highly expressed in ovule primordia, the entire female gametophyte and in early embryos (Hecht et al., 2001). Low expression has been detected in adult vascular tissue. One criterion for GF14λ-*At*SERK1 interaction *in vivo* is that GF14λ expression has to coincide with *At*SERK1 expression. RT-PCR showed that the *GF14λ* gene is constitutively expressed (results not shown). Therefore GF14λ expression is likely to overlap with *At*SERK1 expression.

We tested whether the interaction between *At*SERK1 and GF14λ occurs in *Arabidopsis* cells. To this end we isolated proteins from tissue-cultured *Arabidopsis* cells (Menges and Murray, 2002) and performed immunoprecipitations with *At*SERK1-specific antibodies (αSP1) or αGF14λ antiserum (αGF14λ). The presence of 14-3-3 proteins in

tissue-cultured *Arabidopsis* cells was determined by Western analysis using αGF14λ antiserum (Fig. 5, lane 1). The αGF14λ antiserum recognises two proteins that might represent different members of the 14-3-3 family. The 14-3-3 protein family in *Arabidopsis* contains proteins with a calculated molecular weight ranging from 27.9 kD (GF14λ and GF14κ) to 30.1 kD (GF14υ) (Rosenquist et al., 2001). When used in an immunoprecipitation assay, the αGF14λ antiserum precipitates a protein corresponding to the lower molecular weight band in lane 1 (Fig. 5, lane 2). Probably the αGF14λ antiserum can only detect other members of the *Arabidopsis* 14-3-3 family when they are denatured. The lower molecular weight protein also co-precipitates with *At*SERK1 using the *At*SERK1 specific antibodies αSP1 (Fig. 5, lane 3). From these results we conclude that *At*SERK1 associates with a member of the 14-3-3 family *in vivo*. In comparison with

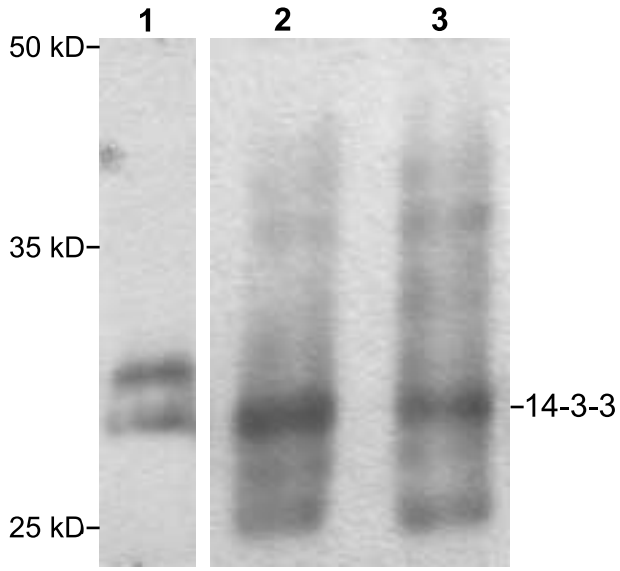

Figure 5: *In vivo* interaction between GF14λ and *At*SERK1. **Lane 1**: Direct Western analysis on protein extract isolated from tissue cultured *Arabidopsis* cells using αGF14λ antiserum to detect the presence of 14-3-3 protein. For the immunoprecipitation assays, immobilised αGF14λ antiserum or *At*SERK1 αSP1 was incubated with the protein extract and immune-complexes were pelleted, washed, subjected to SDS-PAGE and detected by Western blotting using αGF14λ antiserum. **Lane 2**: Immunoprecipitation assay with αGF14λ antiserum. **Lane 3**: Immunoprecipitation assay with αSP1.

the other members of the *Arabidopsis* 14-3-3 family, GF14λ is one of the smallest members (Rosenquist et al., 2001). In addition, no other member of the 14-3-3 family than GF14λ was recovered from the yeast interaction screen. Therefore we suggest that the interacting protein is indeed GF14λ.

Co-localisation of *At*SERK1 and GF14λ and confirmation of the interaction *in vivo*

The occurrence of an *in vivo* interaction and co-localisation of GF14λ and *At*SERK1 was also determined at the cellular level in plant protoplasts. For this we fused the entire coding region of GF14λ to the C-terminus of CFP and YPF (CFP-GF14λ and YFP-GF14λ). The entire coding region of *At*SERK1 was fused to the N-terminus of YFP (*At*SERK1-YFP). The fusion proteins were then transiently expressed in cowpea mesophyll protoplasts. Confocal Laser Scanning Microscopy (CLSM) was used to image cells expressing the fusion proteins. Figure 6 shows the localisation of fluorescent signals for *At*SERK1-YFP (Fig. 6a-c), YFP-GF14λ (Fig. 6d-f) and CFP-GF14λ together with *At*SERK1-YFP (Fig. 6g-j). For *At*SERK1-YFP it has previously been shown that a fluorescent signal can be detected at the plasma membrane and to a lesser extent at endomembranes (Shah et al., 2001a). Figure 6a and 6c confirm the previously noted location of the *At*SERK1 protein. For YFP-GF14λ, fluorescence can be detected in the nucleus and the cytosol. Nuclear localisation of YFP-GF14λ was confirmed by co-localisation of YFP-GF14λ fluorescence and DAPI staining (Fig. 6k-m). 14-3-3 proteins do not contain any obvious cellular localisation signal, although many differences in cellular organisation exist among the 14-3-3 proteins. For example the *Arabidopsis* GF14κ isoform is found in the nucleus and the plasma membrane region, and GF14υ is found in the cytoplasm and in the chloroplast stroma (Sehnke et al., 2002). To examine the subcellular localisation of *At*SERK1 in the presence of GF14λ, we co-expressed CFP-GF14λ with *At*SERK1-YFP in protoplasts. The superimposed image (Fig. 6j) shows co-localisation of the proteins at the plasma membrane. No obvious difference in localisation of either protein is observed upon co-expression. The occurrence of a molecular interaction between *At*SERK1-YFP and CFP-GF14λ was studied by measuring the Fluorescence Resonance Energy Transfer (FRET) between the CFP (donor)

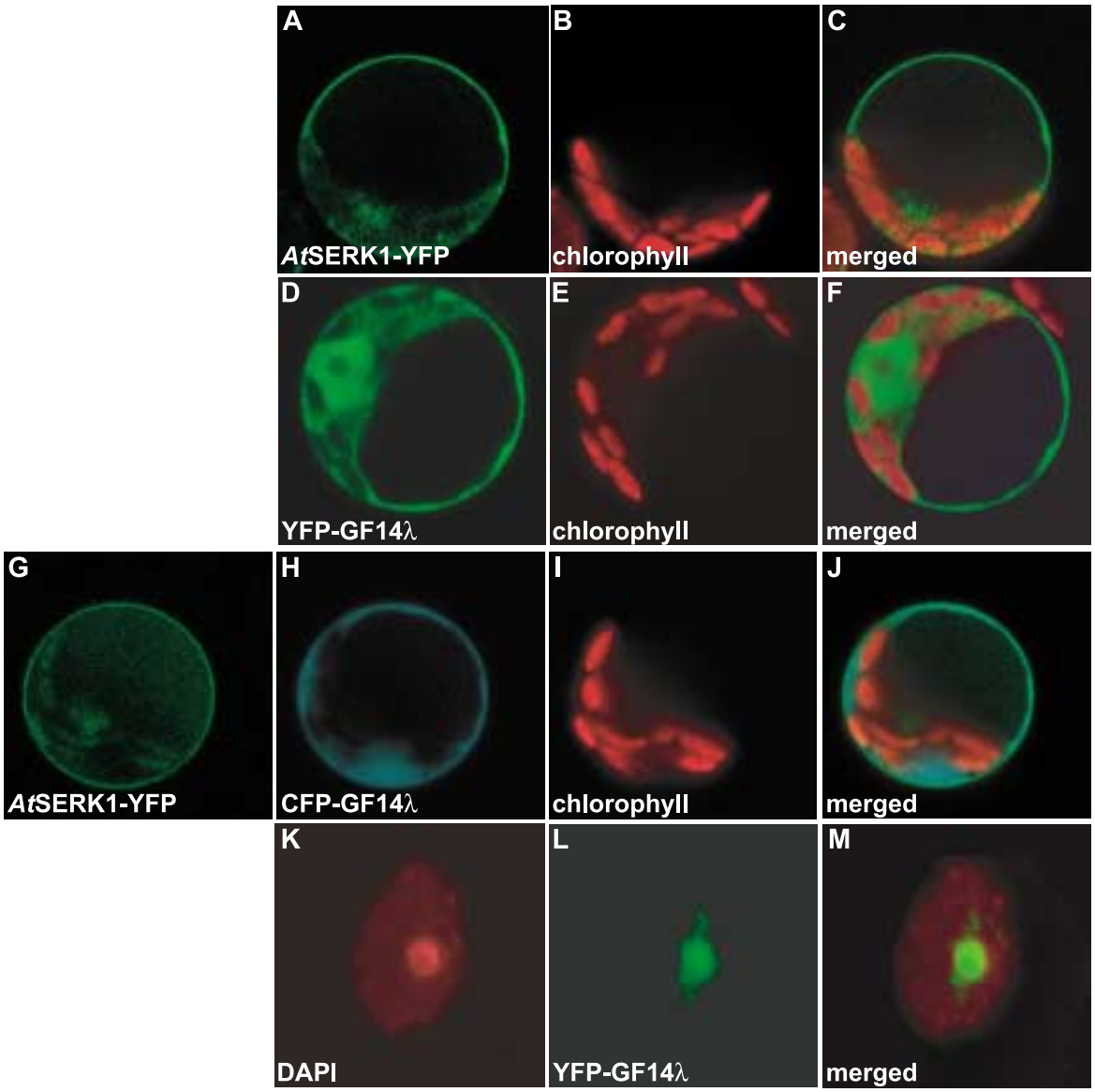

Figure 6: Cellular localisation of fluorescently labelled *At*SERK1 and GF14λ in cowpea mesophyll protoplasts. All images were obtained by Confocal Laser Scanning Microscopy (CLSM). Chlorophyll auto-fluorescence is shown in red, YFP fluorescence is shown in green and CFP fluorescence is shown in cyan. **A-C**: Cellular localisation of *At*SERK1-YFP. The superimposed images of A and B result in C. **D-F**: Cellular localisation of YFP-GF14λ. The superimposed images of D and E result in F. **G-J**: Co-localisation of *At*SERK1-YFP and CFP-GF14λ. The superimposed images of G, H and I result in J. **K-M**: DAPI staining in a fixated protoplast (K) and localisation of YFP-GF14λ fluorescence in the nucleus of the same protoplast (L). The superimposed images of K and L result in M.

and YFP (acceptor) fluorophores. FRET occurs when the fluorophores interact based on dipole-dipole interaction, which is only possible at close proximity (5 nm for CFP and YFP). In the case of FRET, the CFP fluorescence will be quenched and the YFP fluorescence will be increased, which indicates a molecular interaction of the proteins that are fused to the fluorophores. FRET was measured using Fluorescence Spectral Imaging Microscopy (FSPIM) as a detection method. Spectral images were taken from small regions of protoplasts co-expressing *At*SERK1-YFP and CFP-GF14λ at approximately equal expression levels and fluorescence emission spectra were generated. As a control spectral images were taken from protoplasts expressing only CFP-GF14λ. Figure 7 shows the emission spectra of CFP and YFP fluorescence in protoplasts. The peak at 480 nm corresponds to CFP emission and the peak at 520 nm to YFP fluorescence. The ratio of 480 to 520 nm emission intensity acts as an indicator of the extent of FRET. In order to compare the different measurements, the CFP emission peak at 480 nm is normalised to 1. The emission intensity ratios deduced from measurements at several sites in the cell, but not at the plasma membrane, of 35 different protoplasts co-expressing *At*SERK1-YFP and CFP-GF14λ is close to 1.2 (Fig. 7a). This is comparable with measurements obtained from 10 protoplasts only expressing CFP-GF14λ. The emission intensity ratios obtained from measurements at the plasma membrane of the 35 different protoplasts co-expressing *At*SERK1-YFP and CFP-GF14λ showed that there was an increase in YFP fluorescence in 46% of the measurements performed with a maximum of 1.6 (Fig. 7b). The FRET energy transfer efficiency was calculated from the emission intensity ratios between Fig. 7a and 7b and is roughly 23%. These results show that at the plasma membrane *At*SERK1 and GF14λ can interact. These results were confirmed using Acceptor Photobleaching (APB) (Wouters and Bastiaens, 1999). APB was performed by bleaching the YFP fluorophore in a defined region. Images of the protoplast were taken before and after the bleaching event. When YFP is inactivated due to bleaching, the CFP fluorescence energy can no longer be transferred to YFP in the case of FRET between CFP-GF14λ and *At*SERK1-YFP. This will result in an increase of the 480 nm CFP emission signal and a decrease of the 520 nm YFP emission signal. When we bleached the YFP fluorescence signal at a small portion of the plasma membrane where CFP-GF14λ and *At*SERK1-YFP co-localise, an increase in CFP intensity was observed (Fig. 7c). As a control we bleached YFP at a region in the cell where the two proteins do not interact (Fig. 7d). This did not result in an increase of CFP

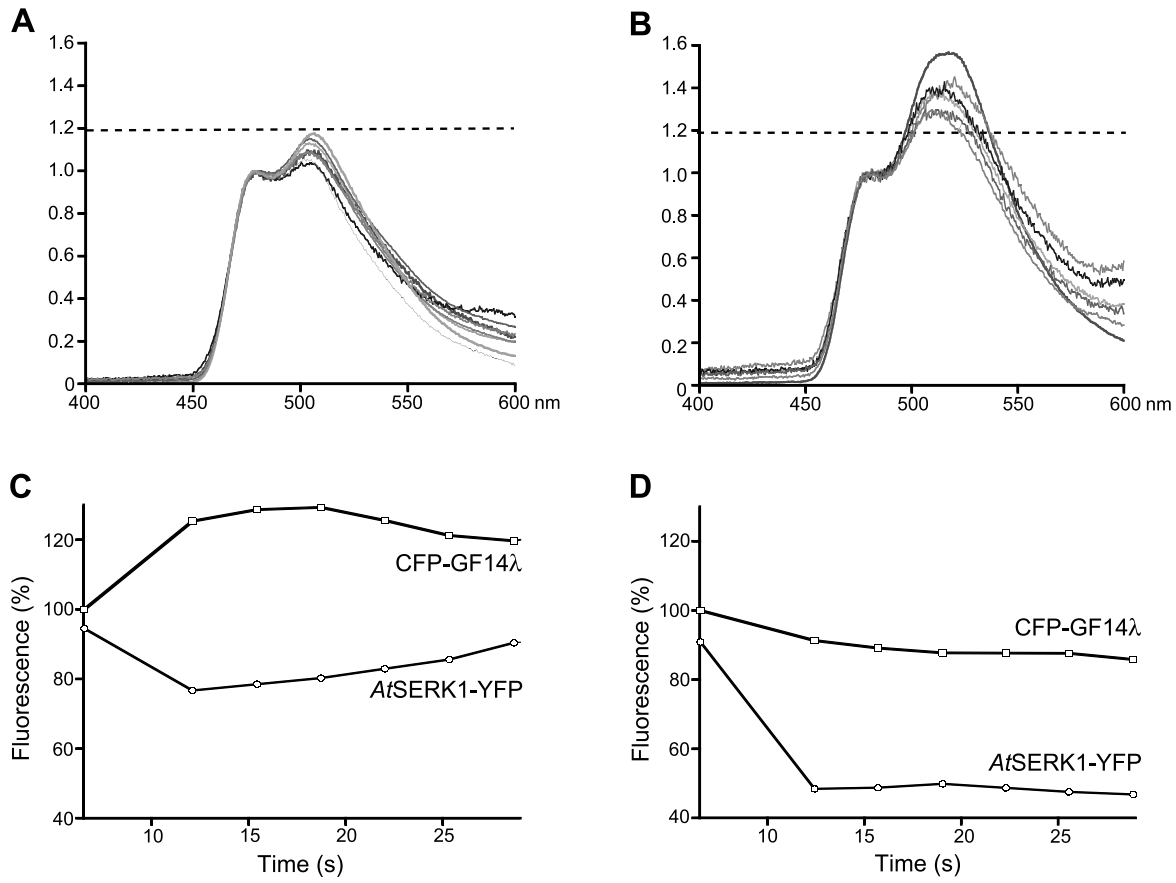

Figure 7: FRET-SPIM and FRET-APB analysis of protoplasts expressing GF14λ and *At*SERK1 fused to CFP and YFP. For the FRET-SPIM analysis fluorescent intensities were normalised to 1.0 at 480 nm for comparison. Emission spectra from protoplasts co-expressing *At*SERK1-YFP and CFP-GF14λ obtained at several sites in the cell where the two proteins do not interact (**A**) and at the plasma membrane where they do interact (**B**). Typical FRET-APB analysis of protoplasts co-expressing *At*SERK1-YFP and CFP-GF14λ at the plasma membrane (**C**) and at an intracellular site where they do not interact (**D**). For comparison of the figures, the fluorescent counts were recalculated to the percentage of fluorescence. The original intensities of CFP-GF14λ and *At*SERK1-YFP fluorescence in Fig. C were at 2025 and 1840 counts respectively and in Fig. D at 840 and 790 counts respectively.

intensity. To compare the changes in fluorescence intensity between Figure 7c and 7d, the fluorescent counts were converted to fluorescent percentages. Energy transfer efficiencies can be calculated based on the release of quenching of donor fluorescence due to FRET, measured by comparing the intensity of donor fluorescence before and after complete

photobleaching of the acceptor (Bastiaens et al., 1996). For *At*SERK1-YFP and CFP-GF14λ the FRET energy transfer efficiency is roughly 20%. However, the fluorescence of *At*SERK1-YFP was not completely bleached, therefore the actual energy transfer will be higher. The FRET efficiency using acceptor photobleaching or FSPIM as a detection method is comparable. Therefore, we conclude that the interaction between GF14λ and *At*SERK1 as first observed in yeast, is feasible at the plasma membrane in plant cells as well using two different approaches.

Discussion

A yeast two-hybrid screen was performed to identify proteins interacting with the Somatic Embryogenesis Receptor-like Kinase 1 (*At*SERK1). As a result of this screen GF14λ, a member of the *Arabidopsis* family of 14-3-3 proteins, was found to interact with the kinase domain of *At*SERK1. GF14λ is the only *Arabidopsis* 14-3-3 isoform that was found as a putative interacting protein in two independent yeast two-hybrid screens using *At*SERK1 as bait. The interaction between *At*SERK1 and GF14λ was confirmed *in vitro* by pull down assays. With immunoprecipitation assays we showed that a 14-3-3 protein co-precipitates with *At*SERK1, indicating that these two proteins interact in *Arabidopsis* plant cells. The *in vivo* interaction was confirmed at the cellular level by a molecular interaction between fluorescently labelled GF14λ and *At*SERK1 at the plasma membrane in protoplasts. Taken together we conclude that the interaction between *At*SERK1 and GF14λ is indeed a specific one.

GF14λ belongs to the 14-3-3 protein family which consists of highly conserved 28-31 kD proteins. 14-3-3 proteins are broadly expressed in a wide range of eukaryotes and at least 13 different isoforms have been identified in *Arabidopsis* (Rosenquist et al., 2001). Interaction of plant 14-3-3 proteins with kinase domains was found for the calcium-dependent protein kinases CDK1 and CDPK2 (Camoni et al., 1998; Moorhead et al., 1999) and for the wheat protein kinase WPK4 (Ikeda et al., 2000). So far no interaction with a transmembrane-located receptor kinase has been reported in plants.

Binding of a 14-3-3 protein can alter the confirmation of the interacting protein, resulting in a change in activity of that protein. An example is the plant plasma membrane

H^+-ATPase that is activated upon 14-3-3 binding at the C-terminus by displacement of the auto-inhibitory domain, thus forming an H^+-ATPase-14-3-3 complex that can be stabilised by the fungal toxin fusicoccin (Svennelid et al., 1999). The *Arabidopsis* calcium-dependent kinase CDK1 is stimulated by 14-3-3 proteins after Ca^{2+}-dependent activation (Camoni et al., 1998). 14-3-3 proteins can also function as a competitive inhibitor preventing the binding of other proteins or as a scaffold protein by promoting the assembly of signalling complexes. Binding of 14-3-3 proteins to the phosphorylated mammalian cell death protein BAD prevents apoptosis by prohibiting BAD from heterodimerising to the Bcl-XL family of apoptosis inducing proteins. (Tan et al., 2000). In addition, several human 14-3-3 isoforms can facilitate coupling of protein kinase C-ζ (PKC-ζ) to Raf-1 (van der Hoeven et al., 2000). In plants, another calcium-dependent kinase (CDPK2) that has been purified from cauliflower binds to 14-3-3 and is able to phosphorylate nitrate reductase (Moorhead et al., 1999), thereby creating a 14-3-3 binding site. No effect of 14-3-3 binding on activity of the kinase was observed, but 14-3-3 proteins are found to act as an inhibitor of nitrate reductase (Pigaglio et al., 1999). 14-3-3 binding can also affect cellular translocation of the target protein by blocking a nuclear localisation or export signal. For example, interaction of 14-3-3 with the yeast cell cycle protein phosphatase Cdc25 may negatively regulate Cdc25 function by inhibiting nuclear import of Cdc25, thereby preventing mitotic progression (Lopez-Girona et al., 2001). Taken together, it is becoming clear that these proteins have a regulatory function via direct protein-protein interactions.

Many of the interactions with a 14-3-3 protein are mediated through binding of the 14-3-3 protein to a sequence specific 14-3-3 binding domain. Motifs are based on the consensus motif [RK]$X_{(2,3)}$pSXP, where X stands for any amino acid and p denotes a phosphorylated amino acid (Muslin et al., 1996; Yaffe et al., 1997; Sehnke et al., 2002). Phosphorylation of the serine residue in the 14-3-3 binding domain has been shown to be important for the interaction with a 14-3-3 protein (Muslin et al., 1996; Yaffe et al., 1997). However, some 14-3-3 binding proteins have been identified whose sequences deviate significantly from the motifs mentioned above, or do not require phosphorylation for binding (Jaspert and Oecking, 2002). In the kinase domain of *At*SERK1 we identified the sequence RPPS^{394}QPP as putative 14-3-3 binding motif. GST pull down assays showed that binding of full-length GF14λ increased five fold upon phosphorylation of *At*SERK1.

Phospho-amino acid analysis of HCl-hydrolysed autophosphorylated *At*SERK1 showed that *At*SERK1^{kinase} is not only autophosphorylated on threonine residues, but also to a lesser extent on serine residues (Shah et al., 2001b). In the *At*SERK1 activation loop (A-loop) no serine residue is present. The serine phosphorylation that was observed in the phospho-amino acid analysis of autophosphorylated *At*SERK1 protein might therefore represent Ser^{394} in the 14-3-3 binding domain.

Under native conditions 14-3-3 proteins predominantly exist as dimers (Wu et al., 1997). The crystal structure of the 14-3-3 dimer was resolved for the human isoforms 14-3-3ζ and 14-3-3τ (Liu et al., 1995, Xiao et al., 1995). The 14-3-3 dimer forms an U-shaped structure. The N-terminal domains of the two 14-3-3 monomers form the dimerisation interface and the floor of the cleft; the C-terminal domains form the sides of the channel. Each monomer produces a cleft that is sufficient in size and shape to accommodate the interaction with a phosphorylated peptide from a binding partner (Yaffe et al., 1997). The residues that line the interior of the protein-binding channel, including many of the residues that form direct ligand contacts, are highly conserved between the different isoforms. More variable residues are distributed over the outer surface of the protein. Part of the N-terminal domain containing the first two α-helices, is deleted in GF14λ$^{39-248}$. We showed that for phosphorylation-dependent interaction with the kinase domain of *At*SERK1 the complete 14-3-3 protein is needed. A complete GF14λ protein may provide all the residues that are in contact with the interacting protein, thereby influencing the stability of the interaction, or increasing the affinity for the 14-3-3 binding sequence on *At*SERK1. In addition, the formation of a 14-3-3 dimer may be important for an optimal interaction with *At*SERK1 (Yaffe, 2002).

14-3-3 isoforms are very homologous to each other and the residues involved in ligand binding are highly conserved. However, there are differences in binding specificity. It was shown by surface plasmon resonance analysis that there are large differences in affinity between nine *Arabidopsis* 14-3-3 isoforms and a target peptide representing the binding motif present in the C-terminus of the plant plasma membrane H^+-ATPase (Rosenquist et al., 2000). We showed that *At*SERK1 is able to transphosphorylate GF14λ. Phosphorylation of the animal 14-3-3β and 14-3-3ζ isoforms by casein kinase 1α (CK1α) affects their ability to bind specific targets, such as Raf kinase (van der Hoeven et al., 2000). Phosphorylation of plant 14-3-3 proteins has been described as well but a role was

not identified. The *Arabidopsis* GF14ω 14-3-3 isoform is phosphorylated by endogenous membrane kinase activity (Lu et al., 1994) and an oat 14-3-3 protein was also found to be phosphorylated (Korthout and de Boer, 1998). Phosphorylation of 14-3-3 adds a charged phoshate-group to the protein, which can influence the specificity of interaction with its current interactor and/or with another interactor. It is possible that phosphorylated GF14λ is able to interact with another component of the *At*SERK1 signalling complex. *At*SERK1 was shown to form homodimers in plant protoplasts (Shah et al., 2001a). Although GF14λ seems to be phosphorylated by *At*SERK1 at the same rate as *At*SERK1 autophosphorylation, there may be a role for GF14λ as adaptor protein stabilising the putative *At*SERK1 homo- or heterodimeric status.

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Chapter 4

*At*SERK1 receptor-like kinase signalling involves a protein complex containing *At*CDC48

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Abstract

Leucine-rich repeat containing transmembrane receptor-like kinases (LRR-RLKs) are important components of plant signal transduction. The Somatic Embryogenesis Receptor-like Kinase 1 (*At*SERK1) is an RLK containing five leucine-rich repeats. *At*SERK1 is proposed to participate in a signal transduction cascade involved in ovule and embryo development. In a yeast two-hybrid screening, *At*CDC48 was found as a putative *At*SERK1 interacting protein. *In vitro* binding assays showed that *At*CDC48 preferentially binds to the phosphorylated *At*SERK1 kinase domain. Upon binding, *At*SERK1 is able to transphosphorylate *At*CDC48 on a threonine within the C-terminal 62 amino acid residues. In yeast, *At*CDC48 is also able to interact with the *At*SERK1-binding partners GF14λ and KAPP. Co-immunoprecipitation studies revealed that in *Arabidopsis* cells *At*CDC48 is in complex with *At*SERK1 and a 14-3-3 protein.

Introduction

Receptor-like protein kinases (RLKs) are key elements in many signal transduction chains. RLKs consist of an extracellular domain, a transmembrane domain and an intracellular catalytic kinase domain. We have previously identified the *Arabidopsis* Somatic Embryogenesis Receptor-like Kinase (*At*SERK), which is an RLK containing a leucine zipper, five leucine-rich repeats, a single transmembrane domain and a functional serine-threonine kinase (Hecht et al., 2001; Shah et al., 2001b). *At*SERK1 is highly expressed during female gametophyte development and early embryogenesis (Hecht et al., 2001). Ovule primordia, the entire female gametophyte and early embryos show high *At*SERK1 expression while low expression has been detected in adult vascular tissue. Ectopic expression of *At*SERK1 does not result in obvious phenotypes in *Arabidopsis* plants but increases the efficiency of somatic embryo formation in culture. Based on its specific expression pattern and its involvement in embryo formation *At*SERK1 is proposed to participate in an uncharacterised signal transduction cascade involved in ovule and embryo development (Hecht et al., 2001). Previous work has shown that *At*SERK1 interacts *in vitro* and *in vivo* with the kinase-associated protein phosphatase (KAPP; Shah et al., 2002).

To identify additional components of the *At*SERK1 signalling complex, we used the yeast two-hybrid system to screen for proteins interacting with the kinase domain of *At*SERK1. Three different proteins were found as valid *At*SERK1 interacting proteins encoding the 14-3-3 protein GF14λ, the AAA-ATPase *At*CDC48 and a putative RNA binding protein (RBP) (Chapter 2). In this chapter we will analyse the interaction between *At*CDC48 and *At*SERK1. Based on interaction studies in yeast we also show that, besides binding to the kinase domain of *At*SERK1, *At*CDC48 can interact with GF14λ and KAPP. This shows analogy to the mammalian CDC48 homologue p97/VCP, which can be phosphorylated by the JAK-2 kinase and dephosphorylated by the phosphatase PTPH1 that associates with a 14-3-3 protein (Zhang et al., 1997; Zhang et al., 1999).

Materials and methods

Yeast two-hybrid interaction tests

The DuplexA yeast two-hybrid system (Origene Technologies Inc.) was used to verify the interaction between *At*SERK1 (Genbank accession nr. A67827) and *At*CDC48. The *At*SERK1 kinase domain (*At*SERK1^{kinase}, a.a. 266-625), the LRR domain (*At*SERK1^{LRR}, a.a. 26-234) and the mature *At*SERK1 protein minus the signal sequence (*At*SERK1$^{\Delta SS}$, a.a. 31-625) were cloned in the bait vector pEG202 and in the prey vector pJG4-5 (Origene Technologies Inc.) as translational fusions to LexA and B42 respectively (Shah et al., 2001a). The yeast two-hybrid library plasmid pJG4-5-*At*CDC48$^{749-809}$ was co-transformed with pEG202-*At*SERK1^{kinase}, pEG202-*At*SERK1^{LRR} or pEG202-*At*SERK1$^{\Delta SS}$ into EGY48, plated on selective induction plates (his$^-$, ura$^-$, trp$^-$) containing Xgal and grown for two days at 30°C. A comparable experiment was done with a longer part of *At*CDC48 encoded by EST cDNA clones 104O24 (accession nr. T22005) and 180L9 (accession nr. H36923). EST cDNA clones were obtained from the *Arabidopsis* Biological Resource Centre. Interaction of the EST encoded peptides with *At*SERK1 was also tested using *At*SERK1 cloned in pJG4-5 as prey, and the EST encoded peptides cloned in pEG202 as bait. To make in frame fusions a 900 bp cDNA fragment of EST 104O24 and a 1413 bp cDNA fragment of EST 180L9, encoding *At*CDC48$^{549-809}$ and *At*CDC48$^{386-809}$ respectively, were amplified by PCR from the corresponding λZiplox plasmid using a gene-specific forward primer (5'-CCG CTC GAG CTT CTG ACA ATG TGG-3', 5'-CCG CTC GAG GGA CGT CCT GAA GTT CTG AGG-3') and a reverse primer located on vector λZiplox (5'-CCG CTC GAG GGC CAG TGA ATT GAA TTT AGG-3'). Both primers contained an *XhoI* site. PCR products were digested and cloned into the *XhoI* sites of pEG202 and pJG4-5.

The yeast two-hybrid system was also used to determine whether *At*CDC48 could interact with the *At*SERK1 interacting proteins GF14λ, RBP and KAPP. Therefore pair wise interaction tests were performed using *At*SERK1^{kinase}, *At*CDC48$^{549-809}$, *At*CDC48$^{386-809}$, GF14λ, RBP$^{118-357}$ (Chapter 2), KAPP and KI-KAPP (Shah et al., 2002) cloned in pJG4-5 and pEG202. After incubation at 30°C for two days, the occurrence of an interaction was determined. The occurrence of an interaction between RBP and the *At*SERK1 interaction partners was determined after incubation at 30°C for five days.

Protein expression and affinity purification

To express the peptide encoded by pJG4-5-*At*CDC48$^{747-809}$ and the peptides encoded by the EST cDNA clones, translational fusions were made to glutathione-S-transferase (GST) and to the maltose binding protein (MBP). A 290 bp cDNA fragment encoding *At*CDC48$^{747-809}$ was amplified by PCR from the corresponding pJG4-5 plasmid using pJG4-5 derived primers containing a *BamHI* restriction site in the forward primer (5'-CGC GGA TCC TAC CCT TAT GAT GTG CC-3') and a *SalI* site in the reverse primer (5'-ACG CGT CGA CTC TGG CGA AGA AGT CC-3'. The PCR fragment was digested with *BamHI* and *SalI*, and inserted in the corresponding sites of pGEX-4T1 and pMal-c2x. The fragments encoding *At*CDC48$^{549-809}$ and *At*CDC48$^{386-809}$ that were cloned into vectors pEG202 and pJG4-5 were also cloned into vector pGEX4T-1. A 900 bp fragment was digested from pGEX-4T1-*At*CDC48$^{549-809}$ using *BamHI* and inserted in the corresponding sites of pMal-c2x resulting in the construct pMal-*At*CDC48$^{549-809}$. A 1400 bp cDNA fragment of ESTs 180L9, encoding *At*CDC48$^{386-809}$ was amplified by PCR from the λZiplox plasmid using a gene-specific forward primer containing a *SalI* site (5'-ACG CGT CGA CGG ACG TCT TGA AGT TCT GAG G-3') and the reverse primer on the λZiplox vector containing an *XhoI* site. The PCR fragment was digested with *SalI* and *HindIII* and inserted in the corresponding sites of pMal-c2x. The GST-*At*SERK1^{kinase} construct was provided by Shah et al. (2001a). All plasmid constructs were transformed to *E. coli* BL21 for expression of fusion proteins.

Protein expression of GST-fusion proteins and GST was induced with 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG). The proteins were solubilised by sonification and bound to glutathione sepharose 4B beads following the manufacturer's instructions (Pharmacia Biotech, Inc). GST-fusion proteins were eluted from the beads with 15 mM reduced glutathione in 50 mM Tris pH 8.0 and 150 mM NaCl. Protein expression of MBP-fusion proteins and MBP was induced with 0.3 mM IPTG. The proteins were solubilised by sonification and bound to amylose resin (New England Biolabs) following the manufacturer's instructions. MBP-fusion proteins were eluted from the resin with 15 mM maltose in column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA and 1 mM DTT). All fusion proteins were dialysed against 50 mM Tris pH 7.4, 1

mM DTT and 0.1 mM EDTA after purification. Protein concentrations were determined by Bradford micro-assay (Bio-Rad) using BSA as a standard.

Phopshorylation assays

Transphosphorylation of *At*CDC48$^{747-809}$ and *At*CDC48$^{386-809}$ was tested by incubating 500 ng protein in the presence of 500 ng GST-*At*SERK1^{kinase} for 45 minutes at 30°C in phosphorylation buffer (20 mM Tris, pH 7.5, 150 mM KCl, Triton, 10 mM $MgCl_2$) containing 50 µM unlabeled ATP and $3.7 \cdot 10^5$ Bq of [γ-^{32}P]ATP in a final volume of 30 µL. The reaction was stopped by adding SDS-PAGE sample buffer and incubated for five minutes at 100 °C. The proteins were separated by 10% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue to verify equal loading. After drying the gel, radioactivity was detected with a PhosphoImager using the Image Quant program (Molecular Dynamics). *At*SERK1^{kinase}-specific phosphorylation was tested by incubating the MBP-*At*CDC48$^{747-809}$ fusion protein with ATP in the absence of GST-*At*SERK1^{kinase}.

Production of αGST-*At*SERK1^{kinase} antibodies

αGST-*At*SERK1^{kinase} antibodies were generated by immunising rabbits with the purified fusion protein GST-*At*SERK1^{kinase}. The rabbit serum was purified from antibodies directed against bacterial proteins by passing the serum over immobilised bacterial protein extract. The αGST-*At*SERK1^{kinase} antiserum was used at a 1:15,000 dilution for Western analysis.

***In vitro* binding studies**

MBP-*At*CDC48$^{747-809}$ and MBP-*At*CDC48$^{386-809}$ were used for *in vitro* binding studies to confirm the interaction with the *At*SERK1 kinase domain using both phosphorylated and non-phosphorylated *At*SERK1^{kinase}. GST-*At*SERK1^{kinase} was phosphorylated by adding 100 µM ATP in a total volume of 30 µL protein binding buffer (PBB: 20 mM Tris pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.1% Triton X-100) and incubating for 30 minutes at 30°C. 300 ng MBP-fusion protein was bound to amylose resin and incubated for two hours at 4°C with 100 ng GST-*At*SERK1^{kinase} in PBB in a total volume of 300 µL. The immobilised protein complexes

were washed three times with 1 mL PBB. Bound proteins were removed from the beads by adding SDS-PAGE sample buffer to the beads and incubating at 100°C for five minutes. The proteins were then separated by 12% SDS-PAGE and transferred to nitrocellulose membrane (Schleicher and Schuell), followed by Western analysis using αGST-*At*SERK1^{kinase} antiserum. Proteins on the Western blots were visualised using an alkaline phosphatase-based detection system with standard conditions.

Immunoprecipitation of *At*CDC48

Protein extracts were obtained from *Arabidopsis thaliana* cell suspension (Menges and Murray, 2002) by grinding the tissue in extraction buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% Glycerol, 5 mM EGTA and 1% Triton X-100) containing protease inhibitors (1mM PMSF, 1% aprotinin and 10 µg/mL leupeptin) and phosphatase inhibitors (5 mM EDTA, 100 mM NaF). For the co-immunoprecipitation 20 µL of *At*SERK1-specific peptide antibodies (αSP1) or αGF14λ antiserum (Chapter 3), bound to sepharose-protein A, was incubated with 50 µg of plant protein extract at 4°C for 1.5-2 hours. The immune-complexes were washed once with high salt buffer (50 mM Tris pH 7.5, 0.1% Triton-X100, 0.5 M NaCl), then once with medium salt buffer (50 mM Tris pH 7.5, 0.1% Triton X100, 0.15 M NaCl) and finally twice with low salt buffer (50 mM Tris pH 7.5, 0.1% Triton X100). Immunoprecipitated proteins were eluted from the sepharose by adding sample buffer and incubating at 100°C for five minutes. The proteins were separated on 12% SDS-PAGE and analysed by Western analysis using α*At*CDC48 antibodies (Rancour et al., 2002). Visualisation of proteins on the Western blots was performed using a horseradish peroxidase-based detection system (ECL, Amersham) according to standard conditions.

Results

*At*SERK1 interacts with *At*CDC48A in yeast

A LexA version of the yeast two-hybrid system was used to identify proteins that interact with the kinase domain of *At*SERK1 (*At*SERK1^{kinase}, a.a. 266-625). One of the putative *At*SERK1-interacting proteins that was found was identified as the *At*CDC48A protein (At3g09840). The *Arabidopsis* genome encodes three CDC48 isoforms:

AtCDC48A, *AtCDC48B* (At3g53230) and *AtCDC48C* (At5g03340) (AGI, 2000). The gene encoding *At*CDC48A is located on BAC F8A24 on chromosome 3. *At*CDC48A is a protein of 809 residues with a predicted molecular mass of 89 kD. It is a member of the AAA-ATPase family of ATPases associated with diverse cellular activities and contains two AAA-domains (aa 207-414 and aa 481-691; Feiler et al., 1995), which are conserved regions of about 220 amino acids that contain an ATP-binding site.

The two yeast two-hybrid clones that were isolated encode only the C-terminal 62 (*At*CDC48$^{747\text{-}809}$) and 65 (*At*CDC48$^{744\text{-}809}$) residues of *At*CDC48A respectively. To rule out a potential artefact, EST 180L9 (accession nr. T22005) and EST 104O24 (accession nr. H36923) encoding the C-terminal 260 (*At*CDC48$^{549\text{-}809}$) and 423 (*At*CDC48$^{386\text{-}809}$) residues of *At*CDC48 respectively were chosen to confirm the interaction with *At*SERK1. Together with *At*CDC48$^{747\text{-}809}$ they were tested for interaction in yeast with the human Lamin C protein, the extracellular LRR domain of *At*SERK1 (*At*SERK1^{LRR}, a.a. 26-234) and the *At*SERK1^{kinase} domain. Table 1 gives an overview of the yeast two-hybrid interaction tests we performed. It shows that in yeast all *At*CDC48 peptides interact with the kinase domain of *At*SERK1 and do not interact with the human Lamin C protein or *At*SERK1^{LRR}.

Table 1: Interaction of *At*CDC48 with the kinase domain of *At*SERK1 was confirmed with *At*CDC48$^{386\text{-}809}$ (EST 180L9) and *At*CDC48$^{549\text{-}809}$ (EST 104O24) using the yeast two-hybrid system. Interaction was determined using *At*SERK1 either as bait or as prey. pEG202 = bait vector (AD), pJG4-5 = prey vector (BD). Compared are the interactions of *At*SERK1 with the peptides encoded by the yeast two-hybrid clone and by the ESTs. ++ strong interaction, + interaction, +/- poor interaction, - no interaction, n.d.= not determined.

	BD-*At*CDC48 (747-809)	BD-*At*CDC48 (549-809)	AD-*At*CDC48 (549-809)	BD-*At*CDC48 (386-809)	AD-*At*CDC48 (386-809)
*At*SERK1^{kinase}	+	+	+	++	n.d.
*At*SERK1^{LRR}	-	-	-	-	n.d.
Lamin C	-	-	-	-	n.d.

To confirm the existence of a direct interaction between *At*SERK1 and *At*CDC48, *At*CDC48$^{747\text{-}809}$ and *At*CDC48$^{386\text{-}809}$ were used for *in vitro* binding studies. For this the *At*SERK1 kinase domain (*At*SERK1^{kinase}) was expressed as C-terminal fusion protein with glutathione-S-transferase (GST) in *E. coli* and purified by affinity chromatography on glutathione resin. Because GST is known to form homodimers by itself, we expressed *At*CDC48$^{747\text{-}809}$ and *At*CDC48$^{386\text{-}809}$ as C-terminal fusion proteins with maltose binding protein (MBP). MBP-*At*CDC48$^{747\text{-}809}$ and MBP-*At*CDC48$^{386\text{-}809}$ were expressed in *E. coli* and purified by affinity chromatography on amylose resin. GST-*At*SERK1^{kinase} was added to amylose resin with immobilised *At*CDC48 and tested for its ability to bind to *At*CDC48.

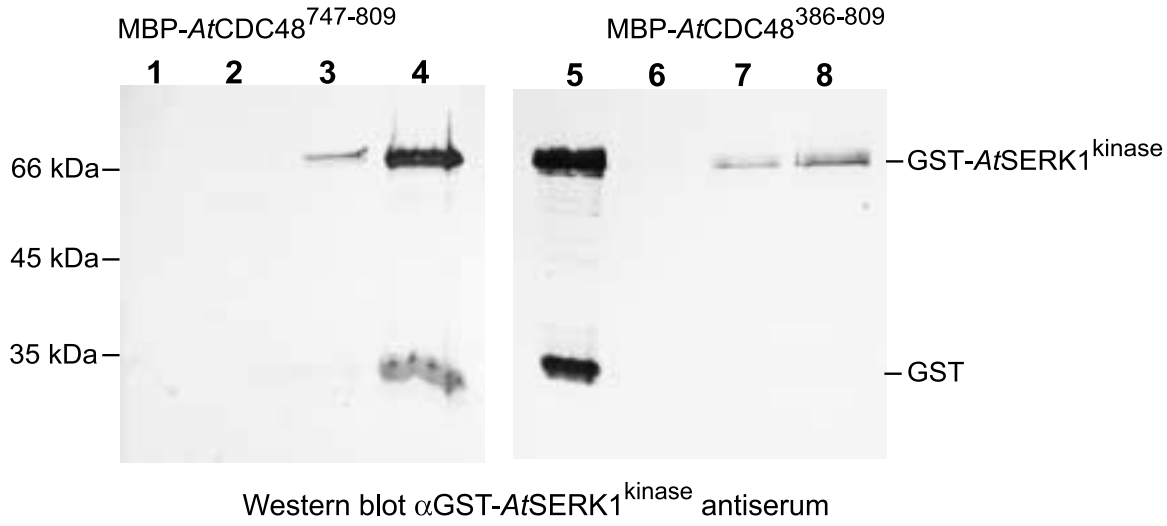


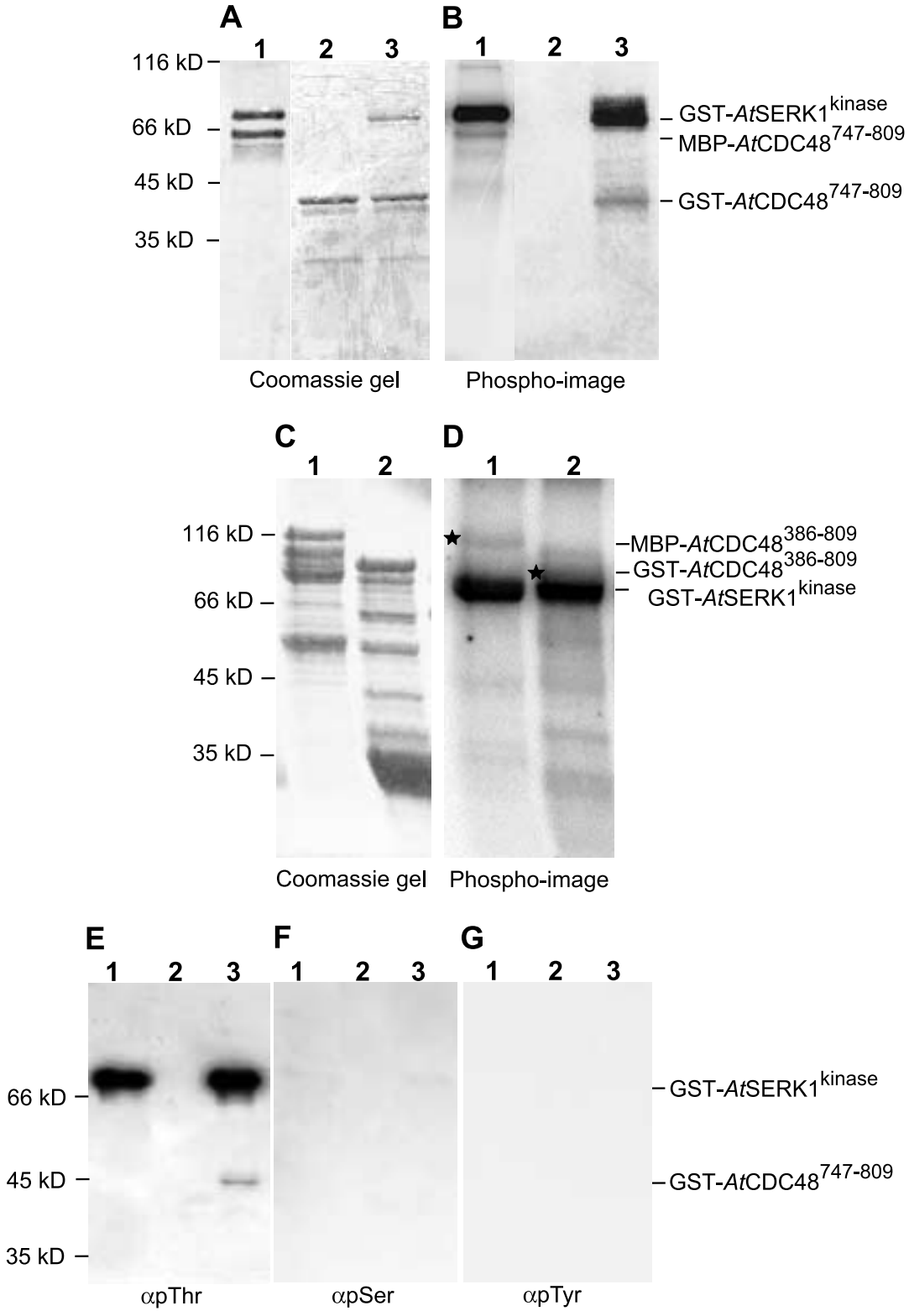
Figure 1: *In vitro* binding assays for the binding of *At*CDC48 to the kinase domain of *At*SERK1. GST-*At*SERK1^{kinase} was incubated with immobilised MBP, MBP-*At*CDC48$^{747\text{-}809}$ or MBP-*At*CDC48$^{386\text{-}809}$. Proteins bound to the sepharose beads were pelleted, washed, eluted, subjected to SDS-PAGE and detected by Western blotting using αGST-*At*SERK1^{kinase} antiserum. This antiserum recognises, besides *At*SERK1^{kinase}, also GST. **Lane 1:** Binding assay of GST and MBP-*At*CDC48$^{747\text{-}809}$. **Lane 2**: Binding assay of non-phosphorylated GST-*At*SERK1^{kinase} and MBP-*At*CDC48$^{747\text{-}809}$. **Lane 3**: Binding assay of non-phosphorylated GST-*At*SERK1^{kinase} and MBP-*At*CDC48$^{747\text{-}809}$. **Lane 4** and **5**, *At*SERK1^{kinase} (100 ng), **Lane 6:** Binding assay of GST and MBP-*At*CDC48$^{386\text{-}809}$. **Lane 7**: binding assay of non-phosphorylated GST-*At*SERK1^{kinase} and MBP-*At*CDC48$^{386\text{-}809}$. **Lane 8**: binding assay of phosphorylated GST-*At*SERK1^{kinase} and MBP-*At*CDC48$^{386\text{-}809}$.

Binding assays were performed using phosphorylated or non-phosphorylated *At*SERK1^{kinase}. Bound proteins were eluted and subjected to SDS-page and Western analysis using αGST-*At*SERK1^{kinase} antiserum (Figure 1). Although hardly visible, MBP-*At*CDC48$^{747-809}$ interacts with non-phosphorylated GST-*At*SERK1^{kinase} (Fig. 1, lane 2), but also slightly with GST (Fig. 1, lane 1). Upon phosphorylation of *At*SERK1, MBP-*At*CDC48$^{747-809}$ and GST-*At*SERK1^{kinase} clearly interact (Fig. 1 lane 3). MBP-*At*CDC48$^{386-809}$ does not interact with GST (Fig. 1, lane 6) but interacts both with non-phosphorylated (Fig. 1, lane 7) and phosphorylated GST-*At*SERK1^{kinase} (Fig. 1, lane 8). Upon phosphorylation of *At*SERK1^{kinase}, there was an increase in interaction with *At*CDC48$^{386-809}$. Lanes 4 and 5 contain GST-*At*SERK1^{kinase}. From these results we conclude that MBP-*At*CDC48 binds to the kinase domain of *At*SERK1 *in vitro.* Although phosphorylation of *At*SERK1 increases the interaction *in vitro*, it is not clear whether the interaction of *At*SERK1 with the C-terminal amino acids of *At*CDC48 is phosphorylation dependent.

*At*SERK1^{kinase} is able to transphosphorylate *At*CDC48 *in vitro*

*At*SERK1^{kinase} is able to transphosphorylate proteins *in vitro* (Shah et al., 2001b). Therefore, a phosphorylation assay was performed to further substantiate the interaction between *At*SERK1 and *At*CDC48. *At*CDC48$^{747-809}$ and *At*CDC48$^{386-809}$ were expressed as fusion protein with MBP, but also as fusion proteins with GST. To allow *At*SERK1 to transphosphorylate *At*CDC48, both the MBP- and GST-derived fusion proteins were incubated with GST-*At*SERK1^{kinase} in the presence of labelled ATP. The phospho-images in Figure 2 show that *At*SERK1 is able to transphosphorylate MBP- and GST-

Figure 2: Transphosphorylation assays with *At*SERK1^{kinase} of the *At*CDC48 peptides encoded by the two-hybrid protein fragment and the EST cDNA 180L9. After electrophoresis, gels were stained with Coomassie Brilliant-Blue (**A** and **C**) to verify equal loading. After drying the gels, radioactivity was detected with a PhosphoImager (**B** and **D**). **A** and **B**. **Lane 1**: GST-*At*SERK1^{kinase} + MBP-*At*CDC48$^{747-809}$. **Lane 2**: GST-*At*CDC48$^{747-809}$. **Lane 3**: GST-*At*SERK1^{kinase} + GST-*At*CDC48$^{747-809}$. **C** and **D**: **Lane 1**: GST-*At*SERK1^{kinase} + MBP-*At*CDC48$^{386-809}$. **Lane 2**: GST-*At*SERK1^{kinase} + GST-*At*CDC48$^{386-809}$. Transphosphorylation of *At*CDC48$^{747-809}$ was detected by Western analysis using α(p)Thr antibodies (**E**), α(p)Ser antibodies (**F**) and α(p)Tyr antibodies (**G**). **Lane 1**: GST-*At*SERK1^{kinase}. **Lane 2**: GST-*At*CDC48$^{747-809}$. **Lane 3**: GST-*At*SERK1^{kinase} + GST-*At*CDC48$^{747-809}$.


A
B
1
2
3
116 kD
66 kD
45 kD
35 kD
GST-AtSERK1kinase
MBP-AtCDC48747-809
GST-AtCDC48747-809
Coomassie gel
Phospho-image
C
D
MBP-AtCDC48386-809
GST-AtCDC48386-809
GST-AtSERK1kinase
E
F
G
αpThr
αpSer
αpTyr

*At*CDC48$^{747\text{-}809}$ (Fig. 2b, lane 1 and 3). Transphosphorylation on the larger *At*CDC48$^{386\text{-}809}$ peptide seems less in intensity (Fig. 2d). *At*SERK1^{kinase} does not transphosphorylate GST nor MBP (Chapter 2) and *At*CDC48 is not phosphorylated in the absence of *At*SERK1^{kinase} (Fig. 2b, lane 2).

We determined which residues in *At*CDC48 are phosphorylated by *At*SERK1 using α(p)Thr, α(p)Ser and α(p)Tyr antibodies. α(p)Thr antibodies recognised the GST-*At*CDC48$^{747\text{-}809}$ peptide after *in vitro* phosphorylation by GST-*At*SERK1^{kinase} (Fig. 2e) while α(p)Ser (Fig. 2f) and α(p)Tyr (Fig. 2g) antibodies did not. According to the prediction of phosphorylation sites by NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos) there are two putative threonine phosphorylation sites within the C-terminal 62 amino acids of *At*CDC48. One of these sites is conserved amongst the *Arabidopsis* *At*CDC48 family (Thr^{796} of *At*CDC48A) but not in yeast. Because *At*SERK1 is able to phosphorylate both *At*CDC48$^{747\text{-}809}$ and *At*CDC48$^{386\text{-}809}$ fusion proteins, we conclude that *At*SERK1 phosphorylates *At*CDC48 on at least one threonine residue within the C-terminal 62 amino acids.

*At*CDC48 and *At*SERK1 are members of a protein complex

In addition to *At*CDC48, the kinase domain of *At*SERK1 can also bind the 14-3-3 protein GF14λ (Chapter 3), a putative RNA binding protein (Genbank accession nr. BAA97154, Chapter 2) and the kinase-associated protein phosphatase KAPP (Shah et al., 2002). Full length GF14λ and the C-terminal part of the putative RNA binding protein (RBP) were isolated as *At*SERK1 interacting proteins from the yeast two-hybrid screening described in Chapter 2. Interaction of *At*SERK1 with RBP$^{118\text{-}357}$ was verified in yeast. Interaction with GF14λ was verified by *in vitro* binding assays and *in vivo* by a direct interaction in cowpea protoplasts and by co-immunoprecipitation of *At*SERK1 and 14-3-3 protein from *Arabidopsis* seedling protein extract. The phosphatase KAPP was determined to physically and functionally interact with the *At*SERK1 kinase domain, as it is able to dephosphorylate phosphorylated *At*SERK1 and actively prevents autophosphorylation of the kinase (Shah et al., 2002). Yeast two-hybrid interaction tests showed that KAPP binds to *At*SERK1 with its kinase interaction domain (KI-KAPP). *In vitro* phosphorylation assays showed that *At*SERK1 is able to use GF14λ, RBP and KAPP as substrate for phosphorylation. To determine whether *At*SERK1 functions in a complex containing one

or more of the *At*SERK1 interacting proteins we performed an extensive pair wise yeast two-hybrid interaction test using *At*SERK1^{kinase}, *At*CDC48$^{549-809}$, GF14λ, RBP$^{118-357}$, KAPP and KI-KAPP (Shah et al., 2002) cloned in pJG4-5 and pEG202. Unfortunately, not all interactions could be tested due to autoactivation of the reporter genes by KI-KAPP, KAPP and GF14λ when fused to the LexA DNA-binding protein of pEG202. As summarised in Table 2, *At*CDC48 appears to interact not only with *At*SERK1 also with itself, with GF14λ and with KAPP. While *At*SERK1 interacts with the kinase interaction domain of KAPP (Shah et al., 2002), *At*CDC48 only interacts with the complete KAPP protein. *At*CDC48 homohexadimerises *in vivo,* which explains the interaction between the *At*CDC48 peptides. Although RBP does interact with the kinase domain of *At*SERK1, we observed only a slight activation of the reporter genes for the interaction between RBP and *At*CDC48 and between RBP and KAPP after prolonged incubation. From these results we propose that a protein complex can be formed containing at least *At*SERK1, *At*CDC48, GF14λ and KAPP.

Table 2: Pair wise interaction tests between *At*SERK1^{kinase}, *At*CDC48$^{549-809}$, GF14λ, RBP$^{118-357}$, KAPP and KI-KAPP cloned in pEG202 (BD) and/or pJG4-5 (AD) using the yeast two-hybrid system. ++ strong interaction, + interaction, +/- poor interaction, - no interaction, n.d. = not determined.

	AD-*At*SERK1^{kinase}	AD-*At*CDC48$^{549-809}$	AD-RBP$^{118-357}$
BD-*At*CDC48$^{549-809}$	+	++	+/-
BD-GF14λ	+	++	-
BD-RBP$^{118-357}$	+	+/-	-
BD-KAPP	++	+	+/-
BD-KI-KAPP	++	-	-

*At*CDC48 interacts with *At*SERK1 and a 14-3-3 protein *in vivo*

We next tested whether the interactions between *At*CDC48 and *At*SERK1 and between *At*CDC48 and GF14λ occur in *Arabidopsis* cells. To this end we isolated proteins from tissue-cultured *Arabidopsis* cells (Menges and Murray, 2002) and performed

immunoprecipitations with *At*SERK1-specific peptide antibodies (αSP1) or αGF14λ antiserum (αGF14λ). αGF14λ antiserum was raised against the complete GF14λ protein and is therefore not absolutely specific for GF14λ. The presence of *At*CDC48 in tissue-cultured *Arabidopsis* cells was determined by Western analysis using α*At*CDC48 antiserum (Fig. 3, lane 1). α*At*CDC48 recognises a protein with a molecular weight of 97 kD that corresponds to the previously determined molecular weight of *At*CDC48 (Rancour et al., 2002). When the *At*SERK1 specific antibody αSP1 is used in an immunoprecipitation assay, a 97 kD protein is co-precipitated that is recognised by the α*At*CDC48 antiserum (Fig. 3, lane 2). When instead GF14λ antiserum is used in the

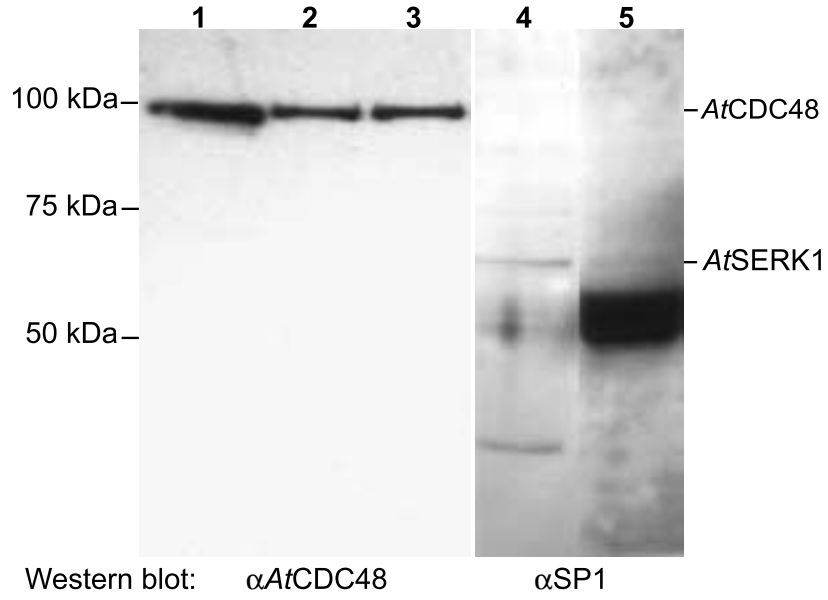


Figure 3: *In vivo* interaction between *At*CDC48 and *At*SERK1 and between *At*CDC48 and a 14-3-3 protein. **Lane 1**: Direct Western analysis with α*At*CDC48 on protein extract isolated from tissue cultured *Arabidopsis* cells to detect the presence of *At*CDC48 protein. For the immunoprecipitation assays, immobilised *At*SERK1 αSP1 or αGF14λ antiserum was incubated with the protein extract and immune-complexes were pelleted, washed, subjected to SDS-PAGE and detected by Western analysis using α*At*CDC48 antiserum. **Lane 2**: Immunoprecipitation assay with *At*SERK1 αSP1. **Lane 3**: Immunoprecipitation assay with αGF14λ antiserum. **Lane 4**: Direct Western analysis with *At*SERK1 αSP1 on protein extract isolated from tissue cultured *Arabidopsis* cells to detect the presence of *At*SERK1 protein. **Lane 5**: Immunoprecipitation assay with *At*SERK1 αSP1 and detection of *At*SERK1 by Western analysis using αSP1.

immunoprecipitation assay, the same 97 kD protein that is recognised by α*At*CDC48 is co-precipitated (Fig. 3, lane 3). The presence of *At*SERK1 in the immune-complexes was confirmed by Western analysis on the same blot using αSP1 (Fig. 3 lane 4 and 5). The band that co-migrates at a molecular weight of roughly 50 kD corresponds to the αSP1 antibody that is recognised by the secondary α-rabbit antibody. In contrast, the immunopurified secondary α-chicken antibody that was used to detect α*At*CDC48 does not recognise αSP1 and gives less background. From these results we conclude that *in vivo* *At*CDC48 not only interacts with *At*SERK1, but also with a member of the *Arabidopsis* 14-3-3 protein family.

Discussion

*At*CDC48 was found as putative *At*SERK1 interacting protein in a yeast two-hybrid screening. Here we confirmed the interaction between *At*CDC48 and *At*SERK1 *in vitro*. The interaction is phosphorylation-dependent, because *At*CDC48 preferentially binds to phosphorylated *At*SERK1. Upon binding, *At*SERK1 is able to transphosphorylate *At*CDC48 on at least one threonine residue within the C-terminal 62 amino acid residues. Pair wise interaction tests between *At*SERK1 and its binding partners showed that besides binding to *At*SERK1^{kinase}, *At*CDC48 can also bind to GF14λ and KAPP in yeast. In addition, *At*CDC48 also interacts with *At*SERK1 and 14-3-3 protein in *Arabidopsis* cells. We propose that *At*CDC48 functions together with *At*SERK1, GF14λ and KAPP in a protein complex.

CDC48 function

*At*CDC48 was originally described by Feiler et al. (1995) as an *Arabidopsis thaliana* cell division cycle protein based on functional complementation studies in yeast Cdc48 mutants. *S. cerevisae Cdc48-1* mutants arrest as large budded cells with an undivided nucleus and microtubules spreading aberrantly throughout the cytoplasm from a single spindle plaque (Fröhlig et al., 1991). The protein is also annotated as a putative endoplasmic reticulum ATPase (accession nr. AC015985) due to its homology with the mammalian CDC48 isoform p97 or VCP, which was shown to regulate membrane fusion

and assembly of endoplasmic reticulum *in vitro* together with the adaptor protein p47 (Pécheur et al., 2002; Rabinovich et al., 2002). Like yeast Cdc48, p97/VCP is also involved in targeted proteolysis of ubiquitinated retro-translocated proteins from the ER to which it is targeted by a protein complex, Ufd1/Npl4 (Jarosch et al., 2002), which competes with p47 for binding p97/VCP (Meyer et al., 2000). Cdc48/p97/VCP was proposed to affect different cellular processes via specific adaptor proteins. The commonality between these processes seems to be the ATP-dependent protein folding/unfolding activity of Cdc48/p97/VCP (Golbik et al., 1999).

Arabidopsis CDC48 expression pattern and co-localisation with *At*SERK1

In *Arabidopsis*, *in situ* hybridisation experiments showed that CDC48 is highly expressed in proliferating cells of the vegetative shoot, root, floral inflorescence and flowers and in rapidly growing cells (Feiler et al., 1995). *AtCDC48* mRNA expression is upregulated in developing ovules and microspores and down-regulated in most differentiated cell types, although high transcript levels are maintained in tapetum and vascular tissues. The localisation of the *At*CDC48 protein in plant tissues corresponds to mRNA expression (Feiler et al., 1995). At the subcellular level, the *At*CDC48 protein is localised at the division plane in dividing *Arabidopsis* cells and in punctate, cytoplasmically distributed structures in both dividing and interphase cells (Rancour et al., 2002). During interphase *At*CDC48 was also found to be associated with the nuclear envelope. *At*CDC48 was detected in both soluble and membrane fractions and was found to cofractionate with the SNAREs KNOLLE and SYP31 in a sucrose density gradient (Rancour et al., 2002). KNOLLE can be found at the cell plate and in cytoplasmic puntate structures (Lauber et al., 1997). SYP31 localises to the division plane during cytokinesis (Rancour et al., 2002). Disruption of the *KNOLLE* gene results in an embryonic defect with cells containing incomplete cross walls and enlarged cells with polyploid nuclei (Lukowitz et al., 1996). At the division plane *At*CDC48 co-localises and interacts with the SYP31 (Rancour et al., 2002). *At*CDC48 also co-localises but does not interact with the KNOLLE protein at the division plane nor in the cytoplasmic punctate structures. In these cytoplasmic punctate structures *At*CDC48 also co-localises with the endosomal-specific marker protein SNARE SYP21 (Rancour et al., 2002).

The *At*SERK1 gene is expressed during female gametophyte development, during early embryogenesis and in adult vascular tissue (Hecht et al., 2001). *At*CDC48 expression is therefore overlapping with *At*SERK1 expression. However, *At*SERK1 is a transmembrane receptor protein that localises to the plasma membrane (Shah et al., 2001a). *At*CDC48 can be found in the cytoplasm as well as attached to membranes (Rancour et al., 2002). Fluorescently labelled *At*SERK1 and KAPP proteins both localise to the plasma membrane in protoplasts when they are separately expressed. When the proteins are co-expressed, *At*SERK1 and KAPP become sequestered into intracellular vesicles similar to early endosomes (Shah et al., 2002). *At*SERK1 and KAPP only physically interact in these intracellular vesicles, which suggests that KAPP is required for *At*SERK1 endocytosis. *At*CDC48 also localises to endosomes, therefore an interaction between *At*SERK1 and *At*CDC48 is possible after *At*SERK1 internalisation at the endosomes. However, interaction between *At*SERK1 and cytosolic *At*CDC48 is also possible.

Phosphorylation regulates shuttling of Cdc48/p97/VCP

In yeast, Cdc48 is essential for cell cycle progression and is presumably involved in spindle pole body duplication or separation (Fröhlig et al., 1991). In addition, *Sc*Cdc48 participates in homotypic fusion of ER membranes (Latterich et al., 1995). *Sc*Cdc48 shuttles between cytoplasm and nucleus in a cell cycle-dependent manner. This is regulated by phosphorylation of the tyrosine residue in the C-terminal DDDLYS sequence, resulting in the exposure of a N-terminal nuclear localisation signal (Madeo et al., 1998). *At*CDC48 can replace the essential cell division cycle function of the endogenous *S. cerevisiae* Cdc48 protein in yeast (Feiler et al., 1995). Like in *Sc*Cdc48, the N-terminal region of *At*CDC48 contains two putative nuclear localisation signals ($KKX_{(8)}RKK$ and KGKRKD). *At*CDC48 was found to contain two copies of the conserved nucleotide-binding domain (AAA-module) of AAA-ATPases that are important in ATP binding and hydrolysis (a.a. 207-414 and 481-691). *At*CDC48 lacks membrane-spanning regions. The C-terminus contains the conserved negatively charged sequence DDDDLYN (Feiler et al., 1995).

*At*SERK1 phosphorylates a threonine residue of *At*CDC48 within the C-terminal 62 amino acid residues. The mammalian CDC48 homologue p97/VCP is tyrosine

phosphorylated in response to T cell antigen receptor activation (Egerton et al., 1992). p97 phosphorylation coincides with its relocalisation from the ER to the centrosome (Lavoie et al., 2000). Although *At*SERK1 does not phosphorylate the tyrosine residue in the sequence DDDDLYN of *At*CDC48 (Fig. 2g), phosphorylation of a threonine residue at the C-terminus might have a comparable function as phosphorylation of tyrosine residue in the yeast DDDLYS sequence in changing the three-dimensional structure of the CDC48 protein, possibly resulting in cellular relocalisation.

_At_SERK1 may function in a protein complex containing _At_CDC48, GF14λ and KAPP

Pair wise interaction tests and co-immunprecipitations between *At*SERK1 and its interacting partners showed that besides binding to *At*SERK1^{kinase}, *At*CDC48 can also interact with GF14λ and KAPP. The mammalian CDC48 homologue p97/VCP was found to be phosphorylated by the protein tyrosine kinase JAK-2 (Lavoie et al., 2000) and dephosphorylated by the phosphotyrosine phosphatase PTPH1 (Zhang et al., 1999). PTPH1 dephosphorylates the C-terminal tyrosines (Tyr706 and Tyr805) of VCP that are required for the recognition of VCP as substrate by PTPH1 When expression of wild-type PTPH1 was induced, cell growth was strongly inhibited and VCP was specifically dephosphorylated (Zhang et al., 1999). Like JAK-2, *At*SERK1 phosphorylates *At*CDC48. Analogous to the function of PTPH1, KAPP might be the phosphatase that negates *At*SERK1 phosphorylation of *At*CDC48.

In mammals it was shown that 20-50% of PTPH1 is associated with the 14-3-3β protein in a manner that is dependent on serine phoshorylation of PTPH1 (Zhang et al., 1997). The interaction between PTPH1 and 14-3-3β is regulated by mitogenic signals. Two motifs in PTPH1 were shown to be involved in 14-3-3β binding (RSLS^{359}VEH and RVDS^{853}EP). KAPP does not contain these sequences, but contains several sequences that resemble 14-3-3 binding domains including one corresponding to the consensus 14-3-3 binding motif [RK]X$_{(2,3)}$pSXP, where X stands for any amino acid and p denotes a phosphorylated amino acid (Sehnke et al., 2002). This 14-3-3 binding sequence (RLPSS^{105}SP) lies in the cytoplasmic domain of the KAPP protein located upstream of the

FHA domain that is necessary for the interaction of KAPP with *At*SERK1 (Shah et al., 2002). Therefore, KAPP may also be able to bind a 14-3-3 protein.

*At*SERK1 binds GF14λ and is able to phosphorylate GF14λ (Chapter 3). *At*CDC48 is also able to interact with GF14λ in yeast and interacts with a 14-3-3 protein in *Arabidopsis* cells. A putative 14-3-3 binding domain, KARQS^{572}AP, corresponding to the consensus 14-3-3 binding motif can be found in the *At*CDC48 protein. The sequence is located in the second AAA-module (Feiler et al., 1995) and is conserved in all three *Arabidopsis* CDC48 family members but not in yeast Cdc48. Whether this sequence is indeed involved in 14-3-3 binding has to be confirmed. Based on our results concerning the interaction partners of *At*SERK1, we propose that *At*SERK1 functions together with *At*CDC48, GF14λ and KAPP in a protein complex similar to the mammalian complex containing p97/VCP, PTPH1 and 14-3-3β. In this model, *At*SERK1 may fulfil the role of the JAK-2 kinase by phosphorylating *At*CDC48. Analogous to phosphorylation of p97/VCP by JAK-2, phosphorylation of *At*CDC48 by *At*SERK1 may enhance cell-cycle activity. In dividing *Arabidopsis* cells, *At*CDC48 is found at the phragmoplast (Rancour et al., 2002). Phosphorylation of *At*CDC48 by *At*SERK1 may relocalise the *At*CDC48 protein to the plane of division. Dephosphorylation of *At*SERK1 and *At*CDC48 by KAPP may suppress cell growth by reversing the effects of phosphorylation. We propose that *At*SERK1 is involved in one of the many controlling pathways of the cell cycle.

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Chapter 5

14-3-3 binding sequences in *Arabidopsis thaliana* protein kinases

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Abstract

Adaptor or scaffold proteins like 14-3-3 proteins help protein kinases with the recruitment of their substrate proteins. 14-3-3 proteins bind to their target molecules in a sequence specific manner. In plants, the general binding sequence was determined as [RK]$X_{(2,3)}$SXP. We previously showed that the receptor-like kinase *At*SERK1 binds to the 14-3-3 protein GF14λ. In the kinase domain of *At*SERK1 we found a sequence resembling the 14-3-3 binding motif in a loop between helices αD and αE. Analysis of the kinase domains of all *Arabidopsis thaliana* membrane receptor-like kinases and receptor-like cytoplasmic kinases revealed that among the protein kinases that contain a putative 14-3-3 binding sequence in their kinase domain there exists a subset that has preference for the loop between helices αD and αE as location for a 14-3-3 binding sequence. Besides these receptor-like kinases, several other *Arabidopsis* kinases also contain a 14-3-3 binding sequence in this loop.

Introduction

Hundreds of protein kinases control a wide range of cellular events such as cell division, cell signalling, cell differentiation and cell death. Receptor kinases are essential to perceive the signals that induce these cellular events. In general, receptor kinases consist of an extracellular domain, a transmembrane domain and an intracellular catalytic kinase domain. In the *Arabidopsis* genome a large number of proteins is present that have the predicted topologies of a receptor kinase but for which the receptor function has not been demonstrated yet (AGI, 2000). These proteins are referred to as receptor-like kinases (RLKs).

The catalytic domains of protein kinases range from 250 to 300 amino acid residues corresponding to about 30 kD. Crystal structures of several kinases have been determined showing that the kinase fold is extremely well conserved among serine/threonine and tyrosine kinases. The kinase domain is divided into two subdomains, or lobes (Johnson et al., 1998). The smaller N-terminal lobe is composed of a five-stranded β-sheet (β1-β5) and one α-helix called helix αC. The larger C-terminal lobe is predominantly helical. ATP is bound in a deep cleft between the two lobes located beneath a highly conserved loop connecting strands β1 and β2. This phosphate-binding loop (P-loop) contains a glycine-rich sequence motif and is important for fixing the orientation of the phosphates of ATP. A centrally located loop, the activation loop, provides a protein-binding platform for the peptide substrate (Huse and Kuriyan, 2002). The bound peptide substrate is then located across the ATP-binding cleft, close to the γ-phosphate of ATP.

Genomic sequence analysis revealed that *Arabidopsis* contains 417 genes encoding RLKs (Shiu and Bleecker, 2001a). Like all plant kinases these kinases contain serine/threonine kinase consensus sequences. Analysis of plant kinases revealed that they are derived from a common ancestor. Amongst the group of descendents from this ancestral form are the Pelle cytoplasmic kinases of animals and a large number of cytoplasmic plant kinases called receptor-like cytoplasmic kinases (RLCKs) (Shiu and Bleecker, 2001a). Most plant RLKs are members of distinct families. Shiu and Bleecker showed that there is a strong tendency for proteins that are grouped together based on the homology of their kinase domains to also have similar extracellular domains. A large family among the plant RLKs are the leucine-rich repeat (LRR) containing transmembrane

receptors. The LRR motif is believed to be involved in mediating protein-protein interactions. In receptors it may interact directly or indirectly with the ligand (Kobe and Deisenhofer, 1994).

Once a receptor kinase is activated, its phosphorylation activity can be used for transduction of the signal. In order to recruit substrate proteins to a membrane located receptor kinase several mechanisms have evolved. One of these mechanisms involves adaptor or scaffold proteins (Burack and Shaw, 2000). These proteins do not have enzymatic activity themselves, but function in mediating protein-protein interactions. 14-3-3 proteins can function as adaptor or scaffold molecules. They bind in a sequence specific manner to phosphoserine or -threonine residues of a diverse range of proteins. In plants, they generally recognise proteins containing the sequence motifs [RK]$X_{(2,3)}$pSXP, where X stands for any amino acid and pS denotes phosphoserine residues (Sehnke et al., 2002). However, some 14-3-3 interacting proteins have been identified whose sequences deviate significantly from the motifs mentioned above or do not require phosphorylation of their targets for binding (Aitken, 2002).

*At*SERK1 interacts with the 14-3-3 protein GF14λ (Chapter 3). In the kinase domain of *At*SERK1 we found a putative 14-3-3 binding site surrounding Ser-394 with the sequence RPPS^{394}QPP. This sequence is located in a loop opposite to the active site of the kinase molecule. Sehnke et al. (2002) stated that roughly 40% of all *Arabidopsis* proteins contain a putative 14-3-3 binding sequence. In order to determine how many protein kinases contain a putative 14-3-3 binding sequence in their kinase domain and whether there is a preference for the location of these 14-3-3 binding sequences, we performed a survey of all *Arabidopsis* protein kinases using standard bioinformatics tools.

Methods

Motif search

Sehnke et al. (2002) proposed the motif [RK]$X_{(2,3)}$SXP as the consensus 14-3-3 binding sequence in plant 14-3-3 target proteins. The pattern matching facility at The *Arabidopsis* Information Resource (TAIR; http://www.*Arabidopsis*.org./cgi-bin/patmatch/nph-patmatch.pl) was used to scan all known and predicted *Arabidopsis* proteins with this motif.

Protein accession numbers and sequences were extracted from the multiple alignment of 610 RLKs and RLKCs provided by Shiu and Bleecker (2001a). A protein pattern search on these sequences using the motif [RK]$X_{(2,3)}$SXP was performed using the program 'Fuzzpro' provided by the European Molecular Biology Open Software Suite (EMBOSS).

In the *Arabidopsis* genome 1055 protein kinases could be found using Interpro entry IPR000719 that was last modified on 8 November 2000. Interpro IPR00719 combines the results obtained using the protein kinase catalytic domain signature (PS50011) and the protein kinase ATP binding domain signature (PS00107) from PROSITE (http://www.expasy.org/prosite/), the protein kinase family in Pfam (PF00069; http://www.sanger.ac.uk/Software/Pfam/) and the protein kinase family in ProDom (PD000001; http://prodes.toulouse.inra.fr/ prodom/2002.1/html/home.php). The accession number list containing the 1055 *Arabidopsis* protein kinases was compared with the accession number list containing the 10767 14-3-3 binding domain hits. The accession numbers of protein kinases that contained a sequence corresponding to the 14-3-3 binding motif were then compared with the list of 610 RLK and RLCK accession numbers. The complete sequences of the protein kinases that were not RLKs or RLCKs were aligned to the profile of the multiple alignment of RLK and RLCK kinase domains provided by Shiu and Bleecker (2001a) using Clustal X software (Thompson et al., 1997). A motif search was then performed on the peptide sequences that aligned to the loop between helices αD and αE. Using the pattern search facility at PROSITE we determined the location of the kinase domain in the proteins that contained a putative 14-3-3 binding sequence in the loop between helices αD and αE. To verify the location of the 14-3-3 binding sequence, the kinase domains of the protein kinases were aligned to the 21 RLK and RLCK kinase domains with a putative 14-3-3 binding sequence in the loop between helices αD and αE.

Occurrence frequencies

Relative amino acid occurrence frequencies were calculated using the program 'Compseq' provided by EMBOSS. To calculate the relative occurrence frequencies in *Arabidopsis* proteins the protein FASTA file ATH1.pep was used that was made available by TIGR on 25-7-02. For kinases the set of 610 RLKs and RLCKs was used (Shiu and Bleecker, 2001a). The occurrence frequency of a sequence resembling the 14-3-3 binding

motifs [RK]XXXSXP or [RK]XXSXP was calculated using the formula 1/(2*((R+K)*S*P)), where each letter stands for the relative amino acid occurrence frequency in a specific dataset.

Profile search

The multiple alignment of the sequence in the loop between helices αD and αE and part of the adjacent helices of 21 RLK and RLCK sequences containing a putative 14-3-3 binding sequence in this region was made using Clustal X software. The alignment was manually edited to correct for proper alignment of the putative 14-3-3 binding sequences. The sequence alignment was then used to generate a Hidden Markov Model (HMM) profile (Eddy, 1998) using the HMM package that can be obtained at http://hmmer.wustl.edu/. A profile search with the 'αD-αE loop' profile was performed on the set of 610 RLK and RLCK protein kinase sequences and on the complete set of *Arabidopsis* proteins present in the protein FASTA file ATH1.pep.

Results

The *Arabidopsis* genome encodes 25498 sequences that are either known or predicted to encode polypeptides. Sehnke et al. (2002) screened all these protein sequences with a combined search of the motifs [RK]XXSXP (Mode 1) and [RK]XXXSXP (Mode 2) and identified ten thousand peptide sequences corresponding to either one of the motifs. On this Sehnke et al. (2002) predicted that 40% of all *Arabidopsis* proteins are potential 14-3-3 binding targets. When we performed this screen, we found 10767 peptide sequences corresponding to either one of the motifs present in 7951 proteins that contain one or more of these putative 14-3-3 binding sequences. This corresponds to 31% of the 25498 proteins predicted to be encoded by the *Arabidopsis* genome. A greater part of the 7951 proteins (74%) contains a single 14-3-3 binding sequence, the rest of the proteins contain two (19%) or more 14-3-3 binding sequences with a maximum of 15. The average number of 14-3-3 binding sequences per protein is 1.4.

On average, the frequency of each amino acid in a random sequence with 20 different amino acids is once every 20 residues (0.05). However, in eukaryotic proteins

some amino acids are preferred above others. Table 1 gives the relative frequency of each separate amino acid residue in eukaryotic proteins (Arquès and Michel, 1997), in *Arabidopsis* proteins and in *Arabidopsis* kinase domains of RLKs and RLCKs. Using these residue occurrence frequencies, a sequence resembling the motif [RK]XXSXP is expected once per stretch of 1986 residues in a protein sequence the size of the *Arabidopsis* proteome. The same is true for the sequence resembling the motif [RK]XXXSXP. A combined motif search using [RK]$X_{(2,3)}$SXP is therefore expected to find a sequence resembling one of these motifs once per stretch of 993 residues. 21% of

Table 1: Observed frequencies of amino acid residues in protein sequences of eukaryotes in general, in *Arabidopsis* proteins and in *Arabidopsis* protein kinase domains (RLKs and RLCKs).

Amino acid	Relative frequency in eukaryotes	Relative frequency in *Arabidopsis*	Relative frequency in protein kinase domains
Ala, A	0.0712	0.0627	0.0614
Cys, C	0.0201	0.0183	0.0185
Asp, D	0.0522	0.0545	0.0591
Glu, E	0.0650	0.0677	0.0654
Phe, F	0.0414	0.0428	0.0325
Gly, G	0.0686	0.0635	0.0687
His, H	0.0233	0.0227	0.0325
Ile, I	0.0536	0.0532	0.0565
Lys, K	0.0620	0.0640	0.0554
Leu, L	0.0918	0.0948	0.0119
Met, M	0.0230	0.0243	0.0267
Asn, N	0.0449	0.0440	0.0409
Pro, P	0.0519	0.0478	0.0417
Gln, Q	0.0415	0.0346	0.0263
Arg, R	0.0501	0.0537	0.0591
Ser, S	0.0756	0.0895	0.0701
Thr, T	0.0564	0.0510	0.0446
Val, V	0.0632	0.0670	0.0705
Trp, W	0.0123	0.0126	0.0123
Tyr, Y	0.0317	0.0286	0.0387

the *Arabidopsis* genome contains coding sequences (AGI, 2000), corresponding to roughly 11.2 million amino acid residues. When the coding sequence of the *Arabidopsis* genome is random, a sequence that resembles the motif sequence [RK]$X_{(2,3)}$SXP is expected to be found 11310 times. The motif search on the proteins encoded by *Arabidopsis* using [RK]$X_{(2,3)}$SXP resulted in 10767 sequences, which is not significantly different from the amount of hits that can be found when using a random sequence of the same size. Therefore, in the absence of additional experimental data, the biological significance of most of the ten thousand putative 14-3-3 binding sequences and the prediction that 40% of all *Arabidopsis* proteins are potential 14-3-3 binding targets as stated by Sehnke et al. (2002) is highly doubtful.

Our next question was whether we could refine the prediction for the occurrence of 14-3-3 binding sequences based on our previous experimental evidence concerning the interaction between *At*SERK1 and GF14λ. Shiu and Bleecker (2001a) made a multiple alignment of kinase domains of 610 receptor-like kinases (RLKs) and receptor-like cytoplasmic kinases (RLCKs). We compared the accession number list containing the 610 *Arabidopsis* RLKs and RLCKs with the accession number list containing the 10767 putative 14-3-3 binding sequences. 271 of the 610 RLKs and RLCKs (44%) contained a 14-3-3 binding sequence. Because the percentage of RLKs and RLCKs found to have a putative 14-3-3 binding sequence is roughly similar to that observed for the entire proteome, the biological significance of this result is still quite doubtful. In addition, beside the presence of a putative 14-3-3 binding sequence in the kinase domain of *At*SERK1, another binding sequence was found in the extracellular part of the protein. Experiments in yeast showed that GF14λ does not bind to the extracellular domain of *At*SERK1 (Chapter 3). Therefore, we only looked at kinase domains for the prediction of the occurrence of putative 14-3-3 binding sequences.

Of the 271 protein kinases only 51 contained a putative 14-3-3 binding sequence in their kinase domain. A three-dimensional model of the *At*SERK1 kinase domain was made previously (Fig. 1; Shah et al., 2001) based on homology with the kinase domain of the insulin receptor (accession nr. P06213; Hubbard et al., 1994). According to this model, the putative 14-3-3 binding sequence of *At*SERK1 is present in a loop extending from the kinase domain. The same loop is present in the insulin receptor although it does not

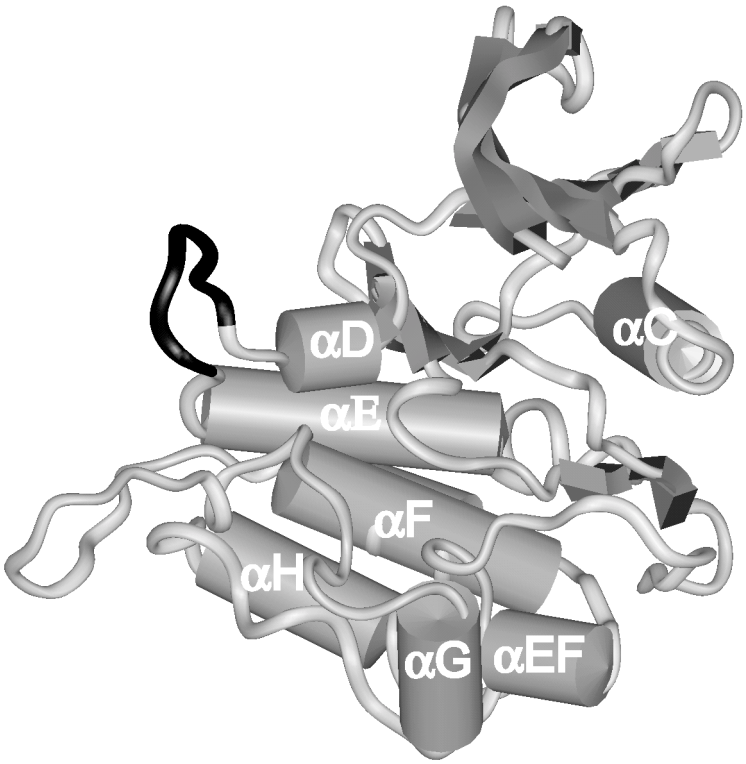

Figure 1: Homology based model of the kinase domain of *At*SERK1 with names assigned to the α-helices. The kinase domain of the human insulin receptor (accession nr. P06213) was used as template (Shah et al., 2001b). The 14-3-3 binding domain is located in a loop extending from the kinase and is shown in black.

contain a 14-3-3 binding motif and does not bind 14-3-3 (Furlanetto et al., 1997). Based on the literature (Hanks et al., 1988; Hanks and Hunter, 1995; Johnson et al., 1998) and the alignment between the kinase domains of *At*SERK1 and the insulin receptor we assigned names to the helices in the three-dimensional model of *At*SERK1. Based on the alignment of the 610 RLKs and RLCKs including *At*SERK1, we determined the location of the 54 putative 14-3-3 binding sites in the 51 RLK and RLCK kinase domains by comparison with the *At*SERK1 three-dimensional model (Table 2).

According to the alignment only one sequence contains a putative 14-3-3 binding sequence in the loop between αEF and αF (Table 2). Seven sequences contain one in the loop between αF and αG. Also seven sequences contain one in the loop between αG and αH and 13 sequences contain one at the end of the kinase domain after αH. Besides these loops, there are also five sequences that contain a putative 14-3-3 binding sequence in the

Table 2: Spreading and the number of sequences corresponding to the 14-3-3 binding motif [RK]$X_{(2,3)}$SXP in 51 protein kinases containing 54 putative 14-3-3 binding sequences in total. The location is based on the alignment of kinase domains (Shiu and Bleecker, 2001a) in comparison with the homology-based model of the kinase domain of *At*SERK1.

Amount	Loop between helices	Comment
21	αD - αE	14-3-3 loop *At*SERK1
5	αE - αEF	Activation loop
1	αEF- αF	
7	αF - αG	
7	αG - αH	
13	After αH	End kinase domain

activation loop between αE and αEF. Most putative 14-3-3 binding sequences can be found in the loop between helix αD and αE (21 out of 54, Table 3a), which is the loop where the putative binding sequence for GF14λ is located in *At*SERK1. All 51 sequences have only one putative 14-3-3 binding sequence, except for three sequences that have two (Table 3). In all three sequences that contain two putative 14-3-3 binding sequences, one of the two binding sequences is located in the loop between helices αD and αE. No putative 14-3-3 binding sequences can be found in the small N-terminal lobe of the kinase domain.

The 610 RLKs and RLCKs, with 417 proteins being RLKs, can be divided into 21 subfamilies based on their extracellular domain (Shiu and Bleecker, 2001a). As in *At*SERK1, 216 out of the 417 RLKs (52%) have an extracellular domain with leucine-rich repeats (LRR). Of the 21 protein kinases that contain a putative 14-3-3 binding sequence between helices αD and αE, 12 are RLKs (Table 3), six of which have an extracellular domain with LRRs (50%). From this we conclude that there is no preference for the presence of 14-3-3 binding sequences in LRR-RLKs. Therefore, the presence of a 14-3-3 binding domain in the LRR-RLK *At*SERK1 does not indicate an LRR-RLK specific signalling mechanism.

Table 3: RLKs and RLCKs containing a sequence corresponding to the 14-3-3 binding motif [RK]$X_{(2,3)}$SXP. Protein kinase subfamilies are according to Shiu and Bleecker (2001b). **Table 3a**: Proteins with putative 14-3-3 binding sequence in the loop between helices αD and αE.

Accession number	Subfamily	14-3-3 binding sequence	Protein name
At5g35580	RLCKVII	RRCSLP	*Arabidopsis thaliana* ser/thr protein kinase-like
At2g05940	RLCKVII	RYSASLP	*Arabidopsis thaliana* putative protein kinase
At1g61590	RLCKVII	RISLSLP	*Arabidopsis thaliana* ser/thr protein kinase
At3g02810	RLCKVII	KADSDP	*Arabidopsis thaliana* putative protein kinase
At3g58690	Extensin	RSGSVP	*Arabidopsis thaliana* ser/thr-specific protein kinase-like
At1g71830	LRR II	RPPSQP	*At*SERK1
At4g33430	LRR II	RPESQP	*At*SERK3
At4g30520	LRR II	KLKSKP	*Arabidopsis thaliana* receptor like kinase homolog somatic
At1g11050	RKF3L	KMPLSWP	*Arabidopsis thaliana* Ser/Thr protein kinase isolog
At5g38990	*Cr*RLK1L1	RDKASDP	*Arabidopsis thaliana* receptor-like protein kinase
At5g39000	*Cr*RLK1L1	RDKTSDP	*Arabidopsis thaliana* receptor-like protein kinase
At2g23300	LRR III	RKGGSSP	*Arabidopsis thaliana* receptor-like protein kinase
At5g67280	LRR III	RKVGSSP	*Arabidopsis thaliana* receptor like protein kinase
At3g28040	LRR VII	REPSTP	*Arabidopsis thaliana* unknown protein
At5g60310	L-Lectin	KPVLSWP	*Arabidopsis thaliana* receptor like protein kinase
At5g42120	L-Lectin	KKPSSDP	*Arabidopsis thaliana* receptor lectin kinase-like protein
At2g24370	RLCK IX	RLGNSPP	*Arabidopsis thaliana* putative protein kinase
At5g26150	RLCK IX	RTGNSPP	*Arabidopsis thaliana* putative protein various predicted protein
At5g12000	RLCK IX	RRGNSPP	*Arabidopsis thaliana* putative receptor-like kinase
At2g45910	RLCK IX	KDNSPP	*Arabidopsis thaliana* putative protein kinase
At5g25440	RLCK II	RPKSLP	*Arabidopsis thaliana* putative protein kinase

Table 3b: Proteins with putative 14-3-3 binding sequence elsewhere in the kinase domain.

Accession number	Subfamily	14-3-3 binding sequence	Protein name
At2g28940	RLCKVII	KQPKSRP	*Arabidopsis* thaliana putative protein kinase
At5g56790	PERKL	RDPNSRP	*Arabidopsis* thaliana putative protein
At3g51990	CR4L	KVKSTP	*Arabidopsis* thaliana putative ser/thr protein kinase Pto kinase
At4g03390	LRR V	RCVQSEP	*Arabidopsis* thaliana putative LRR receptor like protein kinase
At2g20850	LRR V	RCVQSEP	*Arabidopsis* thaliana putative LRR receptor protein kinase
At4g34500	TAKL	KIKTSPP	*Arabidopsis* thaliana putative ser/thr protein kinase
At4g31100	WAKL	RDDSKP	*Arabidopsis* thaliana ser/thr-specific protein kinase like
At1g80640	RLCK X	KPSSEP	*Arabidopsis* thaliana putative protein kinase
At1g49730	URK I	KEGRSRP	*Arabidopsis* thaliana hypothetical protein predicted by genemark.hmm
At5g51770	RLCK XI	KSPVSRP	Putative protein
At3g21630	LysM	RLGDSYP	*Arabidopsis* thaliana putative protein kinase
At3g50230	LRR III	KSTDSRP	*Arabidopsis* thaliana Receptor-like protein kinase
At2g42290	LRR III	RLSVSAP	*Arabidopsis* thaliana putative receptor like protein kinase
At1g67510	LRR III	KSPDSSP	*Arabidopsis* thaliana putative receptor like protein kinase
At3g08680	LRR III	KHPDSRP	*Arabidopsis* thaliana putative protein kinase
At5g05160	LRR III	RNPESRP	*Arabidopsis* thaliana receptor like protein kinase
At5g65700	LRR XI	RLSSIP	*Arabidopsis* thaliana receptor protein kinase like protein
At3g49670	LRR XI	RLSSVP	*Arabidopsis* thaliana receptor protein kinase
At3g19700	LRR XI	RDFSAP	*Arabidopsis* thaliana receptor like protein kinase
At2g35620	LRR XIII	KCVSSSP	*Arabidopsis* thaliana putative receptor like protein kinase
At1g56140	LRR VIII-2	RPNSSP	*Arabidopsis* thaliana receptor like protein kinase
At5g01550	L-Lectin	RPTSRP	*Arabidopsis* thaliana receptor like protein kinase
At5g01560	L-Lectin	KPESRP	*Arabidopsis* thaliana receptor like protein kinase
At5g01540	L-Lectin	KPASRP	*Arabidopsis* thaliana receptor like protein kinase
At2g29220	L-Lectin	RSPESRP	*Arabidopsis* thaliana putative protein kinase
At5g61550	RLCK IX	KKAGSWP	*Arabidopsis* thaliana putative protein kinase 1
At2g16250	N.A.	KLGISSP	*Arabidopsis* thaliana putative LRR receptor protein kinase
At1g70450	PERKL	KTVSHP	*Arabidopsis* thaliana putative protein kinase

At3g26700	RLCK IX	RAESEP	*Arabidopsis* thaliana putative protein kinase
At1g06840	LRR VIII-1	KRMSSVP	*Arabidopsis* thaliana receptor protein kinase
At5g38990	*Cr*RLK1L1	RMQSVP	*Arabidopsis* thaliana Receptor-like protein kinase
At5g39000	*Cr*RLK1L1	RMQSVP	*Arabidopsis* thaliana Receptor-like protein kinase
At5g67280	LRR III	RTGGSAP	*Arabidopsis* thaliana receptor like protein kinase

The catalytic domains of protein kinases range from 250 to 300 amino acid residues. Compared to the complete *Arabidopsis* proteome, the relative occurrence frequencies of amino acid residues in the 610 protein kinases is different (Table 1). Based on the occurrence frequencies of residues in kinase domains we expect 89 peptide sequences corresponding to the motif sequence [RK]$X_{(2,3)}$SXP in a set of 610 proteins with a random sequence and a size comparable to protein kinase domains (Table 4). In the set of 610 RLKs and RLCKs we only found 51 sequences with a putative 14-3-3 binding sequence. The amount of putative 14-3-3 sequences that we found in protein kinases is lower than the number we expected to occur by chance alone, which might be due to the high degree of sequence conservation in kinase domains.

Table 4: Expected and observed amount of sequences corresponding to the 14-3-3 binding motif [RK]$X_{(2,3)}$SXP in different protein sets. The expected amount of sequences is calculated based on relative amino acid occurrence frequencies (Table 1).

Protein set	Expected	Observed
25498 *Arabidopsis* proteins	11310	10767
610 RLKs and RLCKs	258	271
610 RLK / RLCK kinase domains	89	51
End kinase domain >αH	4	13
Loop αD-αE	4	21

We found 21 protein kinases that have a putative 14-3-3 binding sequence in the loop between helices αD and αE and 13 protein kinases that have a putative binding sequence at the end of the kinase domain after helix αH (Table 2). On average, these loops both contain roughly ten amino acids. Based on the relative amino acid frequencies in kinases, four sequences corresponding to the 14-3-3 binding motif [RK]$X_{(2,3)}$SXP can be

expected in a set of 610 peptides containing ten amino acids with a random sequence (Table 4). When an alternative motif like [RK]$X_{(2,3)}$PXS or P$X_{(2,3)}$[RK]S is used for a motif search on the sequence in the loop between helices αD and αE no sequences can be found that contain either one of these motifs. This is in contrast to results obtained with other parts of the kinase domain including the sequence at the end of the kinase domain where these alternative motifs can be found. In addition, *At*SERK1 does not have a putative 14-3-3 binding sequence at the end of the kinase domain after helix αH. Because at present we only have experimental evidence available for the interaction between GF14λ and the kinase domain of *At*SERK1 (Chapter 3), we considered the presence of a 14-3-3 consensus binding sequence between helices αD and αE as a potential biologically relevant position in RLKs and RLCKs.

In order to optimise the consensus 14-3-3 binding sequence, we made a profile of the sequence in the loop between helices αD and αE and part of the adjacent helices based on a multiple alignment of 21 RLK and RLCK sequences that contain a putative 14-3-3 binding sequence at that location (Fig. 2). Part of the adjacent helices was also taken to build the profile, due to the high level of sequence divergence in the loop. This profile was tested against the dataset of the 610 RLKs and RLCKs and returned all 21 sequences used to make the profile. In addition, it also returned many other protein kinases that did not contain a putative 14-3-3 binding sequence in the loop between helices αD and αE. Apparently these kinases were retrieved based on homology with helices αD and αE only. The same was true when the profile was searched against the complete set of proteins encoded by the *Arabidopsis* genome. From this we conclude that by using a profile based on the loop between helices αD and αE no additional protein kinases can be found containing a putative 14-3-3 binding sequence in this region.

A reason we did not find any other protein kinases with a putative 14-3-3 binding sequence in the loop between helices αD and αE might be the absence of such protein kinases in *Arabidopsis*. In the *Arabidopsis* proteome 1055 putative protein kinases can be found using Interpro entry IPR000719, which searches for proteins containing the protein kinase catalytic domain signature, the protein kinase ATP binding domain signature and proteins in the protein kinase families of Pfam and ProDom. Of these 1055 proteins 468 contained a sequence corresponding to the 14-3-3 binding motif. 199 of the 468 proteins were not RLKs or RLCKs. The complete sequences of these 199 protein kinases were

```
HMM-Profile YEYMPNGSLEDHLFEP----RPPLSDP----IPLSWQTRLKA-

  At2g05940 YEFMPRGSLENQLFR-----RYSASLP--------WSTRMKA-
  At1g61590 YEFMPRGSLENHLFR-----RISLSLP--------WATRLKA-
  At5g35580 YEFMPRGSLESQLF------RRC-SLP------LPWTTRLNA-
  At2g23300 YDFVPNGSLVNARY------RKGGSSP----CHLPWETRLKA-
  At5g67280 YDFVPNGSLANARY------RKVGSSP----CHLPWDARLKA-
  At5g25440 SEYFPNGSLGSYISRDS---RPK-SLP--------WETRLKA-
  At4g33430 YPYMANGSVASCLRE-----RPE-SQP-----PLDWPKRQRA-
  At1g71830 YPYMANGSVASCLRE-----RPP-SQP-----PLDWPTRKRA-
  At4g30520 YPYMPNGSVAS---------KLK-SKP-----ALDWNMRKRA-
  At3g28040 SEYIPNGNLQSKLHE-----REP-STP-----PLSWDVRYKA-
  At5g38990 YEYMPHGTLKDHLFR-----RDKASDP-----PLSWKRRLEA-
  At5g39000 YEYMPHGTLKDHLFR-----RDKTSDP-----PLSWKRRLEA-
  At1g11050 YDYMSNGNLDDHLFPRGETTKMPLSWP---------QRKSA--
  At5g60310 SDYMTNGSLDEHLDDQ----KPVLSWP---------QRLVA--
  At5g26150 YEFMENGSLEDRLF------RTGNSPP------LSWRKRFEA-
  At5g12000 YEFMDNGSLEDRLF------RRGNSPP------LSWRKRFQA-
  At2g24370 YEFMANGSLEDRLF------RLGNSPP------LSWQMRFRA-
  At2g45910 YEYLPGGSLEDRLTC-----K-DNSPP------LSWQNRVRA-
  At5g42120 FEYLPNGSLSEFLHK-----K-PSSDPSEEIIVLSWKQRVN--
  At3g02810 YDYISGGSLQDHLHEP----KAD-SDP------MDWTTRMQA-
  At3g58690 YEFMANGGLQEHLYLPN---RSG-SVP----PRLDWETRMRA-
                      αD          [RK]X(2,3)SXP        αE
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Figure 2: Multiple alignment of the protein sequences in the loop between helices αD and αE and part of the adjacent helices of 21 protein kinase sequences. All sequences contain a putative 14-3-3 binding sequence in this loop.

aligned to the profile of the multiple alignment of RLK and RLCK kinase domains provided by Shiu and Bleecker (2001a). A pattern search with the motif [RK]$X_{(2,3)}$SXP on the protein sequence that aligned to the loop between helices αD and αE resulted in another 15 proteins that might contain a putative 14-3-3 binding sequence in this loop. However, three sequences were not kinases. To confirm the location of the 14-3-3 binding site, the kinase domains of the remaining 12 sequences were aligned to the kinase domains of the 21 RLK and RLCK kinase domain sequences with a putative 14-3-3 binding sequence in the loop between helices αD and αE. Two out of the 12 sequences did not contain a putative 14-3-3 binding sequence in the loop between helices αD and αE. Thus, there are at least 10 additional protein kinases with a putative 14-3-3 binding sequence in this loop that we did not find with the αD-αE loop based profile search (Table 5).

Table 5: Additional protein kinases containing a sequence corresponding to the 14-3-3 binding motif [RK]$X_{(2,3)}$SXP.

Accession number	14-3-3 binding sequence	Protein name
At5G01920	KDRSFP	Putative protein
At1G01450	KNTLSLP	Hypothetical protein
At1G64300	RYLFSVP	Putative phytochrome
At5G41730	RYLSSIP	Putative protein
At2G38620	RKGSNP	Cyclin-dependent kinase B1;2
At3G48750	KAVKSMP	Cyclin-dependent kinase A;1
At2G36350	RCFSEP	Putative protein kinase
At2G43930	KFGSFP	Putative protein kinase
At3G06030	KFGSFP	NPK1-related protein kinase 3
At3G50730	RFMHSRP	Protein kinase *At*N1-like protein

Discussion

In the kinase domain of *At*SERK1 we found a putative 14-3-3 binding sequence with the sequence RPPS^{394}QPP. This sequence is located in a loop situated between helices αD and αE. Upon performing a pattern search with the 14-3-3 binding motif [RK]$X_{(2,3)}$SXP on the kinase domains of 610 RLKs and RLCKs, we found 21 protein kinases with a putative 14-3-3 binding sequence in the loop between helices αD and αE. This is more than can be expected in a random set of 610 peptides with the same size as the loop. Among the protein kinases of *Arabidopsis* that are not RLK or RLCK another 10 proteins were found with a putative 14-3-3 binding sequence in the same loop.

Based on a pattern search with the 14-3-3 binding motif [RK]$X_{(2,3)}$SXP on the *Arabidopsis* proteome, Sehnke et al. (2002) stated that roughly 40% of all *Arabidopsis* proteins contain a putative 14-3-3 binding sequence. Due to the degeneracy of the motif [RK]$X_{(2,3)}$SXP, the amount of possible sequences corresponding to this motif is enormous. This will highly increase the change that a sequence corresponding to the motif can be found at random. Therefore the biological significance of a sequence corresponding to the

motif is highly doubtful. We tried to refine the prediction for the occurrence of 14-3-3 binding sequences based on our previous experimental evidence concerning the interaction between *At*SERK1 and GF14λ (Chapter 3). We restricted our data set to protein kinase domains and tried to find a relation between the location of 14-3-3 binding sequences and their biological significance. In protein kinase domains of RLKs and RLCKs we found a higher amount of 14-3-3 binding sequences between helices αD and αE than can be expected in a comparable set of proteins the size of the loop. Based on the experimental evidence of an interaction between *At*SERK1 and GF14λ and the presence of a putative GF14λ binding sequence in the *At*SERK1 kinase domain only at this location, we considered the presence of a 14-3-3 consensus binding sequence between helices αD and αE as a potential biologically relevant position in RLKs and RLCKs. From this we conclude that putative 14-3-3 binding sites can only be found when location and biological relevance of the binding sites are included in the analysis. However, experimental evidence is still necessary to verify whether 14-3-3 proteins can indeed bind to these putative binding sites.

As the sequence of the 14-3-3 binding motif is very degenerative we tried to optimise this motif and find kinases that are not RLK or RLCK with a 14-3-3 binding sequence in the loop between helices αD and αE. Therefore, the alignment of the region containing the loop between helices αD and αE and part of the helices was used to make a profile. The borders of the loop between these helices are not strictly defined. Minor and Kim (1996) determined that the context of a protein sequence is important for the formation of secondary structures like α-helices. Therefore, the precise location of helices αD and αE may vary a little resulting in a variation in the loop between the helices containing the 14-3-3 binding sequences. With the profile made from the alignment we could not find the 10 kinases with a putative 14-3-3 sequence that we did find using a pattern search with the original motif. This might be due to the high level of sequence homology between the helices αD and αE and the low level of sequence homology in the loop between these helices. Therefore, a profile search based on the sequence in the loop between helices αD and αE and part of the adjacent helices is not a reliable method to find protein kinases with putative 14-3-3 binding sequences in this loop. If an optimisation of the 14-3-3 binding motif in plant proteins is possible, it probably has to wait for more experimentally identified 14-3-3 binding sequences in these proteins.

The number of putative 14-3-3 binding sequences we found in kinase domains is based on the motif [RK]$X_{(2,3)}$SXP. However, some 14-3-3 interacting proteins have been identified whose sequences deviate significantly from the motifs mentioned above, or do not require phosphorylation of their targets for binding (Aitken, 2002). Therefore, the actual number of 14-3-3 binding sequences in protein kinase domains will deviate from what can be found by performing a pattern match using the motif [RK]$X_{(2,3)}$SXP. For example, plant plasma membrane H^+-ATPases use a unique three-residue 14-3-3 binding site, YpTV, at the very C-terminus of the proteins (Jelich-Ottmann et al., 2001). However, we did not find this sequence at the C-terminus of 1055 putative *Arabidopsis* protein kinases.

14-3-3 proteins exist as protein families in many species (Rosenquist et al., 2000). They exist as dimers in a native situation and also *in vitro* they readily form heterodimers (Wu et al., 1997). The crystal structure that was determined for two human 14-3-3 proteins (Xiao et al., 1995; Liu et al., 1995) shows that each molecule of the 14-3-3 dimer consists of nine antiparallel α-helices, together resulting in a U-shaped structure. The N-terminal domains form the dimerisation interface and the floor of the cleft; the C-terminal domains form the sides of the channel. Each monomer produces a cleft that is sufficient in size and shape to accommodate the interaction with a phosphorylated peptide from a binding partner (Yaffe et al., 1997). Thus, the 14-3-3 dimer is able to interact with two different phosphorylated peptides.

Like *At*SERK1, *At*SERK3 contains a putative 14-3-3 binding sequence in the loop between helices αD and αE. BAK1, which is identical to SERK3, was shown to interact with the BRI1 receptor kinase (Nam and Li., 2002). BRI1 does not contain a putative 14-3-3 binding motif in its kinase domain. BRI1 was shown to interact with BAK1 in the absence of 14-3-3 proteins (Li et al., 2002) indicating that 14-3-3 proteins are not necessary for heterodimerisation of *At*SERK3 and BAK1. Shah et al. (2001) showed that *At*SERK1 is able to homodimerise in plant cells in the absence of 14-3-3 proteins indicating that 14-3-3 proteins are not necessary for *At*SERK1 homodimerisation, although they might have a stabilising role.

The loop between helices αD and αE is located opposite to the active site of the kinase molecule and is therefore not directly involved in substrate binding or catalytic activity. However, binding of a 14-3-3 protein may influence the structural conformation

of the *At*SERK1 protein thereby changing its catalytic activity or its substrate binding properties. As 14-3-3 proteins form dimers, the target binding site of the other half of the 14-3-3 dimer that binds to *At*SERK1 may bind a substrate protein. In this way the 14-3-3 dimer may function as an adaptor/scaffold protein bridging the interaction between a substrate protein and the *At*SERK1 protein kinase thereby increasing the efficiency of trans-phosphorylation. This has been demonstrated for the interaction between Raf-1 and protein kinase C (PKC), where a 14-3-3 dimer facilitates the coupling between these proteins as a response of the cell to mitogenic stimuli (Van der Hoeven et al., 2000; Chapter 1).

Protein kinases with a putative 14-3-3 binding domain in the loop between helices αD and αE (Table 2a) might be substrates of *At*SERK1. Among the proteins that were found are family members of the *Cr*RLKs first isolated from *Catharanthus roseu*s that share homology to the kinase domain of the Pto kinase, which is essential for the onset of disease resistance after binding of the avrPto pathogen from *Pseudomonas syringae* (Tang et al., 1996). Therefore, *Cr*RLK family members might function in pathogen response like the legume lectin (L-Lectin) kinase-like proteins and a member of the extensin family (Shiu and Bleecker, 2001b) that also contain a 14-3-3 binding sequence. Among the protein kinases with a putative 14-3-3 binding sequence that were not RLKs or RLCKs, we also found a hypothetical protein (At1G01450), a putative phytochrome, a putative protein (At5g41730) and an ATN1-like protein (Table 5). According to the PlantsP Kinase Classification (PPC: http://plantsp.sdsc.edu/plantsp/family/class.020318. html) these proteins are family members of the light-sensor kinases. NPK1 is a MAPKKK that was shown to regulate the lateral expansion of the cell plate at cytokinesis (Nishihama et al., 2002). Therefore, like the cyclin-dependent kinases, that are Cdc2-like kinases and the NPK1-related protein kinase 3, several kinases containing a 14-3-3 binding sequence may be involved in cell division. *At*SERK1 is proposed to function in a signal transduction cascade involved in embryo formation (Hecht et al., 2001). Previously, we found that *At*CDC48, which is proposed to have a role in cell division (Rancour et al., 2002), is a potential target of *At*SERK1 signalling (Chapter 4). Therefore, the binding of 14-3-3 proteins may link *At*SERK1 signalling to cell cycle regulation. While clearly further experiments are essential to confirm or reject this hypothesis, this bioinformatical approach may provide an initial set of candidate proteins that putatively interact with

*At*SERK1 via a 14-3-3 dimer. In this way bioinformatics can direct the experimental approach for the identification of the biological function of *At*SERK1.

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Chapter 6

Summarising discussion

Protein kinases control a wide range of cellular events, such as cell division, cell growth, cell differentiation or cell death. Receptor-like protein kinases (RLKs) contain an extracellular domain, a transmembrane domain and an intracellular catalytic kinase domain. They percept extracellular stimuli and transduce these signals via many phosphorylation and dephosphorylation events to their effectors inside the cell. Phosphorylation of a residue might create a new protein-interaction site that may or may not include the phosphorylated residue itself Phosphorylation may also induce rearrangements in the 3D-conformation of the phosphorylated protein resulting in a change of catalytic activity or enabling protein-protein interactions (Schlessinger, 2000). Through these protein-protein interactions and with possible help of adaptor or scaffold proteins an individual receptor kinase may be involved in the regulation of multiple processes.

The *Arabidopsis* Somatic Embryogenesis Receptor-like Kinase 1 (*At*SERK1) was identified as an RLK containing a leucine zipper, five leucine-rich repeats (LRRs), a single transmembrane domain and a functional serine-threonine kinase (Hecht et al., 2001; Shah et al., 2001b). *At*SERK1 is expressed in ovule primordia, during early embryogenesis and vascular tissue of the adult plant (Hecht et al., 2001). Ectopic expression of *At*SERK1 increases somatic embryo formation in culture. Based on this expression pattern and the involvement in embryo formation, *At*SERK1 was proposed to participate in a signal transduction cascade involved in ovule and embryo development (Hecht et al., 2001).

The subject of the research described in this thesis is the identification of proteins that interact with the kinase domain of *At*SERK1 and may be involved in the signal transduction cascade mediated by the *At*SERK1 receptor. Over the last decade, yeast two-hybrid systems have become a general approach to identify protein-protein interactions (Serebriiskii et al., 2000). Therefore, we used this system to search for proteins interacting with the kinase domain of *At*SERK1. As a result of this screen in yeast we found six different putative *At*SERK1 interacting proteins. These proteins were predicted to encode the 14-3-3 protein GF14λ, the AAA-ATPase *At*CDC48, a putative RNA binding protein (RBP), the potassium transporter KEA1, the aquaporin *At*SIP1 and a racemase-like protein. Of these putative interacting proteins, *At*SERK1 can transphosphorylate only GF14λ, *At*CDC48 and RBP. Due to homology with a nuclear RNA binding protein, the

interaction between RBP and *At*SERK1 is not obvious. Therefore, only GF14λ and *At*CDC48 were selected for further analysis. RBP, *At*SIP1 and KEA1 can still be valid *At*SERK1 interaction partners, although the interaction has to be confirmed with a system different from yeast two-hybrid analysis.

Beside the putative *At*SERK1 interacting proteins that we did find, there were also proteins that we expected to find but that did not came out of the two-hybrid screening. For example, we did not find a protein phosphatase. Receptor kinase activity and subsequent signalling is regulated by phosphorylation and dephosphorylation events. Therefore, we expected to find a protein phosphatase that is able to dephosphorylate *At*SERK1. Shah et al. (2002) determined that the kinase-associated protein phosphatase KAPP is able to dephosphorylate *At*SERK1 *in vitro*. In addition they showed that the fluorescently labelled *At*SERK1 and KAPP proteins co-localise and interact in endosomes, which suggests that KAPP is involved in *At*SERK1 endocytosis. We showed that KAPP is also able to interact with the *At*SERK1 interaction partner *At*CDC48 in yeast. From these results, we propose that KAPP may indeed function as a component of the *At*SERK1 signalling cascade.

A yeast two-hybrid screen with the kinase domain of the brassinosteroid receptor BRI1 resulted in the isolation of the protein kinase BAK1, which binds with its C-terminal domain to the BRI1 kinase domain (Nam and Li., 2002). BAK1 has high sequence identity with *At*SERK1 and is identical to the *At*SERK family member *At*SERK3 (accession nr. AF384970). The two LRR-receptor kinases BAK1 and BRI1 bind *in vitro* and *in vivo* and can transphosphorylate each other *in vitro* (Li et al., 2002). Most likely they function together through heterodimerisation to mediate BR signalling in *Arabidopsis*. Because interaction studies showed that the *At*SERK1 kinase domain could not form homodimers by itself (Shah et al., 2001a), we did not expect to find *At*SERK1 as interaction partner. However, based on the results with BRI1, we expected to find a transmembrane heterodimerisation partner for *At*SERK1.

As yeast two-hybrid screenings are never saturating, most probable not all *At*SERK1^{kinase}-interacting proteins have been found. This could be due to weak interactions, the lack of accessory proteins or the need of post-translational modifications that are essential for protein function and their interaction but cannot be provided by yeast due to the absence of the relevant modifying enzymes (Serebriiskii et al., 2000). For

example, some proteins that bind to the *At*SERK1 kinase domain might need the presence of the adaptor protein GF14λ. In addition, the phosphorylation status of the *At*SERK1 kinase domain in the nucleus of yeast is unknown. For these reasons, proteins that we expected to find were probably not found.

The interaction between *At*SERK1 and GF14λ was confirmed *in vitro* by pull down assays that showed that GF14λ preferably binds to the phosphorylated *At*SERK1 kinase domain. Besides binding GF14λ, *At*SERK1 can also transphosphorylate GF14λ *in vitro*. The *in vivo* interaction was confirmed with immunoprecipitation assays and at the cellular level by a molecular interaction between fluorescently labelled GF14λ and *At*SERK1 at the plasma membrane of protoplasts. GF14λ belongs to the 14-3-3 protein family, which consists of highly conserved 28-31 kD proteins that predominantly exist as dimers under native conditions (Wu et al., 1997). 14-3-3 proteins are broadly expressed in a wide range of eukaryotes and at least 13 different isoforms have been identified in *Arabidopsis* (Rosenquist et al., 2001). Although 14-3-3 isoforms are very homologous to each other and the residues involved in ligand binding are highly conserved, there are differences in binding specificity (Rosenquist et al., 2000). Binding of a 14-3-3 protein can alter the confirmation of the interacting protein, resulting in a change in activity of that protein (Svennelid et al., 1999). 14-3-3 proteins can function as a competitive inhibitor preventing the binding of other proteins or as a scaffold protein by promoting the assembly of signalling complexes (Pigaglio et al., 1999). 14-3-3 binding can also affect cellular translocation of the target protein by blocking a nuclear localisation or export signal (Lopez-Girona et al., 2001). Taken together, it is becoming clear the 14-3-3 proteins have a regulatory function via protein-protein interactions. However, the role GF14λ performs upon binding to *At*SERK1 is unknown. GF14λ may function as an adaptor protein, facilitating phosphorylation of substrate proteins by the *At*SERK1 kinase domain upon binding of these proteins to the other half of the 14-3-3 dimer. We determined that GF14λ is being transphosphorylated as soon as *At*SERK1 starts autophosphorylation. Therefore, GF14λ binding does not inhibit the catalytic activity of *At*SERK1. Phosphorylation of 14-3-3 adds a charged phoshate-group to the protein, which can influence the specificity of interaction with its current interactor and/or with another interactor. It is possible that GF14λ is able to interact with another component of the *At*SERK1 signalling complex after transphosphorylation by *At*SERK1.

The interaction between *At*SERK1 and *At*CDC48 was confirmed *in vitro* by binding assays and in plant cells by co-immunoprecipitation. The binding assays showed that *At*CDC48 preferably binds to the phosphorylated *At*SERK1 kinase domain. Upon binding, *At*SERK1 is able to transphosphorylate *At*CDC48 on at least one threonine residue within the C-terminal 62 amino acid residues. Pair wise interaction tests between *At*SERK1 and its binding partners in yeast showed that besides binding to *At*SERK1^{kinase}, *At*CDC48 can also bind to GF14λ and the phosphatase KAPP. Interaction between *At*CDC48 and GF14λ was confirmed in plant cells. In *Arabidopsis*, *At*CDC48 is highly expressed in proliferating and rapidly growing cells (Feiler et al., 1995), where *At*CDC48 can be found in the cytoplasm as well as attached to membranes. At the subcellular level, the *At*CDC48 protein is localised at the division plane in dividing *Arabidopsis* cells, in endosomes in both dividing and interphase cells and with the nuclear envelope during interphase (Rancour et al., 2002). *At*SERK1 and the phosphatase KAPP were shown to interact in early endosomes (Shah et al., 2002). Therefore, an interaction between *At*SERK1 and *At*CDC48 is possible after *At*SERK1 internalisation at the endosomes. However, interaction between *At*SERK1 and cytosolic *At*CDC48 is also possible.

Yeast Cdc48 and its mammalian homologues p97 and VCP are proposed to affect different cellular processes like cell division (Madeo et al., 1998), homotypic membrane fusion (Pécheur et al., 2002) and targeted proteolysis of ubiquitinated retro-translocated proteins from the ER (Rabinovich et al., 2002) using specific adaptor proteins. The commonality between these processes seems to be the ATP-dependent protein folding/unfolding activity of Cdc48/p97/VCP (Golbik et al., 1999). The Cdc48 protein shuttles between the cytoplasm and the nucleus in a cell cycle-dependent manner, which makes it possible to couple the different cellular activities. In yeast, this shuttling is regulated by phosphorylation of a tyrosine residue in the C-terminus of the protein (Madeo et al., 1998). The mammalian CDC48 homologue p97/VCP was found to be phosphorylated by the protein tyrosine kinase JAK-2 (Lavoie et al., 2000) and dephosphorylated by the phosphotyrosine phosphatase PTPH1 (Zhang et al., 1999). p97 phosphorylation coincides with its relocalisation from the ER to the centrosome (Lavoie et al., 2000). Phosphorylation of a threonine residue in the C-terminus of *At*CDC48 by *At*SERK1 might have a comparable function as phosphorylation of the tyrosine residue in the C-terminus of yeast Cdc48 in changing the three-dimensional structure of the Cdc48

protein. During cell division *At*CDC48 is found at the division plane (Rancour et al., 2002). Phosphorylation of *At*CDC48 by *At*SERK1 might result in cellular relocalisation of *At*CDC48 to the phragmoplast. Analogous to the function of PTPH1, KAPP might be the phosphatase that negates *At*SERK1 phosphorylation of *At*CDC48, thereby inhibiting cellular relocalisation of *At*CDC48.

*At*CDC48 was shown to be able to interact with GF14λ. Analogous to PTPH1 that binds 14-3-3β (Zhang et al., 1997), KAPP may also bind a 14-3-3 protein. Like 20 other RLKs and cytoplasmic RLK-homologues, *At*SERK1 contains a putative 14-3-3 binding domain based on the motif [RK]$X_{(2,3)}$SXP (Sehnke et al., 2002) in a loop between two helices located at the opposite site of the molecule from where the catalytic site of the kinase domain is located. Both *At*CDC48 and KAPP also contain a putative 14-3-3 binding sequence. Based on our results, we propose that *At*SERK1 and its interaction partners are involved in one of the many controlling pathways of the cell cycle. The observation that several *Arabidopsis* kinases predicted to be involved in cell division also contain a putative 14-3-3 binding sequence supports this hypothesis.

Besides its function in cell cycle progression, *At*CDC48 is also involved in targeted proteolysis of ubiquitinated retro-translocated proteins from the ER (Rabinovich et al., 2002). Proteins in the secretory pathway that are misfolded or unassembled, are dislocated from the ER to the cytosol upon which they are ubiquitinated (Bonifacino et al., 1998). *At*CDC48, with the help of specific adaptor proteins, is proposed to mediate the dislocation of ubiquitinated proteins and to target them for proteolysis. Like other misfolded or proteins, newly synthesised *At*SERK1 proteins that are misfolded during the secretory pathway will be degradated by this mechanism. Endocytosis in combination with regulated proteolysis is a mechanism to downregulate receptor signalling (Strous and Gent, 2002). *At*SERK1 was shown to be internalised when co-expressed with the phosphatase KAPP in protoplasts (Shah et al. 2002). In analogy to the mammalian p47, Ufd1 and Npl4 Cdc48 adaptor proteins (Meyer et al., 2002), the kinase domain of *At*SERK1 may contain a domain that is structurally similar to ubiquitin. *At*CDC48 may bind to this domain and target *At*SERK1 for degradation. To verify our hypotheses about the role of *At*CDC48 in *At*SERK1 signalling more experimental evidence is needed. The molecular interaction of fluorescently labelled *At*SERK1 and *At*CDC48 should indicate where these proteins interact in the cell.

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Summary

Receptor kinases are essential to perceive the signals that induce cellular events such as cell division, cell differentiation and cell death. The Somatic Embryogenesis Receptor-like Kinase 1 (*At*SERK1) is a receptor-like kinase (RLK) containing five leucine-rich repeats in its extracellular domain. *At*SERK1 is proposed to participate in a signal transduction cascade involved in ovule and embryo development. The downstream components of the *At*SERK1 signal transduction cascade are mostly unknown. Therefore we used the yeast two-hybrid system to screen for proteins interacting with the kinase domain of *At*SERK1. As a result of the screen we found six different putative *At*SERK1 interacting proteins. These proteins were predicted to encode a member of the *Arabidopsis thaliana* family of 14-3-3 proteins called GF14λ, the AAA-ATPase *At*CDC48, a putative RNA binding protein (RBP), the potassium transporter KEA1, the aquaporin *At*SIP1 and a racemase-like protein. As we were looking for proteins that may be involved in the signal transduction cascade mediated by the *At*SERK1 receptor kinase we performed i*n vitro* kinase assays to determine which proteins *At*SERK1 can use as a substrate. GF14λ, *At*CDC48 and RBP can be transphosphorylated by *At*SERK1 *in vitro*. Therefore, only the interaction of *At*SERK1 with these proteins was studied in further detail.

In vitro binding assays were performed to confirm the interaction with *At*SERK1 and showed that GF14λ preferably binds to the phosphorylated *At*SERK1 kinase domain. The interaction between *At*SERK1 and GF14λ *in vivo* was demonstrated by co-immunoprecipitation assays and in plant cells by the occurrence of FRET between fluorescently labelled *At*SERK1 and GF14λ proteins. In the kinase domain of *At*SERK1 we found a putative 14-3-3 binding motif with the sequence RPPSQPP surrounding Ser-394. This motif is located in a loop opposite to the active site of the kinase molecule. Beside *At*SERK1 another 20 *Arabidopsis* receptor-like kinases contain a putative 14-3-3 binding domain in this loop.

Like GF14λ, the ATPase *At*CDC48 preferably binds to the phosphorylated kinase domain in an *in vitro* binding assay and the *in vivo* interaction was demonstrated by co-immunoprecipitation assays. Upon binding, *At*SERK1 is able to transphosphorylate *At*CDC48 on a threonine residue within the C-terminal 62 residues of the protein. In yeast,

*At*CDC48 is also able to interact with the *At*SERK1-binding partners GF14λ and the protein phosphatase KAPP. The interaction between *At*CDC48 and a 14-3-3 protein was confirmed in plant cells by co-immunoprecipitation. This shows analogy to the mammalian CDC48 homologue p97/VCP, which can be phosphorylated by the JAK-2 kinase and dephosphorylated by the phosphatase PTPH1 that associates with a 14-3-3 protein. Based on the function and the cellular localisation of *At*CDC48 we propose that *At*SERK1 is involved in one of the many controlling pathways of the cell cycle. The observation that several *Arabidopsis* protein kinases predicted to be involved in cell division also contain a putative 14-3-3 binding sequence supports this hypothesis.

Eiwitten die aan de *Arabidopsis thaliana* Somatische Embryogenese Receptor Kinase 1 binden

Samenvatting

Receptor kinases kunnen signalen van buiten de cel opvangen en deze doorgeven naar effector eiwitten in de cel. Op deze manier zijn ze betrokken bij de regulatie van veel cellulaire processen, zoals celdeling, celdifferentiatie en celdood. De Somatische Embryogenese Receptor Kinase (*At*SERK1) is een receptor kinase van *Arabidopsis* (zandraket) die waarschijnlijk betrokken is bij een signaaltransductie route die zorgt voor de ontwikkeling van ovules en embryos. Het is tot nu toe nog onduidelijk naar welke effector eiwitten *At*SERK1 zijn signaal doorgeeft. Daarom hebben we met behulp van het twee-hybride systeem in gist geprobeerd om eiwitten te vinden die aan het intracellulaire kinase gedeelte van de receptor binden. Met dit systeem hebben we zes mogelijke interactoren gevonden. Dit zijn de eiwitten GF14λ, een lid van de *Arabidopsis* familie van 14-3-3 eiwitten, *At*CDC48, een ATPase, RBP, een RNA bindend eiwit, KEA1, een kalium transport eiwit, *At*SIP1, een waterkanaal, en tenslotte een racemase-achtig enzym. Omdat we zochten naar eiwitten die het signaal van *At*SERK1 kunnen ontvangen hebben we een fosforylatie experiment uitgevoerd, waarmee we kunnen bepalen of deze eiwitten door *At*SERK1 als substraat gebruikt kunnen worden. GF14λ, *At*CDC48 en RBP kunnen door *At*SERK1 gefosforyleerd worden, dus hebben we alleen van deze eiwitten de interactie verder geanalyseerd.

De interactie van GF14λ met *At*SERK1 hebben we bevestigd door middel van *in vitro* bindingsexperimenten. Hieruit bleek dat GF14λ bij voorkeur bindt aan *At*SERK1 als het kinase domein is geactiveerd door middel van fosforylatie. De interactie tussen deze twee eiwitten hebben we ook aangetoond in planten cellen. Dit hebben we gedaan door middel van co-immunoprecipitatie experimenten, maar ook door het aantonen van een moleculaire interactie tussen fluorescent gelabeld GF14λ en *At*SERK1. In het kinase domein van de *At*SERK1 receptor hebben we een mogelijke 14-3-3 bindingssequentie gevonden. Deze sequentie bevind zich in een lus van het eiwit, precies aan de andere kant van het molecuul als waar het katalytische centrum zich bevindt. Naast *At*SERK1 hebben we dit motief ook nog in 20 andere receptor kinases van *Arabidopsis* gevonden.

De interactie van *At*CDC48 met *At*SERK1 hebben we aangetoond *in vitro* met bindinsexperimenten en in planten cellen door middel van co-immunoprecipitatie. Net als GF14λ, bindt *At*CDC48 bij voorkeur aan *At*SERK1 als het kinase domein is geactiveerd door fosforylatie. Als *At*CDC48 heeft gebonden kan het door *At*SERK1 worden gefosforyleerd. Dit gebeurt dan op een threonine aminozuur ergens in de laatste 62 aminozuren van *At*CDC48. Proeven in gist met het twee-hybride systeem laten zien dat niet alleen *At*SERK1, maar ook *At*CDC48 aan de eiwitten GF14λ en de fosfatase KAPP kan binden. Dit is analoog aan het CDC48 eiwit in dierlijke cellen, waar het p97 of VCP wordt genoemd. p97/VCP kan door het kinase eiwit JAK-2 worden gefosforyleerd en door de fosfatase PTPH1 worden gedefosforyleerd. Aan de fosfatase PTPH1 is het grootste gedeelte van de tijd een 14-3-3 eiwit gebonden. Gebaseerd op de functie en de lokalisatie van het CDC48 eiwit in *Arabidopsis* stellen wij voor, dat *At*SERK1 is betrokken bij een van de vele signaaltransductie routes die de celdeling regelen. Het feit dat enkele kinase eiwitten van *Arabidopsis*, die waarschijnlijk functioneren tijdens de celdeling, ook een mogelijke 14-3-3 bindingssequentie hebben ondersteunt deze hypothese.

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Lieve mensen, dank jullie wel !!!!!

Curriculum vitae

Ingrid Maijelle Rienties werd geboren op 2 maart 1976 te Emmen. In 1988 behaalde zij het VWO diploma aan de Gemeentelijke Scholen Gemeenschap (GSG) te Emmen. Vervolgens begon ze met de studie biologie aan de Katholieke Universiteit Nijmegen. Al snel was duidelijk dat de voorkeur uit ging naar het bestuderen van de moleculaire biologie en dan het liefst van planten. De eerste stage was dan ook bij de vakgroep Experimentele plantkunde in Nijmegen in de groep van Titi Mariani, waar zij gewerkt heeft aan de isolatie and karakterisatie van genen die specifiek tot expressie komen in de secretorische zone van stampers uit de bloemen van de tabaksplant. Een tweede stage werd gedaan bij het CPRO-DLO in Wagenigen, tegenwoordig Plant Research International, in de groep van Harry Verhoeven, waar zij gewerkt heeft aan het zoeken naar genen van Petunia die betrokken zijn bij de productie van vluchtige stoffen. In november 1994 werd de studie afgerond en haalde zij het doctoraal examen met genoegen. In september van datzelfde jaar begon ze al als OIO bij de vakgroep Moleculaire Biologie van Wageningen Universiteit om eiwitten te vinden die aan de receptor kinase *At*SERK1 kunnen binden en mogelijk een rol spelen in de signaal-transductie keten die wordt gemedieerd door *At*SERK1. Dit onderzoek heeft na ruim 4 jaar geresulteerd in dit proefschrift

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