Characterization of the *Arabidopsis thaliana* somatic embryogenesis receptor-like kinase 1 protein

Khalid Shah

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receptor-like kinase 1 protein

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
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prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
op woensdag 6 juni 2001
des namiddags te vier uur in de Aula

Statements

1. The activated receptor dimer is in a transient rather than a stable state in the plasma membrane.
   Verveer, P.J., 2000 *Science* **290**, 1567-1570

2. The phosphorylation or dephosphorylation of a protein is a flexible way of reversibly altering its confirmation and hence its ability to function.
   Cohen, P., 2000 *Trends Biol. Sciences* **25**, 596-601

3. AtSERK1 N-linked glycosylation sites in the extracellular LRR domain are important for targeting the AtSERK1 protein to the plasma membrane.
   This thesis.

4. The phosphorylation of Thr-468, an amino-acid conserved in most plant RLKs, is essential for both auto- and transphosphorylation activity of AtSERK1.
   This thesis.

5. Reason, Observation, and Experience, the Holy Trinity of Science.
   Robert G. Ingersoll.

6. Evolution is cleverer than you are.
   Francis Crick.

7. Chance favors the trained mind.
   Louis Pasteur.

8. With each passing day, my belief in achieving my research goals gets stronger and stronger although most of the days yield miniscule returns.

Statements accompanying the thesis: 'Characterization of the *Arabidopsis thaliana* somatic embryogenesis receptor-like kinase 1 protein' by Khalid Shah, Wageningen, June 6 2001.

For my parents to whom I owe the gift of life

Contents

SCOPE

One of the most exciting developments in molecular cell biology is the elucidation of many signalling cascades that transduce an extracellular stimulus from its perception at the membrane to its effectors inside the cell. Transmembrane receptors are prime components of many these signalling chains and thus help determine cell fate, growth, differentiation, migration and death. The plant embryo develops from the fertilized egg cell via characteristic morphological stages into plants Somatic embryogenesis is the process where by somatic cells develop into plants via the same morphological stages. The *Somatic Embryogenesis Receptor Kinase (SERK)* gene was isolated from *Daucus carota* somatic cells as a marker to monitor the transition of somatic into embryogenic cells. During zygotic embryogenesis, expression of *SERK* gene is found in globular zygotic embryos and not in later embryo stages. The *Arabidopsis thaliana Somatic Embryogenesis Receptor Kinase* 1 *(AtSERK1)* gene is the most closely related *Arabidopsis SERK* gene and is expressed in developing ovules, early embryos and in the vascular tissues of seedlings and adult plants. The predicted AtSERK1 protein contains an extracellular domain with a leucine zipper (LZ) motif followed by five leucine-rich repeats (LRRs), a proline-rich region, a single transmembrane region and an intracellular kinase domain. The major goal of the study presented in this thesis is the biochemical characterization of the AtSERK1 protein in order to reveal some of the early steps in the signal transduction cascade mediated by AtSERK1.

In Chapter 1, an introduction is given concerning the role of single transmembrane serine/threonine receptor kinases in signal transduction. Besides the description of well-known animal serine/threonine receptor kinases, its plant counterparts are discussed in detail.

In Chapter 2, the functional studies performed on different LRR transmembrane proteins and their role in protein-protein interactions is reviewed.

In Chapter 3, the biochemical properties, based on phosphorylation assays of affinity purified AtSERK1 and AtSERK1 mutant proteins produced *in vitro* is presented. In addition comparative modelleing of the AtSERK1 kinase domain using the insulin receptor kinase domain as a template is reported. Based on the predicted AtSERK1 structure, threonine residues in the AtSERK1 activation loop of catalytic sub-domain VIII were postulated to be targets for autophosphorylation activity. Single amino acid mutations replacing threonine residues in the AtSERK1 A-loop reveal the importance of Thr-468 in both AtSERK1 auto- and

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transphosphorylation. The ability of the AtSERK1 protein to transphosphorylate a catalytically inactive AtSERK1 protein shows that AtSERK1 catalyzes an intermolecular mechanism of autophosphorylation. Based on these observations, similar to the mechanism employed by animal receptors, the presence of a minor population of catalytically active AtSERK1 molecules *in vitro*, or ligand induced conformational changes *in vivo*, the activation of AtSERK1 requires intermolecular autophosphorylation. Activation of the receptor may require the movement of the AtSERK1 threonine containing A-loop followed by release of transphosphorylation activity and protein substrate binding. In this model, phosphorylation of Thr-468 in the A-loop of one AtSERK1 monomer is also essential for releasing catalytic activity of that same monomer.

In Chapter 4, the experiments are presented that reveal the sub-cellular localization and oligomerization of the AtSERK1 protein. These experiments were performed using transient transfections in cowpea mesophyll protoplasts. In addition, the potential for oligomerization of the AtSERK1 protein was determined by fluorescence resonance energy transfer (FRET) between GFP-derivative-AtSERK1 fusion in plant cells and in the yeast two hybrid based protein interaction system. The AtSERK1 protein is targeted to plasma membranes of plant cells. The extracellular LRRs, and in particular the N-linked oligosaccharides that are present on them are essential for correct localization of the AtSERK1 protein. The AtSERK1 protein exists as a monomer in the membrane. Only a minority of the AtSERK1 receptor molecules on the plasma membrane is in a predimerized state. In the absence of the LZ domain, the small population of predimerized AtSERK1 receptors are not detectable anymore which shows the importance of the LZ domain in dimerization. While identification of the ligand for AtSERK1 is clearly essential for determining its precise mode of action, it is proposed that AtSERK1 exhibits ligand-induced homodimerization

In chapter 5, experiments are described that demonstrate the interaction of the AtSERK1 protein with the *Arabidopsis* kinase associated protein phosphatase (KAPP). Using *in vitro* phosphorylation experiments and transient transfections in cowpea mesophyll protoplasts, it was shown that the kinase domain of AtSERK1 interacts with the kinase interaction domain of KAPP in a phosphorylation-dependant manner. The kinase interaction domain of KAPP does not interact with the catalytically inactive kinase mutant or the mutants lacking the essential threonines in the AtSERK1 A-loop for phosphorylation of AtSERK1 kinase. Using GFP-derivative fusions, we also show that the KAPP and AtSERK1 proteins are colocalized in plant

cells at the plasma membrane and in intracellular vesicles, but only interact physically intracellularly. It is proposed that KAPP plays an essential role in internalization and inactivation of the AtSERK1 protein.

In Chapter 6, a summarizing discussion of the research presented in the thesis is provided.

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General Introduction

Signal transduction mediated by single membrane spanning serine/threonine kinase receptors

Khalid Shah and Sacco C. de Vries. Manuscript prepared for submission in *Trends in plant Sciences*

All cells are continuously subjected to a multitude of biotic and abiotic stimuli. Variations in e.g. day length, mechanical stress, and sugar level or hormones need to be perceived, integrated and acted upon. Essential components in such perception mechanisms are receptors. Receptors come in many different types, ranging from cell surface located plasma membrane spanning receptors such as those that perceive growth factors, light or sugars to receptors that act intracellularly in e.g. calcium sensing, recycling of ER resident proteins or enhancer activation by steroid hormones.

The same or highly analogous receptors and their corresponding ligands often exhibit striking cell specificity in terms of their cellular effects, so that the same signal can elicit a different response depending on how it is 'wired' into the cellular transduction mechanisms (Cohen et al., 1995). Such is the importance of signalling systems that it is estimated, based on complete genome sequence, that about 20% of the cellular activities in intracellular organisms have to do with signal perception and transduction (Wolfsberg et al., 2000). Novel transduction pathways are continuously reported while different mechanisms for receptor activity and functioning are being proposed (Verveer et al., 2000), indicative of rapidly progressing understanding of signalling mechanisms and pathways

In this review we will focus on a specific class of receptors, the plasma membrane located receptor kinases, that are widespread in all organisms studied are essential components in a wide variety of signal transduction pathways (Hanks et al., 1988). This class of receptors is characterized by an intracellular domain that possesses protein kinase activity that is attached via a transmembrane domain to an extracellular signal perception domain. Signalling pathways through these receptors involve an extracellular and a cytoplasmic phase. Binding of a ligand to the extracellular domain induces oligomerization of two or three or more receptor monomers. This is then followed by receptor autophosphorylation that in turn leads to phosphorylation of cytoplasmic proteins. Eventually, activation of these second messengers then directly or indirectly initiates the physiological process in the cell that was the target of the particular signalling pathway.

We will now first describe mechanisms of receptor activation and inactivation and different types of transmembrane receptors before turning to discussing in detail the serine/threonine receptor kinase signalling and the protein families that function as downstream partners in

serine/threonine receptor kinase signalling. We will end with the description of plant receptors of the plasma membrane receptor kinase type.

Receptor dimerization

Receptors that have a single transmembrane domain appear to be primarily activated by ligand induced dimerization or oligomerization (Heldin, 1995). Dimerization can take place between two identical receptors (homodimerization); between different members of the same receptor family (heterodimerization); or between a receptor and an accessory protein (heterodimerization) (Lemmon and Schlessinger, 1994) (Fig. 1). Although activation by heterodimerization appears to be the most common mechanism (Heldin, 1995), there are many receptors that are activated by homodimerization e.g., the receptors for the growth hormones, erythropoietin, prolactin and granulocyte colony stimulating factor. Different receptor ligand interactions leading to dimerization are illustrated in Fig. 2. Several of the ligands are dimers themselves and thus contain two surfaces for receptor binding. For example platelet derived growth factor (PDGF) is a disulfide-bonded dimer that induces PDGF receptor dimerization and can occur in three different isoforms: A chain homodimers that bind to PDGFα receptors with high affinity, B chain homodimers that bind to both PDGF α and β receptors with equal affinity and AB chain heterodimers. Thus, AA PDGF produces PDGFα- PDGFα receptor homodimers and BB produces all possible combinations, αα, αβ and ββ PDGF receptors (Kanakaraj, 1991). In contrast, monomeric human growth hormone (hGH) ligand uses two different sites on its surface to interact with two receptor molecules to form a ligand receptor complex (Wells, 1996). The crystal structure of a trimeric ligand reveals that the tumor necrosis factor (TNF)-β binds to three TNF receptor molecules in a ligand-receptor complex (Banner 1993). Another situation arises when a monomeric ligand itself is incapable of inducing dimerization of its receptor unless it forms a multivalent complex with an accessory protein. A classical example of this is the acidic fibroblast growth factor (aFGF), which forms a multivalent complex with heparin sulfate proteoglycans. In turn FGF complex can then bind to two or more receptors (Spivak-Kroizman, 1994). In many cases receptor molecules are in a predimerized state and the ligand binding changes the receptor confirmation and leads to their activation. An example of this is the activation of insulin receptor which is a disulfide bonded dimer and may require a conformational change by ligand binding to initiate signalling (Klemm et al., 1998). Different receptor-ligand

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interactions leading to dimerization are illustrated in Fig. 2. The general oligomerization model holds that inactive receptor monomers are in equilibrium with the active receptor dimers, and that ligand binding stabilizes the active dimeric form (Schlessinger, 1988).
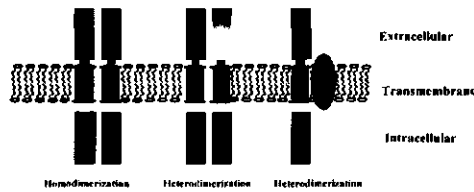
Figure 1. Dimerization of two similar receptors (**A**) two related receptors (**B**) and a receptor and an accessory protein (**C**).

Three general lines of evidence suggest that transmembrane receptors are activated by monomer-to-dimer transition. The most convincing evidence for a dimerization mechanism of activation is that ligand binding leads to receptor dimerization (Heldin et al., 1989). A second line of evidence is that artificially induced dimerization or naturally occurring mutations lead to dimerization, recapitulating signalling in the absence of the physiological ligand (Schlessinger, 1988; Weiner et al., 1989). Finally, oligomerizing the intracellular regions of receptors with cell-permeable synthetic ligands can lead to dimerization (Spencer et al., 1993).

Dimerization represents a powerful and flexible regulatory mechanism that can achieve a variety of consequences (Klemm et al., 1998). After dimerization the receptor molecules are brought in close proximity with one another, which may allow them to act in trans on one another. The most common example of this strategy is the ligand-induced dimerization of the TGF-β receptor or the EGF receptor whose intracellular kinase domains phosphorylate the dimer partner when brought in closer proximity (Heldin, 1995).

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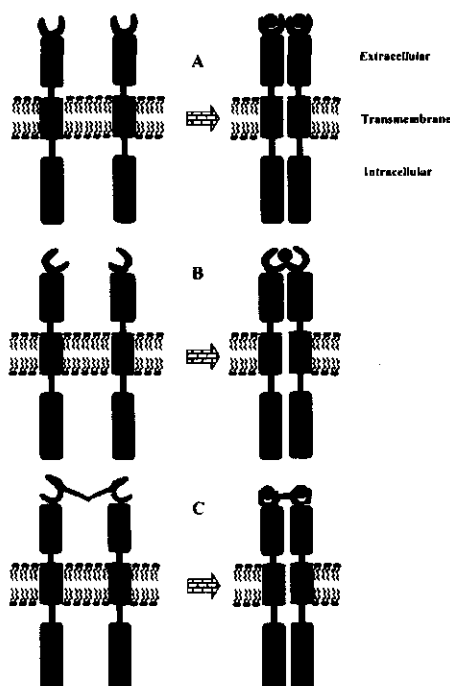
Figure 2. Ligand induced dimerization. **A**: Two monovalent ligand molecules (small rounds) bind to two similar or related receptor molecules. **B**: A divalent ligand molecule can bind to two similar or related receptor molecules. **C**: Two monovalent ligand molecules bind to two predimerized receptor molecules.

Autophosphorylation

The next step in the signal transduction cascade is receptor "autophosphorylation" which occurs when one receptor molecule phosphorylates intracellular kinase domain of the other in a dimer (Ullrich and Schlessinger, 1990). The kinase catalytic domains range from 250-300 amino acid residues, corresponding to about 30 kD. By virtue of the analysis of conserved sequences (Hunter, 1986) and by assays of truncated enzymes (Levinson, 1984; Wilkerson, 1986) fairly precise boundaries of kinase domains have been defined. The kinase domain is divided into twelve smaller sub-domains and each contains a characteristic pattern of conserved residues. The smaller N-terminal lobe containing sub-domain I-IV is primarily involved in anchoring and

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orienting the ATP molecule (Hanks et al., 1991). The larger C-terminal lobe containing sub-domains VIa-IX is largely responsible for binding the substrate and initiating phosphotransfer (Brugge and Darrow 1984; Yaciuk and Shalloway, 1986). Sub-domain V residues span the C-terminal and the N-terminal lobe whereas the function of most poorly conserved domains, X and XI is obscure (Hanks and Hunter, 1996). The mechanism of initiation of autophosphorylation is still unknown; one possibility is that the monomeric receptor has a low basal kinase activity, which is sufficient to phosphorylate and activate the companion receptor after dimerization. The reciprocal phosphorylation would then rapidly follow. An alternative mechanism would be that the interaction between the intracellular domains of the receptors in the dimer might induce the conformational change that leads to an increased kinase activity (Heldin, 1995). The recent studies on ErbB1 receptor using EGF ligand coated beads have shown the rapid and extensive propagation of receptor phosphorylation over the entire cell surface after focal stimulation with the ligand (Verveer et al., 2000). Autophosphorylation is commonly seen on conserved residues within the kinase domain. Depending on the amino acid residues that are phosphorylated, the receptor kinases have been classified into two families: those that phosphorylate on tyrosine residues and those that phosphorylate on serine and/or threonine residues. The catalytic domains of tyrosine and serine/threonine kinases appear to have common features and a basically identical mode of action (Schenk and Jagalska, 1999).

Biochemical experiments have provided immense information on the mechanism of phosphorylation catalyzed by kinases. Crystal structures of several receptor kinases have begun to reveal precise molecular mechanisms by which receptors recognize their cognate ligands, become activated through dimerization and autophosphorylation, and discriminate between potential substrates. For example crystallographic studies of the unphosphorylated form of the insulin receptor kinase (IRK) have provided information on the molecular mechanism by which receptor kinases are maintained in a low activity state prior to ligand binding and receptor autophosphorylation (Hubbard et al., 1994). Three tyrosine residue autophosphorylation sites are present in a conformationally flexible segment (Mohammadi et al., 1996) called the A-loop of the insulin receptor. The A-loop is defined by the parts of sub-domains VII and VIII (Johnson et al., 1996). Autophosphorylation of the tyrosine residues in the IRK A-loop results in a change in its confirmation which then permits unrestricted access to the binding sites for ATP and for protein substrates (Hubbard, 1997).

Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) are important transducers of a variety of extracellular signals that control cell growth and differentiation (Schlessinger and Ullrich, 1992). The epidermal growth factor receptor (EGFR), the platelet derived growth factor receptor (PDGFR) and the insulin receptor (IRK) are the classical examples of RTKs. Fig 3 shows the downstream signalling pathways mediated by these RTKs. After activation of RTKs by ligand binding, the tyrosine residues in the intracellular domain are phosphorylated. Of the two types of phosphorylation sites characterized, one type of tyrosine phosphorylation occurs on tyrosines inside the kinase domains. For the other types, the phosphorylation occurs on sites localized outside the kinase domains and serve as docking sites for downstream signal transduction molecules (Heldin, 1995). These downstream molecules contain conserved non-catalytic domains such as the src homology 2 and 3 (SH2 and SH3) that serve as adaptor molecules of signalling complexes (Koch et al., 1991). For example SH2 domains bind to specific phosphotyrosyl residues on activated receptors and SH3 domains bind to polyproline motifs on a separate set of target proteins (Pawson, 1995; Cohen et al., 1995). This results in the simultaneous association of a single protein that contains both SH2 and SH3 domains with two or more partners, and thus ensures assembly of complexes of signalling proteins around the activated cell surface receptor (Pawson and Scott, 1997). There can be a direct or an indirect connection of these domains to downstream signalling molecules, including phospholipases, Ras-like guanosine triphosphatases (GTPases), protein kinases, transcription factors, and polypeptides that regulate cell adhesion and cytoskeletal architecture. For example, the SH2 domain containing proteins, the shc (Src homology) and Grb2 (collagen and growth factor receptor bound protein bind to phosphorylated RTKs and are involved in the downstream recruitment of proteins like son of sevenless (Sos), which is a guanidine nucleotide exchange factor (GEF) towards the plasma membrane (Bonfini et al., 1996) (Fig 3). This stimulates the exchange of GDP for GTP on the G-protein, Ras, which gets activated and in turn stimulates a protein kinase cascade leading to activation of the mitogen activated protein kinase cascade (MAPK) also known as extracellular signal regulated kinase (ERK) (Guan, 1994).
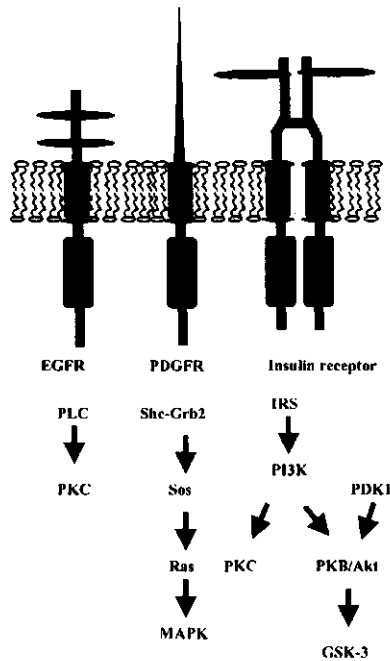
Figure 3. Schematic diagrams of EGF, PDGF and Insulin receptors and their downstream signalling. The extracellular domain, the transmembrane domain and the intracellular domains are shown. Open ovals indicate the cysteine rich regions in the extracellular domain. Downstream proteins binding to the activated form of each of these receptors are shown.

Receptor serine/threonine kinases

A number of transmembrane serine/threonine kinase receptors have been identified in sea urchins, nematodes, flies, vertebrates and plants. The best characterized serine threonine kinases are the receptors that are activated by the binding of three different isoforms of TGF-β. The TGF-β superfamily includes activins, inhibins, bone morphogenic proteins (BMPs) and other secreted factors. These have been shown to direct a wide range of cellular responses, including proliferation, changes in cell shape, apoptosis and specification of cell fate (Raftery and

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Sutherland, 199). Many of them have important functions during embryonal development in pattern formation and tissue specification (Heldin et al., 1997). This receptor family consists of two sub-types, I and II. Both receptor types are structurally similar, with cysteine rich extracellular regions and intracellular protein kinase domain. A glycine and serine rich domain (GS) in the juxtamembrane domain is the characteristic of type I, but not of type II receptors (Heldin et al., 1997) (Fig. 4). Studies on the TGF-β receptors have provided a model for the activation of these serine/threonine transmembrane receptor complexes TGF-β related factors signal through TGF-β type I (TβR-I) and type II (TβR-II) receptors, the TβR-II being capable of binding to TGF-β independently of TβR-I. The TGF-β first binds to the TβR-II, which is present in the plasma membrane in an oligomeric form with activated kinase activity (Henis et al., 1994; Chen et al., 1994). A monomeric or an oligomeric form of TβR-I is then recruited into the TβR-II complex and is phosphorylated at serine and threonine residues in the GS domain by the activated TβR-II (Heldin et al., 1997). The similar sites in activin receptor, ActR-IB are phosphorylated by activin type II receptors (Attisano et al., 1996; Willis et al., 1996)). Several growth factors like activins and BMPs are known to bind to the same type II receptor. Except a few minor variations in the receptor activation, as shown by the BMPs and the TGF-β isoforms, it is very likely that the other serine/threonine transmembrane receptors are activated by a similar mechanism (Attisano et al., 1996; Willis et al., 1996).

Signalling through serine/threonine receptor kinases

There are a number of downstream proteins involved in serine/threonine receptor kinase signalling that have been identified so far and provide an intriguing parallel with tyrosine kinase signalling events. Several proteins have been shown to associate with serine/threonine transmembrane receptors. Among the most studied ones are the 14-3-3 proteins, SMADS and the WD-40 repeat containing proteins. The latter contain the conserved sequences of 40 amino acids that typically end in tryptophan-aspartate (WD-40 repeats). TRIP-1, a WD-40 repeat cytoplasmic protein is known to bind to and become phosphorylated by TβR-II, the interaction being dependent on the functional TβR-II kinase (Chen et al., 1995).
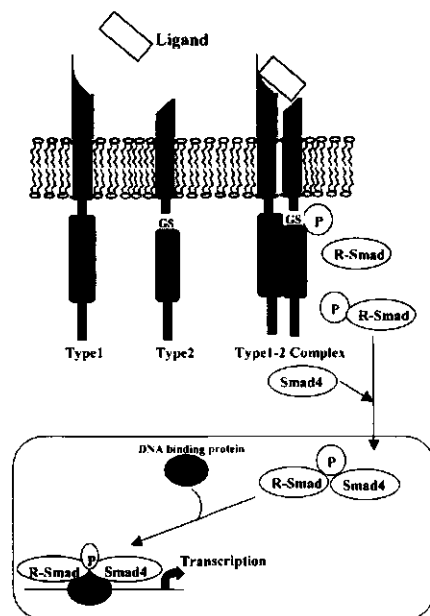
Figure 4. The transforming growth factor-β (TGF-β) signal transduction pathway. Binding of the ligand (TGF-β family member) to its type II receptor in presence of type I receptor leads to the formation of a receptor complex and phosphorylation of type I receptor. Downstream phosphorylation of SMADS and their translocation into the nucleus leading to their association with a DNA-binding partner and the activation of transcription of target genes is shown.

14-3-3 proteins

There is a growing evidence that signalling pathways utilizing serine phosphorylation employ members of the 14-3-3 family of adaptor proteins (Morrison et al., 1994). These proteins were originally identified in the mammalian brain tissue (Moore and Perez, 1967) and named after their migration pattern on two dimensional protein gels. Similar to the SH2 domains that bind to phosphotyrosine in a sequence-specific manner, 14-3-3 proteins can bind to specific phosphoserine-containing motifs (Braselmann and McCormick. 1995). This could result in the assembly of a signalling complex. The discovery that specific 14-3-3 isoforms interact with Raf kinase (reviewed by Morrison, 1994; Aitken, 1995; and Brubelo and Hall, 1995) providing a link

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to control of proliferation via the Ras-Raf pathway has generated an immense interest in the 14-3-3 protein family. The crystal structure of 14-3-3 provides some clues to its role in association with cellular proteins. 14-3-3 is a dimer consisting of two subunits separated by a long negatively charged channel (Xaio et al., 1995; Liu et al., 1995). This dimer can simultaneously bind two ligands, which may allow 14-3-3 protein to operate as an adaptor/scaffolding protein for inducing protein-protein interactions in a multimeric complex (reviewed by Fu et al., 2000). The crystal structure of the 14-3-3 protein (Xaio et al., 1995) shows that the phosphorylation site sits exposed in a region where it could be readily phosphorylated by a kinase with the correct recognition features. It further strengthens the idea that phosphorylation regulates interaction of 14-3-3 proteins with kinases active in signal transduction pathways (reviewed by Aitken, 1996). The ability of the 14-3-3 proteins to recognize the pSer motif in Cdc25C, a phosphatase that regulates the activity of the Cdc2 protein kinase, thereby controlling the entry into mitosis provides an additional clue for a role of 14-3-3 proteins in cell cycle control (Peng et al., 1997). A plant 14-3-3 was found to have a role in controlling nitrogen metabolism in spinach by binding and inhibiting the phosphorylated nitrate reductase (Moorhead et al., 1996). It has also been shown that the phosphorylation status of the plant plasma membrane H+-ATPase is important for its interaction with a 14-3-3 protein (Camoni et al., 2000). There are many unresolved issues regarding the precise physiological function of 14-3-3 proteins. In eukaryotes, there is usually a family of 14-3-3 encoding genes of which individual members show some cell specificity. 14-3-3 proteins could therefore act as adaptor or scaffolding proteins that take part in the cell or tissue specific 'wiring' of serine/threonine phosphorylation dependent signalling pathways.

SMAD proteins

The proteins of SMAD family are the first identified substrates of type I serine/threonine receptor kinases and play a central role in the transduction of receptor signals to target genes in the nucleus (Fig. 4). For the simplification of the nomenclature, the designation SMADS has been suggested for vertebrate homologues of *sma* and *Mad* genes (Heldin et al., 1997). A number of members of the SMAD family have been identified and their role in cell signalling has been described. SMADs are molecules of relative molecular mass with the Mad homology domains MH-1 and MH-2 at the amino and the carboxy terminals connected by an intervening linker region that is of variable length and sequence. After ligand binding in the extracellular milieu, the

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activated type I and type II serine/threonine kinase receptors both phosphorylate Smad proteins. The phosphorylated Smad proteins associate with other Smads and form hetero-oligomeric complexes. These complexes translocate into the nucleus, thereby influencing the transcription of target genes (reviewed by Zimmerman and Padget, 2000) (Fig. 4). Based on structural and functional considerations, SMADS fall into three categories (reviewed by Massauge, 1998): (a) SMADS that are direct substrates of serine threonine kinase receptors like TβR-I and II (b) SMADS that associate with receptor kinase binding SMADS and participate in signalling and (c) SMADS which act antagonistically on receptor kinase binding to SMADS and other SMADS binding Smads.

The different members of the SMAD family have different roles in signalling. Among the receptor regulated SMADs (R-Smads), Smad1 and presumably its close homologues Smad5 and Smad8, are targets for the bone morphogenic receptor, (BMPR-I), (Kretzschmar et al., 1997) and mediators of BMP signalling (Hoodless et al., 1996; Graff et al., 1996; Liu et al., 1996; Thomsen, 1996; and Suzuki et al., 1997). Smads2 and Smad3 mediate in TGF-β and activin signalling by binding to the TβR-I and activin serine threonine receptors (Graff et al., 1996; Eppert et al., 1996; Chen et al., 1996; Lagna et al., 1996).

The mode of action of Smad4, an accessory SMAD to the R-Smads, differs from those of the other members of SMAD family. Smad4 becomes associated with R-Smads (Fig. 4) when these are phosphorylated by the corresponding receptors (Kretzschmar et al., 1997; Zhang et al., 1996; and Lagna et al., 1996). Smad4 has been shown to be required for Smad2 or Smad3 growth inhibitory responses in mammalian cells, and a dominant negative Smad4 construct interferes with Smad1 and Smad2 signalling in frog embryos and mammalian cells (Zhang et al., 1996; Lagna et al., 1996). Thus Smad4 participates in TGF-β, activin, and BMP signalling pathways as a partner of R-Smads. The anti-Smads, Smad6 and Smad7 are inhibitors of signalling by R-Smads. Smad6 preferentially inhibits BMP signalling and Smad7 inhibits both BMP and TGF-β signalling. One of the mechanisms that has been proposed on how anti-Smads exert their inhibitory role is that anti-Smads can bind to receptor serine/threonine complexes and prevent phosphorylation of R-Smads (Hayashi et al., 1997; Inamura et al., 1997), which in turn prevents Smad heterotrimer formation and nuclear accumulation.

More recent studies have been directed at understanding intracellular regulation of the Smad pathways and have addressed the role of a number of Smad binding proteins that either initiate or

inhibit Smad-mediated signalling through serine threonine kinases, particularly in the TGF-β pathway (Zimmerman and Padget, 2000). These comprise mostly of positive regulators of receptor serine/threonine kinase signalling, including both an upstream accessory protein called Smad anchor for receptor activation (SARA) and many downstream effectors that function as either general or tissue specific transcriptional regulators (Tsukazaki et al., 1998).

Serine threonine phosphatases

The dephosphorylation of a receptor protein is essential for signal transduction pathways that utilize receptor kinases. Phosphatases that dephosphorylate serine and threonine residues are encoded by the PPP and the PPM gene families, which are defined by distinct amino acid sequences and crystal structures. The PPP family includes the signature phosphatases, PP1, PP2A and PP2B while the PPM family comprises the Mg^{2+} dependent protein phosphatases that include PP2C and pyruvate dehydrogenase phosphatase (Cohen, 1997). PP2A is one of the major serine/threonine phosphatases which regulates several processes including receptor kinase mediated signalling (Sontag et al., 1993) and cell cycle progression (Clarke et al., 1993; Sontag et al., 1995). As discussed earlier TRIP-1, a WD-40 repeat protein is associated with serine/threonine kinase receptors. The same WD-40 repeats have been identified in the Bα subunit of the serine/threonine protein phosphatase 2A (PP2A) (Griswold-prenner et al., 1998). It has been demonstrated that the WD-40 repeats of the Bα regulatory subunit of the PP2A phosphatase interacts directly with the cytoplasmic domains of the type I TGF-β receptors and are a direct target for the kinase activity (Griswold-Prenner et al., 1998). Recently a Smad4-independent phosphatase-mediated inhibition of mitogen-activated extracellular regulated kinase (ERK)-2 has shown the existence of a novel effector pathway contributing to suppression of epithelial pancreatic carcinoma cell proliferation by TGFβ-1 (Giehl et al., 2000). This suggests a careful fine-tuning of phosphorylation and dephosphorylation reactions involved in receptor mediated signalling.

Plant receptor like kinases

Plant genomes encode a variety of protein kinases, and while some are functional homologs of animal and fungal kinases, others have a novel structure (reviewed by Saterlee and Sussman, 1998). All plant receptor like kinases (RLKs) published to date, except for PRK1, encode proteins with serine/threonine protein kinase signature rather than tyrosine-specific signature sequences (reviewed by Saterlee and Sussman, 1998). It will be of interest to determine if they employ similar components as TGF-β signalling pathways in animals. Based on the structure of their putative extracellular domains RLKs fall into several classes (Torii, 2000): the S domain class of RLKs have distinctive extracellular domains homologous to the S-locus glycoprotein; the leucine rich repeat (LRR) class of RLKs have extracellular domains with a varying number of LRRs. Protein kinases like rice Xa21, *Brassica* BRI1, *Arabidopsis* TMK1, CLAVATA1, ERECTA, HAESA and AtSERK1 belong to the LRR-RLKs; the lectin class represented by Arabidopsis LecRK1 gene product has an extracellular domain homologous to carbohydrate binding lectins; the EGF class of RLKs represented by Wall-Associated receptor protein kinase, WAK1 that has two epidermal growth factor-like repeats in its extracellular domain; the TNFR class represented by maize CRINKLY4 receptor kinase has an extracellular domain with homology to tumor necrosis factor receptor as well as seven repeated motifs; the PR class represented by Arabidopsis PR5K that has an extracellular domain which exhibits sequence similarity to PR5 (pathogenesis related protein 5). The plant RLKs with different types of extracellular domains are shown in Fig. 5. It is quite clear that a huge structural diversity in the extracellular receptor domains exists which perhaps reflects their functional diversity (Torii et al., 2000). The structure of the receptor domains of some RLKs suggests that their respective ligands may be proteins, as similar extracellular domains in animals all bind protein ligands (Kobe and Deisenhofer, 1994; Smith et al., 1994). Owing to the presence of cell wall that sets apart plasma membrane of two adjacent cells, ligands for plant RLKs are presumed to be secreted proteins rather than the membrane bound cell surface proteins (Becraft et al., 1998).
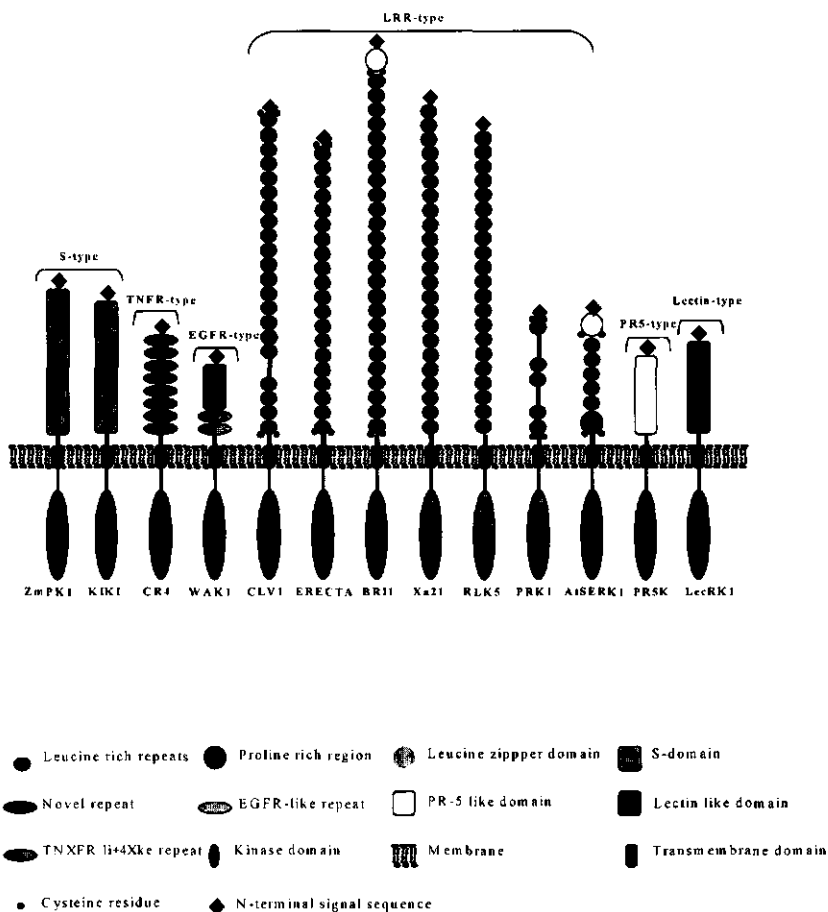
Figure 5. Classification and structural topology of plant RLKs. The domains and the residues represented are shown below the diagrams of RLKs.

Based on the amino acid sequence alignment of the protein kinase catalytic domains (Hanks and Quinn, 1991), plant RLKs are most related to each other and thus comprise a distinct family of protein kinases. Predicted aminoacid sequences of RLKs indicate that they possess serine/threonine substrate specificity. Assays with affinity purified plant RLKs expressed in *E.*

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coli and other expression systems have shown that RLKs autophosphorylate on serine and/or threonine residues. The only exception is the *petunia PRK1*, which is a dual specificity kinase, autophosphorylating both on serine and tyrosine residues (Mu et al., 1994). RLK autophosphorylation has been shown to occur predominantly by intermolecular mechanisms (trans) and it has been suggested that oligomerization may be involved in the regulation of the kinase activity (Horn et al, 1994). The kinase domain of RLKs appears to be essential for their function. Mutations that remove the C-terminal region of the kinase domain after sub-domain VII and VIII result in severe phenotypes (Li and Chory, 1997). Based on the analysis of molecular lesions in *bri1* and *clv1* mutant alleles, most of the missense mutations in the kinase domain lie in the activation loop (Torii, 2000). In addition it is of interest to note that all three phosphorylation sites in sub-domain VIII of BRI1 (Oh et al., 2000) lie within the predicted A-loop region given that the activation of many protein kinases occurs by autophosphorylation of one to three residues within the activation loop of sub-domain VII and VIII (Johnson et al., 1996). It is therefore likely that such point mutations severely compromise the proper substrate recognition and in turn influence signal transduction.

Genetic evidence has placed BRI1 as a sole, nonredundant component of plant steroid hormone brassinolide (BR) perception, supporting a hypothesis that BRI1 may be a BR receptor (Torii et al, 2000). The recent chimeric studies using the extracellular domain of BRI1 and the intracellular domain of Xa21 from rice have revealed the importance of the BRI1 extracellular domain in signal transduction (He et al., 2000). However it seems unlikely that a large LRR domain with 25 repeats is the binding surface for a small molecule like BR. Unlike other LRR-RLKs, BRI1 LRRs are interrupted by a 70 aminoacid loop out island which has been shown to be essential for BRI1 function. Mutations in this region confer a strong phenotype (Li and Chory, 1997). It is speculated that this loop out island is a binding site for BRs and that the LRRs of BRI1 perhaps interact with other nonredundant components of the BR complex.

The studies on the action of S-locus receptor kinase (SRK), a receptor-like kinase involved in the self-incompatibility response in *Brassica* have shown that phosphorylation occurs in trans, suggesting the existence of constitutive homo-oligomers of membrane-anchored SRK (Giranton et al., 2000). The oligomeric status of SRK *in planta* has shown that SRK is associated both with other SRK molecules and with other stigma proteins in non-pollinated flowers.

In *Arabidopsis*, the LRR-RLK, CLAVATA1 (CLV1) is the best characterized RLK (Lease et al.,

1998). There other *CLAVATA* genes encode a small secreted protein, CLAVATA3 (CLV3) and an LRR-RLK, CLAVATA2 (CLV2) which forms a receptor complex with CLV1 (Trotochaud et al., 1999). Genetic approaches by double mutant analysis have shown the involvement of CLV1, CLV2 and CLV3 in the same signalling pathway (Clarke et al., 1997). Until recently it had been proposed that CLV3 interacts with CLV1/CLV2 receptor complex (Fletcher et al., 1999; Jeong et al., 1999). The recent studies by Trotochaud et al., 2000 on CLV1 and CLV2 expressed in yeast cells and CLV3 from plant extracts reveal that CLV3 indeed acts as a ligand to form CLV1/CLV2 receptor complex. CLV3 mature protein has no cysteines and thus is unlikely to form a structure related to any of the disulfide linked ligand-like molecules like pollen coat protein (PCP-A1), a 55 aminoacid molecule with 8 cysteines that binds to SLG of *Brassica* (Fletcher et al., 1999).

There is also evidence for the existence of downstream components that interact with plant RLKs. The first evidence came from studies by Stone et al (1994) who showed that RLK5 (HAESA) *in vitro* interacts with a type 2C protein phosphatase called kinase associated protein phosphatase (KAPP). Subsequently, it has been shown that many but not all RLKs interact with KAPP *in vitro*. Williams et al (1997) showed that KAPP also binds CLV1 both *in vitro* and using co-immunoprecipitation in plant extracts. Providing functional significance for the role of KAPP in CLV signalling, they showed that overexpression of KAPP in wild type plants yields a *clv*1 phenotype (Lease et al., 1998). In addition, Stone et al (1996), have shown that in a homozygous clv1 mutant background, the clv1 phenotype was suppressed by reducing the amount of KAPP expression. These studies suggest that KAPP is a negative regulator of CLV1 probably functioning through modulation of CLV activity. Recent biochemical studies put RhoGTPase (Rop) related proteins from plants as potential candidates (Trotochaud et al., 1999). A Rop protein has been found to be associated with the active CLV1 complex but not with the inactive complex (Trotochaud et al., 1999). It is very well possible that Rop proteins are plant equivalent of the Ras GTPase super-family which relays animal RTK signals aimed at the activation of MAPK pathways (Torii et al., 2000).

It is likely that signalling mediated by plant RLKs eventually regulates gene expression through the activation of transcription factors. The *Arabidopsis* WUSCHEL (WUS) protein, a homeodomain transcription factor is a strong candidate for a target of the CLV1-mediated signalling pathway. The phenotype of the wus mutants is characterized by the incorrect

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specification of stem cells during embryogenesis and disruption of the maintenance of shoot apical meristems (Laux et al., 1996). This phenotype resembles the transgenic plants that constitutively express CLV3. Recent studies demonstrate that the CLV1 pathway acts by repressing the activity of WUS (Brand et al., 2000). This evidence came from the observation that overexpression of CLV3 protein results in a loss of expression of the WUS gene (Brand et al., 2000) leading to a loss in the population of stem cells in the shoot apical meristem. In clv mutants, increased WUS expression results in the accumulation of stem cells (Brand et al., 2000). The CLV1 signalling complex and its proposed target WUS appear to control the proliferation and differentiation status of the stem cells in the shoot apical meristem.

Recent findings from the studies of CLV1 and other RLKs have shed light on one mechanism of RLK activation and downstream signalling: the association of CLV3, a ligand to the CLV1 receptor appears to activate the receptor complex. In turn this binds KAPP, a negative regulator, and Rop GTPase that may relay signals to downstream targets such as MAPK cascade that eventually could regulate transcription factors such as WUS. It is interesting to note that like the animal TGF-β receptors, plant RLKs also act as hetero-oligomers, including a 'primary receptor' that binds the ligand and acts as a 'transducer' (Torii, 2000). Apparently, plant and animal serine threonine receptors posses very similar basic mechanisms to transduce signals across the membranes. Based on the recent studies on plant RLKs and the knowledge from the well known serine threonine kinases from animals, a model has been proposed for the signal transduction mechanism employed by plant RLKs to transduce signals across the membranes (Fig. 6).

The AtSERK1 gene

Somatic embryogenesis is the process whereby somatic cells develop *in vitro* mature embryos via characteristic embryogenic morphological stages. The later stages of somatic embryogenesis closely resemble zygotic embryo development, thus passing through the globular, heart and torpedo stages. The possibility to generate large numbers of somatic embryos of the same developmental stage has made somatic embryogenesis an attractive system to identify genes specifically expressed during early stages of embryo development. A number of genes have been isolated that are expressed during the early stages of *Daucus carota* somatic embryo development. Although several genes have been reported (Lin et al, 1996) that are putative markers for the embryogenic cell clusters but none of them has been described as a reliable

marker for the preceding stage of competent cells.

A search for the marker genes to monitor the transition of somatic into competent and embryogenic cells in established carrot cell cultures resulted in the identification of *SERK* gene that is transiently expressed up to a 100 cell globular stage during somatic embryogenesis (Schmidt et al., 1997). In plants SERK mRNA could only be detected transiently in the zygotic embryo up to early globular stage and not in later stages (Schmidt et al., 1997). The most closely related *SERK* gene from *Arabidopsis*, *AtSERK1* was isolated. It encodes an LRR receptor kinase that is expressed during ovule development and early embryogenesis and like *DcSERK, AtSERK1* marks cells competent to form embryos in culture (Hecht et al., 2001 submitted). The predicted primary structure of AtSERK1 protein consists of an N-terminal signal peptide followed by a leucine zipper domain, 5 LRR units, a proline rich domain containing the SPP motif, a single transmembrane domain, and the 12 conserved domains of the serine-threonine kinase. The main distinguishing feature of AtSERK1 protein from other RLKs is the presence of the proline-rich domain containing SPP motif, located between the LRRs and the transmembrane domain. From the recent studies on AtSERK1 expression patterns, it is speculated that the AtSERK1-mediated signalling pathway, as it occurs during somatic embryogenesis, is recruited from a pathway that operates normally during ovule development and early zygotic embryogenesis (Hecht et al 2001, submitted). Therefore it was proposed that AtSERK1 could be a component of more general embryogenesis-signalling pathway. Competent cells may contain an inactive receptor, which is activated by the presence of the proper ligand in order to switch on the embryogenesis program. To identify the signalling mediated by AtSERK1, it is of prime importance to reveal the different steps and identify the components of the AtSERK1 signalling pathway both using functional analysis (Hecht et al., unpublished data) as well as biochemical studies (this thesis).
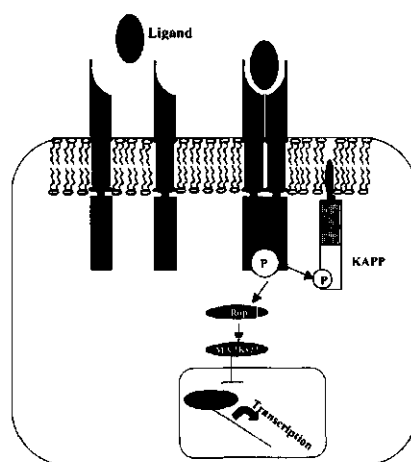
Figure 6. Model for the activation of plant RLKs. Ligand binding activates the homo or hetero dimerization of RLKs that leads to the phosphorylation of its kinase domains. A negative regulator of RLKs, KAPP associates with heterodimeric receptor complex. The association and activation of Rop may in turn transduce signals through MAPK cascade and finally regulate the activity of transcription factors like WUS.

References

1. Cohen, G.B., Ren, R. and Baltimore, D. (1995) Modular binding domains in signal transduction proteins. *Cell* **80**, 237-248

2. Wolfsberg, T.G., McEntyre, J. and Schuler, G.D. (2000) Guide to draft human genome. *Nature* **409**, 824-826.

3. Verveer, P.J., Wouters, F.S., Reynolds, A.R. and Bastiaens, P.I. (2000) Quantitative imaging of lateral ErbB1 receptor signal propagation in the plasma membrane. *Science* **290**, 1567-1570.

4. Hanks, S.K., Quinn, A.M. and Hunter, T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241**, 42-52. Review.

5. Heldin, C.H. (1995) Dimerization of cell surface receptors in signal transduction. Cell 80, 213-23. Review.

6. Lemmon, M.A. and Schlessinger, J. (1994) Regulation of signal transduction and signal diversity by receptor oligomerization. *Trends Biochem. Sci.* **19**, 459-63. Review.

7. Kanakaraj, P., Raj, S., Khan, S.A. and Bishayee, S. (1991) Ligand-induced interaction between alpha- and beta-type platelet-derived growth factor (PDGF) receptors: role of receptor heterodimers in kinase activation. *Biochemistry* **30**, 1761-67.

8. Wells, J.A. (1996) Binding in the growth hormone receptor complex. *Proc. Natl. Acad. Sci.* U. S. A. **93**,1-6. Review.

9. Banner, D.W, D'Arcy, A., Janes, W., Gentz, R., Schoenfeld, H.J., Broger, C., Loetscher, H. and Lesslauer, W.

(1993) Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. *Cell* **73**, 431-45.

10. Spivak-Kroizman, T., Lemmon, M.A., Dikic, I., Ladbury, J.E., Pinchasi, D., Huang, J., Jaye, M., Crumley, G., Schlessinger, J. and Lax, I. (1994) Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell proliferation. *Cell* **79**, 1015-24.

11. Klemm, J.D., Schreiber, S.L. and Crabtree, G.R. (1998) Dimerization as a regulatory mechanism in signal transduction. *Annu. Rev. Immunol.* **16**, 569-92. Review.

12. Schlessinger, J. (1988) Signal transduction by allosteric receptor oligomerization. Trends *Biochem. Sci.* **13**, 443-7.

13. Heldin, C.H, Ernlund, A., Rorsman, C., Ronnstrand, L. (1989) Dimerization of B-type platelet-derived growth factor receptors occurs after ligand binding and is closely associated with receptor kinase activation. *J. Biol. Chem.* **264**, 8905-12

14. Weiner, D.B., Kokai, Y., Wada, T., Cohen, J.A., Williams, W.V. and Greene, M.I. (1989) Linkage of tyrosine kinase activity with transforming ability of the p185neu oncoprotein. *Oncogene* **4**, 1175-83.

15. Spencer, D.M., Wandless, T.J., Schreiber, S.L. and Crabtree, G.R. (1993) Controlling signal transduction with synthetic ligands. *Science* **262**, 1019-24.

16. Ullrich, A. and Schlessinger, J. (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203-12. Review.

17. Hunter, T. (1986) Cancer Cell growth control mechanisms. Nature 322,14-16.

18. Levinson, A.D., Courtneidge, S.A. and Bishop, J.M. (1981) Structural and functional domains of the Rous sarcoma virus transforming protein (pp60src*). Proc. Natl. Acad. Sci. U. S. A.* **78**, 1624-8.

19. Wilkerson, V.W., Bryant, D.L. and Parsons, J.T. (1985) Rous sarcoma virus variants that encode src proteins with an altered carboxy terminus are defective for cellular transformation. *J. Virol.* **55**, 314-21.

20. Hanks, S.K, Quinn, A.M. (1991) Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. *Methods Enzymol* **200**, 38-62.

21. Brugge, J.S. and Darrow, D. (1984) Analysis of the catalytic domain of phosphotransferase activity of two avian sarcoma virus-transforming proteins. *J. Biol. Chem.* **259**, 4550-57.

22. Yaciuk, P. and Shalloway, D. (1986) Features of the pp60v-src carboxyl terminus that are required for transformation. *Mol. Cell. Biol.* **6**, 2807-19.

23. Hanks, S.K., Hunter T. (1996) Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J.* **9**, 576-96

24. Schenk, P.,W and Jagalska, S. (1999) Signal perception and transduction: the role of protein kinases. *Biochimica. Biophysics. Acta.* **1449**, 1-24

25. Hubbard, S.R., Wei, L., Ellis, L. and Hendrickson, W.A. (1994). Crystal structure of the tyrosine kinase domain of the human insulin receptor. *Nature* 372,746-54.

26. Mohammadi, M., Schlessinger, J. and Hubbard, S.R. (1996) Structure of the FGF receptor tyrosine kinase domain reveals a novel autoinhibitory mechanism. *Cell* **86**, 577-87

33

27. Johnson, L.N., Noble, M.E., and Owen, D.J. (1996) Active and inactive protein kinases: structural basis for regulation. *Cell* **85**, 149-158.

28. Hubbard, S.R. (1997) Structure Crystal structure of the activated insulin receptor tyrosine kinase in complex with peptide substrateand ATP analog. *EMBO. J.* **16**, 5572-81.

29. Schlessinger, J., Ullrich, A. (1992) Growth factor signalling by receptor tyrosine kinases. *Neuron* **9**, 383-391.

30. Koch, C.A., Anderson, D., Moran, M.F., Ellis, C. and Pawson, T. (1991) SH2 and SH3 domains: elements that control interactions of cytoplasmic signalling proteins. *Science* **252**, 668-74.

31. Pawson T. (1995) Protein modules and signalling networks. *Nature* **373**, 573-80. Review.

32. Cohen, G.B., Ren, R. and Baltimore, D. (1995) Modular binding domains in signal transduction proteins. *Cell* **80**, 237-48. Review.

33. Pawson, T. and Scott, J.D. (1997) Signalling through scaffold, anchoring, and adaptor proteins. *Science* **278**, 2075-80. Review.

34. Bonfini, L., Migliaccio, E., Pelicci, G., Lanfrancone, L. and Pelicci, P.G. (1996) Not all Shc's roads lead to Ras. *Trends. Biochem. Sci.* **21**, 257-61. Review.

35. Guan, K.L., (1994) The mitogen activated protein kinase signal transduction pathway: from the cell surface to the nucleus, *Cell. Signal.* **6**, 581-589

36. Raftery, L.A. and Sutherland, D.J. (1999) TGF-β signal transduction in Drosophila development: From Mad to Smads *Dev. Biol.* **210** 251-268

37. Heldin, C.H., Miyazono, K. and ten Dijke, P. (1997) TGF-β signalling from cell membrane to nucleus through SMAD proteins. *Nature* **390**, 465-71. Review.

38. Henis, Y.I., Moustakas, A., Lin, H.Y. and Lodish, H.F. (1994) The types II and III transforming growth factor-β receptors form homo-oligomers. *J. Cell Biol.* **126**, 139-54.

39. Chen, R.H. and Derynck, R. (1994) Homomeric interactions between type II transforming growth factor-β receptors. *J Biol. Chem.* **269**, 22868-74.

40. Attisano, L., Wrana, J.L., Montalvo, E. and Massague, J. (1996) Activation of signalling by the activin receptor complex. *Mol. Cell Biol.* **16**, 1066-73.

41. Willis, S.A., Zimmerman, C.M., Li, L.I. and Matthews, L.S. (1996) Formation and activation by phosphorylation of activin receptor complexes. *Mol Endocrinol.* **10**, 367-79.

42. Chen, R.H., Miettinen, P.J, Maruoka, E.M., Choy, L. and Derynck, R. (1995) A WD-domain protein that is associated with and phosphorylated by the type II TGF-β receptor. *Nature* **377**, 548-52.

43. Morrison, D. (1994) 14-3-3: modulators of signalling proteins? *Science.* **266**, 56-57. Review.

44. Moore, B.W. and Perez, V.J. (1967) Specific acid proteins of the nervous system. *In Physiological and Biochemical aspects of Nervous Integration ed. FD Carlson*, pp.343- 359 Englewood Cliffs, NJ: Prentice-Hall

45. Braselmann, S. and McCormick, F. (1995) Bcr and Raf form a complex in vivo via 14-3-3 proteins. *EMBO. J.* **14**, 4839-48.

46. Aitken, A., Jones, D., Soneji, Y. and Howell, S. (1995). 14-3-3 proteins: biological function and domain

34

structure. *Biochem. Soc. Trans.* **23**, 605-11. Review.

47. Burbelo, P.D. and Hall, A. (1995) 14-3-3 proteins. Hot numbers in signal transduction. *Curr. Biol.* **5**, 95-96. Review.

48. Xiao, B., Smerdon, S.J., Jones, D.H., Dodson, G.G., Soneji, Y., Aitken, A. and Gamblin, S.J. (1995) Structure of a 14-3-3 protein and implications for coordination of multiple signalling pathways. *Nature* **376**, 188-91.

49. Liu, D., Bienkowska, J., Petosa, C., Collier, R.J., Fu, H. and Liddington, R. (1995) Crystal structure of the zeta isoform of the 14-3-3 protein. *Nature* **376**, 191-94.

50. Fu, H. Subramaniam, R.R. and Masters, S.C. (2000)14-3-3 proteins: structure, function, and regulation. *Annu Rev Pharmacol Toxicol.* **40**, 617-47. Review

51. Peng, C.Y., Graves, P.R., Thomas, R.S., Wu, Z., Shaw, A.S. and Piwnica-Worms, H. (1997) Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. *Science* **277**, 1501-5.

52. Moorhead, G., Douglas, P., Morrice, N., Scarabel, M., Aitken, A. and MacKintosh, C. (1996) Phosphorylated nitrate reductase from spinach leaves is inhibited by 14-3-3 proteins and activatedby fusicoccin. *Curr. Biol.* **6**, 1104-13.

53. Camoni, L., Iori, V., Marra, M. and Aducci, P. (2000) Phosphorylation-dependent interaction between plant plasma membrane H+-ATPase and 14-3-3 proteins. *J. Biol. Chem.* **275**, 9919-99123.

54. Zimmerman, C.M. and Padget, R. W. (2000) Transforming growth factor β signalling mediators and modulators. *Gene* **249**, 17-30. Review.

55. Massague, J. (1998) TGF-β signal transduction. *Annu. Rev. Biochem.* **67**, 753-91. Review.

56. Kretzschmar, M., Liu, F., Hata, A., Doody, J. and Massague, J. (1997) The TGF-β family mediator Smad1 is phosphorylated directly and activated functionally by the BMP receptor kinase. *Genes Dev.* **11**, 984-95.

57. Hoodless, P.A, Haerry, T., Abdollah, S., Stapleton, M., O'Connor, M.B., Attisano, L. and Wrana, J.L. (1996) MADR1, a MAD-related protein that functions in BMP2 signalling pathways. *Cell* **85**, 489-500.

58. Graff, J.M, Bansal, A. and Melton, D.A. (1996) Xenopus Mad proteins transduce distinct subsets of signals for the TGF β superfamily. *Cell* **85**, 479-87.

59. Liu, F., Ventura, F., Doody, J. and Massague, J. (1995) Human type II receptor for bone morphogenic proteins (BMPs): extension of the two-kinasereceptor model to the BMPs. *Mol. Cell Biol.* **15**, 3479-86.

60. Thomsen, G.H. (1996) Xenopus mothers against decapentaplegic is an embryonic ventralizing agent that acts downstream of the BMP-2/4 receptor. *Development* **122**, 2359-66.

61. Suzuki, A., Chang, C., Yingling, J.M., Wang, X.F. and Hemmati-Brivanlou A. (1997) Smad5 induces ventral fates in Xenopus embryo. *Dev. Biol.* **184**, 402-5.

62. Eppert, K., Scherer, S.W., Ozcelik, H., Pirone, R., Hoodless, P., Kim, H., Tsui, L.C., Bapat, B., Gallinger, S., Andrulis, I.L., Thomsen, G.H., Wrana, J.L. and Attisano, L. (1996) MADR2 maps to 18q21 and encodes a TGF β-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell* **86**, 543-52.

63. Chen, Y., Lebrun, J.J. and Vale, W. (1996) Regulation of transforming growth factor β- and activin-induced transcription by mammalian Mad proteins. *Proc. Natl. Acad. Sci. U .S .A.* **93**, 12992-7.

35

64. Lagna, G., Hata, A., Hemmati-Brivanlou, A. and Massague, J. (1996) Partnership between DPC4 and SMAD proteins in TGF-β signalling pathways. *Nature* **383**, 832-6.

65. Zhang, Y., Feng, X., We, R. and Derynck, R. (1996) Receptor-associated Mad homologues synergize as effectors of the TGF-β response. *Nature* **383**, 168-72.

65. Hayashi, H., Abdollah, S., Qiu, Y.B., Cai, J.X. and Xu, Y.Y (1997) The MAD-related protein Smad7 associates with the TGF β receptor and functions as an antagonist of TGFbeta signalling. *Cell*, **89**, 1165-1173

66. Imamura, T., Takashe, M., Nishihara, A., Oeda, E. and Hanai, J. (1997) Smad6 inhibits signalling by the TGF-β superfamily. *Nature* **839**, 622-626

67. Tsukazaki, T., Chiang, T.A., Davison, A.F., Attisano, L., Wrana, J.L. (1998) SARA, a FYVE domain protein that recruites Smad2 to the TGF β receptor. *Cell* **95**, 779-791

68. Cohen, P.T.W (1997). Novel protein serine/threonine phosphatases: variety is the spice of life. *Trends Biochem. Sci.* **22**, 245-251.

69. Sontag, E., Fedorov, S., Kamibayashi, C., Robbins, D., Cobb, M. and Mumby, M. (1993). The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the map kinase pathway and induces cell proliferation. *Cell* **75**, 887-97.

70. Clarke, P.R., Hoffmann, I., Draetta, G. and Karsenti, E. (1993) Dephosphorylation of cdc25-C by a type-2A protein phosphatase: specific regulation during the cell cycle in Xenopus egg extracts. *Mol. Biol. Cell* **4**, 397-411

71. Sontag, E., Nunbhakdi-Craig, V., Bloom, G.S. and Mumby, M.C. (1995) A novel pool of protein phosphatase 2A is associated with microtubules and is regulated during the cell cycle. *J. Cell Biol.* **128**, 1131-44.

72. Griswold-Prenner, I., Kamibayashi, C., Maruoka, E.M., Mumby, M.C. and Derynck R. (1998) Physical and functional interactions between type I transforming growth factor β receptors and B alpha, a WD-40 repeat subunit of phosphatase 2A. *Mol. Cell Biol.* **18**, 6595-604.

73. Giehl, K., Seidel, B. Gierschik, P., Adler G. and Menke A. (2000) TGF β1 represses proliferation of pancreatic carcinoma cells which correlates with Smad4-independent inhibition of ERK activation. *Oncogene* **19**, 4531-41.

74. Satterlee, J.S. and Sussman, M.R. (1998) Unusual membrane-associated protein kinases in higher plants. *J. Membr. Biol.* **164**, 205-13. Review.

75. Torii, K.U. (2000) Receptor kinase activation and signal transduction in plants: an emerging picture. *Curr. Opin. Plant Biol.* **3**, 361-367.

76. Kobe, B and Deisenhofer, J. (1994). The leucine rich repeat-a versatile binding motif. *Trends. Biochem. Scienc.* **19**, 415-21

77. Smith, C. A., Farrah, T. and Goodwin, R.G. (1994) The TNF receptor family of cellular and viral proteins: activation, costimalation and death. *Cell* **76**, 956-62

78. Becraft, P.W., Stinard, P.S. and McCarty, D.R. CRINKLY4: (1996) A TNFR-like receptor kinase involved in maize epidermal differentiation. *Science* **273**, 1406-9.

79. Mu, J.H., Lee H.S. and Kao, T.H., (1994) Characterization of a pollen-expressed receptor-like kinase gene of *Petunia inflata* and the activity of its encoded kinase. *Plant Cell* **6**, 709-721.

80. Horn, M.A, and Walker, J.C. (1994) Biochemical properties of the autophosphorylation of RLK5, a receptor-like protein kinase from Arabidopsis thaliana. *Biochim. Biophys. Acta.* **1208**, 65-74.

81. Li, J., Chory, J. (1997) A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. *Cell* **90**, 929-38.

82. Oh, M.H., Ray, W.K., Huber, S.C., Asara, J.M., Gage, D.A. and Clouse SD (2000) Recombinant brassinosteroid insensitive 1 receptor-like kinase autophosphorylates on serine and threonine residues andphosphorylates a conserved peptide motif in vitro. *Plant Physiol.* **124**, 751-766

83. He, Z., Wang, Z., Li, J., Zhu, Q., Lamb, C., Ronald, P., and Chory, J. (2000). Perception of Brassinosteroids by the Extracellular Domain of the Receptor Kinase BRI1. *Science* **288**, 2360-63.

84. Giranton, J.L., Dumas, C., Cock, J.M. and Gaude, T. (2000) The integral membrane S-locus receptor kinase of *Brassica* has serine/threonine kinase activity in a membranous environment and spontaneously forms oligomers in planta. *Proc. Natl Acad. Sci.*, U S A. **97**, 3759-64

85. Trotochaud, A.E., Hao, T., Wu, G., Yang, Z. and Clark, S.E. (1999) The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signalling complex that includes KAPP and a Rho-related protein. *Plant Cell* **11**, 393-406.

86. Clarke, S.E., Williams, R.W. and Meyerowitz, E.M. (1997)The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. *Cell* **16**, 575-85.

87. Fletcher, J.C., Brand, U., Running, M.P., Simon, R. and Meyerowitz, E.M. (1999) Signalling of cell fate decisions by CLAVATA3 in Arabidopsis shoot meristems. *Science* **283**, 1911-14.

88. Jeong, S., Trotochaud, A.E. and Clark, S.E. (1999) The Arabidopsis CLAVATA2 gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase. *Plant Cell* **11**, 1925-34.

89. Trotochaud, A.E., Jeong, S. and Clark, S.E. (2000) CLAVATA3, a multimeric ligand for the CLAVATA1 receptor-kinase. *Science* **289**, 613-7

90. Stone, J.M, Collinge, M.A., Smith, R.D., Horn, M.A. and Walker, J.C. (1994) Interaction of a protein phosphatase with an Arabidopsis serine-threonine receptor kinase. *Science* **266**, 793-795.

91. Lease, K., Ingham, E. and Walker, J.C. (1998) Challenges in understanding RLK function. *Curr. Opin. Plant Biol.* **1**, 388-92

92. Williams, R.W., Wilson, J.M. and Meyerowitz, E.M. (1997) A possible role for kinase-associated protein phosphatase in the Arabidopsis CLAVATA1 signalling pathway. *Proc Natl Acad Sci* U S A. **94**, 10467-72.

93. Laux, T., Mayer, K.F., Berger, J. and Jurgens, G. (1996) The WUSCHEL gene is required for for shoot and floral meristem integrity in Arabidopsis. *Development* **122**, 87-96

94. Brand, U., Fletcher, J.C., Hobe, M., Meyerowitz, E.M. and Simon, R. (2000) Dependence of stem cell fate in Arabidopsis on a feedback loop regulated by CLV3 activity. *Science* **289**, 617-19.

95. Lin, X., Hwang, G.J., and Zimmerman, J.L. (1996) Isolation and characterization of a diverse set of genes from carrot somatic embryos. *Plant Physiol.* **112**, 1365-74.

96. Schmidt, E.D., Guzzo, F., Toonen, M.A. and de Vries, S.C. (1997) A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. *Development* **124**, 2049-2062.

97. Hecht V., Vielle-Calzada, J.P, Hartog, M.V., Schmidt, E. D. L., Boutilier, K., U. Grossniklauss and de Vries, S.C. (2001) The Arabidopsis *somatic embryogenesis receptor kinase 1 (AtSERK1)* gene is expressed in developing ovules and enhances embryogenic competence in culture (submitted)
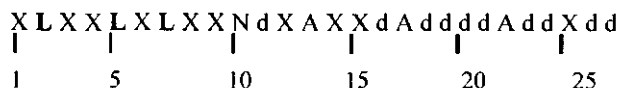
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Leucine rich repeats of transmembrane proteins: roles in signal transduction and development

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Introduction

Leucine rich repeats (LRRs) are short sequence motifs that were first discovered in leucine-rich α 2-glycoprotein, a protein of unknown function from human serum (Takahashi, 1985). They have subsequently been found in many different proteins and are considered to be an important motif in interactions between proteins and between proteins and other molecules (Kobe and Deisenhofer, 1994). LRRs are mostly present in tandem array and have been described in an increasing number of proteins, giving rise to an LRR superfamily. LRRs most often are 24 aminoacids in length, but may contain between 20 and 29 residues. The number of repeats ranges from one, as in the platelet glycoprotein-1b (Roth, 1991), to as much as 38, as in the tomato-disease resistance protein Cf-2 (Dixon et al., 1996). A typical LRR consensus sequence is shown in Fig. 1.

$$X\ L\ X\ X\ L\ X\ L\ X\ X\ N\ d\ X\ A\ X\ X\ d\ A\ d\ d\ d\ d\ A\ d\ d\ X\ d\ d$$

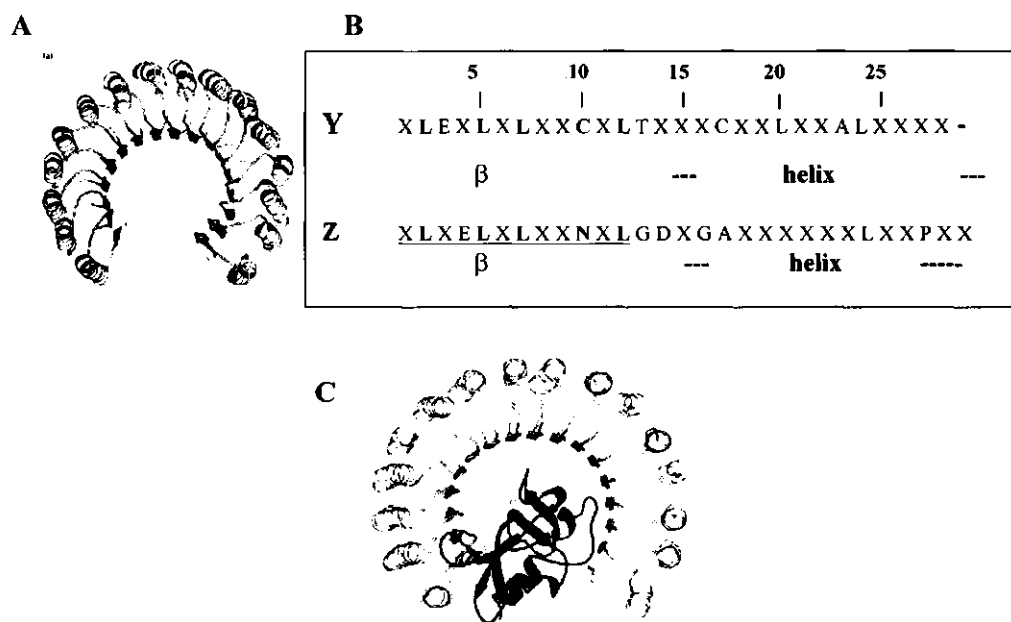$$1\qquad 5\qquad\quad 10\qquad\quad 15\qquad\quad 20\qquad\quad 25$$

Figure 1. The LRR consensus sequence. L is leucine, A is any aliphatic amino acid, N is an asparagine, X is any amino acid and d indicates possible deletions (Kobe and Deisenhofer, 1994).

In the LRR motif, only the leucines are completely conserved, while the highly conserved asparagine at position 10 is sometimes replaced by a cysteine or a threonine (Kobe and Deisenhofer, 1994) and some LRR proteins (trk receptors) contain glutamine at this position. On either side of the asparagine at position 9, glutamine and at position 11, asparagine are common. The asparagine residues in position 9 frequently form part of a potential N- glycosylation site (Buchanan and Gay, 1996).

General structure and subfamilies

The first report of the detailed structure of an LRR containing protein was given by Kobe and Deisenhofer in 1993 for the crystal structure of the ribonuclease inhibitor (RI) protein. This protein contains 15 LRRs and may provide a prototype structure for all LRR proteins (Hocking et al., 1998). Each LRR consists of a β-strand (L X X L X L X X N X L) parallel to an α-helix (X A X X d A d d d d A d d X d d) resulting in a non-globular horse shoe shaped protein. The outer

circumference of the horse shoe consists of α helices while the inner is a parallel sheet. The ligand (ribonuclease A), (Fig. 2C) interacts with the β sheet of the concave face of the inhibitor. Binding of the ligand results in a conformational change of the entire structure of the inhibitor and increases the surface area for binding (Kobe and Deisenhofer in 1995), consistent with the idea that LRRs are ideal for mediating protein-protein interaction (Hocking et al., 1998). The eleven-residue segment corresponding to the β strand region is conserved in LRRs whereas the remaining parts of the repeat may be very different (Kobe and Deisenhofer, 1994; Kajava et al., 1995).

A

B

```
            5       10      15      20      25
            |        |       |       |       |
Y    X L E X L X L X X C X L T X X X C X X L X X A L X X X X -
     β                          ---        helix          ---

Z    X L X E L X L X X N X L G D X G A X X X X X X X L X X P X X
     β                          ---         helix        -----
```

C

Figure 2. Structure of porcine ribonuclease inhibitor (A) Portrayed as a ribbon diagram (Kobe and Deisenhofer, 1994), (B) consensus sequence and secondary structure of LRRs of porcine RI. The sequence of RI is aligned so that the two types of repeats (Y and Z) alternate in the sequence. One letter amino acid code is used. 'X' denotes any amino acid and 'A' denotes an aliphatic amino acid. The part of the repeat that is strongly conserved in all LRR proteins is underlined and the conserved residues are shown in bold. The solid lines below the sequence mark the core region of the sheet and helix; the dashed lines denote extensions of helix in different repeats, (C) the structure of RNase A-RI complex. Taken from Kobe and Deisenhofer, (1995)

Thus, while the invariant sequence of the β region is a characteristic function of LRR superfamily, the sequence of the variable part suggests several specific subfamilies may differ in the three dimensional structure, possibly important for the specificity of interaction with legends. One of the examples of such specificity as conferred by the variable residues of the LRR domain is shown by the Trk family (TrkA, B and C) of neurotrophin receptors (Windisch et al., 1995). The second LRR repeat in the TrkA and TrkB, to which nerve growth factor (NGF) and neurotrophin respectively binds, have 13 identical residues and 11 variable residues that determine the specificity and the affinity of the respective receptor (Hocking et al 1998).

The LRR superfamily consists of widely divergent proteins with diverse functions. These proteins include many which participate in biologically important processes, such as receptors for hormones, transcription factors, enzymes, enzyme inhibitors, proteins for cell adhesion, nucleases and ribosome-binding proteins that have been isolated from a broad range of organisms. The large LRR superfamily is ordered into six large classes on the basis of repeat consensus sequence (Table 1) (Kajava, 1998). The bacterial LRRs are the shortest and are all extracellular and of Gram negative bacterial origin. These LRR containing proteins are essential for bacterial virulence, playing an important role in the initial stages of an infection (Reisner and Sterley, 1992). Plant LRRs have a length similar to the typical 24-residue LRR but their consensus sequences in the variable part differ from the typical LRR consensus (Lt/sGXlP compared to LXXLP; Table 1) (Kajava, 1998). These LRR domains are extracellular parts of the multidomain membrane proteins. The proteins of PS-LRR sub-family also share a common function of transducers of signals across membranes and some of them mediate plant resistance to pathogens (Jones and Jones, 1997).

The LRRs of extracellular proteins are generally flanked by cysteine rich domains on both N- and C-terminal sides. The N-flank-LRR-C-flank motif has been described as an LRM-cassette (Schneider and Schweiger, 1991; Kobe and Deisenhofer, 1994). These domains are characterized by four similarly spaced cysteines in a stretch of about 20 amino acids for the amino flanking region and about 50 amino acids for the carboxy-flanking region (Kobe and Deisenhofer, 1994). On the basis of alignment of 40-70 proteins, the consensus sequence of the C and N-flanking region has been refined. Currently four different types of C flanks have been identified (Table 2, Kajava et al., 1998). The CF1 is the most common one, while the second known type of C-flanking sequence with two conserved cysteine residues, CF2, is found in the small proteoglycans

(Fischer et al., 1991). The CF3 flank has three conserved cysteine residues and is found in about 50 sequences of G protein coupled receptors .The CF4 flank is found in plant LRR proteins. The NF domain is found in 72 different proteins with typical LRRs, including that having either CF1, CF2 or CF3 domain (Kajava, 1998).

LRR subfamily	Length residue	Range residue	Consensus sequence	Origin	Location
Typical	24	(20-27)	+ + - LxxLxxLxLxxNxLxxLpxxoFxx	Animals, fungi	Extracellular
RI- like	28-29	(28-29)	+ xxLxxLxLxxN/CxLxxxgoxxLxxoLxx -----------	Animals	Intracellular
PS	26	(23-25)	+ +- LxxLxxLxLxxNxLt/sGxIPxxLG --------	Plants, primary eukaryotes	Extracellular
CC	26	(25-27)	+ C/LxxLxxLxLxxCxxITDxxoxxLa/gxx ------------	Animals, plants, fungi	Intracellular
SD22- like	22	(21-23)	+- LxxLxxLxLxxNr/kIr/kr/kIEN/GLEx -------------	Animals, fungi	Intracellular
Bacterial	20	(20-22)	+ PxxLxxLxVxxNxLxxLPD/EL -- --------	Gram-negative bacteria	Extracellular

Table 1. LRR subfamilies. Bold and upper case letters indicate, more than 70% and 40% occurrence of a given residue in a certain position respectively. Lowercase letters indicate more than 30% identity. o denotes a non-polar residue; and x, any residue. The sites of insertions and deletions are indicated by + and -. The underlined residues indicate the constant part of the consensus sequence while the broken lines indicate the distinctive regions of each superfamily. (RI) Ribonuclease inhibitor, (CC) Cysteine-containing, (PS) Plant specific. Taken from Kajava, 1998

The consensus flanks discussed in Table 2 are only found in the sections of proteins that occur or are presumed to occur extracellularly so the cysteine residues probably form disulphide bonds with a structural role (Buchanan and Gay, 1996). In small animal proteoglycans whose most of the sequence consist of LRM cassettes, the cysteine residues of the flanks form interchain disulphide loops (Kresse et al., 1993). The LRM cassette has been mapped as a ligand binding site of human platelet glycoprotein Ib (Vicente et al., 1990).

CF1 domain	**LxxLxLxxNP**(w/f)x$\underline{C}$x$\underline{C}$xoxWLxxw(9-24)ox$\underline{C}$(0-18)$\underline{C}$xxp...
CF2 domain	**LxxVYLxxN**nl(s/t)xogxxdF$\underline{C}$xoxxxxo(4-5)y(4)LxxNpo(6)Pxxfx$\underline{C}$o
CF3 domain	**LxxAxL**(s/t)YPSH$\underline{CC}$AFxN95-19)nosx$\underline{C}$nxsxxR...
CF4 domain	**LxxLnLsx**Nx$L\underline{c}G\underline{c}$oP(12-16)oxxn(1-5)$\underline{C}$(1-11)$\underline{C}$...
NF domain	(7-10)$\underline{C}$P(2-25)$\underline{C}$x$\underline{C}$(4-17)ox$\underline{C}$(2-4)oxxoPxxopxx**oxxLxL**

Table 2. Consensus sequence of the cysteine-rich flanking domains. C-terminal cysteine containing flanking domains: CF1, CF2, CF3, CF4 and N-terminal cysteine containing flanking region (NF). Bold and upper case letters indicate, respectively, more than 70% and 40% occurrence of a given residue in a certain position. Lowercase letters indicate more than 30% identity. o denotes a non-polar residue; and x, any residue. The sequences at the beginning of the CF domains and at the end of NF domain that are shown in bold correspond to the conserved part of LRRs. The conserved cysteine residues are underlined. Taken from Kajava, 1998

Functional role of LRR domain

Tandem arrays of a characteristic LRR motif have been found in the primary structure of a number of proteins that for instance participate in cell adhesion and hormone perception (reviewed by Kobe and Deisenhofer, 1995; Buchanan and Gay, 1996). There are no obvious biological properties that are shared between all members of the LRR family. However most LRR-containing proteins appear to be part of multisubunit complexes and more than half have a postulated role in signal transduction. Proteins composed entirely or almost entirely of LRRs offer a good clue as to function of LRR domain. These include RI, *sds22+*, *chaoptin*, *cf-9*, leucine-rich alpha 2-glycoprotein (*LRG*), small proteoglycans and glutamic acid/alanine-rich protein (*GARP*). For example, chaoptin, a membrane adhesion protein is anchored to the membrane via a glycophospholipid anchor. chaoptin promotes homophilic cell aggregation in Drosophila non-adhesive Schneider line 2 (S2) cells (Krantz and Zipursky, 1990). This leads to the idea that the LRRs of chaoptin molecules from different cells adhere to one another (Buchanan and Gay., 1996). Mutations in the *chaoptin* gene result in reduced developmental competence of cells to generate normal rhabdomeric membranes. The mutant protein exhibits altered distribution in both developing and adult photoreceptor neurons (Pollock et al., 1990). Furthermore, phenotype of Drosophila deficient in chaoptin indicates that this protein is required for the development of photoreceptor cells. Mutations in a single LRR of sds22[+], a fission yeast

nuclear protein, destroys the ability to rescue an sd22 mutant and the deletion of 4-5 LRR repeats abolishes the ability to bind to $dis2^+$ and $sds21^+$ protein phosphatase catalytic subunits (Stone et al., 1993). The crystal structure of the RI-RNaseA complex reveals the contribution from different regions of the repeats and makes clear the function of LRRs of RI (Kobe and Deisenhofer, 1995).

From the classification shown in Table 2, it is known that LRR proteins have a different cellular location. In the following paragraphs we will discuss only the role of those LRR proteins that are either transmembrane receptors or are attached to the membrane via different anchors (Table 3). Fig. 3 gives a schematic representation of these LRR proteins, their repeat size, the number of repeats and their proposed roles. In transmembrane receptors, LRRs and their flanks always occur in the presumed extracytoplasmic section, with a very few exceptions (p34 protein). The current hypothesis on the role of LRRs is that they participate in mediating heterotrophic interactions that may range from direct cell-cell contact or receptor-ligand interactions. Expression patterns, phenotypic analysis of LRR mutant alleles and site directed mutagenesis of aminoacid residues in the LRRs have also elucidated the role of many of the LRR proteins in the development of both plants and animals.

Glycoprotein hormones influence the development and function of the ovary, testis and thyroid by binding to specific high-affinity receptors. A group of these high affinity receptors are the extracellular domain LRR containing G protein-coupled receptors (LGRs). LGR6 and LGR7 form glycoprotein hormone receptors. The high affinity binding sites of the LGRs have been mapped to the LRR region (Braun et al., 1991) suggesting that LRRs mediate receptor specificity. Human chorionic gonadotropin (hcG) binds to the N-terminal half (exodomain) of the leutinizing hormone/chorionic gonadotropin receptor (LH/CG-R), after which a second contact is made with the membrane associated C-terminal half (endodomain) of LH/CG-R. This exodomain binds hormone with high affinity and specificity and contains eight LRRs. Site-directed mutagenesis have revealed the importance of LRR2 and LRR4 in of LH/CG-R in hormone binding (Song et al., 2000a). From ribonuclease binding to RI studies (Fig. 1), it is known that the inner lining of the horseshoe consists of the β-stranded Leu/Ile-X-Leu/Ile motif and the RI β-strands interact with ribonuclease.

Protein	Source	Proposed function, observation	Repeat size	Repeats
Toll	Drosophila	Embryo development	24	19
18 wheeler	Drosophila	Morphogenesis	24	27
gp150	Drosophila	Cell adhesion?	24	18
Connectin	Drosophila	Synapse development	24	7
Chaoptin	Drosophila	Photoreceptor cell development	24	30
MATER	Drosophila	Embryogenesis	29	14
DLGR-2	Drosophila	Development	23	17
Caps	Drosophila	Synapse formation	24	14
Inlb	Bacterial	Plant defense	22	8
GP1bα	human	Platelet glycoprotein	24	7
5T4	human	Cell adhesion	24	7
Trk	human	Signal transduction	23	2
LH/CG-R	human	Signal transduction	24	8
FSH-R	human	Signal transduction	24	8
pal	rat	Photoreceptor cell dev.	23	5
ERECTA	Arabidopsis	Morphogenesis	24	20
CF-9	Arabidopsis	Disease resistance	24	25
AtSERK1	Arabidopsis	Embryo development	24	5
Xa21	Arabidopsis	Disease resistance	24	22
CLAVATA1	Arabidopsis	Differentiation	23	23
BRI1	Arabidopsis	Brassinosteroid signal transduction	24	25

Table 3. Extracellular LRR containing proteins bound to the plasma membrane.

Mutations of Leu-103 and Ile-105 in the LRR4 of the exodomain of the LH/CG-R highly influence hormone binding, folding and proper surface localization. Mutations in the Asn-107, Thr-108 and Gly-109 in LRR4 reveal affect in downstream cAmp induction. This implicates the involvement of Asn-107, Thr-108 and Gly-109 residues of LRR4 in contacting the endodomain of LH/CG-R (Song et al., 2000a). In contrast to LH/CG-R, the important LRRs are spread throughout the exodomain of FSHR (Song et al., 2001b). Despite the 94% homology in the Leu/Ile-X-Leu/Ile sequences between follicle stimulating hormone receptor (FSHR) and LH/CG-R, they are capable of recognizing their cognate ligands, LH/CG and FSH, respectively, with a high affinity and no cross-reactivity. This suggests different contact points in LRRs of the two
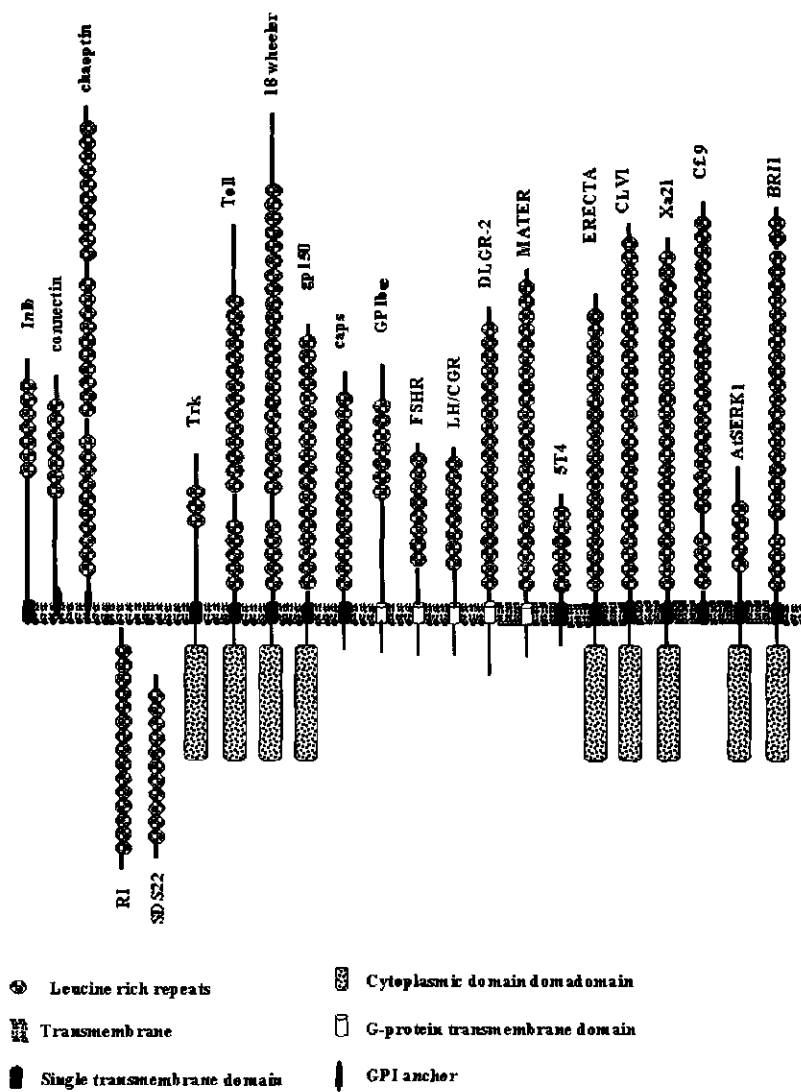
Fig. 3. Shemetic diagram depecting the modular organization of transmembrane LRR proteins

receptors, which may explain the distinct hormone specificity despite the structural similarity of the two receptors (Song et al 2001b).

Mutations in surface glycoproteins have also revealed the importance of LRRs in protein localization. The extracellular domains of the mammalian platelet glycoproteins in the GPIb-V-IX complex have LRRs with cysteine flanks on both sides by consensus. A disorder of the platelet glycoprotein GPIb-IX-V complex leads to Bernard-Soulier Syndrome (BSS). A single base pair mutation, resulting in the Phe-55 Ser substitution within the LRR of GbIX polypeptide was shown to result in the impaired surface expression and to cause BSS (Hayashi and Suzuki, 2000). Mutations of the conserved charged residues in the first and the sixth LRR domain of GP1bα, the ligand binding sub-unit of GP1b-IX-V complex, binding to the von Willebrand factor (Vwf) receptor, have shown changes in the conformation, synthesis and vWf-binding capacity of the GP Ib/IX/V complex (Kharghan et al, 2000).

Disease resistance in plants is often controlled by gene for gene mechanism in which avirulance (avr) gene products encoded by pathogens are specifically recognized, either directly or indirectly, by plant disease resistance (R) gene products. Most variation between resistance genes and their closely related homologs occurs within the LRR, particularly within the xxLxLxx (where L indicates a conserved leucine or other aliphatic residue and x represents any amino acid) motif of the repeat units, and evidence suggests that this region has been subject to diversifying selection (Dodds et al. 2000). Many of the cloned *R* genes contain cytoplasmic LRRs except for a few that have an extracellular LRR domain. These include tomato *Cf*-9 and *Cf*-2 and rice *Xa*21 (Fig. 3). *Xa*21D, a member of the *Xa*21 multigene family codes for a secreted LRR receptor like protein lacking the transmembrane and kinase domains due to retrotransposon insertion (Wang et al, 1998). The introduction of *Xa*21D into a susceptible rice strain conferred partial disease resistance to *X. oryzae* (Wang et al, 1998). It is speculated that secreted Xa21D protein employs its LRRs to form heterodimer with the endogenous LRR-RLK lacking recognition specificity. The kinase domain of the endogenous LRR-RLK will be activated upon ligand binding to the Xa21D (Torii, 2000). However no direct interaction between an LRR and a ligand produced by a pathogen has been demonstrated to date for any of the extracellular LRR containing R genes, the involvement of LRRs of cytoplasmic R-genes in mediating resistance to bacterial pathogens has been shown (Warren et al, 1998). Both bacterial and host LRR proteins have been shown to be involved in mediating entry of bacteria into host cells. Flagellin, the main protein of the bacterial

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flagella, elicits defense responses and alters growth in *Arabidopsis* seedlings. The *Arabidopsis FlAGELLIN SENSING2* (FLS2), an LRR-receptor like kinase (RLK), confers sensitivity to bacterial flagella. In particular a region of 15 amino acids in third LRR region appears to be necessary for binding of flagellin protein with the FLS2 receptor (Gomez and Boller, 2000).

For many microbial pathogens, entry into host mammalian cells is an important step during infection (Dramsi and Cossart, 1998). *Listeria monocytogenes* InlB protein is an LRR surface protein that mediates entry of bacteria into a wide variety of cell types, and stimulates tyrosine phosphorylation of three adaptor proteins, Gab1, Cbl and Shc in addition to stimulating changes in actin cytoskeleton (Ireton et al., 1999). Studies with monoclonal antibodies recognizing the LRR region in InlB have shown that LRRs of InlB protein are necessary for the entry of Listeria into mammalian cells and the activation of downstream P13-kinase (Braun et al., 1999).

The early studies on Drosophila genes and the later studies in other animal and plant genes have revealed the role of LRRs in plant and animal development. Many transmembrane proteins have been shown to influences the regulation of gene expression and morphogenesis of cells during embryonic development (McDonald, 1989). Based on the expression pattern and the mutational analyses, transmembrane LRR proteins have been shown to mediate in localization, binding and signal transduction events during the early stages of development (Table 3).

A plausible role for the LRRs of the maternal transmembrane protein Toll is suggested by the finding that it functions in heterotypic cell adhesion (Keith and Gay, 1990). Dorsoventral polarity of the Drosophila embryo is established by a signal transduction pathway in which Toll appears to function as the receptor for a ventrally localized extracellular ligand (Schneider et al., 1991). Recessive mutations that lower the activity of the Toll alter either the extracellular or the cytoplasmic domain of the protein. Tl^{rm9} allele has Lys-Ser mutation in the 8th LRR of Toll and causes an unusual embryonic phenotype. The embryos are laterlized; both dorsal and lateral structures are absent, and all cells adopt lateral fates (Anderson, 1985). Tl^{r26} allele has a Val-Ala mutation in the cysteine flanking motif following the 12th LRR and causes a strong dorsalizing phenotype. The dominant ventralizing alleles of Toll fall into two genetic classes that differ in their requirement for wild type allele of Toll. The class 1 dominant alleles, Tl^{l} Tl^{10b} and Tl^{9Q} all have a mutation in the C-terminal cysteine flank adjacent to the last (15th) LRR. These mutants encode proteins that are active in all dorsoventral regions, regardless of the presence or absence of the wild type protein or its ligand. The dominant class II alleles of Toll, Tl^{84c} Tl^{5B} and Tl^{DB2}

have mutations in 12th, 13th and the 15th LRR respectively. All these 3 alleles encode stable truncated polypeptides, resulting in ventralized embryos. Microinjection studies of mutant transcripts containing amino acid mutations in the LRR region caused the same phenotypes as described for each mutation (Schneider et al., 1991). This supports the view that the LRRs of Toll have a major role to play in its function (Winans and Hashimoto, 1995). 18 wheeler with 27 LRRs has a similar primary structure like Toll, promotes heterophilic cell adhesion in non adhesive S2 cells (Eldon et al., 1994). connectin is a cell-surface molecule that mainly consists of LRRs. During synapse formation, the protein localizes to synaptic sites; afterward, it largely disappears. Like chaoptin this protein has also been shown in mediating homophilic cell adhesion in S2 cells (Krantz and Zipursky, 1990).

Many other transmembrane proteins whose extracellular domain consists mainly of LRR have been shown to be involved in early stages of development (Fig 2. and Table 3). However, no functional analysis has been performed to show the involvement of LRRs of these proteins in their function. These include Drosophila capricious (caps) protein which is involved in selective synapse formation (Shishido et al., 1998); DLGR-2. a leucine rich repeat containing G-protein coupled receptor, which is expressed only in embryos and pupae and is absent in the later stages of development (Eriksen et al., 2000); the mice MATER protein, the absence of which in genetically engineered mice results in a striking failure of the mouse embryo to progress beyond early cleavage stages (Tong, et al 2000); a rat transmembrane glycoprotein Pal is expressed in photoreceptor cells of the retina with expression correlated with the development of photoreceptor outer segments (Gomi et al., 2000); and mouse 5T4 protein, the transcripts of which are highly expressed in embryos and in adult tissue transcripts are restricted to brain and ovary (King et al, 1999).

There is increasing evidence that transmembrane receptors also play important roles in cellular signalling and developmental regulation in plants. These plant genes are called receptor-like kinases (RLKs). Plant RLKs like CLAVATA1, ERECTA, BRI1 and the SERK proteins have an LRR containing extracellular domain (Fig. 3). Various studies have implicated the role of their LRR in early plant development (Torii, 2000) and protein targeting (Shah et al., 2001) and also speculated their role in ligand binding and signal transduction (Lease et al 1998). The predicted site for this interaction is the solvent exposed parallel β-sheet (Kobe and Deisenhofer, 1995). The LXXLXLXX domain within the LRR corresponds with the solvent face of the protein with the

leucine residues facing away from the solvent face (Jones and Jones, 1997). The X or the variable amino acid could lead to specificity of the protein binding to the LRR (Freidrichsen, 2000) BRI1. The strong clv1 alleles, *clv1-4* and *clv1-8* alleles have a G-to-D and D-to-N substitution within this solvent face domain (the 6th and the 10th LRR respectively) (Clarke et al., 1997). In contrast to this bri1-9 is a weak allele (Noguchi et al, 1999) altering a serine in the conserved, BRI1 solvent face, LXXLXLSX (Li and Chory, 1997). *er-103* has a M-to-I substitution in the 10th LRR of ERECTA that results in a weak phenotype (Torii et al., 1996). These missense mutations in the LRR region possibly perturb the proper ligand-receptor interactions and compromise activation of these receptors. Alternatively, such mutations may interfere with receptor dimer formation.

SERK with 5 extra cellular LRRs is transiently expressed during *Daucus carota* somatic embryogenesis, the expression disappearing after the 100-celled globular stage (Schmidt et al., 1997). To date the elements concerning the origin and targets of processes of cell-to-cell communication in early stages of development in plants, the SERK gene may represent a significant part of a mechanism that is essential for the formation of plant cells destined to become embryos. LRR deletion studies on the Arabidopsis homolog of SERK, AtSERK1 have revealed the role of AtSERK1 LRRs in receptor targeting to the cell surface. LRR2 and LRR4 each with 2 glycosylation sites have been shown to have a profound influence on AtSERK1 protein targeting (Shah et al. 2001, submitted). Plant transmembrane receptor LRRs have also been shown to be important in photoperiod induction. The alternate transcript initiation of an LRR transmembrane receptor like protein, *inrpk1* in morning glory (*Ipomoea nil*) indicates the role of LRRs in photoperiodic-induced flowering (Basset et al., 2000).

In this review we have attempted to give an in depth overview of the cell surface LRR proteins with major emphasis on the role they play in protein-protein interactions and signal transduction pathways in the context of plant and animal development. This seems just to be the beginning of understanding of how and where LRRs function, the further structural and functional understanding of these proteins will provide a wealth of information in this regard. The future studies will mostly concentrate to reveal the structural basis of protein-protein interactions mediated by LRR proteins.

References

1. Takahashi, N., Takshashi, Y. and Putnam, F.W. (1985) Periodicity of leucine and tandem repetition of a 24-amino acid segment in the primary structure of leucine-rich alpha 2-glycoprotein of human serum. *Proc Natl. Acad. Sci.* U. S. A. **82**, 1906-10.

2. Kobe, B., and Deisenhofer, J. (1994) The leucine rich repeat: aversatile binding motif *TIBS* **19**, 415-421

3. Roth, G.J. (1991) Developing relationships: arterial platelet like adhesion, glycoproteins Ib and leucine rich glycoproteins. *Blood* 77, 5-19

4. Dixon M.S., Jones, D.A., Thomas, C.M, Harrison K. and Jones, J.D. (1996): The tomato Cf-2 disease resistance locus comprises two functional genes encoding leucine rich repeat proteins. *Cell* **84**, 451-459

5. Buchanan Sean G. ST. C. and Nicholas J. Gay (1996) Structural and functional diversity in the leucine-rich repeat family of proteins *Prog. Biophys. Mol. Biol.* **65**, 1-44

6. Hocking, A.M., Shinomura T. and McQuillan, D.J. (1998) Leucine rich repeat glycoproteins of extracellular. *Matrix Biol.*17, 1-19

7. Kobe, B., and Deisenhofer, J. (1995) A structural basis for interactions between leucine rich repeats and protein ligands. *Nature* 374, 183-186

8. Kajava, A.V., Vassart, G. and Wodak, S.J. (1995) Modelling of the three-dimensional structure of proteins with the typical leucine-rich repeats. *Structure* **3**, 867-77.

9. Windisch, J.M., Marksteiner, R., Lange, M.E., Anuer, B. and Schneider, R. (1995) Brain derived neurotrophic factor, neurotrophin-3, and neurotrophin-4 bind to a single leucine rich repeat of TrkB. *Biochemistry* **34**, 11256-63

10. Kajava, A.V. (1998) Structural diversity of leucine- rich repeat proteins. *J. Mol. Biol.* **277**, 519-27.

11. Reisner, B.S and Straley, S.C. (1992) *Yersinia pestis* YopM: thrombin binding and overexpression. *Infect. Immun.* **60**, 5242-52

12. Jones, D and Jones, J. (1997) The role of leucine-rich repeat proteins in plant defence *Ad. Bot.Res.* **24**, 90-167

13. Schneider, R. and Schweiger, M. (1991) The yeast repair proteins RAD1 and RAD7 share similar putative functional domains. *Fedn. Eur. Biochem. Socs. Lett.* **283**, 203-6

14. Fischer, I.W., Heegard, A.M., Vetter, U., Vogel, W., Just, W., Termine, J.D. and Young, M.F. (1991) Human biglycan gene. *J.Biol. Chem.* **266**, 14371-77

15. Kreese, H., Hausser, H. and Schoenherr, E (1993) Small proteoglycans. *Experimenta* **49**, 403-416

16. Vicente, V., Houghten, R.A. and Ruggeri, Z.M. (1990) Identification of a site in the alpha chain of platelet glycoprotein Ib that participates in von Willebrand factor binding. *J. Biol. Chem.* **265**, 274-80.

17. Schneider, D.S., Hudson, K.L., Lin, T.Y. and Anderson, K.V. (1991)Dominant and recessive mutations define functional domains of Toll, a transmembrane protein required for dorsal-ventral polarity in the Drosophila embryo. *Genes Dev.* **5**, 797-807.

18. Kobe, B., and Deisenhofer, J. (1995) Proteins with leucine rich repeats. *Curr. Opin. Struct. Biol.* **5**, 409-416

19. Krantz, D and Zipursky, S. (1990) Drosophila chaoptin, a member of the leucine rich repeat family is a photoreceptor cell-specific adhesion molecule. *Eur. Molec. Biol. Org. J.* **9**, 1969-77

20. Pollock, J.A., Ellisman, M.H. and Benzer, S. (1990) Subcellular localization of transcripts in Drosophila photoreceptor neurons: chaoptic mutants have an aberrant distribution.*Genes Dev.* **4**, 806-821.

21 Stone, E., Yamano, H., Kinoshita, N. and Yanagida, M. Mitotic regulation of protein phosphatases by the fission yeast sds22 protein. *Curr. Biol.* **3**, 13-26.

22. Braun, T., Schofield, P.R. and Sprengel, R. (1991) Amino-terminal leucine-rich repeats in gonadotropin receptors determine hormone selectivity. *EMBO J.***10**, 1885-90

23. Song, Y.S, Ji, I., Beauchamp, J., Isaacs, N.W. and Ji, T.H. (2000) Hormone Interactions to Leu Rich Repeats in the Gonadotropin Receptors: II. Analysis of Leu Rich Repeat 4 of Human Luteinizing Hormone/Chorionic Gonadotropin Receptor. *J Biol Chem.* Jul 6

24. Song, Y.S, Ji, I., Beauchamp, J., Isaacs, N.W. and Ji, T.H. (2001) Hormone Interactions to Leu-rich Repeats in the Gonadotropin Receptors. I. Analysis of Leu Rich Repeat 4 of Human Luteinizing Hormone/Chorionic Gonadotropin Receptor and Follicle stimulating hormone receptor. *J. Biol. Chem.* **276**, 3426-3435

25. Hayashi, T. and Suzuki, K. (2000) Molecular pathogenesis of Bernard-Soulier syndrome. *Semin. Thromb. Hemost.* **26**, 53-9.

26. Afshar-Kharghan, V., Gineys, G., Schade, A.J., Sun, L., Li, C.Q., McIntire, L.V., Dong, J.F. and Lopez J.A. (2000) Necessity of conserved asparagine residues in the leucine-rich repeats of platelet glycoprotein Ib alpha for the proper conformation and function of the ligand-binding region. Biochemistry **39**, 3384-91.

27. Dodds, P.N., Lawrence, G.J. and Ellis, J.G. (2000) Six Amino Acid Changes Confined to the Leucine-Rich Repeat beta-Strand/beta-Turn Motif Determine the Difference between the P and P2 Rust Resistance Specificities in Flax. *Plant Cell* **13**, 163-178.

28. Wang, G.L., Ruan, D.L., Song, W.Y., Sideris, S., Chen, L., Pi, L.Y., Zhang, S., Zhang, Z., Fauquet, C., Gaut, B.S., Whalen, M.C. and Ronald, P.C. (1998) Xa21D encodes a receptor-like molecule with a leucine-rich repeat domain that determines race-specific recognition and is subject to adaptive evolution. *Plant Cell* **10**, 765-79.

29. Torii, K. (2000) Receptor kinase activation and signal transduction in plants: an emerging picture *Curr. Opin. Plant. Biol.* **3**, 361-367.

30. Warren, R.F., Henk, A., Mowery, P., Holub, E. and Innes, R.W. (1998) A mutation within the leucine-rich repeat domain of the Arabidopsis disease resistance gene RPS5 partially suppresses multiple bacterial and downy mildew resistance genes. *Plant Cell* **10**, 1439-52.

31. Gomez-Gomez, L. and Boller, T. (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Mol. Cell* **5**, 1003-11.

32. Dramsi, S. and Cossart, P. (1998) Intracellular pathogens and the actin cytoskeleton. *Annu. Rev. Cell Dev. Biol.* **14**, 137-166.

33. Ireton, K., Payrastre, B. and Cossart, P. (1999) The Listeria monocytogenes protein InlB is an agonist of mammalian phosphoinositide 3-kinase. *J. Biol. Chem.* **274**, 17025-32.

34. Keith, F. and Gay, N. (1990) The Drosophila membrane receptor Toll can function to promote cellular adhesion. EMBO J.**9**, 4299-306

35. Anderson, K.V., Bokla, L. and Nusslein-volhard, C. (1985) Establishment of dorsal-ventral polarity in the Drosophila embryo: the induction of polarity by the Toll gene product. *Cell* **42**, 791-798.

36. Winans, K.A. and Hashimoto, C. (1995) Ventralization of the Drosophila embryo by deletion of extracellular leucine-rich repeats in the Toll protein. *Mol Biol Cell.* **6**, 587-96.

37. Eldon, S., Kooyer, S., D'Evelyn, D., Duman, M., Lawinger, P., Botas, J. and Bellen, H. (1994) the Drosophila 18 wheeler is required for morphogenesis and has striking similarities to Toll. Development 120, 885-899.

38. Shishido, E., Takeichi, M. and Nose, A. (1998) Drosophila synapse formation: regulation by transmembrane protein with Leu-rich repeats, CAPRICIOUS. *Science* **280**, 2118-21.

39. Eriksen, K.K., Hauser, F., Schiott, M., Pedersen, K.M., Sondergaard, L. and Grimmelikhuijzen, C.J. (2000) Molecular cloning, genomic organization, developmental regulation, and a knock-out mutant of a novel leu-rich repeats-containing G protein-coupled receptor (DLGR-2) from Drosophila melanogaster. *Genome Res.* **10**, 924-38.

40. Tong, Z.B., Nelson, L.M. and Dean, J. (2000) Mater encodes a maternal protein in mice with a leucine-rich repeat domain homologous to porcine ribonuclease inhibitor. *Mamm Genome* **11**, 281-7.

41. Gomi, F., Imaizumi, K., Yoneda, T., Taniguchi, M., Mori, Y., Miyoshi, K., Hitomi, J., Fujikado, T., Tano, Y. and Tohyama, M. (2000) Molecular cloning of a novel membrane glycoprotein, pal, specifically expressed in photoreceptor cells of the retina and containing leucine-rich repeat. *J Neurosci.* **20**, 3206-13.

42. King, K.W, Sheppard, F.C., Westwater, C., Stern, P.L. and Myers K.A. (1999) Organisation of the mouse and human 5T4 oncofoetal leucine-rich glycoprotein genes and expression in foetal and adult murine tissues. *Biochim Biophys Acta.***1445**, 257-70.

43. Shah, K. Gadella, T.W.J., Hecht, V and deVries, S.C. (2001) Subcellular localization and dimerization of AtSERK1 (submitted)

44. Lease, K., Ingham, E. and Walker, J.C. (1998) Challenges in understanding RLK function.
Curr Opin Plant Biol **15**, 388-92.

45. Friedrichsen, D.M., Joazeiro, C.A., Li, J., Hunter, T. and Chory, J. (2000) Brassinosteroid-insensitive-1 is a ubiquitously expressed leucine-rich repeat receptorserine/threonine kinase. *Plant Physiol.* **123**, 1247-56.

46. Clark, S.E., Williams, R.W. and Meyerowitz, E.M. (1997) The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. *Cell* **89**, 575-85

47. Noguchi, T., Fujioka, S., Choe, S., Takatsuto, S., Yoshida, S., Yuan, H., Feldmann, K.A. and Tax, F.E. (1999) Brassinosteroid-insensitive dwarf mutants of Arabidopsis accumulate brassinosteroids. *Plant Physiol.* **121**, 743-52.

48. Li, J. and Chory, J. (1997) A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. *Cell* **90**, 929-38.

49. Torii, K.U., Mitsukawa, N., Oosumi, T., Matsuura,Y., Yokoyama, R., Whittier, R.F. and Komeda, Y. (1996) The Arabidopsis ERECTA gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. *Plant Cell* **8**, 735-46.

50. Schmidt, E.D., Guzzo, F., Toonen, M.A. and de Vries, S.C. (1997) A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. *Development* **124**, 2049-62.

51. Bassett, C.L., Nickerson, M.L., Cohen, R.A. and Rajeevan, M.S. (2000) Alternative transcript initiation and novel post-transcriptional processing of a leucine-rich repeat receptor-like protein kinase gene that responds to short-day photoperiodic floral induction inmorning glory (Ipomoea nil). *Plant Mol Biol.* **43**, 43-58.

3

Role of threonines in the AtSERK1 activation loop in auto- and transphosphorylation

Abstract

The *AtSERK1* gene encodes a receptor-like protein kinase that is transiently expressed during embryogenesis. To determine the intrinsic biochemical properties of the AtSERK1 protein, we have expressed the intracellular catalytic domain as a glutathione S-transferase (GST) fusion protein in *E. coli*. The AtSERK1-GST fusion protein mainly autophosphorylates on threonine residues ($K_m=4x10^{-6}M$) and the reaction is Mg^{2++} dependent and inhibited by Mn^{2++}. A lysine to glutamic acid substitution (K330E) in the kinase domain of AtSERK1 abolishes all kinase activity. The active AtSERK1^{kin} can autophosphorylate inactive AtSERK1^{K330E} protein demonstrating an intermolecular mechanism of autophosphorylation. The AtSERK1 kinase protein was modelled using insulin receptor kinase as template. Based on this model, threonine residues in the AtSERK1 activation loop of catalytic sub-domain VIII are predicted to be targets for autophosphorylation. Replacing Thr-468 with either alanine or glutamic acid completely obliterated the AtSERK1 ability to be phosphorylated. Transphosphorylation on MBP and casein showed tyrosine, serine and threonine as targets, demonstrating that AtSERK1 is a dual specificity kinase. The AtSERK1^{T468A} was not able to transphosphorylate, showing that only phosphorylated AtSERK1 kinase can transphosphorylate.

Introduction

Perception and transduction of external stimuli is essential for the growth and development of multicellular organisms. In many cases this involves membrane-associated receptor proteins of which the ligand binding domains perceive the extracellular signal molecule. Information is then transmitted through the kinase domains of the receptor that in turn lead to phosphorylation of target proteins [1,2]. Most animal receptor kinases have tyrosine kinase activity, only a few are serine/threonine kinases [2,3] or can phosphorylate serine/threonine in addition to tyrosine [4]. There is increasing evidence that protein kinases also play important roles in cellular signalling and metabolic regulation in plants. Rapid and transient changes in the phosphorylation pattern of plant membrane associated proteins have indicated the involvement of protein kinases in signalling events [5,6]. Many plant genes encode receptor kinases sharing the same basic domain structures as shown in animal receptors and are called receptor-like kinases (RLKs). Plant RLKs are involved in diverse processes such as hormone response [7] mitogenic induction [8], self-incompatibility [9], shoot meristem development [10] and disease resistance [11]. Expression studies have implicated RLKs to be involved in embryogenesis, pollen function and light responses [12]. Based on the amino acid sequence of their extracellular domains, the RLKs reported so far have been categorized into 6 different types [13] (a) the S-domain type, with similarities to S-locus glycoproteins in *Brassica*; (b) the epidermal growth factor receptor (EGFR)-like; (c) the extracellular lectin domain type (d) the TNFR (tumor necrosis factor receptor) domain type; (e) PR (pathogenesis related protein 5) domain type and (d) the leucine-rich repeat (LRR) type, with 3-26 leucine-rich repeats. The LRR motif occurs not only in receptor kinases but in many other proteins as well and may be involved in protein-protein interactions [14]. Analysis based on mutant phenotypes has revealed that in particular the LRR type, are involved in plant development [13,15]. The *ERECTA* gene was the first putative receptor containing extracellular LRRs to be cloned from *Arabidopsis* and is involved in organ elongation [16]. The best characterized of the plant LRR RLKs is the *CLAVATA1* (*CLV1*) gene from *Arabidopsis* that is similar in structure to *ERECTA*. *CLV1* is involved in maintaining the balance between cell proliferation and organ formation in shoot and the inflorescence meristems [10]. Another example is the brassinosteroid receptor gene *BRI1* [17].

Expression of plant RLKs in *E. coli* or in insect cells yielded purified proteins suitable for biochemical studies. Plant RLKs were thus shown to autophosphorylate on serine and/or threonine residues. A possible exception is the *Petunia PRK1* gene that autophosphorylates on serine and tyrosine residues [18]. Autophosphorylation has been shown to occur predominantly by intermolecular mechanisms (trans) and it has been suggested that oligomerization may be involved in the regulation of the kinase activity [19]. The sites where phosphorylation occurs have so far only been identified in CrRLK1 from *Catharanthus roseus*. Site directed mutagenesis has identified Thr-720 in the sub-domain XI important both for auto and transphosphorylation of CrRLK1 [20].

This report describes the biochemical characterization of an LRR-RLK from *Arabidopsis thaliana*, AtSERK1. The encoding gene is expressed in ovules, zygotic embryos and in embryogenic suspension cultures of *Arabidopsis* [21]. During somatic embryogenesis AtSERK1 expression continues up to early globular stage embryos and is absent in later stages of embryo development. This transient expression pattern is also observed during zygotic embryogenesis. DNA sequence analysis suggests that AtSERK1 consists of an extracellular domain that has a leucine zipper domain followed by five tandemly repeated leucine rich motifs and a specific proline-rich region. The extracellular domain is linked, via, a transmembrane hydrophobic region to a protein kinase catalytic domain that is homologous to serine/threonine kinases. The catalytic kinase domain of the AtSERK1 protein was expressed as a recombinant fusion protein in *E. coli*. After assaying the activity of the AtSERK1 recombinant fusion protein we found that AtSERK1 has an intrinsic protein kinase activity and autophosphorylates mainly on threonine, slightly on serine but not on tyrosine residues. We report the identification of a threonine residue, Thr-468 that is important both for auto- and transphosphorylation. The kinetics of the autophosphorylation reaction suggests that it proceeds via an intermolecular mechanism.

Materials and Methods

Expression of AtSERK1 kinase catalytic domain in *E. coli*

The cDNA sequence encoding the AtSERK1 (acc no: A67827) kinase catalytic domain, corresponding to nucleotides 1000-2068 (Fig. 1), was amplified by PCR using the 2.1 kb cDNA cloned in vector pBluescriptII SK⁻ as a template. Primer *Sma*I1000 (5' TCCCCCGGGTATTTTCTTCGATGTCCCTG 3') and primer *Not*I2068 (5'ATAAGAATGC GGCCGCCCTTGGACCAGATA 3') amplified a PCR fragment of 1.1 kb that was cloned into a *Sma*I and *Not*I cleaved pGEX-4T1 (Pharmacia) resulting in AtSERK1^{kin} construct. Site-directed mutagenesis of highly conserved lysine, Lys-330 essential for phosphotransfer in protein kinases, to glutamic acid was performed according to manufacturer's instructions (Stratagene), resulting in the AtSERK1^{K330E} construct. Site directed mutagenesis of all three threonine residues; Thr-462, Thr-463 and Thr-468 to glutamic acid resulted in a AtSERK1$^{3T \rightarrow E}$ construct whereas the single amino acid mutations were made resulting in AtSERK1^{T459E}, AtSERK1^{T462E}, AtSERK1^{T463E} and AtSERK1^{T468E} constructs. The same amino acids were also mutated to alanine residues resulting in AtSERK1^{T459A}, AtSERK1^{T462A}, AtSERK1^{T463A} and AtSERK1^{T468A}. All plasmids were verified by restriction endonuclease digestion and DNA sequencing before being transformed into the *E. coli* Bl21 strain for protein expression. A 2 ml overnight culture was transferred to 200 ml of LB medium and the cells were grown at 37°C to an OD_{600} of 0.6 and then induced with 0.1 mM isopropyl-D-thiogalactopyranoside (IPTG) for 3-4 hrs at 30° C. The cells were collected by centrifugation, resuspended in 6 ml of phosphate buffered saline (PBS) lysis buffer containing protease inhibitor cocktail (Mini^{Tm}, Boehringer Manhiem), sonicated and cleared by centrifugation at 12,000 g. The soluble GST-fusion proteins were purified from the supernatant by Glutathione Sepharose 4B (Pharmacia) chromatography according to the manufacturer's instructions.

Computer modelling studies

The X-ray structures of IRK (inactive insulin receptor kinase, determined at 1.9 A° resolution) [22] and of IRK3P (activated human insulin receptor kinase, refined to 2.1 A° resolution) [23] were used as templates for modelling AtSERK1 kinase domain. These two templates produced the best E value when using BLAST against the PDB database. The sequence of the kinase

domain of AtSERK1 was aligned with the sequence of the insulin receptor kinase using the advanced BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Models of the kinase domain of AtSERK1 in its activated and inactive form were built using the Modeller-5 program of Andrej Sali [24]. Modeller is an excellent programme for comparative structural modelling as it results in a very accurate, detailed and explicit models of protein structure [25,26].

The quality of the 3D-models was evaluated using PROCHECK and Prosa II version 3.0 [27]. The two programmes can evaluate the quality of 3D homology models very well [26]. Improvement of the models could be obtained by an iterative sequence-structure alignment procedure, yielding finally the sequence alignment between the AtSERK1 kinase domain and IRK. This improvement in the sequence-structure alignment procedure was especially important for the loop (aa 498-515) which is present in the AtSERK1 kinase domain but not in the insulin receptor kinase.

Auto- and transphosphorylation assays

The kinase activity of the AtSERK1^{kin} and the AtSERK1^{kin} mutant GST fusion proteins was demonstrated by incubating 500 ng (100 μg ml^{-1}) of protein in 20 mM Tris (pH 7.5), 50 mM NaCl, 0.01% Triton X-100, 10 mM MgCl$_2$, 1 mM Dithiothreitol (DTT), 50 μM unlabeled ATP and 10 μCi of [γ^{32}P] ATP in a final volume of 30 μl. After incubation for 30 minutes at 30°C, the reaction was quenched by adding Laemmli SDS-PAGE sample buffer, boiled at 95° C for 5 minutes and separated by 10% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue (CBB) to verify equal loading and then dried. The radioactivity was quantified with a PhosphoImager using the ImageQuant program (Molecular Dynamics).

To remove the GST tag from AtSERK1^{kin} and AtSERK1$^{3T\rightarrow E}$ proteins, 50 μg of each was treated with 10 units of thrombin (Pharmacia) and incubated at 16° C for 4 hrs. Phosphorylation assays were performed in a similar way by incubating the cleaved AtSERK1^{kin} or AtSERK1$^{3T\rightarrow E}$ proteins with AtSERK1^{K330E} in concentrations ranging from 0-500 ng. For transphosphorylation assays 1 μg of casein (Sigma) or myelin basic protein (MBP, Sigma) was incubated with 500 ng of AtSERK1^{kin} or with AtSERK1^{kin} mutant proteins. The reaction conditions were the same as described for the autophosphorylation experiments.

Phosphoaminoacid analysis

The CBB stained phosphorylated bands of AtSERK1^{kin}, casein and MBP were excised from the gel, rehydrated in 50 mM $(NH_4)_2CO_3$, 0.1% SDS and 0.25% β-mercaptoethanol (2-ME), ground with a small pestle, boiled for 5 min and extracted by agitation for 90 min. The proteins were precipitated by adding 20 µg of BSA and 20% (w/v) of solid trichloro-acetic acid (TCA) and incubated overnight at -20°C. The precipitate was collected by centrifugation and the TCA was removed by lyophilization for 30 min. The sample was hydrolysed in 50 µl 6M HCl for 1 h at 120°C. The HCl was evaporated, and the pellet was resuspended in the ascending solvent of ethanol, glacial acetic acid and water (1:1:1 v/v/v) containing 20 nmol each of phosphoaminoacid standards. The sample was applied to a 10 x 10 cellulose TLC plate (Merck) as described [28] using the ascending solvent for 90 min. The first dimension fractionation was followed by a second dimension chromatography in a phosphopeptide buffer containing isobutyl alcohol, formic acid and water (8:3:4 v/v/v) for 1 hr. Phosphoaminoacid standards were visualized by spraying the plate with 0.25% ninhydrin in acetone, and heating it at 65°C for 30 min. The radiographic film was exposed to the TLC plate for 12 h and the radioactivity quantified with ImageQuant program (Molecular Dynamics).

Results

The deduced protein sequence of AtSERK1 (Fig. 1A) consists of 605 amino acids with a calculated M_r of 68,000 Daltons. The predicted domain structure has the general structure of the RLK type kinases [29]: it consists of a hydrophobic N-terminal putative signal peptide, an extracellular domain consisting of a leucine zipper domain, 5 leucine-rich repeats and a proline-rich region unique for the AtSERK1 family [30] followed by a single hydrophobic membrane-spanning domain of 24 amino acids and an intracellular C-terminal kinase catalytic domain. AtSERK1 has 7 putative N-glycosylation sites, 5 of which are located in the extracellular LRR domain (Fig. 1). This distribution is characteristic for RLKs and there is evidence that RLKs are indeed glycoproteins [31]. The AtSERK1 protein kinase catalytic domain has all of the 11 sub-domains as described for protein kinases [32] and is predicted to have a serine/threonine protein kinase activity based on conserved amino acids found in the sub-domains VIb and VIII.

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ggatttttattttattttttactcttcgtttatttttaatgctaatgggttttcaaaaggg      60
ttatcgaaaaatgagtgagtttgtgtctgaggttgtctctgcaaagtgttaatggtggtg     120
attttcggaagttagggtttttctcggaatctgaagagatcaaaatcaagatttggaaattac  180
cattgttgtttgaaATGGAGTCGAGTTATGTGGGTGTTTATCTTACCTTCACTGATCTTAC     240
              M  E  S  S  Y  V  V  F  I  L  L  S  L  I  L       16
TTGGGAATCATTCAGTGTGGCTTGCTTCTGCTAATTTGGAAGGTGATGCTTTGCATACTT     300
 G  N  H  S  L  W  L  A  S  A  N  L  E  G  D  A  L  H  T  L      36
TCAGYAGTTACTCTAGTTGATCCAAACAATGTCTTGCAGAGCTGGGATCCTAACGCTAGTGA   360
 L  R  V  T  L  V  D  P  N  N  V  L  Q  S  W  D  P  T  L  V      56
ATCCTTGCACATGGTTCCATGTCACTTGCAACAACGAGAACAGTGTCAATAAGACTTGATT   420
 N  P  C  T  W  F  H  V  T  C  N  N  E  N  S  V  I  R  V  D      76
TGGGGAATGCAGAGTTATCTGGCCATTPAGTTCCAGAGCTTGGTGTGCTGAAGAATTTGC     480
 L  G  N  E  L  S  G  H  L  V  P  E  L  G  V  L  K  N  L  Q      96
AGTATTTGGAGCTTTACAGTAACAACATAACTGGCCCGATTCCTAGTTAATCTTTGGAAATC   540
 Y  L  E  L  Y  S  N  N  I  I  G  P  I  P  S  N  L  G  N  L     116
TGACAAACTTAGTGAGTTTGGATCTTTACTTAAACAGCTTCTCCGGTCCTATTCCGGAAT    600
 T  N  L  V  S  L  D  L  Y  L  N  S  F  S  G  F  I  P  E  S     136
CATTGGGAAAGCTTTCAAAGCTGAGATTTCTCCGGCTTAACAACAACAGTCTCACTGGGT    660
 L  G  F  L  S  K  L  R  F  L  R  L  N  K  M  S  L  T  G  S     156
CAATTCCTATGTCACTGACCAATATTACTACCCTTCAAGTGTTAGATCTATCAAATAACA    720
 I  P  N  S  L  T  N  I  T  T  L  C  V  L  D  L  S  N  N  R     176
GACTCTCTGGTTCAGTTCCTGACAATGGCTCCTTCTCAGTCTTCACACCCCATCCAGTTTTG   780
 L  S  G  S  V  P  D  N  G  S  F  S  L  F  T  F  I  S  F  A     196
CTAATAACTTAGACCTATGTGGACCTGGTTACAAGTCACCCATGTCCTGGATCTCCCCGGT    840
 N  N  L  D  L  C  G  P  V  T  S  H  P  C  P  G  S  P  P  F     216
TTTCTCCTCCACCACCTTTTATTCAAACCTCCCCCAGTTTCCACCCGGAGTGGGTATGGTA    900
 S  P  P  P  F  I  Q  P  P  P  V  S  T  P  S  G  Y  G  I       236
TAACTGGAGCAATAGCTGGTGGAGTTGCTGCAGGTGCTGCTTTGCCTTTGCTGCTCCTG     960
 T  G  A  I  A  G  G  V  A  A  G  A  A  L  P  F  A  A  F  A     256
CAATAGCCTTTGCTTGGTGGCGACGAAGAAGCCTACTAGATATTTTCTTTGGATGTTCCTG    1020
 I  A  P  A  N  W  R  R  R  G  P  L  D  I  F  S  G  V  P  A     276
CCCCAACAAGATCCGAAGTTCATCTGGGACAGCTCAAGAAGTTTTTCTTTCCGGCAGCTAC   1080
 E  E  D  P  E  V  H  L  G  Q  L  K  R  F  S  L  R  E  L  Q     296
AAGTGGCGAGTGATGGGTTTAGTAACAAGAACATTTTGGGCAGAGGTGGGTTTGGGAAAG    1140
 V  A  S  D  G  F  S  N  K  H  I  L  G  R  G  G  P  G  K  V     316
TCTATAAGGGAGTGTTTGGCAGAAGGAACTCTTNTTAGCTGTCAAGAGACTGAAGGAAGAGC   1200
 Y  K  G  R  L  A  D  G  T  L  V  A  V  E  R  I  K  R  E  F     336
GAACTCCAGGTGGAGAACTCCAGTTTCAAAACAGAAGTAGAGATGGAAAGTATGGCAGGTC   1260
 T  P  G  G  E  L  Q  F  Q  T  E  V  E  M  I  S  N  A  V  H     356
ATCGAAACCTGTTGAGATTACGAGGTTTCTGTATGACACCGACCGAGGAGATTGCTTTTGT   1320
 P  M  L  L  F  L  R  G  F  C  N  T  P  T  E  R  L  L  V  Y     376
ATCCTTACATGGCCAATGGCAAGTGTTGGCTTCGTGTCTCAGAAGAGAGGCCACCGGTCACAAC   1380
 P  Y  M  A  N  G  S  V  A  S  C  L  R  E  R  F  P  S  Q  P     396
CTCCGCTTGATTGCCCAACGGGCAAGAGAATCGGGCTAGGCTCACCTCGAGGTTTGTCTT   1440
 P  L  D  W  P  T  R  K  R  I  A  L  G  S  A  R  G  L  S  Y     416
ACCTACATGATCACTGTGATCCGAAGATCATTCACCGTGACCTAAAAGCAGCAAACATCC   1500
 L  H  D  H  C  D  P  K  I  T  H  R  D  V  K  A  A  N  I  L     436
TCTTTAGACGAAGAATTCTGAAGTGGTTGTTGGATATTTCCGGGTTGGGCAAAGTTTATGTAGT   1560
 L  D  S  E  F  E  A  K  V  V  G  D  G  G  L  A  K  L  M  D  Y   455
ATAAAGACACTCACGTGACCACAGCAGTCCGTGGCACCATCGGTCACATCGCTCCAGAAT   1620
 K  D  T  H  V  T  T  A  V  R  G  T  I  G  H  I  A  P  E  Y     476
ATCTCTCAACCGGAAAATCTTCAGAGAAAACCGACGTTTTCGGATACGGAATCATGCTTC   1680
 L  S  T  G  N  S  S  E  K  T  D  Y  F  G  Y  G  I  M  L  L     496
TAGAAACTAATTCACAGGACAAAGAGCTTTGGATCCCGCTGGGCTAGCTAACGACGACGACG   1740
 E  L  I  T  G  Q  P  A  F  G  L  A  R  L  A  N  D  D  D  Y     516
TCATGTTACTTGACTGGGTGAAAGGATTGTTGAAGGAGAAGAAGCTAGAGATGTTAGTGG   1800
 M  L  L  D  W  V  K  G  L  L  K  E  K  K  L  E  M  L  V  D     536
ATCCAGATCTTCAAACAAACTACGAGGAGAGGAAACTGGAACAGCTGATACAAGTGGCGT   1860
 P  D  L  Q  T  N  Y  E  E  R  E  L  E  Q  V  I  Q  V  A  L     556
TGCTAGCCACGTCATCTATCACCAATGCAAAGCATGAAAGTGTTGTAAGGATGTC         1920
 L  C  T  G  G  S  P  M  S  R  P  K  H  S  E  V  V  R  M  L     576
TGGGAAGGCAGATGGGCTTGGCGGAGAAATGGGACGAATGGCAAAAAATTCAGAATTTTGAGGGG  1980
 R  G  G  L  A  E  K  H  D  E  N  Q  K  V  E  I  L  P  E       596
AAGAGATTGATTTGAGCCCTAATCCTAACTCTGATTGGATTCTTGATTCTACTTACAATT    2040
 E  I  D  L  S  P  N  P  N  S  D  N  I  L  Q  S  T  Y  N  L     616
TGCAGGCCGTTGAGTTACTTGGTCCAAGGTAAaaaaaaaaaaaaaaaaaa              2094
 P  A  V  E  L  L  G  P  R  *                                    625
```

Figure 1. Nucleotide sequence and predicted amino acid sequence of AtSERK1. The dark gray shaded amino acid sequences represent the N terminal signal peptide and the membrane spanning region. The light gray shaded region (amino acid 30-74) represents the leucine zipper domain, followed by 5 LRRs (74-202) in the extracellular domain. The potential N-linked glycosylation sites are underlined and the lysine and threonine residues in the kinase domain that were mutated in this study are shown in bold. The 15 invariant amino acids found in almost all eukaryotic protein kinases are boxed.

Fusion proteins were constructed in which Glutathione S-transferase (GST) was fused in frame to the N-terminal end of the AtSERK1 kinase domain. After expression in *E. coli* BI21, GST-fusion proteins were affinity-purified on glutathione sepharose. In a standard assay with 10 mM divalent cations, the autophosphorylation was complete after 25-35 min (Fig. 2A), and therefore, 30 min incubations were used routinely. There was no autophosphorylation when $MgCl_2$ was left out of the reaction mixture. The addition of $MnCl_2$ considerably reduced the activity while addition of $CaCl_2$ had no influence on the autophosphorylation activity (Fig. 2B). Optimal autophosphorylation activity was achieved at 10 mM $MgCl_2$ (data not shown). The AtSERK1^{kin} protein exhibited standard Michaelis-Menten kinetics with respect to ATP, with a K_m of 4×10^{-6} M and a V_{max} of $4.6 \times 10^{-9} mg^{-1} min^{-1}$ (data not shown).
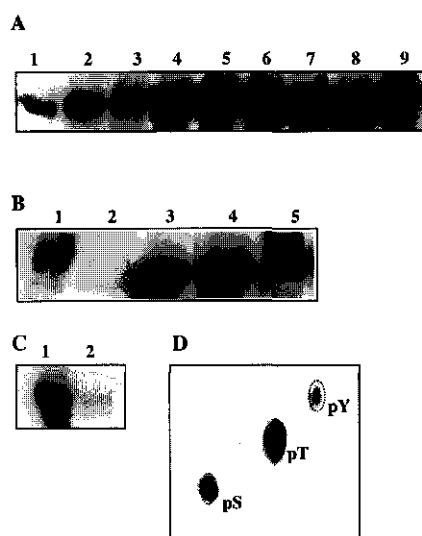
Figure 2. Autophosphorylation characteristics of AtSERK1. Bacterially produced AtSERK1^{kin} protein was purified and aliquots of 500 ng were incubated with [γ-^{32}P] as described in methods. After separation on 10% SDS-PAGE, the gels were autoradiographed using a PhosphoImager. A: Time course of autophosphorylation activity. AtSERK1 protein was incubated for 5 min (lane 1), 10 min (lane 2), 15 min (lane 3), 25 min (lane 4), 35 min (lane 5), 45 min (lane 6), 60 min (lane 7), 75 min (lane 8) and 90 min (lane 9). B: Divalent cation requirement of autophosphorylation activity. AtSERK1^{kin} protein was incubated with 10 mM of divalent cations, $MnCl_2$ (lane 1), $CaCl_2$ (lane 2), $MgCl_2$ (lane 3), $MgCl_2$ and $CaCl_2$ (lane 4) and $MgCl_2$ and $MnCl_2$ (lane 5). C: Autophosphorylation of the AtSERK1^{kin} (lane 1) and AtSERK^{K330E} (lane 2) proteins. D: Phosphoaminoacid analysis of the HCl-hydrolyzed, autophosphorylated

AtSERK1^{kin}. The hydrolyzed protein was spotted onto TLC plate and resolved by chromatography in two dimensions. The autoradiograph of the phosphorylated amino acids after hydrolysis of AtSERK1^{kin} and the position of the standard amino acids (dotted circles), visualized by spraying with 0.25% ninhydrin, phosphoserine (pS), phosphothreonine (pT) and phosphotyrosine (pY) respectively are shown.

To further confirm the specific kinase activity of the AtSERK1^{kin} protein, the AtSERK1^{K330E} mutant protein in which the essential lysine required for the phosphotransfer was replaced by glutamic acid and was analyzed for kinase activity. Fig. 2C shows that the AtSERK1^{K330E} mutant protein no longer autophosphorylates, confirming that AtSERK1 is a functional receptor kinase. To determine which amino acids are autophosphorylated, the ^{32}P- labeled proteins were subjected to total hydrolysis. The subsequent analysis of the radioactive amino acids showed that the autophosphorylation was almost exclusively on threonines (80%) and partly on serines (20%). Radiolabelled tyrosine was not detected (Fig. 2D).

To identify the precise residues on which AtSERK1 autophosphorylates, we compared the predicted three-dimensional models of the AtSERK1 kinase domain with the well-characterized structure of the kinase domain of human insulin receptor. The Z scores of the AtSERK1 models as determined by Prosa II were –7.1 and –7.0. These values imply that both AtSERK1 models according to the quality assessment criteria developed by Sanchez et al [25] are very reliable (pG=0.99). The Procheck evaluation of the models showed that 98.9% of residues are in the Phi/Psi most favored/additionally allowed regions of the Ramachandran plot. In addition the side chain parameters Chi-1 and Chi-2 determined by Procheck are better than a protein X-ray crystal structure model with 2.0 A° resolution. Comparing the X-ray structures of the active (IRK3P) and the inactive human insulin receptor kinase (IRK) has revealed that major structural changes occur upon phosphorylation [22, 23]. In the inactive IRK structure, the activation A-loop (residues 1149-1170) traverses the cleft between the N- and C- terminal lobes so that the protein substrate-binding site is largely blocked. Autophosphorylation of Tyr-1158, Tyr-1162 and Tyr-1163 results in a dramatic change in the confirmation of the IRK A-loop. One of the tyrosines, Tyr-1158 is displaced ~30 A° from its position in the unphosphorylated A-loop. The conformational change of the phosphorylated A-loop permits unrestricted access to the binding sites for ATP and protein substrates [23]. While the homology between the predicted the AtSERK1 kinase sequence and the IRK is no more than 26%, a comparison of the model of the AtSERK1 kinase domain and the inactive insulin receptor kinase (IIRK) shows that they superimpose very well except for an extra

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loop region in the AtSERK1 kinase (amino acids 498-515). Since it is known that the activation of the insulin receptor kinase occurs by autophosphorylation on residues present within the activation loop of sub-domain VII and VIII [33, 34], we designated the same region in the AtSERK1 kinase domain as the AtSERK1 A-loop (Fig. 3A and Fig. 3B). The AtSERK1 A-loop begins with an invariant asparagine in sub-domain VII (Asp-447 in AtSERK1) and terminates with an invariant glutamic acid in sub-domain VIII (Glu-475 in ASERK1) [35]. The AtSERK1 A-loop contains 4 threonine residues, Thr-459, Thr-462, Thr-463, Thr-468 and the tyrosine, Tyr-456 that are potential phosphorylation sites. In the AtSERK1 kinase model, the major region of the AtSERK1 A-loop is quite identical to the A-loop of the inactive insulin receptor kinase.

A


```
      447                      475
      |                        |
AtS  DFGLAKLMDYKDTHVTTAVRGTIGH-IAPE
IRK  DFGHTRDIYETDYYRKGGKGLLPVRNMAPE
     |                         |
     1150                      1179
```
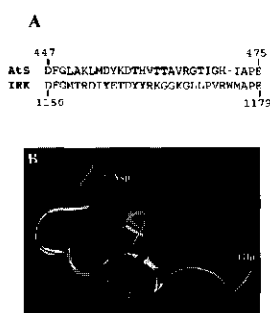

Figure 3. Sequence alignment and ribbon diagrams of the IRK and AtSERK1^{kin} activation loop **A:** Sequence alignment of the IRK activation loop (aa 1150-1179) and the predicted AtSERK1 (AtS) A-loop (aa 447-475). Both AtS and the IRK activation loop begin with Asp (D) and terminates with Glu (E). The position of the threonines, Thr-459, Thr-462, Thr-463, Thr-468 in AtS and the tyrosine, Tyr-1159, Tyr-1162, Tyr-1165 in IRK are shown in bold. The AtS A-loop spans aa 447-460 of sub-domain VII and aa 461-475 of sub-domain VIII. **B:** Superposition of the activation loops of IRK structure (cyan) and AtSERK^{kin} structure (orange) indicating the close proximity of the tyrosines (cyan) of IRK and the threonines (orange) of AtSERK1^{kin}. The beginning and the end of the loop are marked by Asp and Glu respectively.

Based on the prediction of the presence of the threonines in the AtSERK1 A-loop and the predominantly serine/threonine autophosphorylation properties (Fig. 2D), we performed site directed mutagenesis on the three threonines that might be involved in the autophosphorylation of AtSERK1. This led us to making a mutant construct, AtSERK1$^{3T \to E}$, in which Thr-462, Thr-463 and Thr-468 in AtSERK1 A-loop were changed to glutamic acid. Assays with bacterially expressed affinity purified AtSERK1$^{3T \to E}$ mutant protein showed an almost complete absence of phosphorylated residues (lane 2, Fig. 4A). Only a long exposure of the film revealed a radioactive

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band but it was too weak to attempt to identify the phosphorylated amino acids. It may represent the weak phosphorylation of serine that was observed after total hydrolysis of AtSERK1^{kin} (Fig. 2D).

To investigate the role of the individual threonine residues, Thr-462, Thr-463 and Thr-468 in AtSERK1 autophosphorylation, all the three threonine residues were mutagenized separately to glutamic acid or to alanine. To ensure that the fourth threonine residue, Thr-459 in the AtSERK1 A-loop did not have any influence on the phosphorylation status of AtSERK1 kinase, we also mutagenized, Thr-459 either to glutamic acid or to alanine. The threonine mutations either to glutamic acid or to alanine residues were performed to ensure that the substitution of threonines did not influence the protein structure and thus the kinase activity. The assays with purified proteins showed that there was a reduced phosphorylation in the AtSERK1^{T462E} mutant (lane 4, Fig. 4A) and an almost complete absence of phosphorylation in the AtSERK1^{T468E} mutant protein (lane 6, Fig. 4A). The reduced phosphorylation was also observed in AtSERK1^{T462A} (lane 4, Fig. 4B) while the AtSERK1^{T468A} mutant protein was completely devoid of phosphorylated amino acids (lane 6, Fig. 4B). The effect on phosphorylation activity with both the types of substitutions was similar which reveals that the resulting kinase activity is due to the change of threonine residues and not due to the newly introduced amino acid residues.
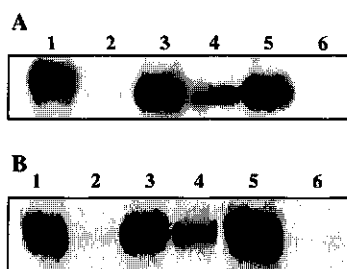
Figure 4. Autophosphorylation properties of AtSERK1^{kin} and AtSERK1^{kin} mutants. Bacterially produced AtSERK1^{kin} proteins were purified and aliquots of 500 ng incubated with [γ-^{32}P] as described in methods. After separation on a 10% SDS-PAGE the resulting gels were autoradiographed using a PhosphoImager. A: Autoradiographs of AtSERK1^{kin} (lane 1), AtSERK1$^{3T→E}$ (lane2), AtSERK1^{T459E} (lane 3), AtSERK1^{T462E} (lane 4), AtSERK1^{T463E} (lane 5) and AtSERK1^{T468E} (lane 6) proteins. B: Autoradiographs of AtSERK1^{kin} (lane 1), AtSERK1$^{3T→E}$ (lane2), AtSERK1^{T459A} (lane 3), AtSERK1^{T462A} (lane 4), AtSERK1^{T463A} (lane 5) and AtSERK1^{T468A} (lane 6) proteins.

The ability of AtSERK1 proteins to transphosphorylate was tested with common substrates as casein and MBP (Fig. 5). Both proteins were clearly transphosphorylated by AtSERK1^{kin}. The phosphorylation of casein (Fig. 5A) and MBP (Fig. 5C) was considerably reduced when they were incubated with mutant AtSERK1$^{3T \rightarrow E}$ or AtSERK1^{K330E} proteins. To further determine if the individual threonines influenced the phosphorylation of casein or MBP, we incubated AtSERK1^{T459A}, AtSERK1^{T462A}, AtSERK1^{T463A} and AtSERK1^{T468A} mutant proteins with casein (Fig. 5B) and MBP (Fig. 5D). Both casein and MBP were not phosphorylated by AtSERK1^{T462A} and AtSERK1^{T468A} (lanes 2 and 4 in Fig. 5B and 5D). This suggests that the phosphorylation status of Thr-468 and is essential for AtSERK1 transphosphorylation activity .
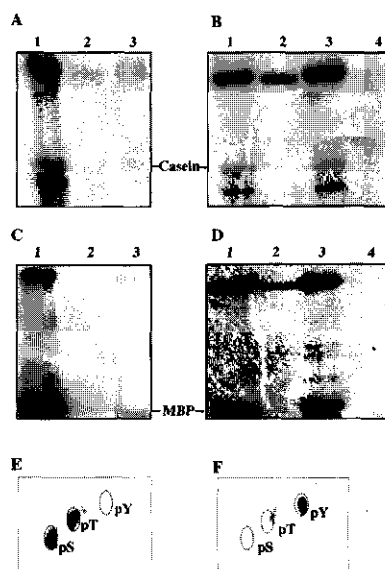
Figure 5. Transphosphorylation properties of AtSERK1^{kin} and AtSERK1^{kin} mutants. Aliquots of 500 ng of purified AtSERK1^{kin} proteins were incubated with 1 μg of casein (A, B) or MBP (C, D) in the presence of [γ-^{32}P] as described in Materials and Methods. After separation on a 10% SDS-PAGE the resulting gels were autoradiographed using a PhosphoImager. A: Autoradiographs of casein incubated with AtSERK1^{kin} (lane 1), AtSERK1^{K330E} (lane 2) and AtSERK1$^{3T \rightarrow E}$ (lane3) proteins. B: Autoradiographs of casein incubated with AtSERK1^{T459A} (lane 1), AtSERK1^{T462A} (lane 2), AtSERK1^{T463A} (lane 3) and AtSERK1^{T468A} (lane 4) proteins. C: Autoradiographs of MBP incubated with AtSERK1^{kin} (lane 1), AtSERK1^{K330E} (lane 2) and AtSERK1$^{3T \rightarrow E}$ (lane 3) proteins. D:

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Autoradiographs of MBP incubated with AtSERK1^{T459E} (lane 1), AtSERK1^{T462E} (lane 2), AtSERK1^{T463E} (lane 3) and AtSERK1^{T468E} (lane 4) proteins. E and F: Phosphoaminoacid analysis of HCl hydrolyzed, phosphorylated casein and MBP. The autoradiograph of the phosphorylated amino acids after hydrolysis of casein (E) and MBP (F) and the position of the standard amino acids (dotted circles), phosphoserine (pS), phosphothreonine (pT) and phosphotyrosine (pY) respectively are shown.

To determine the identity of the phosphorylated amino acids in casein and MBP, the ^{32}P-labeled proteins were subjected to total hydrolysis. The subsequent analysis of the radioactive amino acids showed that the transphosphorylation of casein was mostly on serine and threonine (Fig. 5E) whereas MBP was transphosphorylated on tyrosine residues (Fig. 5F).

These results identified threonine residues, Thr-462 and mainly Thr-468, in the AtSERK1 kinase domain that are important for autophosphorylation. In addition the phosphorylation status of Thr-468 seems to be important for transphosphorylation. Furthermore, AtSERK1 transphosphorylation on MBP and casein shows tyrosine, serine and threonine as targets, suggesting that AtSERK1 is a dual specificity kinase.

The AtSERK1 reaction showed second order kinetics (Fig. 6A) which suggests that AtSERK1 autophosphorylates by intermolecular phosphorylation [19]. To provide additional evidence for intermolecular phosphorylation of AtSERK1, we tested whether AtSERK1^{kin} was able to phosphorylate AtSERK1^{K330E} proteins. AtSERK1^{K330E} protein was phosphorylated only in the presence of AtSERK1^{kin} (lane 2, Fig. 6B). These results clearly demonstrate the intermolecular character of the AtSERK1 reaction. We also incubated the mix of AtSERK1$^{3T\rightarrow E}$ and AtSERK1^{K330E} proteins together with or without casein or MBP in a phosphorylation assay. The results show that AtSERK1$^{3T\rightarrow E}$ protein is not able to phosphorylate the AtSERK1^{K330E} protein (lane 1, Fig. 6C) regardless of the presence of a potentially active catalytic site in the AtSERK1$^{3T\rightarrow E}$ protein. Furthermore, this mutant protein mix is not able to transphosphorylate casein or MBP (lane 2 and lane 3, Fig. 6C).
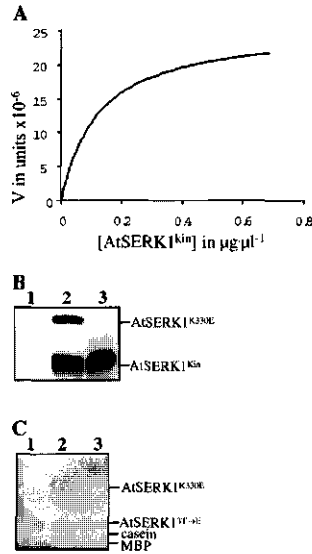
Figure 6. A: Plot of AtSERK1 phosphorylation versus AtSERK1^{kin} protein concentration **B**: Phosphorylation assay of thrombin cleaved AtSERK1^{kin} protein and AtSERK1^{K330E}. 500 ng of either protein were incubated alone or together in the presence of [γ-^{32}P] as described in Materials and Methods. After separation on a 10% SDS-PAGE the resulting gels were autoradiographed using a Phospholmager. Autoradiographs of AtSERK1^{K330E} (lane 1), AtSERK1^{K330E} and AtSERK1^{kin} proteins (lane 2) and AtSERK1^{kin} (lane 3). **C**: Autoradiographs of phosphorylation assays of a mix of AtSERK1^{K330E} and thrombin cleaved AtSERK1$^{3T→E}$ (lane 1) incubated with casein (lane 2) or MBP (lane 3). The phosphorylation assays performed as described above.

These results suggest that under *in vitro* conditions, phosphorylation of Thr-468 and perhaps also of Thr-462 requires the presence of another AtSERK1 oligomer and that the catalytic activity of AtSERK1 in turn depends on the presence of a phosphorylated threonine residue in the AtSERK1 A-loop. We suggest that the AtSERK1 A-loop phosphorylation is required both for auto-activity and trans-activity. The general validity of this model for plant receptor like kinases is supported by the observation that in a wide range of receptors including the AtSERK family [30] a threonine at position 7 amino acids from the end of the A-loop is conserved (Fig. 7).

```
                Sub-domain VII     Sub-domain VIII

           447              459 462 463  468      475
            |                |    | |    |         |
AtSERK1     DFGLAKLMDYKDTHV-TTAVRGTIGHIAPE

AtSERK2     DFGLARLMDYKDTHV-TTAVRGTIGHIAPE
AtSERK3     DFGLAKLMDYKDTHV-TTAVRGTIGHIAPE
AtSERK4     DFGLAKLMNYNDSHV-TTAVRGTIGHIAPE
AtSERK5     DFGLAKLMNYNDSHV-TTAVRGTIGHIAPE
BRI1        DFGMARLMSAMDTHLSVSTLAGTPGYVPPE
OsPK10      DFGLAKLMTREQSHVFTTL-RGTRGYLAPE
DcSERK      DFGLARLMDYKDTHV-TTAVRGTLGYIAPE
ZmPk1       DFGLVKLLNRGGSTQNVSHVRGTLGYIAPE
ARK1        DFGMARIFGREETEANTRRVVGTYGYMSPE
TMK1        DFGLVRLAPEGKGSI ETRIAGTFGYLAPE
RLK4        DFGLAKLLGRDSFR-VLATMRGTWGYVAPE
```

Figure 7. Sequence alignment of AtSERK1 A-loop compared with the corresponding region of the sequence of AtSERK1 homologs and closely related plant RLKs. The AtSERK1 A-loop spans aa 447-460 of sub-domain VII and aa 461-475 of sub-domain VIII. The conserved threonine in most plant serine/threonine type of RLKs is shown in bold letters.

Discussion

The *Arabidopsis AtSERK1* gene is a member of the diverse family of serine/threonine receptor kinases that have been identified in plants. In this study we have shown that the AtSERK1 catalytic domain indeed exhibits phosphorylation activity on serine and threonine. A single amino acid substitution of Lys-330 to Glu-330, an invariant lysine found in the catalytic site of all protein kinases abolishes the AtSERK1 kinase activity completely. We have also identified Thr-462 and Thr-468 as the prime intermolecular targets of AtSERK1 kinase activity. Finally we have shown that AtSERK1 is capable of transphosphorylating artificial substrates such as casein and MBP on both serine/threonine as well as tyrosine respectively. This classifies AtSERK1 as a dual specificity-type receptor kinase with respect to its transphosphorylation activity and as a serine/threonine kinase with respect to its autophosphorylation activity.

Thus AtSERK1[kin] resembles CrRLK1 [20], RLK4 [36], KIK1 [37], TMK1 [38] and OsTMK [39] plant RLKs which have a preference for threonine autophosphorylation. To our knowledge, only two previous studies have identified specific target site for autophosphorylation in plant RLKs. Using formic acid digests and site-directed mutagenesis, it was shown that Thr-720 was required for phosphorylation of CrRLK1 [20]. Employing MALDI mass spectrometry on the BRI1-kinase domain, the presence of five phosphorylation sites was shown, one each in the sub-domains I and

VIa and three in sub-domain VIII [40]. Employing computer assisted molecular modelling in combination with the functional studies of various kinase domains of transmembrane receptors has given an insight into the conformational changes that take place once the receptors are activated [41]. In our studies we used the X-ray crystal structure of the unphosphorylated [22] and phosphorylated [23] insulin receptor kinase (IRK) as templates for the modelling experiments. The computer modelled structure of the AtSERK1^{kin} compared to the IRK structure showed a striking similarity except for the region that is present on sub-domain IX of AtSERK1. This similarity is remarkable given the very low homology at protein sequence level and the fact that the IRK protein has tyrosine kinase activity. Apparently structural conservation amongst kinases is much stronger than sequence conservation or biochemical properties. This notion is further strengthened when the A-loop in IRK that is formed from sub-domains VII and VIII of the protein kinase catalytic core [34] is examined in more detail. In IRK, this loop contains three tyrosine residues that can be phosphorylated and that are of importance for receptor kinase signalling activity [23]. In the AtSERK1 kinase model the same kind of A-loop in the AtSERK1 kinase domain is observed. The major difference is the presence of threonines in the AtSERK1 A-loop instead of tyrosines in the IRK A-loop. Based on the prediction that the threonines in AtSERK1 might fulfill the same role as tyrosines in IRK, mutant proteins were constructed in which Thr-459, Thr-462, Thr-463 and Thr-468 were mutated individually to either alanine or to glutamic acid residues. After kinase activity measurements, it appeared that Thr-468 is the most likely single target for intermolecular AtSERK1 kinase activity. Confirmation of the role of Thr-468 in vivo will require mass spectrometry of phosphorylated peptide fragments of AtSERK1 and site-specific antibodies directed against the A-loop region, experiments that are currently in progress.

Nevertheless, it is of interest to note that all three phosphorylation sites in sub-domain VIII of BRI1 [40] lie within the predicted A-loop region given that the activation of many protein kinases occurs by autophosphorylation of one to three residues within the activation loop of sub-domain VIII [35]. Like animal receptor kinases, the sub-domains representing the AtSERK1 A-loop may also be important for the phosphorylation of plant RLKs in general. Not only do many RLKs have serine or threonine residues, some of which were shown to be phosphorylated *in vitro*, in the corresponding A-loop regions (Fig. 7), also genetic analysis points to the functional importance of this region. Numerous mutants affecting brassinosteroid signalling fall within the

proposed A-loop region [12], including bri1-104 and bri1-115 [17]. The importance of A-loop phosphorylation is supported by our finding that upon mutation of Thr-468 (conserved in most of the plant RLKs, Fig. 7), all AtSERK1 phosphorylation activity is lost.

In addition to the intermolecular autophosphorylation reaction, we were also interested in AtSERK1 phosphorylation on exogenous substrates. AtSERK1 can phosphorylate both serine/threonine and tyrosine containing substrates, which widens the spectrum of downstream proteins it might interact with. So far tyrosine kinase activity has not been widely encountered in plants, although the identification of the rolB protein as a tyrosine phosphatase [42] suggests that it does occur. AtSERK1 transphosphorylation activity is abolished when the threonine mutants are employed. It is known that the phosphorylation of the A-loop shifts the equilibrium towards a conformation that accommodates protein substrate binding. Point mutations in the A-loop of various receptor kinases are presumed to alter this equilibrium [41] and thus influence protein substrate binding and subsequent transphosphorylation. Indeed in the AtSERK1 threonine mutant proteins transphosphorylation activity was abolished suggesting that the AtSERK1 A-loop may be involved in protein substrate binding.

Autophosphorylation activity and activation of the transmembrane receptor protein kinases is generally mediated by ligand-induced dimerization [43]. The activation of autophosphorylation of the catalytic domain of the receptor is mediated by an intermolecular mechanism of phosphorylation. Dimerization and intermolecular autophosphorylation of a nuclear serine/threonine kinase from *A. thaliana* has been shown [44]. In our studies we also examined the mechanism of the autophosphorylation reaction of AtSERK1. The observation that the AtSERK1^{kin} can transphosphorylate the AtSERK1^{K330E} protein suggests that the AtSERK1 protein catalyzes an intermolecular autophosphorylation *in vitro*.

Based on the experiments reported here we propose that, either by the presence of a minor population of catalytically active AtSERK1 molecules *in vitro*, or by ligand induced conformational changes *in vivo*, AtSERK1 activation requires intermolecular autophosphorylation. This results in movement of the AtSERK1 threonine containing A-loop followed by improved accessibility of the catalytic domain to commence transphosphorylation activity and protein substrate binding. In this model, A-loop phosphorylation of Thr-468 of one AtSERK1 monomer is also essential for intermolecular autophosphorylation activity of that monomer.

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Acknowledgements

References

1. Posada, J., Yew, N., Ahn, N.G., Vande Woude, G.F. and Cooper, J.A. (1993) Mos stimulates MAP kinase in Xenopus oocytes and activates a MAP kinase kinase in vitro. *Mol. Cell Biol.* **13**, 2546-2553.

2 Ullrich, A. and Schlessinger, J., (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203-212.

3 Lin, H.Y., Wang, X.F., Ng-Eaton, E., Weinberg, R.A. and Lodish, H.F. (1992) Expression cloning of the TGF-beta type II receptor, a functional transmembrane serine/threonine kinase. *Cell* **68**, 775-785.

4 Douville, E.M., Afar, D.E., Howell, B.W., Letwin, K., Tannock, L., Ben-David, Y., Pawson, T. and Bell, J.C. (1992) Multiple cDNAs encoding the esk kinase predict transmembrane and intracellular enzyme isoforms. *Mol. Cell Biol.* **12**, 2681-2689.

5 Raz, V. (1993) Ethylene signal is transduced via protein phosphorylation events in plants. *Plant Cell* **5**, 523-530

6 Reymond, P., Short, T.W., Briggs, W.R. and Poff, K.L. (1992) Light induced phosphorylation of a membrane protein plays an early role in signal transduction for phototropism in *Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA* **89**, 4718-4721

7 Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A. and Ecker, J.R., (1993) CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the raf family of protein kinases. *Cell* **12**, 427-441

8 Duerr, B., Gawienowski, M., Ropp, T. and Jacobs, T. M. (1993) ERK1: a mitogen-activated protein kinase from a flowering plant. *Plant Cell* **5**, 87-96.

9 Nassrallah, J.B. and Nassrallah, M.E., (1993). Pollen-stigma signalling in the sporophytic self-incompatibility response. *Plant Cell* **5**, 1325-1335.

10 Clarke, S.E., Williams, R.W. and Meyerowitz, E.M. (1997) The *CLAVATA1* gene encodes a putative receptor kinase that controls shoot and floral meristem size in *Arabidopsis. Cell* **89**, 575-585.

11 Song, W., Wang, G., Chen, L., Kim, H., Pi L., Holsten, T., Gardner, J., Wang, B., Zhai, W., Zhu, L., Fauquet, C. and Ronald, P., (1995) A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21. *Science* **270**, 1804-1806

12 Lease, K., Ingham, E. and Walker, J.C., (1998) Challenges in understanding RLK function.*Curr. Opin. Plant Biol.* **1**, 388-392

13 Torii, K.U. (2000) Receptor kinase activation and signal transduction in plants: an emerging picture. *Curr. Opin. Plant Biol.* 3, 361-367.

14 Kobe, B. and Deisenhofer, J. (1995) A structural basis of the interactions between leucine-rich repeats and protein ligands. *Nature* 374, 183-186.

15 Jin, P., Guo, T. and Becraft, P.W. (2000) The maize CR4 receptor-like kinase mediates a growth factor-like differentiation response. *Genesis* 27, 104-116.

16 Torii, K.U., Mitsukawa, N., Oosumi, T., Matsuura, Y., Yokoyama, R., Whittier, R.F. and Komeda, Y., (1996) The Arabidopsis ERECTA gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. *Plant Cell* 8, 735-746

17 Li, J. and Chory, J. (1997) A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. *Cell* 90, 929-938.

18 Mu, J.H., Lee H.S. and Kao. T.H., (1994) Characterization of a pollen-expressed receptor-like kinase gene of *Petunia inflata* and the activity of its encoded kinase. *Plant Cell* 6, 709-721.

19 Horn, M.A, and Walker, J.C. (1994) Biochemical properties of the autophosphorylation of RLK5, a receptor-like protein kinase from Arabidopsis thaliana. *Biochim. Biophys. Acta.* 1208, 65-74.

20 Schulze-Muth, P., Irmler, S., Schroder, G. and Schroder, J. (1996) Novel type of receptor-like protein kinase from a higher plant (Catharanthus roseus). cDNA, gene, intramolecular autophosphorylation, and identification of a threonine important for auto- and substrate phosphorylation. *J. Biol. Chem.* 271, 26684-26689.

21 Hecht, V., Vielle-Calzada, J.P., Recklinghausen, I., Hartog, M.V., Zwartjes, C.G.M., Schmidt E. D. L., Boutilier, K., van Lammeren, A., and de Vries, S. C. (2000) The *Arabidopsis somatic embryogenesis receptor kinase 1* gene during ovule and embryo development (submitted)

22 Hubbard, S.R., Wei, L., Ellis, L. and Hendrickson, W.A. (1994) Crystal structure of the tyrosine kinase domain of the human insulin receptor. *Nature* 372,746-754.

23 Hubbard, S.R. (1997) Crystal structure of the activated insulin receptor tyrosine kinase in complex with peptide substrate and ATP analog. *EMBO J.* 16, 5572-5581.

24 Sali, A. and Blundell, T.L. (1993) Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.* 234, 779-815.

25 Sanchez, R., Sali, A. (1998) Large-scale protein structure modelling of the Saccharomyces cerevisiae genome. *Proc. Natl. Acad. Sci.* U.S.A. 95, 13597-13602.

26 Francoijs CJ, Klomp JP, Knegtel RM2000 Sequence annotation of nuclear receptor ligand-binding domains by automated homology modelling. *Prot. Eng.* 13, 391-4.

27 Sippl, M.J. (1993) Recognition of errors in three-dimensional structures of proteins. *Proteins* 17, 355-362.

28 Boyle, W.J., van der Geer, P. and Hunter, T. (1991) Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol.* 201, 110-149.

29 Braun, D.M., and Walker, J.C. (1996) Plant transmembrane receptors:new pieces in the signalling puzzle. *Trends Biochem. Sci.* 21, 70-73

30 Baudino, S., Hansen, S., Brettschneider, R., Hecht, V.F.G., Dresselhaus, T., Lörz, H., Dumas, C. and Rogowsky, P.M. (2000) Molecular characterization of two novel maize LRR receptor-like kinases, which belong to the SERK gene family. *Planta* (in press)

31 Schaller, G.E. and Bleecker, A.B. (1993) Receptor-like kinase activity in membranes of *Arabidopsis thaliana*. *FEBS Lett.* **333**, 306-310

32 Hanks, S.K., Quinn, A.M. and Hunter, T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241**, 42-52.

33 Rosen, O.M., Herrera, R., Olowe, Y., Petruzzelli, L.M. and Cobb, M.H. (1983) Phosphorylation activates the insulin receptor tyrosine protein kinase. *Proc. Natl. Acad. Sci.* U.S. A. **80**, 3237-3240.

34 Ellis, L., Clauser, E., Morgan, D.O., Edery, M., Roth, R.A. and Rutter, W.J. (1986) Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. *Cell* **45**, 721-732.

35 Johnson, L.N., Noble, M.E., and Owen, D.J. (1996) Active and inactive protein kinases: structural basis for regulation. *Cell* **85**, 149-158.

36 Coello, P., Sassen, A., Haywood, V., Davis, K. and Walker, J. (1999) Biochemical characterisation and expression of RLK4, a receptor-like kinase from *Arabidopsis thaliana. Plant Sci.* **142**, 83-91

37 Braun, D.M., Stone, J.M. and Walker, J.C. (1997) Interaction of the maize and Arabidopsis kinase interaction domains with a subset of receptor-like protein kinases: implications for transmembrane signalling in plants. *Plant J.* **12**, 83-95

38 Chang, C., Schaller, G.E., Patterson, S.E., Kwok, S.F., Meyerowitz, E.M. and Bleecker, A.B. (1992) The TMK1 gene from Arabidopsis codes for a protein with structural and biochemical characteristics of a receptor protein kinase. *Plant Cell* **4**, 1263-1271.

39 van der Knaap, E., Song, W.Y., Ruan, D.L., Sauter, M., Ronald, P.C. and Kende, H. (1999) Expression of a gibberellin-induced leucine-rich repeat receptor-like protein kinase in deepwater rice and its interaction with kinase-associated protein phosphatase. *Plant Physiol.* **120**, 559-570.

40 Oh, M.H., Ray, W.K., Huber, S.C., Asara, J.M., Gage, D.A. and Clouse SD (2000) Recombinant brassinosteroid insensitive 1 receptor-like kinase autophosphorylates on serine and threonine residues andphosphorylates a conserved peptide motif in vitro. *Plant Physiol.* **124**, 751-766.

41 Hubbard, S.R., Mohammadi, M. and Schlessinger, J. (1998) Autoregulatory mechanisms in protein tyrosine kinases. *J. Biol. Chem.* **273**, 11987-11990

42 Filippini, F., Rossi, V., Marin, O., Trovato, M., Costantino, P., Downey, P.M., Lo Schiavo F. and Terzi, M.A. (1996) A plant oncogene as a phosphatase. *Nature* **379**, 499-500.

43 Heldin, C.H. (1995) Dimerization of cell surface receptors in signal transduction. *Cell* **80**, 213-223.

44 Roe, J.L, Durfee, T., Zupan, J.R., Repetti, P.P., McLean, B.G. and Zambryski, P.C. (1997) TOUSLED is a nuclear serine/threonine protein kinase that requires a coiled-coil region for oligomerization and catalytic activity. *J. Biol. Chem.* **272**, 5838-5845.

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Subcellular localization and oligomerization of the *Arabidopsis thaliana* Somatic Embryogenesis Receptor Kinase 1 protein

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Abstract

The *Arabidopsis thaliana Somatic Embryogenesis Receptor Kinase* 1 *(AtSERK1)* gene is expressed in developing ovules and early embryos. *AtSERK1* is also transiently expressed during somatic embryogenesis. The predicted AtSERK1 protein contains an extracellular domain with a leucine zipper (LZ) motif followed by five leucine-rich repeats (LRRs), a proline-rich region, a single transmembrane region and an intracellular kinase domain. The *AtSERK1* cDNA was fused to two different variants of green fluorescent protein (GFP), a yellow-emitting GFP (YFP) and a cyan-emitting GFP (CFP), and transiently expressed in both plant protoplasts and insect cells. Using confocal laser scanning microscopy (CSLM) it was determined that the AtSERK1-YFP fusion protein is targeted to plasma membranes   both plant and animal cells. The extracellular LRRs, and in particular the N-linked oligosaccharides that are present on them appear to be essential for correct localization of the AtSERK1-YFP protein. The potential for dimerization of the AtSERK1 protein was investigated by measuring the YFP/CFP fluorescence emission ratio using fluorescence spectral imaging microscopy (FSPIM). This ratio will increase due to fluorescence resonance energy transfer (FRET) if the AtSERK1-CFP and AtSERK1-YFP fusion proteins interact. In 15% of the cells the YFP/CFP emission ratio for plasma membrane localized AtSERK1 proteins was enhanced. Yeast protein interaction experiments confirmed the possibility for AtSERK1 homodimerization Elimination of the extracellular LZ domain reduced the YFP/CFP emission ratio to control levels indicating that without the LZ Domain AtSERK1 is monomeric.

Introduction

Receptor kinases have been shown to play a key role in developmental processes such as cell proliferation, migration and differentiation. Receptor kinase-mediated signal transduction pathways are highly conserved in both vertebrates and invertebrates[1,2]. In plants, similar receptors, designated as receptor like kinases (RLKs) also appear to play a crucial role in development[3].

The classical model for receptor kinase-activation involves ligand binding-induced dimerization of the receptor, resulting in autophosphorylation of both partners in the dimer, mainly on specific tyrosine, serine or threonine residues. The best known examples of this mode of action are the transforming growth factor (TGF-β) receptor and the epidermal growth factor (EGF) receptors[4]. Ligand-induced dimerization of cell surface receptors can bring into proximity downstream proteins associated with these receptors followed by transphosphorylation of such target proteins. Animal receptor kinases can dimerize either by ligand-dependent non-covalent dimerization[5] or by ligand-independent covalent dimerization[6]. Dimerization can involve two identical receptors (homodimerization); different members of the same receptor family or a receptor and an accessory protein (heterodimerization)[7]. Receptor activation through heterodimerization is the most common[4], but certain receptors such as those for growth hormones employ homodimerization.

The evidence for monomer to dimer transition as a mechanism for receptor activation derives from experiments demonstrating that ligand binding leads to receptor dimerization[4]. Secondly, artificially induced dimerization or naturally occurring mutations leading to dimerization both mimic signaling in the absence of the physiological ligand[8,9]. Finally, oligomerization of intracellular regions of receptors using cell permeable synthetic ligands can lead to dimerization[10]. However, one of the most extensively studied eukaryotic receptors, the insulin receptor, exists as an inactive (αβ) dimer maintained by intersubunit disulfide cross bridges[11].

The mechanisms by which plant receptors transduce signals across the cell surface are largely unknown but plant receptors may also dimerize[12]. Studies on the action of the S-locus receptor kinase (SRK), a receptor-like kinase involved in the self-incompatibility response in *Brassica*, have shown that recombinant SRK autophosphorylates constitutively and exists as dimers in

nonpollinated stigmas[13]. The *CLAVATA (CLV)* genes control cell proliferation in the *Arabidopsis* shoot and inflorescence meristems. It has been shown that the CLV3 peptide acts as a ligand in the CLV1/CLV2 receptor complex[14,15,16]. Recent chimeric studies on RLKs, using the extracellular domain of BRASSINOSTEROID INSENSITIVE1 (BRI1) protein, involved in the perception of brassinosteroids and the intracellular domain of the Xa21 protein, involved in pathogen resistance from rice have revealed that the use of chimeric receptors may be highly useful in plants also[17].

Transmembrane receptor proteins, like other cell surface proteins undergo post-translational modifications such as disulfide bonding, glycosylation, palmitoylation and proteolytic cleavage. Many of these modifications play decisive roles in protein maturation and/or intracellular trafficking[18]. In mammalian cells, the correct folding and oligomerization of complex, multi-domain secretory proteins is controlled by N-glycosylation[19]. For example, the human insulin receptor has several glycosylation sites close to the N-terminus of the receptor. Mutational analysis of these sites showed that glycosylation was essential for correct processing and targeting of the protein to the cell surface[20]. In plant RLKs such studies have not been conducted, but the predicted protein sequence of plant RLKs shows that similar domains close to the N-terminus contain N-linked glycosylation sites.

Somatic embryogenesis is the process whereby somatic cells can develop into plants via characteristic morphological stages. In cultured *Daucus carota* suspension cells, the *SERK* (*Somatic Embryogenesis Receptor Kinase*) gene, a leucine rich repeat (LRR)-RLK, was found to be a marker for single suspension cells capable of forming embryos[21]. During somatic and zygotic embryogenesis *SERK* expression continues up to the early globular stage and is absent in later stages of embryo development. The most closely related *Arabidopsis thaliana SERK1* gene is expressed in developing ovules, early embryos and in vascular tissues of seedlings[22]. The predicted AtSERK1 protein contains an N-terminal leucine zipper (LZ), 5 leucine rich repeats (LRRs) and a proline-rich region in its predicted extracellular domain. This is followed by a single transmembrane domain that is attached to an intracellular kinase domain with all eleven conserved sub-domains as found in serine/threonine kinases[22].

Based on the jelly fish green fluorescent protein (GFP) and confocal microscopy, spatiotemporal dynamics of GFP-fusion proteins in intact cells can be investigated[23,24,25]. We have used AtSERK1-GFP fusion proteins to determine the subcellular localization of this receptor. In

addition, dimerization of the chimeric proteins was investigated making use of fluorescence resonance energy transfer (FRET) by employing spectroscopic variants of GFP. FRET is a quantum-mechanical process by which the excitation energy is transferred from a donor fluorochrome to an appropriate acceptor fluorochrome. FRET only occurs when (i) the donor fluorophore emission spectrum overlaps with the absorption spectrum of the acceptor, (ii) the transition dipole moments of the donor and the acceptor are not perpendicularly oriented and (iii) the distance between the donor and the acceptor is less than $1.5 \square$ the Förster radius ($_0$) for energy transfer[26,27]. Different engineered spectral variants of GFP, exhibiting yellow or cyan fluorescence, (YFP and CFP respectively) provide an opportunity to label the same protein with two different fluorophores in the same living cell[28]. More importantly, the emission and the excitation spectrum of these two variants of GFP are suitable for FRET. This allows to probe proximity of proteins in intact living cells[29]. The R_0 for CFP/YFP FRET is 5.5 nm implying that FRET between CFP and YFP will only be detected when the proximity of the fluorophores is less than 10 nm. FRET is manifested in different ways: a decrease in donor fluorescence quantum yield, a decreased donor fluorescence lifetime, an increased stability of the donor towards chemical photobleaching[30] and an increased sensitized acceptor fluorescence emission, if the acceptor is a fluorophore[31,32]. FRET microscopy[29] has been successfully employed to investigate the assembly of the T-cell antigen receptor complex[33,34], the interleukin-1 (IL-1) receptor complex after IL-1 binding[35] and to study EGF receptor oligomerization[36,37].

The results presented here show that the AtSERK1 protein is localized in the plasma membrane, mainly in the form of monomers. Only a small percentage of the AtSERK1 protein may exist as oligomers (e.g. homodimers), suggesting that the AtSERK1 protein belongs to a class of receptors that can undergo ligand-dependent non-covalent homodimerization. Employing truncated AtSERK1 proteins, it was found that the LRR domain is involved in correct targeting of the receptor while the LZ domain is involved in receptor dimerization.

Results

AtSERK1-YFP/CFP fusion proteins

In this study several AtSERK1-YFP/CFP constructs were used. These constructs are shown in detail in Fig. 1.
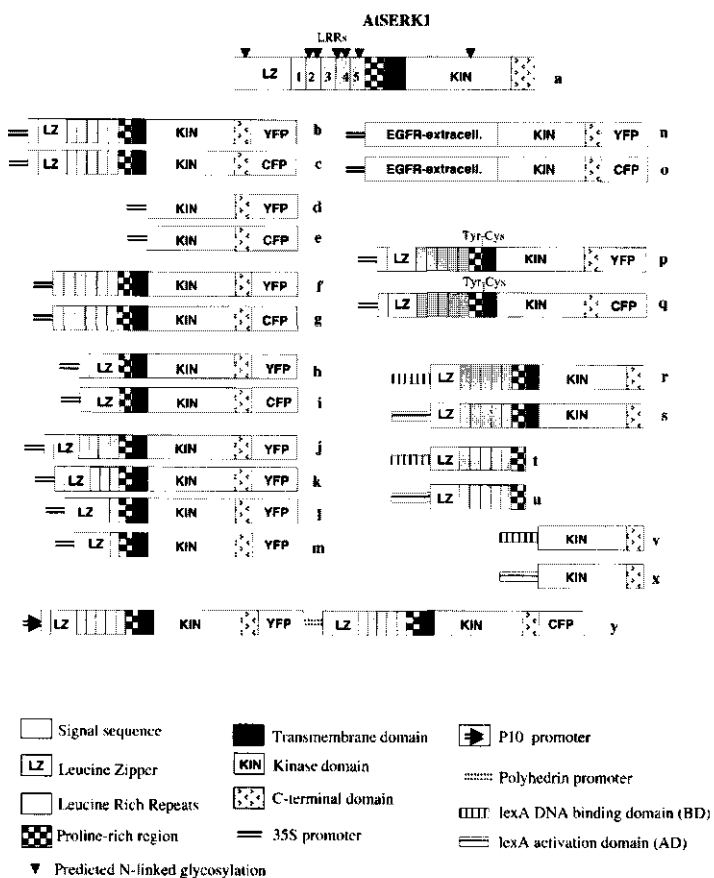
Figure 1. AtSERK1 constructs used in this study. Different domains of AtSERK1 (a) and AtSERK1 fusion constructs used for expression in plant protoplasts (b-q), yeast two hybrid studies (r-x) and expression in Sf21 insect cells (y) are shown. The AtSERK1-YFP/CFP transgenes were constructed in the PMON999 vector under control of the 35S promoter. AtSERK1-YFP/CFP (b, c) contains cDNA encoding the full-length AtSERK1 as a C-terminal

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fusion with the YFP/CFP genes. AtSERK1[kin]-YFP/CFP (d, e) encodes only the intracellular kinase domain of AtSERK1 fused to YFP/CFP. The truncated constructs, AtSERK1ΔLZ-YFP/CFP (f, g) and AtSERK1ΔL - YFP/CFP (h, i) were constructed by removing the leucine zipper and the five extracellular LRRs respectively from the coding sequence of AtSERK1. A series of LRR truncated constructs was made by removing LRRs starting from the N-terminal end and fusing the rest of the coding sequence of AtSERK1 to the YFP. This resulted in the construction of AtSERK1ΔL ^{1}-YFP (j), AtSERK1ΔL ^{1,2}-YFP (k), AtSERK1ΔL ^{1,2,3} -YFP (l), and AtSERK1ΔL ^{1,2,3,4}-YFP (m). EGFR[ex]-AtSERK1[kin]-YFP/CFP (n, o) chimeras were constructed by fusing the extracellular domain of EGFR and the intracellular kinase domain of AtSERK1-YFP/CFP. A single mutation in the AtSERK1 extracellular domain just above the transmembrane domain resulted in AtSERK1^{Y230C}-YFP/CFP (p, q) constructs.

An AtSERK1 (amino acids 31-625) construct lacking only the N-terminal signal sequence, and AtSERK1 constructs consisting of the extra-cellular domain or the intracellular domain only were fused to the activation domain (AD: pJG 4-5) or to the LexA DNA binding protein (BD: pEG202) resulted in AtSERK1$^{(31-625)}$-BD/AD (r, s), AtSERK1$^{(26-234)}$-BD/AD (t, u), AtSERK1$^{(266-625)}$-BD/AD (v, x). For expression in insect cells the full length AtSERK1 fused to YFP and the full-length AtSERK1 fused to CFP were cloned in front of the viral p10 and polyhedrin promoters respectively resulting in the AtSERK[ins]-CFP/YFP (y).

In order to determine the localization of the AtSERK1 protein, several AtSERK1-YFP constructs (Fig.1b, d, f and h) were transfected into cowpea mesophyll protoplasts. After incubation for 12 hrs, soluble and pellet fractions were isolated, separated by denaturing SDS-PAGE and immunoblotted using an antiserum raised against GFP (Fig. 2). The results show that AtSERK1-YFP wild type, AtSERK1ΔLZ-YFP and AtSERK1ΔLRR-YFP proteins are all found exclusively in the pellet fraction. Upon deletion of the entire extracellular domain and the transmembrane domain (Fig. 1d), about 40% of the protein becomes soluble, while the remainder is present in the pellet fraction (Fig. 2). We conclude that the AtSERK1-YFP fusion proteins are correctly expressed and that the majority are present in the insoluble pellet fractions that may represent the membrane fraction.
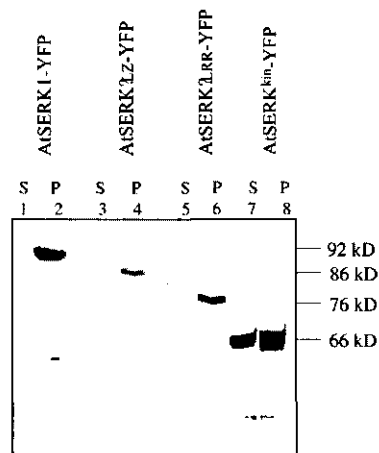
Figure 2. Immunoblot of AtSERK1 fusion proteins in transfected protoplasts. The protoplasts were harvested after 12 hrs of transfection with AtSERK1-YFP transgenes, lysed and separated on a 10% reducing SDS-PAGE. Proteins transferred to nitrocellulose paper were immunoblotted with anti- GFP antibody. S and P denote the soluble cytosolic fractions and the insoluble pellet fractions respectively. Lanes 1 and 2: AtSERK1-YFP; lanes 3 and 4: AtSERK1ΔLZ-YFP; lane 5 and 6: AtSERK1ΔL - YFP; lane 7 and 8: AtSERK1^{kin}-YFP. The molecular mass of the proteins is indicated in kDa.

Localization and targeting of AtSERK1 protein

To determine whether AtSERK1-YFP fusion proteins were localized in the plasma membrane, viable transfected protoplasts were analyzed by confocal laser scanning microscopy (CSLM) 12 hrs after transfection. Protoplasts transfected with an AtSERK1-YFP construct (Fig. 1b) clearly showed fluorescence at the surface of the protoplasts (Fig. 3a). Endomembranes were labeled to a lesser extent, possibly reflecting proteins in transit. In contrast, cytoplasmic expression was observed when the pMON999-YFP vector alone (Fig. 3c) or the AtSERK1^{kin}-YFP construct (Fig. 1d) was transfected into protoplasts (Fig. 3b). The EGFR^{ex}-AtSERK1^{kin}-YFP construct (Fig. 1n) was also expressed in protoplasts and the chimeric protein showed the same predominant surface localization (Fig. 3d) as that of the AtSERK1-YFP protein. The EGF receptor is a true plasma membrane receptor with cysteine-rich repeats in the extracellular domain. The correspondence in

the location of fluorescence between the AtSERK1 and the chimeric EGFR-AtSERK1 receptor strongly suggests that AtSERK1 is a plasma membrane-localized protein.
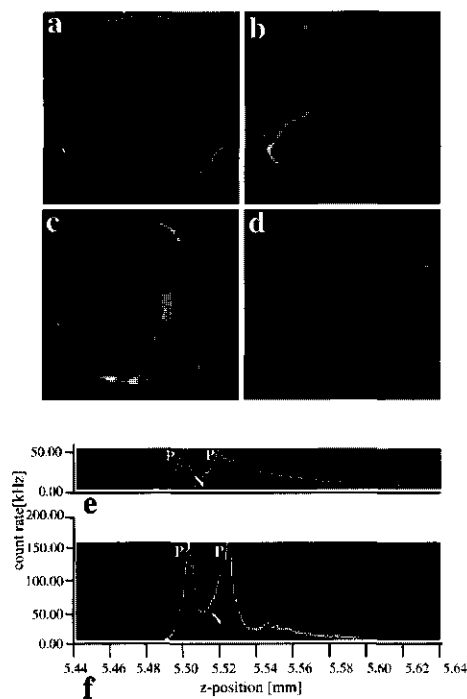
Figure 3. Confocal fluorescence images of protoplasts transfected with (a): AtSERK1- YFP (b): PMON999-YFP (c): AtSERK1^{kin}-YFP (d): EGFR^{ex}-AtSERK1^{kin}-YFP. In green the YFP fluorescence is shown and in red the chlorophyll fluorescence is shown. Fluorescence correlation spectroscopy (FCS) of AtSERK1-YFP-CFP fusion proteins in insect cells (e, f). The profiles show the count rate along the optical z-axis of the non-infected cells (e) and the AtSERK1-YFP expressing Sf21 cells (f). N and P indicate the fluorescence recorded in the nucleus and the plasma membrane respectively.

To confirm the AtSERK1 protein localization in the plasma membrane, the AtSERK1^{ins}-YFP-CFP fusion construct (Fig. 1y) was expressed in *Spodoptera frugiperda* (Sf21) insect cells in order to perform Fluorescence Correlation Spectroscopy (FCS). A line-scan along the optical axis (z-axis) was made to determine the distribution of fluorescent proteins in the cell. Detection of

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fluorescent fusion proteins by the sensitive, single molecule detection, FCS technique is more convenient in insect cells than in plant protoplasts because of the absence of background chlorophyll auto-fluorescence. The AtSERK1^{ins}-CFP-YFP expressing cells showed the same low intensity of 20 kHz (photoelectrons emitted sec^{-1}) in the nuclear region as in the non-infected cells. There was a 3-4 fold (161 kHz) increase in the fluorescence intensity in the plasma membrane region of AtSERK1^{ins}-CFP-YFP expressing cells (Fig. 3f) as compared to highest fluorescence intensity of around 40 kHz in the plasma membrane of the non-infected Sf21 cells (Fig. 3e). This finding indicates that the AtSERK1^{ins}-CFP-YFP proteins are also correctly localized in the plasma membrane in insect cells. In cells expressing the fusion proteins to a very high level, also endomembranes were labelled, possibly reflecting proteins in transit or undergoing endocytosis (data not shown).

To determine whether the extracellular LZ domain and the different LRRs of the AtSERK1 protein have a role in targeting of the AtSERK1 receptor we transfected protoplasts with a series of constructs in which the LZ domain or one or several LRRs were removed (see Fig. 1b, f, h, j-m for an overview of the constructs used). Protoplasts expressing the AtSERK1Δ_{LZ}-YFP protein showed the same surface fluorescence as shown by protoplasts expressing AtSERK1-YFP (Fig. 4a, compare with Fig. 3a). No effect of the removal of the first LRR was evident after transfecting AtSERK1$\Delta_{LRR}{}^{1}$-YFP into protoplasts (Fig. 4b). There was some decrease in surface fluorescence when AtSERK1$\Delta_{LRR}{}^{12}$-YFP (Fig. 4c) or AtSERK1$\Delta_{LRR}{}^{123}$-YFP (Fig. 4d) were transfected into protoplasts. In addition to the plasma membrane, the truncated AtSERK1 proteins were also seen in subcellular organelles. There was a considerable decrease in surface fluorescence when AtSERK1$\Delta_{LRR}{}^{1234}$-YFP was transfected into protoplasts (Fig. 4e). Most of the AtSERK1 protein was seen to be concentrated in subcellular organelles which might represent clogging in ER or Golgi-derived vesicles or sequestering of the AtSERK1 protein in the lysosomal compartments of the protoplasts (Fig. 4e). Upon transfection of AtSERK1Δ_{LRR}-YFP into protoplasts, almost no surface fluorescence was observed (Fig. 4f). Most of the fusion protein was seen internally as in Fig. 4e. We conclude that the LZ domain of AtSERK1 is not involved in protein targeting but that at least four LRRs of AtSERK1 contain specific information for correct targeting of the AtSERK1 protein.
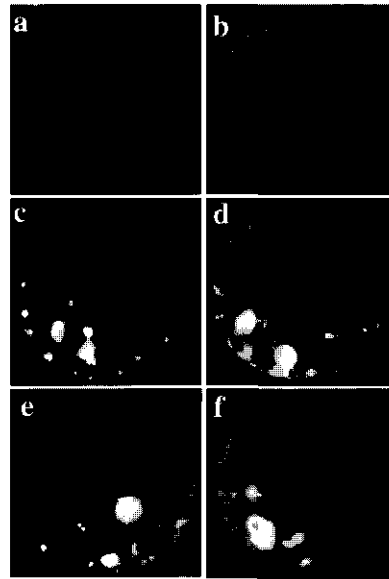
Figure 4. Confocal fluorescence images of protoplasts transfected with LZ and the series of LRR truncated constructs (a): AtSERK1ΔLZ-YFP, (b): AtSERK1ΔL [1] -YFP, (c): AtSERK1ΔL [1,2] -YFP, (d): AtSERK1ΔL [1,2,3]-YFP and (e): AtSERK1ΔL [1,2,3,4]-YFP and (f): AtSERK1ΔL -YFP expression is shown. In green the YFP fluorescence is shown and in red the chlorophyll fluorescence is shown.

The AtSERK1 protein contains 7 predicted N-glycosylation sites, 5 of which are present in the LRRs of the extracellular domain (Fig. 1a). To determine whether these sites are used in the proper targeting of the AtSERK1 protein, we treated the protoplasts with 20 μg ml^{-1} tunicamycin 3 hrs after transfection with AtSERK1-YFP. Tunicamycin prevents the addition of *N*-acetylglucosamine to dolichol phosphate, the first step in the formation of the core oligosaccharide employed in N-linked glycosylation. The transfected protoplasts were visualized with CSLM 12 hrs after the transfection The localization of AtSERK1-YFP in tunicamycin-treated protoplasts (Fig. 5b) showed reduced membrane targeting of the AtSERK1 protein and increased intracellular clogging similar to that observed with the truncated AtSERK1ΔLRR-YFP protein (Fig. 4e, f) when compared with untreated protoplasts (Fig. 5a). Pretreatment of AtSERK[ins]-CFP-YFP expressing insect cells with 20 μg ml^{-1} of tunicamycin also showed intracellular clogging in vesicles (results not shown) comparable to the plant protoplasts. Proteins

from tunicamycin-treated and untreated AtSERK^{ins}-CFP-YFP expressing insect cells were isolated, separated by denaturing SDS-PAGE and immunoblotted using an antiserum against GFP. There was a reduction of the molecular weight of the AtSERK1^{ins}-CFP-YFP fusion protein from 96 kD to 84 kD in the tunicamycin treated cell extract when compared to the untreated AtSERK1^{ins}-YFP-CFP infected Sf21 cells (Fig. 5b). These results suggest that AtSERK1 is glycosylated in both insect cells and cowpea protoplasts and that N-linked glycosylation may be important for targeting the AtSERK1 protein to the plasma membrane.
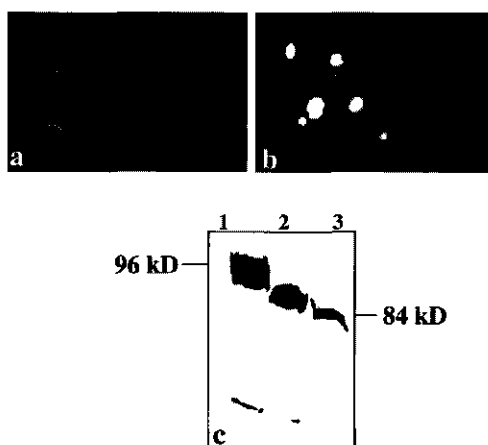
Figure 5. The effect of tunicamycin on protoplasts and Sf21 cells expressing AtSERK1-YFP. (a): Protoplasts transfected with AtSERK1-YFP and visualized after 12 hrs of transfection. (b): Protoplasts treated with 20 μg.ml^{-1} of tunicamycin 3 h after transfection with AtSERK1-YFP and visualized after 12 hrs of transfection. (c): Immunoblot analysis of the Sf21 insect cells, untreated (lane 1) and treated with either 10 μg ml^{-1} (lane 2) or 20 μg ml^{-1} (lane 3) of tunicamycin prior to AtSERK1^{ins}-CFP-YFP infection, harvested after 48 hrs, lysed and separated on 8% reducing SDS-PAGE. Proteins were transferred to nitrocellulose paper and immunoblotted with anti- GFP antibody. The molecular mass of the proteins is indicated in kDa.

Homodimerization of AtSERK1 protein: interaction in yeast

To determine if the AtSERK1 receptor was capable of forming homodimers, the AtSERK1 intracellular kinase domain (AtSERK1$^{(266-625)}$), the extracellular domain (AtSERK1$^{(26-234)}$) and the AtSERK1 protein without signal sequence (AtSERK1$^{(31-625)}$) were fused to the B42 activation domain (AD) in pJG4-5and the lexA DNA-binding domain (BD) in pEG202 (Fig. 1r-x There was no interaction between the two intracellular kinase domains of AtSERK1 (AtSERK1$^{(266-625)}$

in Fig. 6) whereas the AtSERK1 extracellular domains do interact with each other (AtSERK1[(26-234)] in Fig. 6) as indicated by the ß-galactosidase activity and the growth without leucine. The interaction, between two full-length AtSERK1 proteins lacking the N-terminal signal sequence was slightly reduced compared to the interaction shown by the extracellular domains only (AtSERK1[(31-625)] in Fig. 6). This is most likely due to aberrant folding of the full-length membrane receptor protein in the yeast nucleus. From these data we conclude that the AtSERK1 extracellular domains are capable of homodimerization while the kinase domains are unlikely to interact physically.
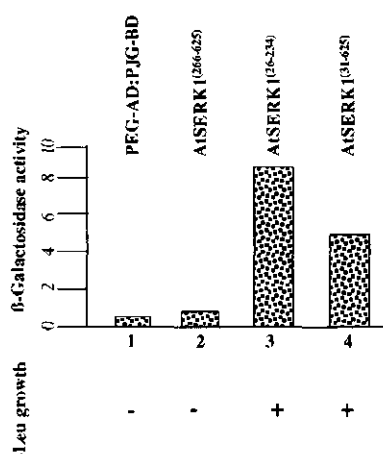
Figure 6. AtSERK1-AtSERK1 interactions in yeast using lexA two hybrid vectors. The AtSERK1[(31-625)], AtSERK1[(26-234)], AtSERK1[(260-625)] fused to the activation domain (AD: pJG 4-5) or with the LexA DNA binding protein (BD: pEG202) were transformed into yeast strain EGY 48 and ß-galactosidase activity and growth on leucine was determined.

Oligomerization of the AtSERK1 protein: Fluorescence Energy Resonance Transfer between CFP and YFP

Because protein-protein interactions demonstrated in the yeast system may not be representative of interactions in living plant cells, nor for interactions in membranes, we used cowpea protoplasts coexpressing AtSERK1-CFP and AtSERK1-YFP fusion proteins. Possible AtSERK1 dimerization was then studied by measuring FRET between the CFP (donor) and the YFP (acceptor) fluorophores. All experiments were based on fluorescence spectral imaging

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microscopy (FSPIM) as a detection system. Spectral images were taken from plasma membrane-regions of protoplasts coexpressing AtSERK1-YFP and AtSERK1-CFP and a fluorescence emission spectrum corrected for background fluorescence was then generated. In case of FRET, the CFP fluorescence will be quenched and the YFP fluorescence would be increased (sensitized). Assuming approximately equal expression levels of both AtSERK1-CFP and -YFP, we consider a ratio of fluorescence intensity at 525 nm over 475 nm (designated as the YFP/CFP emission ratio) of 1.3 or higher as evidence of FRET and hence of AtSERK1-AtSERK1 interactions such as oligomerization or homodimerization. It should be emphasized that with the FSPIM technique it cannot be resolved whether the non-monomeric AtSERK1-receptors are dimeric, trimeric or present as higher oligomeric structures.

A single protoplast coexpressing AtSERK1-CFP and AtSERK1-YFP fusion proteins is shown in Fig. 7a. Five areas of which spectral images are recorded are indicated by rectangles. The YFP/CFP emission ratios deduced from these spectra is close to 1.0 in 4 of the 5 measurements and 1.35 in only one measurement (Fig. 7c). Repeating this experiment in 10 different protoplasts and taking 5 measurements from each protoplast revealed that the YFP/CFP emission ratio is close to 1.0 in about 80% of the spectral measurements and 1.35 in 15% of the measurements. This indicates that receptor oligomerization is not uniformly distributed within the membrane of a single protoplast. The percentage of YFP/CFP emission ratios deduced from single spectral measurements on 60 protoplasts co-expressing AtSERK1-CFP and AtSERK1-YFP are shown in Fig. 7e. About 15% of the measurements show an increased YFP/CFP emission ratio of 1.35, which is similar to the results obtained from a single protoplast (Fig. 7c). The protoplasts co-expressing the intracellular AtSERK1^{kin}-CFP and AtSERK1^{kin}-YFP (Fig. 7b) showed no or little increase in YFP (acceptor) fluorescence (Fig 7d). The YFP/CFP emission ratio was close to 1.0 in almost all the 5 measurements was recorded from the different points (shown by rectangles) in the protoplast (Fig. 7b). Repeating this experiment in 10 different protoplasts and taking 5 measurements from each protoplast revealed a YFP/CFP emission ratio close to 1.0 in about 95% of the spectral measurements. Similar results were obtained when single spectral measurements were performed on 60 protoplasts coexpressing AtSERK1^{kin}-CFP and AtSERK1^{kin}-YFP (Fig. 7e). Collectively these results show that in the protoplasts analyzed, most of the AtSERK1 receptor molecules (~85%) are in the monomeric state while up to 15%

may be oligomerized. No interaction was found between the kinase domains expressed in the cytoplasm, which confirms the yeast two-hybrid experiments.
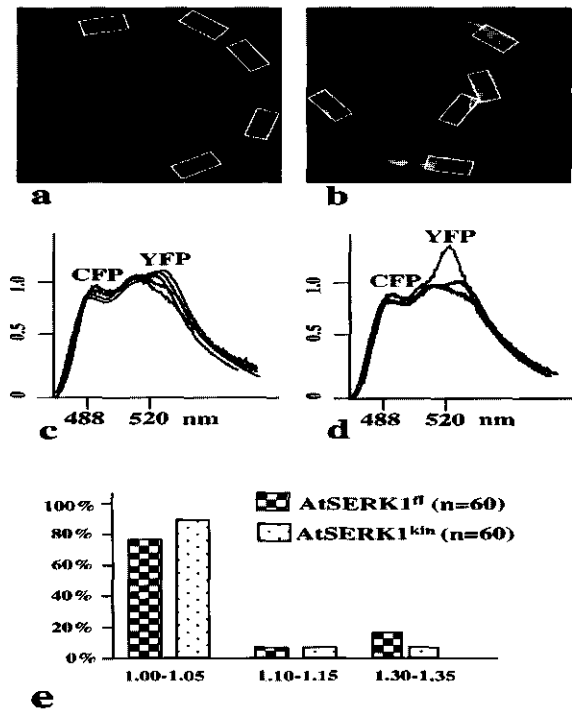
Figure 7. FSPIM analysis of fluorescent AtSERK1 fusion proteins. (a): Confocal image of a protoplast cotransfected with AtSERK1-YFP/CFP showing typical regions (rectangles) used for spectral measurements. (c): Emission spectra of the AtSERK1-CFP/YFP proteins obtained from the spectral images shown in (a). The X-axis represents wavelength (nm) and the Y-axis represents the CFP and YFP fluorescence intensities. (b and d): the same for AtSERK1^{kin}-CFP/YFP, expressing protoplasts. (e): Comparison of the 525/475 nm fluorescence emission ratio (the YFP/CFP ratio), varying form 1.00 to 1.35, of the 60 protoplasts cotransfected with AtSERK1-CFP/YFP, and the protoplasts cotransfected with AtSERK1^{kin}-CFP/ YFP.

The YFP/CFP emission ratios as shown in Fig. 7 may suffer from several potential artifacts unbalanced expression ratio of the donor and the acceptor fluorophores and the absorption of the CFP fluorescence by chlorophyll in plant cells. To exclude the first possibility, we performed more than 10 independent transfection experiments in which we coexpressed AtSERK1-CFP and

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AtSERK1-YFP fusion proteins. In each of these transfections 80% of the protoplasts exhibited a constant YFP/CFP emission ratio indicating a constant expression ratio. In addition, the variation in the YFP/CFP emission ratio within the same cell (in 15% of the cells) indicates that another phenomenon than an unbalanced expression ratio must be responsible for enhanced YFP/CFP emission ratio (i.e. FRET). To avoid the second artifact, FSPIM measurements were performed exclusively over areas lacking chloroplasts. We therefore consider it likely that the results showing an increased donor/acceptor ratio are due to FRET between the CFP and the YFP molecules fused to the AtSERK1 protein, indicating receptor-oligomerization (e.g. homodimerization).

To verify whether AtSERK1 receptor oligomerization was dependent on the extracellular domains, protoplasts were co-transfected with AtSERK1Δ_{LZ}-YFP and AtSERK1Δ_{LZ}-CFP (Fig. 8a). Experiments were performed in the same way as described for the full length AtSERK1 receptor as shown in Fig. 7. The spectra obtained from the area of the protoplast expressing AtSERK1Δ_{LZ}-YFP and AtSERK1Δ_{LZ}-CFP (Fig. 8a) at the plasma membrane (indicated by rectangles) showed that there was no increase in YFP fluorescence in almost all the measurements recorded (Fig. 8c). Repeating this experiment in 10 different protoplasts taking 5 measurements from each protoplast revealed that the intensity ratio is close to 1.0 in about 95% of the spectral measurements. The same results were obtained when single spectral measurements were performed on 60 co-expressing AtSERK1Δ_{LZ}-CFP and AtSERK1Δ_{LZ}-YFP protoplasts (Fig. 8f). Next, protoplasts co-expressing AtSERK1Δ_{LRR}-CFP and AtSERK1Δ_{LRR}-YFP (Fig. 8b) were analyzed. The FSPIM measurements on protoplast expressing these truncated AtSERK1 receptors were performed on both types of receptor molecules, the ones that were properly targeted to the plasma membrane (shown by rectangles) and the ones that remained clogged in the intracellular vesicles (shown by small rectangles) (Fig. 8b). The spectra derived from the five measurements on areas indicated by small rectangles showed an increased fluorescence ratio of 1.35 in 3-4 out of five measurements (Fig. 8d). When this experiment was repeated in 10 different protoplasts, there was an increased YFP/CFP of 1.35 in 70% of the measurements recorded. A YFP/CFP fluorescence intensity ratio close to 1.35 was seen in 1 out of 5 measurements recorded from the different points indicated by rectangles at the plasma membrane of the protoplast. Repeating this experiment in 10 different protoplasts showed that about 15% of the measurements had an increased ratio of 1.35 (Fig. 8e). When these experiments were

performed at the plasma membrane of 60 different protoplasts taking one single measurement from each protoplast, the results coincided with the measurements shown for each individual protoplast (Fig. 8f). The results show that there was an increased YFP/CFP fluorescence intensity ratio again in about 15% of the coexpressing protoplasts suggesting that LRRs are not involved at all in AtSERK1 dimerization at the plasma membrane.
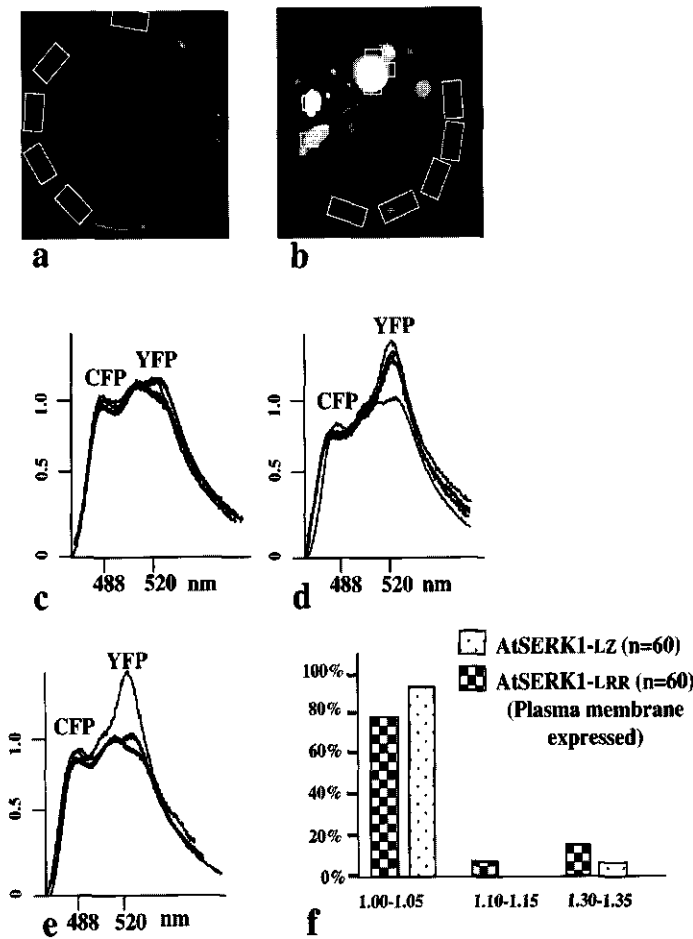
Figure 8. FSPIM analysis of truncated fluorescent AtSERK1 fusion proteins. (a): Confocal image of a protoplast cotransfected with AtSERK1ΔLZ-CFP/YFP showing typical regions (rectangles) used for spectral measurements. b): Confocal image of a protoplast cotransfected with AtSERK1ΔL   -CFP/YFP showing typical plasma membrane regions (rectangles) and intracellular regions (small rectangles) used for spectral measurements (c): Emission spectra

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of the AtSERK1ΔLZ-CFP/ YFP proteins obtained from spectral images shown in (a). The X-axis represents wavelength (nm) and the Y-axis represents the CFP and YFP fluorescence intensities. (d): Emission spectra of the AtSERK1ΔL   -CFP/ YFP proteins obtained from plasma membrane regions. (e): Emission spectra of the AtSERK1ΔL   -CFP/YFP proteins obtained from intracellular regions. (f): Comparison of the fluorescence emission ratio (YFP/CFP ratio) on the plasma membrane, varying form 1.00 to 1.35, of the 60 protoplasts cotransfected with AtSERK1ΔLZ-CFP/YFP, and the protoplasts cotransfected with AtSERK1ΔL   -CFP/YFP.

To determine whether AtSERK1 receptor-dimerization at the plasma membrane could be increased by an intermolecular disulfide bond, we introduced a single unpaired cysteine in the extracellular domain in the region immediately adjacent to the transmembrane domain (Y230C). Protoplasts cotransfected with AtSERK1^{Y230C}-YFP and AtSERK1^{Y230C}-CFP constructs (Fig. 1 p, q) showed protein localization similar to that of AtSERK1-YFP and AtSERK1-CFP (results not shown). The spectra from these protoplasts were obtained in the same way as for AtSERK1-YFP and AtSERK1-CFP. In about 15% of the measurements, the YFP/CFP ratio increased to 1.35 (Fig. 9). Since this is similar to the ratio observed in full-length AtSERK1 proteins, the introduction of an extra cysteine residue does not appear to result in increased AtSERK1 receptor dimerization.
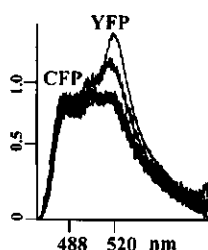
Figure 9. FSPIM analysis of truncated fluorescent AtSERK1 fusion proteins. Emission spectra of the AtSERK1^{Y230C}-CFP/ YFP proteins after excitation at 430 nm.

The synthetic auxin 2,4-dichlorophenoxy acetic acid (2,4-D) is commonly used to initiate somatic embryogenesis. Because of the correspondence between the acquisition of embryogenic potential and SERK expression[21] we investigated if 2,4-D itself can influence AtSERK1 dimerization. Protoplasts were cotransfected with AtSERK-CFP and AtSERK-YFP and were treated with 2,4-D. There was no change in the YFP/CFP emission ratio at the plasma membrane (data not shown).

Taken together, we conclude from these results that the predominant form of AtSERK1 is monomeric and that up to 15% of the AtSERK1-CFP/YFP proteins may be oligomerized (possibly as homodimers) at the plasma membrane of the protoplasts. The oligomerization appears to require only the extracellular LZ domain and not the LRRs or the kinase domain of AtSERK1.

Discussion

The major goals of this work were to determine the subcellular localization of the AtSERK1 protein and to determine whether the AtSERK1 protein was capable of homodimerization *in vivo*. The results show that AtSERK1 is a plasma membrane protein that occurs predominantly as a monomer. In living plant cells, less than 15% of the receptor molecules may exist as a homodimer.

Based on the cDNA sequence, the AtSERK1 protein was predicted to be a membrane associated serine/threonine receptor protein kinase[21]. This prediction was supported by several experiments. Western blot analysis showed that AtSERK1-YFP fusion proteins produced in plant protoplasts are present in a 40,000 g pelletable cell fraction whereas similar YFP fusion proteins lacking the extracellular and transmembrane domains remained in the soluble fraction. Confocal microscopy with AtSERK1-YFP expressed in plant protoplasts showed the presence of the fusion proteins in the plasma membrane in a pattern comparable with that of an EGFR[ex]-AtSERK1[kin]-YFP chimeric protein. Finally, FCS indicated that the AtSERK1-YFP fusion protein was located in the plasma membrane of insect cells. Based on these data we propose that the functional AtSERK1 receptor kinase is located in the plasma membrane.

The correct assembly and localization into the plasma membrane of a glycosylated receptor kinase such as AtSERK1 must involve a series of events. The AtSERK1 predicted proprotein has a 29 aa signal sequence, immediately followed by the LZ domain and the five LRRs[21]. Thus, it is likely that the protein is synthesized on the ER membrane and translocated into the ER lumen where N-glycosylation of the LRRs takes place. The extracellular domains of most of the characterized transmembrane receptors have been shown to be involved in ligand binding, oligomerization and the subsequent activation of the intracellular kinase domain and the initiation of the downstream signal transduction cascade[4]. More recently, it is becoming clear that the

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extracellular domains are also involved in correct targeting of the receptors. For instance, the correct folding, assembly and expression of the human insulin receptor depends on glycosylation of the extracellular domains[20]. Inhibition of N-linked glycosylation by tunicamycin leads to the intracellular accumulation of non-glycosylated insulin proreceptors that fail to fold correctly[38]. The LRRs of AtSERK1 contain of 5 putative N-glycosylation sites. Two of these are present in the second LRR, two in the fourth and one in the fifth LRR starting from the N-terminus of the AtSERK1 protein. Removal of at least two of the LRRs resulted in considerable reduction of the number of AtSERK1 protein molecules being targeted to the plasma membrane. In line with the role of N-linked glycosylation in insulin receptor targeting, AtSERK1 mistargeting would be due to the lack of glycosylation of the extracellular LRRs. Support for this hypothesis comes from tunicamycin studies suggesting a role of the N-linked glycosylation in AtSERK1 protein targeting to the plasma membrane.

Several roles of addition of N- linked oligosaccharides to nascent proteins have been demonstrated; for example, N-linked oligosaccharides attach to lectin-like molecular chaperones such as calnexin and calreticulin[19], facilitating correct protein folding[39]. N-linked oligosaccharides also play a role in the "quality control" system of the ER that ensures selective transportation of the properly folded proteins destined for the Golgi complex[40]. Removal of the N-linked oligosaccharides either by chemical means or through removal of LRRs may thus result in incorrectly folded, non-glycosylated AtSERK1 proteins and these may be trapped in the ER. This suggests that recognition of properly glycosylated proteins by an ER based sorting machinery is an essential element of the assembly and localization process.

Receptor dimerization is employed as a mechanism for both receptor activation and autophosphorylation[8,41]. Well-known examples are the epidermal growth factor receptor (EGFR) and platelet derived growth factor receptor (PDGFR) that undergo dimerization in response to binding of their respective ligands. However, the sensitivity of the PDGFR[42] and some other receptors like calcium sensing receptor (CaR)[43] to reducing agents suggests that one type of intermolecular interaction mediating dimerization is *via* intermolecular disulfide bonds.

The mechanism by which plant receptors transduce signals across the cell surface is largely unknown. There is evidence, which suggests that plant receptors like CLV1 and CLV2 also dimerize[12] and are assembled into a heterodimeric complex to transduce extracellular signals[15].

Recent studies have shown that CLV1 and CLV3 form a potential receptor/ligand pair and that CLV3 binds to the CLV1 and CLV2 heterodimer to form a multimeric protein complex[16].

Our experiments suggest that the AtSERK1 receptor can form oligomerized structures such as homodimers This is based both on yeast interaction experiments and expression of AtSERK1-CFP/YFP fusion proteins in protoplasts. In the latter studies, one potential problem may be that AtSERK1 expression is raised far beyond normal levels due to the use of the strong constitutive 35S promoter. However, because the majority of the protoplasts expressing AtSERK1 fusion proteins do not show FRET and therefore exist as monomers in the membrane, this does not appear to be a wide spread problem. It also appeared that that there are only certain regions on the plasma membrane where AtSERK1 are not monomeric. This could indicate that a minority of AtSERK1 receptor molecules on the plasma membrane is in a predimerized state. This observation is in line with EGF receptor dimerization in mammalian cells. High affinity EGF receptors (12%) dimerize highly in some regions of the plasma membrane. Based on the quantitative determination of FRET on the EGF receptor in mammalian cells, it was suggested that the high affinity subclass of receptors are present in a predimerized state in the absence of the ligand[37]. The predimerized AtSERK1 receptor molecules on the plasma membrane appear to be far less abundant than those observed for the EGF receptor owing to the fact that in only 1 out of 5 measurements, there appeared to be a change in the fluorescence intensity ratio. In addition, the ratio change is on the lower side of FRET detectability. This also indicates that the region of the plasma membrane used for single FRET measurements (indicated by rectangles in Fig. 7 and Fig. 8) mainly contain AtSERK1 receptors that are in the monomeric form. The small population of non-monomeric AtSERK1 receptors are not detected in the absence of the LZ domain in the AtSERK1 receptor, implying that our FSPIM detection system is sensitive enough to detect the changes from a fully monomeric to a state where monomeric and oligomeric AtSERK1 receptors coexist.

LZ domains have been shown to play a role in dimerization of various nuclear and cytoplasmic proteins such as Nek2[44], ZIP kinases[45], MLK-3[46] and TSC-22[47] respectively. There are also recent reports that show the involvement of the LZ domain in receptor dimerization[48]. The presence of cysteines in the N-terminal extracellular domains of transmembrane receptors has been shown to have a role in receptor dimerization by the formation of disulfide bridges[49]. In animals, the type II transforming growth factor receptor (also a receptor serine/threonine kinase)

can form homodimers in the absence of ligands[50,51] whereas the insulin growth factor receptor is found as a disulfide-linked tetramer[52]. Since it is not known whether ligand/s that bind to the AtSERK1 are present in the protoplast culture medium, we cannot answer whether the putative AtSERK1 dimers detected by FSPIM represent ligand-activated dimers or preassociated dimers. The presence of conserved cysteine pairs in the extracellular domains of plant RLKs may imply that a population of predimerized receptors on the cell surface can exist[53]. Among plant RLKs, CLV1 and CLV2 form a disulfide linked heterodimeric receptor complex[15] and also the ethylene receptor, ETR1, is a disulfide-linked dimer[54]. The SRG protein involved in the pollen recognition in *Brassica* stigma is also found as a disulfide-linked dimer[55]. The AtSERK1 protein has four cysteines in the extracellular domain so the small amount of AtSERK1 oligomers found might arise due to the formation of disulfide bridges between the cysteines in the LZ domain. Generation of stable disulfide-bonded receptor-like-protein tyrosine phosphatase-α (RTPT-α) homodimers by introducing a single cysteine in the extracellular domain immediately adjacent to the transmembrane domain has been shown[56]. The introduction of a cysteine residue in a comparable region as RTPT-α does however not change the oligomerization state of the AtSERK1 receptor, so disulfide linkages may be of less importance in AtSERK1 homodimerization.

While identification of the ligand for AtSERK1 is clearly essential for determining its precise mode of action, we propose that AtSERK1 exhibits ligand-induced homodimerization, which would place it in the EGF class of receptors.

Materials and methods

AtSERK1 -YFP/CFP tagged vectors

The entire open reading frame of the *AtSERK1* cDNA was amplified by polymerase chain reaction (PCR) from a *AtSERK1* full-length cDNA (acc. no: A67827)[21] and cloned downstream of the 35S promoter into the *NcoI* site of pMON999-CFP and pMON999-YFP using primers NcoI215f   5'   CATGCCATGGTGGAGTCGAGTTATGTGG 3'   and NcoI2068r   5' CATGCCATGGACCTTGGACCAGATAACTC 3'. This clone and all subsequently described clones are shown schematically in Fig. 1. The AtSERK1 intracellular kinase domain was

similarly cloned into the *NcoI* site of pMON999-CFP and pMON999-YFP using primers nAtSERKC 5' CCATCCGATGGGCCCACTAGATATT TTCTT 3' and NcoI2068r. This resulted in the fusion constructs AtSERK1-YFP;AtSERK1-CFP (Fig.1b,c) and AtSERK1[kin]-YFP;AtSERK1[kin]-CFP (Fig.1d, e). A 60 bp *NcoI-KpnI* fragment corresponding to the signal sequence of AtSERK1 cDNA and a 1420 bp *KpnI-NcoI* fragment corresponding to the coding sequence of the AtSERK1 cDNA without the leucine zipper were prepared by PCR using primer pair NcoI215 and KpnI260 5' CAAA TTAGCAGAAGCAAGCCAC 3'and primer pair KpnI420 5' TGGGGAATGCAGAG TTATCTGGC 3' and NcoI2068 respectively. Both were ligated into the *NcoI* site of PMON999-CFP/YFP plasmids to create truncated AtSERK1 fusion constructs without the leucine zipper domain. The fusion constructs were named AtSERK1ΔLZ-CFP and AtSERK1ΔLZ-YFP (Fig.1f,g). To create the truncated AtSERK1 fusion constructs (without the extracellular LRRs), the AtSERK1-CFP and YFP constructs were digested with *van91* and religated, resulting in AtSERK1ΔLRR-YFP and AtSERK1ΔLRR-CFP constructs (Fig.1h,i). A series of constructs with one or more LRRs deleted were prepared by PCR. DNA fragments of 1580 bp (primers KpnI489 5' CGGGGTACCGAGCTTTACAGTAACAACATAAC 3' and NcoI2068r), 1508 bp (primers KpnI561 5' CGGGGTACCGATCTTTACTTAAACAGCTTCT 3' and NcoI2068r), 1364 bp (primers KpnI633 5'CGGGGTACCCGGCTTAACAACAACAGTCT 3' and NcoI2068r), and 1292 bp (primers KpnI705 5' CGGGTACCGATCTATCAAAT AACAGACTCT 3' and NcoI2068r) generated with 5' *KpnI* and 3' *NcoI* linkers were prepared by PCR. They were ligated together with a 60 bp *NcoI-KpnI* fragment corresponding to the signal sequence of AtSERK1 cDNA into the *NcoI* site of pMON999-YFP plasmids. This resulted in the series of truncated AtSERK1 fusion constructs designated as AtSERK1ΔLRR[1]-YFP (Fig.1j), AtSERK1ΔLRR[1,2]-YFP (Fig.1k), AtSERK1ΔLRR[1,2,3]-YFP (Fig.1l) and AtSERK1ΔLRR[1,2,3,4]-YFP (Fig.1m) respectively.

A 2.1 kb *BglII/NcoI* fragment corresponding to the extracellular and transmembrane domain of human epidermal growth factor receptor (EGFR) was amplified by PCR using primers EGFBglIIf 5' GGAAGATCTGCGATGCGACCCTCCGGG 3' and EGFNcoIr 5' CATGCCAT GGGGCGCTTCCGAACGATGTGG 3' and ligated into the *BglII/NcoI* sites of PMON999-CFP/YFP vectors. The resulting plasmids were digested with *NcoI* and the *NcoI/NcoI* fragment encoding the AtSERK1 intracellular kinase domain was cloned in frame with the C-terminus of EGFR cDNA. This resulted in EGFR[ex]-AtSERK1[kin]-YFP/EGFR[ex]-AtSERK1[kin]-CFP chimeras

(Fig. 1n,o).

For expression in insect cells a Pdual Fast Bac vector, that contains a polyhedrin and a p10 promoter was used. The entire coding sequence of the AtSERK1-CFP using primers KCNotl 5' ATAAGAATGCGGCCGCGGATCCTTACTTGTACAGCTCG 3'and KCSalI 5' ACGCGTCG ACAGAGCCATGGTGGAGTCGAG 3' and the AtSERK1-YFP fusion using primers KYKpnl 5' CGGGGTACCGGATCCTTACTTGTACAGCTCG 3' and KYSmal 5' TCCCCCGGGGAGA GCCATGGTGGAGTCGAG 3' were amplified by PCR and cloned into the *Smal/Kpn*l sites downstream of the polyhedrin promoter and *SalI/Not*l sites downstream of the p10 promoter respectively. This resulted in the AtSERK1^{ins}-CFP-YFP construct (Fig. 1y). All the constructs were confirmed by sequence analysis.

Site directed mutagenesis

This fusion constructs AtSERK1-YFP and AtSERK1-CFP were used for replacing tyrosine[230] with single unpaired cysteine[230] in the extracellular domain immediately adjacent to the transmembrane domain. Site directed mutagenesis was performed according to manufacturer's instructions (Stratagene). This resulted in the AtSERK1^{Y230C}-YFP and AtSERK1^{Y230C}-CFP (Fig. 1p,q) constructs. Clones incorporating the mutations were confirmed by DNA sequence determination.

Transient expression in protoplasts

Cowpea mesophyll protoplasts were prepared essentially as described previously[57] except that 10mM CaCl$_2$ was added to the enzyme solution (0.5 M mannitol, 10 mM CaCl$_2$, 0.1% cellulase and 0.05% pectinase). Ten to fifteen μg of each purified plasmid DNA in 20-75 μl of water was added to 0.5-1x10^6 protoplasts in 75-150 μl of ice cold solution C (0.6 M mannitol, 10 mM CaCl$_2$, pH 5.3-5.8), mixed by gentle shaking and followed immediately by the addition of solution H (40% PEG Mw 6000, 0.6 M mannitol, 0.1 M Ca(NO$_3$) $_2$. The protoplast suspension was incubated for 5-25 sec with gentle shaking followed by the addition of 4.5 ml of solution M (0.5 M mannitol, 15 mM MgCl$_2$ and 0.1%) MES (pH 5.3-5.7) and incubated at room temperature for 20 min. Protoplasts were then washed 3 times with solution M and incubated for 8-24 hrs as described previously[58]. For glycosylation studies, protoplasts were treated with 20 μg·ml^{-1} of tunicamycin 3 hrs after transfection. Protoplasts were mounted on microscopic slides and

visualized under fluorescence microscope. For dimerization studies with 2,4-D, cotransfected protoplasts were treated with 2 μM 2,4-D 12 hrs after transfection.

Expression in insect cells

Monolayers of Sf21cells (1×10^6 cells/35 mm Petri dish) were cotransfected with 2 μg of recombinant bacmid DNA (obtained by transforming DH10BAC *Escherchia coli* cells (GIBCO-BRL)) with AtSERK1[ins]-CFP-YFP Pdual FastBac plasmid DNA using a lipofectin reagent (GIBCO-BRL) as described[59]. After 72 hrs of incubation in Graces's medium (GIBCO-BRL) at 27°C, the supernatant containing extracellular virus particles was collected and Sf21 cell monolayers were infected. For glycosylation studies in insect cells, the tunicamycin treatment was performed on cells prior to the infection with recombinant AtSERK1[ins]-CFP-YFP virus.

SDS-PAGE and Western Blotting

Either two or three days after transfection, the cowpea protoplasts or insect cells were lysed in ice-cold HB buffer (50 mM Tris-HCl pH 7.4, 10 mM KAc, 1mM EDTA) supplemented with single strength proteinase inhibitor cocktail (Boehringer). The lysates were passed through a 25 μm needle and centrifuged at 40,000 g for 30 min at 4°C. Thirty μl of 2x SDS sample buffer (100 mM Tris HCl, pH 6.8, 4% SDS, 0.2 % bromophenol blue and 20% glycerol) and dithiothreitol (DTT) was added to lysates as well as to pellets. After boiling for 3 min, the proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane (IMMOBILON) for 3 hrs at 50V. The membranes were washed in TBS-T buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2% Tween-20) and blocked with 5% non-fat dried milk in TBS-T with for 3 hrs at room temperature, washed with TBS-T and incubated with the anti-rabbit GFP antibody (Clontech; 1:100 final dilution) for 1.5 hrs at room temperature. After 3 washes in TBS-T, membranes were incubated with alkaline phosphatase conjugated anti-rabbit antibody (New England Biolabs; 1:1000 final dilution). Membranes were washed in TBS-T, incubated in Western blue substrate for Alkaline Phosphatase detection (Promega) until staining was visible.

Construction and transformation of yeast two hybrid plasmid vectors

The coding sequence of AtSERK1 cDNA without signal sequence (spanning amino acids 31-625), the kinase domain (amino acids 266- 625) and extracellular domain (amino acids 26-234)

were cloned into either PEG202 bait or PJG4-5 prey yeast expression vectors to generate the translational fusions of AtSERK1 domains with either the *E. coli* peptide (B42) as activation domain (AD: pJG 4-5) or with the LexA DNA binding domain (BD: pEG202) obtained from the DuplexA two-hybrid system (Origene Technologies). All constructs were verified by sequence analysis and are shown in schematic form in Fig. 1. Yeast (*Saccharomyces cerevisae*) strain EGY 48 (lacZ) was transformed with the plasmid constructs and an autoactivation assay and a repression assay of the AtSERK1 fusion constructs was performed according to the users manual to determine possible self-activation and proper entry of the proteins into the nucleus respectively. Yeast cells cotransformed with both bait and prey constructs were grown at 30°C for 2 days. Colonies obtained were scored as positive when they could grow in absence of leucine and show β-galactosidase activity within 72 hrs at 30°C.

Confocal microscopy

Surface fluorescence and intracellular fluorescence was analyzed by confocal laser scanning microscopy (CLSM) using a Zeiss LSM 510 microscopy system (Carl-Zeiss) based on an Axiovert inverted microscope equipped with an Argon ion laser as excitation source. Protoplasts were excited at 514 nm and YFP fluorecsence was separated from chlorophyll fluorescence by using a Zeiss 630 dichroic beamsplitter. YFP emission was selectively detected by using bandpass 535-590 nm filter, and chlorophyll fluorescence was detected simultaneously in another detection channel using a 650 nm longpass filter. A 40x oil immersion objective (numerical aperture 1.3) was used for scanning protoplasts with step (pixel) size of 0.2 μm^2 in the xy plane and a step size of 0.4 μm^2 for serial optical sections in the z-axis. The pinhole setting was 40 μm, which yielded a theoretical thickness (full width at half maximum) of approximately 1 μm.

Fluorescence Correlation Spectroscopy

Three days after infection with AtSERK1[ins]-CFP-YFP, measurements on Sf21 cells were performed with a Zeiss-Evotec ConfoCor[®] system based on an Axiovert 100 inverted confocal microscope (Carl-Zeiss). The sample was excited using an Argon ion laser (output 10 mW). The filter block consisted of a 514 (Δ5) excitation filter, a 530 (Δ20) dichroic mirror and a 570 (Δ40) emission filter. The light was focused into the sample (stored in glass-bottomed 96-well plates [Polyfiltronics]) by a water-immersable objective lens (40x, N.A. 1.2). The emitted light passes a

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size-adjustable pinhole (diameter 40 μm) to reject out-of-focus light, and finally hit an avalanche photodiode. Profile scans along the optical (z) axis through the cells were carried out by piezo-driven movement of the objective lens (step size 5 $\mu m \cdot s^{-1}$). Profile scans through the Sf21 insect cells incubated with Bodipy-labeled lipids indicated the position of the plasma membrane and the nucleus in the profile scan.

Fluorescence resonance energy transfer between AtSERK1-CFP and AtSERK1-YFP on membrane associated AtSERK1 receptors

Fluorescence resonance energy transfer (FRET) between the fluorescently labelled AtSERK1 receptors in the plasma membrane was measured by fluorescence spectral imaging microscopy (FSPIM)[60]. Spectral imaging was done using a Leica DMR epifluorescence microscope equipped with a 250IS imaging spectrograph (Chromex) coupled to a CH250 CCD camera (Photometrics) incorporating a back-illuminated SIT502 chip with 512x512 24 μm square pixels. The excitation light source was a 100 W-mercury arc lamp coupled to an excitation filter wheel. Fluorescent spectral images were acquired using a 20x Plan Neofluar objective (NA 0.5), a bandpass excitation filter (Omega), a 430DCLP dichroic mirror (Omega) and a 455 longpass emission filter (Schott). Spectral images were acquired using a 150 groove/mm grating, set at a central wavelength of 500 nm and a slit width of 200 μm corresponding to 10 μm in the object plane. Typical exposure and CCD integration time was 2-5 sec. Data processing and background subtraction was performed as described[61].

Acknowledgements

References

1. Pawson, T. & Bernstein, A. (1990) Receptor tyrosine kinases: genetic evidence for their role in Drosophila and mouse development. *Trends Genet.* **6**, 350-56.

2. Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. & Soltoff, S. (1991) Oncogenes and signal transduction. *Cell* **64**, 281-302.

3. Lease, K., Ingham, E. & Walker, J.C. (1998) Challenges in understanding RLK function. *Curr. Opin. Plant Biol.* **1**, 388-92.

4. Heldin, C.H. (1995) Dimerization of cell surface receptors in signal transduction. *Cell* **80**, 213-23.

5. Bishayee, S., Majumdar, S., Khire, J. & Das, M. (1989) Ligand-induced dimerization of the platelet-derived growth factor receptor: Monomer-dimer occurs independent of receptor phosphorylation. *J. Biol. Chem.* **264**, 11699-705.

6. Westermark, B. & Heldin, C.H. (1989) Growth factors and their receptors. *Curr. Opin. Cell Biol.* **25**, 1279-85.

7. Lemmon, M.A. & Schlessinger, J. (1994) Regulation of signal transduction and signal diversity by receptor oligomerization. *Trends Biochem. Sci.* **19**, 459-63.

8. Schlessinger, J. Signal transduction by allosteric receptor oligomerization. (1988) *Trends Biochem. Sci.* **13**, 443-47.

9. Weiner, D.B., Kokai, Y., Wada, T., Cohen, J.A., Williams, W.V. & Greene, M.I. (1989) Linkage of tyrosine kinase activity with transforming ability of the p185neu oncoprotein. *Oncogene* **4**, 1175-83.

10. Spencer, D.M., Wandless, T.J., Schreiber, S.L. & Crabtree, G.R. (1993) Controlling signal transduction with synthetic ligands. *Science* **262**, 1019-24.

11. Jiang, G. & Hunter, T. (1999) Receptor signalling: when dimerization is not enough. *Curr. Biol.* **9**, 568-71

12. Williams, R.W., Wilson, J.M., & Meyerowitz, E.M. (1997) A possible role of kinase-associated protein phosphatase in the *Arabidopsis* CLAVATA1 signaling pathway. *proc. Natl Acad. Sci.,* USA **94**, 10467-72

13. Giranton, J.L., Dumas, C., Cock, J.M. & Gaude, T. (2000) The integral membrane S-locus receptor kinase of *Brassica* has serine/threonine kinase activity in a membranous environment and spontaneously forms oligomers in planta. *Proc. Natl Acad. Sci.,* U S A. **97**, 3759-64

14. Fletcher, J.C., Brand, U., Running, M.P., Simon, R. & Meyerowitz, E.M. (1999) Signaling of cell fate decisions by CLAVATA3 in Arabidopsis shoot meristems. *Science* **283**, 1911-14.

15. Jeong, S., Trotochaud, A.E. & Clark, S.E. (1999) The *Arabidopsis* CLAVATA2 gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase. *Plant Cell* **11**, 1925-34.

16. Trotochaud, A.E., Jeong, S. & Clark, S.E. (2000) CLAVATA3, a multimeric ligand for the CLAVATA1 receptor-kinase. *Science* **289**, 613-17.

17. He, Z., Wang, Z., Li, J., Zhu, Q., Lamb, C., Ronald, P. & Chory, J. (2000) Perception of brassinosteroids by the extracellular domain of the receptor kinase BRI1. *Science* **288**, 2360-63.

18. Nagayama, Y., Namba, H., Yokoyama, N., Yamashita, S. & Niwa, M. (1998) Role of asparagine-linked oligosaccharides in protein folding, membrane targeting, and thyrotropin and autoantibody binding of the human

thyrotropin receptor. *J. Biol. Chem.* **273**, 33423-28.

19. Trombetta, E.S. & Helenius, A. (1998) Lectins as chaperones in glycoprotein folding. *Curr Opin Struct Biol.,* **8**, 587-92.

20. Elleman,T.C., Frenkel, M.J., Hoyne, P.A., McKern, N.M., Cosgrove, L., Hewish, D.R., Jachno, K.M., Bentley, J.D., Sankovich, S.E. & Ward, C.W. (2000) Mutational analysis of the N-linked glycosylation sites of the human insulin receptor. *Biochem J.* **347**, 771-79.

21. Schmidt, E.D.L., Guzzo, F., Toonen, M.A.J. and de Vries, S.C., A leucine rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos, Development 124 (1997) 2049-2062

22. Hecht, V., Vielle-Calzada, J.P., von Recklinghausen, I., Hartog, M.V., Zwartjes, C. G. M., Schmidt, E. D. L., Boutilier, K., van Lammeren, A. & de Vries, S.C. (2000) The *Arabidopsis Somatic Embryogenesis Receptor Kinase 1* gene during ovule and embryo development (submitted)

23. Gerdes, H.H. & Kaether, C. (1996) Green fluorescent protein: applications in cell biology. *FEBS Lett.* **389**, 44-47.

24. Ludin, B., Doll, T., Meili, R., Kaech, S. & Matus, A. (1996) Application of novel vectors for GFP-tagging of proteins to study microtubule-associated proteins. *Gene* **173**, 107-11.

25. Carey, K.L., Richards, S.A., Lounsbury, K.M. & Macara, I.G., (1996) Evidence using a green fluorescent protein-glucocorticoid receptor chimera that the Ran/TC4 GTPase mediates anessential function independent of nuclear protein import. *J. Cell Biol.* **133**, 985-96.

26. Förster, T. (1948) Intermolecular energy migration and fluorescence. *Ann. Physik.* **6**, 55-75

27. Johnson, I.D., Kang, H.C & Haugland, R.P. (1991) Fluorescent membrane probes incorporating dipyrrometheneboron difluoride fluorophores. *Anal. Biochem.* **198**, 228-37.

28. Rizzuto, R., Brini, M., De Giorgi, F., Rossi, R., Heim, R., Tsien, R.Y. & Pozzan, T. (1996) Double labelling of subcellular structures with organelle-targeted GFP mutants *in vivo. Curr. Biol.* **6**, 183-88.

29. Gadella Jr., T.W.J. , van der Krogt, G. and Bisseling, T. (1999). GFP-based FRET microscopy in living plant cells. *Trends Plant Sci.* **4**, 287-91.

30. Jovin, T.M. & Arndt-Jovin, D.J. (1989) Luminescence digital imaging microscopy.

Annu. Rev. Biophys. Chem. **18**, 271-308.

31. Wu, P. & Brand, L. (1994). Resonance energy transfer: methods and applications. *Anal. Biochem.* **218**, 1-13.

32. Clegg, R.M. (1995) Fluorescence resonance energy transfer. *Curr. Opin. Biotechnol.* **6**, 103-10.

33. Chuck, R.S., Cantor, C.R. & Tse, D.B. (1990) CD4-T-cell antigen receptor complexes on human leukemia T cells. *Proc. Natl Acad. Sci., U S A,* **87**, 5021-25.

34. Vignali, D.A., Carson, R.T., Chang, B., Mittler, R.S. & Strominger, J.L. (1996) The two membrane proximal domains of CD4 interact with the T cell receptor. *J. Ex. Med.* **183**, 2097-107.

35. Guo, C., Dower, S.K., Holowka, D. & Baird, B. (1995) Fluorescence resonance energy transfer reveals interleukin (IL)-1-dependent aggregation of IL-1 type 1 receptors that correlates with receptor activation. *J. Biol. Chem.* **270**, 27562-68.

36. Carraway, K.L. & Cerione, R.A. (1993) Inhibition of epidermal growth factor receptor aggregation by an antibody directed against the epidermal growth factor receptor extracellular domain. *J. Biol. Chem.* **268**, 23860-67.

37. Gadella, T.W. Jr. & Jovin, T.M. (1995) Oligomerization of epidermal growth factor receptors on A431 cells

studied by time-resolved fluorescence imaging microscopy. A stereochemical model for tyrosine kinase receptor activation. *J. Cell Biol.* **129**, 1543-58

38. Olson, T.S., Bamberger, M.J. & Lane, M.D. (1988) Post-translational changes in tertiary and quaternary structure of the insulin proreceptor. Correlation with acquisition of function. *J. Biol. Chem.* **263**, 7342-51.

39. Bass, J., Chiu, G., Argon,Y. & Steiner, D.F. (1998) Folding of insulin receptor monomers is facilitated by the molecular chaperones calnexin and calreticulin and impaired by rapid dimerization. *J. Cell Biol.* **141**, 637-46.

40. High, S., Lecomte, F.J., Russell, S.J., Abell, B.M. & Oliver, J.D. (2000) Glycoprotein folding in the endoplasmic reticulum: a tale of three chaperones? *FEBS Lett.* **476**, 38-41.

41. Ullrich, A. & Schlessinger, J. (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203-12.

42. Li, W. & Schlessinger, J. (1991) Platlet derived growth factor receptor (PDGFR)-Induced Dimerization of PDGF Receptor in living cells. *Mol. Cell Biol.,* **11**, 3756-3761

43. Bai, M., Trivedi, S. & Brown, E.M. (1998) Dimerization of the extracellular calcium-sensing receptor (CaR) on the cell surface of CaR-transfected HEK293 cells. *J. Biol. Chem.* **273**, 23605-10.

44. Fry, A.M., Arnaud, L. & Nigg, E.A. (1999) Activity of the human centrosomal kinase, Nek2, depends on an unusual leucine zipper dimerization motif. *J. Biol. Chem.* **274**, 16304-10.

45. Baxevanis, A.D. & Vinson, C.R. (1993) Interactions of coiled coils in transcription factors: where is the specificity? *Curr. Opin. Genet. Dev.* **3**, 278-85.

46. Leung, I.W. & Lassam, N. (1998) Dimerization via tandem leucine zippers is essential for the activation of the mitogen-activated protein kinase kinase kinase, MLK-3. *J. Biol. Chem.,* **273**, 32408-15.

47. Kester, H.A., Blanchetot, C., den Hertog, J., van der Saag, P.T. & van der Burg, B. (1999) Transforming growth factor-beta-stimulated clone-22 is a member of a family of leucine zipper proteins that can homo and hetero dimerize and has transcriptional repressor activity. *J. Biol. Chem.* **274**, 27439-47.

48. Behncken, S.N., Billestrup, N., Brown, R., Amstrup, J., Conway-Campbell, B. & Waters M.J. (2000) Growth hormone (GH)-independent dimerization of GH receptor by a leucine zipper results in constitutive activation. *J. Biol. Chem.* **275**, 17000-07.

49. Romano, C., Yang, W.L. and O'Malley, K.L. (1996) Metabotropic glutamate receptor 5 is a disulfide-linked dimer. *J Biol. Chem.* **271**, 28612-6. *Biophys. Chem.* **18**, 271-308.

50. Chen, R.H. & Derynck, R. (1994) Homomeric interactions between type II transforming growth factor-beta receptors. *J. Biol. Chem.* **269**, 22868-74

51. Henis, Y.I., Moustakas, A., Lin, H.Y. & Lodish, H.F. (1994) The types II and III transforming growth factor-beta receptors form homo-oligomers. *J. Cell Biol.* **126**, 139-54.

52. Cheatham, B. & Kahn, C.R. (1995). Insulin action and the insulin signaling network. *Endocrine Rev.* **16**, 117-42

53. Trotochaud, A.E., Hao, T., Wu, G., Yang, Z. & Clark, S. E. (1999) The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signaling complex that includes KAPP and a Rho-related protein. *Plant Cell* **11**, 393-406.

54. Schaller, G.E., Ladd, A.N., Lanahan, M.B., Spanbauer, J.M. & Bleecker, A.B. (1995) The ethylene response mediator ETR1 from Arabidopsis forms a disulfide-linked dimer. *J. Biol. Chem.* **270**, 12526-30.

55. Doughty, J., Dixon, S., Hiscock, S.J., Willis, A.C., Parkin, I.A., & Dickinson, H.G. (1998) PCP-A1, a defensin-like Brassica pollen coat protein that binds the S locus glycoprotein, is the product of gametophytic gene expression. *Plant Cell* **10**, 1333-47.

56. Jiang, G., den Hertog, J., Su, J., Noel, J., Sap, J. & Hunter, T. (1999) Dimerization inhibits the activity of receptor-like protein-tyrosine phosphatase-alpha. *Nature* **401**, 606-10.

57. Rezelman, G., van Kammen, A. & Wellink, J. (1989) Expression of cowpea mosaic virus mRNA in cowpea protoplasts. *J. Gen. Virol.* **70**, 3043-50.

58. Rottier, P.J., Rezelman, G. & van Kammen, A. (1979) The inhibition of cowpea mosaic virus replication by actinomycin D. *Virology* **92**, 299-309.

59. Groebe, D.R., Chung, A.E. & Ho, C. (1990) Cationic lipid-mediated co-transfection of insect cells. *Nucl. Acids Res.* **18**, 4033-36

60. Gadella, T.W. Jr., Vereb, G. Jr., Hadri, A.E., Rohrig, H., Schmidt, J., John, M., Schell, J. & Bisseling, T. (1997) Microspectroscopic imaging of nodulation factor-binding sites on living *Vicia sativa* roots using a novel bioactive fluorescent nodulation factor. *Biophys. J.* **72**,1986-96.

61. Goedhart, J. and Gadella Jr., T.W.J. (2000) Advanced microspectroscopic methods for monitoring fluorescent molecules in living plant root hairs. In *Cell and molecular biology of living plant root hairs*. R.W. Ridge and A.M.C. Emons (Eds.), *pp. 65-93*.

5

Physical and functional interactions between the AtSERK1 and KAPP proteins

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Abstract

The AtSERK1 protein is a plasma membrane-located LRR receptor-like serine threonine kinase that is transiently expressed during embryogenesis. To demonstrate *in vitro* interaction between the AtSERK1 and kinase associated protein phosphatase (KAPP) proteins we have expressed different domains of KAPP cDNA and the kinase domain of AtSERK1 as GST fusion proteins in *E. coli*. Our results show that AtSERK1 interacts with KAPP in a phosphorylation dependent manner. The kinase interaction (KI) domain of KAPP does not interact with a catalytically inactive kinase mutant. Using mutant AtSERK1 proteins lacking Thr-462, Thr-463 and Thr-468 in the AtSERK1 A-loop, we show that all three threonine residues are involved in interaction with KAPP whereas only Thr-468 is required for AtSERK1 autophosphorylation. KAPP cDNA was fused to two different variants of GFP, the yellow fluorescent protein (YFP) or the cyan fluorescent protein (CFP), and transiently coexpressed with AtSERK1 cDNA also fused to YFP or to CFP in plant protoplasts. The interaction between the KAPP-CFP/YFP and AtSERK1-CFP/YFP fusion proteins was investigated with fluorescence spectral imaging microscopy (FSPIM). Our results show that AtSERK1-CFP and KAPP-YFP proteins are colocalized at the plasma membrane but only show fluorescence energy transfer (FRET) indicative of physical interaction in intracellular vesicles. These results suggest that KAPP is an integral part of the AtSERK1 endocytosis.

Introduction

Signal transduction pathways that employ transmembrane receptor kinases are involved in the control of various biological processes within the cell. Phosphorylation, catalyzed by the receptor protein kinases and dephosphorylation of the receptor catalysed by phosphatases are essential control elements in receptor mediated signalling. Two major groups, tyrosine kinases and phosphatases, and serine/threonine kinases and phosphatases are distinguished based on their substrate specificity (Cohen, 1989). Phosphatases that dephosphorylate serine and threonine residues are encoded by the PPP and the PPM gene families that are defined by distinct amino acid sequences and crystal structures. The PPP family includes the signature phosphatases, PP1, PP2A and PP2B while the PPM family comprises the Mg^{2+} dependent protein phosphatases that include PP2C and pyruvate dehydrogenase phosphatase (Cohen, 1997).

Plant genes encoding receptor like proteins with cytoplasmic kinase domains are referred to as receptor-like kinases (RLKs). Plant RLKs are classified into several groups based on the structural features of the predicted extracellular domain. To date, leucine rich repeat (LRR)-RLKs comprise the largest class of RLKs (Torii, 2000) and most of them were shown to be serine/threonine kinases. Examples are the *CLAVATA1* (*CLV1*) gene, that controls cell proliferation in the shoot meristem (Clark et al., 1997), *ERECTA* gene, that regulates organ shape (Torii et al., 1996), and the *BRI1* gene, that is involved in brassinosteroid signalling (Li and Chory, 1997). There is also evidence for phosphatases that interact with different RLKs. Many of the RLKs have been shown to interact with a type 2C protein phosphatase called the kinase associated protein phosphatase (KAPP; Stone et al., 1994; Torii, 2000).

KAPP comprises of an amino terminal type 1 signal anchor followed a kinase interaction (KI) domain and a carboxy terminal type 2C-protein phosphatase catalytic domain (Stone et al., 1994). Type 2C- protein phosphatases are characterized by their substrate preference, insensitivity to various protease inhibitors and an absolute requirement for divalent cations (Shenolikar and Nairn, 1991). The physical interaction between KAPP and the RLKs is thought to be important for their roles in signal transduction (Li et al., 1999). Both genetic and biochemical approaches have shown that KAPP interacts *in vivo* with RLKs suggesting that KAPP negatively regulates

RLK signalling (Williams et al., 1997; Stone et al., 1998). The reduction of KAPP transcript accumulation in an intermediate *clv1* mutant shows the suppression of the mutant phenotype, the degree of suppression being inversely correlated with KAPP mRNA levels. KAPP also co-immunoprecipitates with CLV1 in plant extracts derived from meristematic tissue (Trotochaud et al., 1999). KAPP has also been shown to interact *in vitro* with the CLV1 kinase domain (Williams et al., 1997), the HAESA kinase domain (Stone et al., 1994) and the KIK1 kinase domain (Braun et al., 1997). The KI domain of KAPP binds RLKs *in vitro* in a phosphorylation dependant manner and does not bind kinase inactive mutants of RLKs.

Somatic embryogenesis is the process whereby somatic cells can develop into plants via characteristic morphological stages. In cultured *Daucus carota* suspension cells, the *SERK* (*Somatic Embryogenesis Receptor Kinase*) gene encoding a leucine rich repeat (LRR)-RLK, was found to be a marker for single somatic cells capable of forming embryos (Schmidt et al., 1997). During somatic and zygotic embryogenesis *SERK* expression continues up to the early globular stage and is absent in later stages of embryo development. The most closely related *Arabidopsis AtSERK1* gene encodes a receptor kinase, that is expressed during ovule development and early embryogenesis (Hecht et al., 2001 submitted) and like other known LRR-RLKs, such as *CLAVATA1* and *HAESA* has serine/threonine kinase activity (Shah et al., 2001a, submitted). The predicted AtSERK1 protein contains an N-terminal leucine zipper (LZ), 5 leucine rich repeats (LRRs) and a proline-rich region in its predicted extracellular domain. This is followed by a single transmembrane domain that is attached to an intracellular kinase domain with all eleven conserved sub-domains found in serine/threonine kinases (Shah et al., 2001a, submitted). In our previous studies with affinity purified AtSERK1-GST fusion proteins, we have shown that Thr-468 in the AtSERK1 A-loop, comprising of the amino acids from sub-domain VII and VIII, is essential for transphosphorylating common substrates like casein and MBP (Shah et al., 2001a, submitted).

Different engineered spectral variants of green fluorescent protein (GFP), exhibiting the yellow or the cyan fluorescence, (YFP and CFP respectively) provide an opportunity to label the proteins with two different fluorophores within the same living cell (Rizzuto et al., 1996). These variants retain their characteristic fluorescence when fused to other protein sequences, allowing

fluorescence microscopy to visualize the GFP-fusion protein localization (Gerdes et al., 1996; Ludin et al., 1996, Carey et al, 1996) and the interaction between two proteins by fluorescence resonance energy transfer (FRET; Sorkin et al., 2000) in intact cells. FRET is a quantum mechanical process by which the excitation energy is transferred from a donor fluorophore to an appropriate acceptor fluorophore. FRET only occurs when (i) the donor fluorophore emission spectrum overlaps with the absorption spectrum of the acceptor fluorophore, (ii) the transition dipole moments of the donor and the acceptor are not perpendicularly oriented and (iii) the distance between the donor and the acceptor is less than 1.5x the Forster radius (R_0) energy transfer. The emission and the excitation spectrum of the two variants of GFP are suitable for FRET allowing to probe protein molecular proximity in intact living cells. FRET is manifested in different ways: a decrease in donor fluorescence quantum yield determined by FSPIM, a decreased donor fluorescence lifetime, and an increased stability of the donor towards chemical photo bleaching (Jovin, 1989) and an increased (sensitized) emission acceptor fluorescence emission (if the acceptor is a fluorophore). In our previous studies with AtSERK1-YFP/CFP proteins using FSPIM, we have shown that AtSERK1 is expressed mainly as a monomer at the plasma membrane (Shah et al., 2001b in press).

In this study we have asked if the KAPP protein could be involved in AtSERK1 signalling. To answer this question we used affinity purified AtSERK1 and KAPP proteins to demonstrate the *in vitro* interaction between the two proteins. We have shown that the interaction between the AtSERK1 and KAPP proteins is dependent on the phosphorylation status of the threonine residues in the AtSERK1 A-loop. In addition, to demonstrate the co-localization and the physical interaction between the AtSERK1 and KAPP proteins in live plant cells, we have also fused the whole AtSERK1 cDNA, the whole KAPP cDNA, different domains of AtSERK1 cDNA and KAPP cDNA to two different variants of GFP, CFP and YFP and expressed the chimaeric genes transiently in plant protoplasts. The data here show that AtSERK1-YFP and KAPP-CFP proteins are co-localized and only show FRET in intracellular vesicles and not in the plasma membrane.

Results

Interaction between AtSERK1 and KAPP proteins

In this study AtSERK1-GST, KAPP-GST, AtSERK1-YFP/CFP and KAPP-YFP/CFP constructs were used. These constructs are shown in detail in Fig. 1.
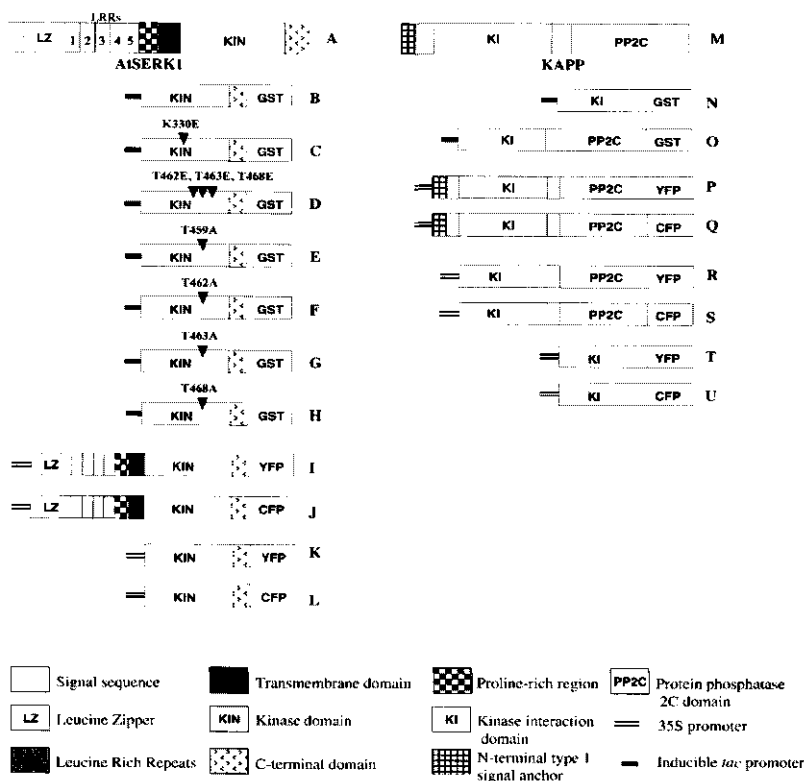
Figure 1. Plasmid constructs used in this study. Different domains of AtSERK1 (A) and KAPP (M) (Stone et a 1994). AtSERK1-GST fusion (B-H) and KAPP-GST fusion (N-O) constructs used for expression in *E. coli*. AtSERK1-CFP/YFP fusion (I-L) and KAPP-CFP/YFP fusion (P-U) constructs used for transient expression in plant protoplasts. The AtSERK1-GST transgenes were constructed in the PGEX-4T1 vector under the control of the inducible *tac* promoter. AtSERK1[kin] (B) contains cDNA encoding for the intracellular kinase domain of AtSERK1 as a N-terminal fusion with the GST. Site directed mutagenesis in the AtSERK[kin] construct generated mutant constructs

(C-H). AtSERK1^{K330E} (C) AtSERK1$^{3T\rightarrow E}$ (D) AtSERK1^{T459A} (E) AtSERK1^{T462A} (F) AtSERK1^{T463A} (G) AtSERK1^{T468A} (H). Each mutation is mentioned on top of the construct. The KAPP-GST transgenes were constructed similarly. The KAPP^{KI} (N) encodes only the kinase interaction domain of KAPP fused to GST. KAPP^{-SA} (O) contains cDNA encoding for the full length KAPP missing only the N-terminal signal anchor fused to GST. The AtSERK1-YFP/CFP transgenes were constructed in the PMON999 vector under the control of the 35S promoter. AtSERK1-YFP/CFP (I, J) contains cDNA encoding for the full length AtSERK1 as a C-terminal fusion with the YFP/CFP genes. AtSERK^{kin}-YFP/CFP encodes only the intracellular kinase domain of AtSERK1 fused to YFP/CFP (K, L). The KAPP-YFP/CFP transgenes were constructed similarly. KAPP-YFP/CFP (P, Q) contains cDNA encoding for the full length KAPP as a C-terminal fusion with the YFP/CFP genes. KAPP^{-SA}-YFP/CFP (R, S), the same as P and Q but missing the N-terminal signal anchor. KAPP^{KI}-YFP/CFP (T, U) encode only the kinase interaction domain of KAPP fused to YFP/CFP.

To determine whether the KAPP KI domain biochemically and physically interacted with AtSERK1 *in vitro*, both the kinase domain of AtSERK1 and the KI domain of KAPP were expressed as GST fusion proteins in *E. coli*. The affinity purified AtSERK1^{kin} and KAPP^{KI} fusion proteins were then used in phosphorylation assays (Fig. 2A). Increasing phosphosphorylation of AtSERK1^{kin} was seen on increasing its concentration (Fig. 2A, lanes 1-3). KAPP^{KI} was phosphorylated in the presence of AtSERK1^{kin} protein (Fig. 2A, lanes 4-8). Since both proteins are substrates for phosphorylation, the level of phosphorylation was determined as a function of protein concentration (Fig. 2A). Above an approximately equimolar ratio, increasing amounts of KAPP^{KI} led to an increase in the phosphorylation of KAPP^{KI} and a decreased phosphorylation of AtSERK1^{kin} (Fig. 2A, lane 6 compared with lane 3). The KAPP^{KI} phosphorylation was less affected when the AtSERK1^{kin} protein concentration was decreased in the incubation mix (compare Fig. 2A, lanes 7 and 8 with lanes 4 and 5). KAPP^{KI} protein was unable to phosphorylate alone (Fig. 2A, lane 9). This demonstrates that KAPP is a substrate for AtSERK1^{kin} and that the two proteins biochemically interact *in vitro*.
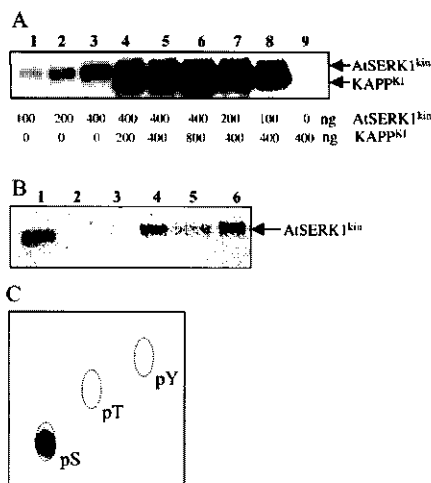
Figure 2. *In vitro* KAPP interactions with AtSERK1^{kin}. **(A)** Bacterially produced, affinity- purified AtSERK1^{kin} and KAPP^{KI} proteins were purified and aliquots ranging from 0-800 ng were used. Different concentrations of AtSERK1^{kin} (top) and KAPP^{KI} (bottom) proteins were incubated with [γ-^{32}P] ATP as described in Materials and methods. After separation on 10% SDS-PAGE, the gels were autoradiographed using a PhosphoImager. **(B)** Immobilized KAPP^{KI} protein was phosphorylated with 10 units of bovine heart muscle kinase (Sigma) in the presence of [γ-^{32}P] ATP. The resulting eluent was used to probe the nitrocellulose filter containing AtSERK1^{kin} (lane 1), AtSERK1^{K330E} (lane 2), AtSERK1$^{3T \rightarrow E}$ (lane 3), AtSERK1^{kin} treated with alkaline phosphatase inhibitor (API) (lane 4), AtSERK1^{kin} treated with alkaline phosphatase (AP) (lane 5) and AtSERK1^{kin} treated with AP and API (lane 6) and then allowed to phosphorylate in presence of ATP and MgCl$_2$. The filters were autoradiographed using a PhosphoImager. **(C)** Phosphoaminoacid analysis of the HCl hydrolyzed KAPP^{KI} protein phosphorylated by AtSERK^{kin}. The hydrolyzed protein was spotted onto a TLC plate and resolved by ascending chromatography in two dimensions. The autoradiograph of the phosphorylated amino acids and the position of the standard amino acids visualized by 0.25% ninhydrin spray, are phosphoserine (pS), phosphothreonine (pT) and phosphotyrosine (pY) respectively.

AtSERK1 A-loop that is formed from sub-domains VII and VIII has three threonine residues that are potential phosphorylation sites (Shah et al., 2001a submitted). To demonstrate if the interaction between AtSERK1^{kin} and KAPP^{KI} is phosphorylation dependent, we tested the

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AtSERK1^{kin}, the catalytically inactive AtSERK1^{K330E} and the AtSERK1$^{3T \rightarrow E}$ mutant proteins in which three threonine residues in the AtSERK1 A-loop have been mutated, for their association with KAPP^{KI} by *in vitro* binding assays. The AtSERK^{kin} proteins were incubated in the presence of unlabeled ATP and transferred after electrophoresis onto a membrane and incubated with [γ ^{32}P] ATP labelled KAPP^{KI} probe. AtSERK1^{kin} (Fig. 2B, lane 1) interacted with KAPP^{KI} probe and there was no interaction observed between the KAPP^{KI} and the AtSERK1^{K330E} and AtSERK1$^{3T \rightarrow E}$ mutant (Fig. 2B, lane 2 and lane 3). To further demonstrate that the interaction between AtSERK1^{kin} and KAPP^{KI} was dependent on the phosphorylation state of the AtSERK1^{kin} protein, the AtSERK1^{kin} was treated with alkaline phosphatase (AP) alone or with AP in addition to the AP inhibitor (API) and incubated in the presence of unlabeled ATP. KAPP^{KI} showed a reduced interaction with the dephosphorylated (AP treated) AtSERK1^{kin} (Fig. 2B, lane 5) as compared to the phosphorylated AtSERK1^{kin} (Fig. 2B, lane 1) or both the AP and API treated AtSERK1^{kin} (Fig. 2B, lane 6). The phosphoaminoacid analysis performed on the KAPP^{KI} phosphorylated by AtSERK1^{kin} protein showed that it was phosphorylated on serines (Fig. 2C).

These results show that KAPP^{KI} interacts with AtSERK1^{kin} in a phosphorylation dependent manner. Furthermore, the threonine residues in the AtSERK1 A-loop seem to play an essential role in the transphosphorylation of the KAPP^{KI} protein.

In our previous work, we have shown that the effect on the phosphorylation activity was similar when AtSERK1 A-loop threonines were mutated to either glutamic acid residues or to alanine residues (Shah et al., 2001a, submitted). To investigate the role of individual threonine residues in the AtSERK1 A-loop in AtSERK1^{kin}-KAPP^{KI} transphosphorylation, all four threonine residues in the AtSERK1 A-loop, Thr-459, Thr-462, Thr-463 and Thr-468 were mutagenized individually to alanine residues. The transphosphorylation assays with affinity purified KAPP^{KI} and AtSERK1^{kin} threonine mutant proteins showed that there was a reduced transphosphorylation of KAPP^{KI} when incubated with AtSERK1^{T459A} (Fig. 3A, lane 2) as compared to KAPP^{KI} phosphorylation by AtSERK1^{kin} (Fig. 3A, lane 1). KAPP^{KI} was not phosphorylated when incubated either with AtSERK1^{T462A} (Fig. 3A, lane 3) or AtSERK1^{T463A} (Fig. 3A, lane 4) or AtSERK1^{T468A} (Fig. 3A, lane 5). The transphosphorylation assays with AtSERK1$^{3T \rightarrow E}$ (Fig. 3A, lane 6) and AtSERK1^{K330E} (Fig. 3A, lane 7) show comparable results as AtSERK1^{T468A}.

These results show that Thr-468 in the AtSERK1 A-loop has a major role in both the autophosphorylation activity of AtSERK1 and transphosphorylation activity on the KI domain of

KAPP. This confirms previous studies using casein and myelin basic protein (MBP) as artificial transphosphorylation targets for AtSERK1 (Shah et al., 2001a, submitted). In addition Thr-463 also influence the transphosphorylation of the KI domain of KAPP. This is a different observation than seen with AtSERK1 transphosphorylation on casein and MBP where only Thr-462 and Thr-468 has influence on the transphosphorylation. (Shah et al., 2001a, submitted). This observation implies the role of other threonine residues in the AtSERK1 A-loop in substrate phosphorylation.
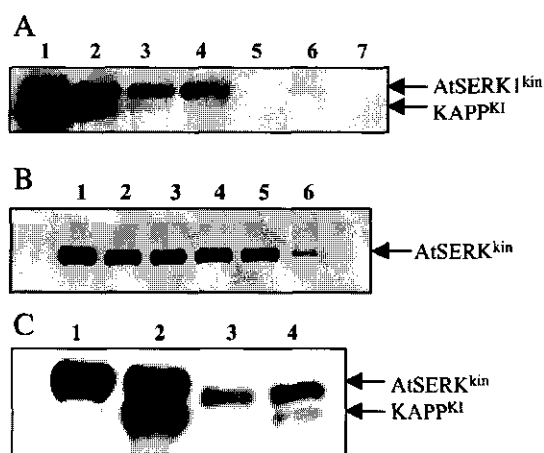
Figure 3. (A). KAPP interactions with AtSERK1^{kin} mutant proteins. 500 ng of affinity purified either AtSERK1^{kin} or AtSERK1^{kin} mutant proteins were incubated with KAPP^{KI} protein in the presence of [γ-^{32}P] ATP in a phosphorylation assay as described in Materials and methods. After separation on 10% SDS-PAGE, the gels were autoradiographed using a PhosphoImager. AtSERK1^{kin} and AtSERK1^{kin} mutants (top) and KAPP^{KI} (bottom) proteins are shown. AtSERK1^{kin} (lane 1), AtSERK1^{T459A} (lane 2), AtSERK1^{T462A} (lane 3), AtSERK1^{T463A} (lane 4), AtSERK1^{T468A} (lane 5), AtSERK1$^{3T \rightarrow E}$ (lane 6) and AtSERK1^{K330E} (lane 7). **(B).** Dephosphorylation of AtSERK1 by KAPP protein. Matrix bound AtSERK1^{kin} was phosphorylated by [γ-^{32}P] ATP as described in Materials and methods. The phosphorylated AtSERK1^{kin} (lane 1) was incubated with KAPP^{-SA} protein and the aliquots were taken after the time intervals of 30, 60, 90, 120 and 240 min (lanes 2-6). After separation on 10% SDS-PAGE, the gels were autoradiographed using a PhosphoImager. (C) Phosphorylation of a mix of 500 ng of each AtSERK1^{kin} and KAPP proteins in presence of [γ-^{32}P] ATP was performed as described in Materials and methods. AtSERK1^{kin} was incubated either alone (lane 1) or with KAPP^{KI} (lane 2) or with KAPP^{-SA} (lane 3), or with both KAPP^{KI} and KAPP^{-SA} proteins (lane 4). The autoradiographs are shown.

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To determine the protein phosphatase catalytic activity of KAPP, the full length KAPP cDNA missing only the N-terminal signal anchor was fused to GST and expressed in *E. coli*. The affinity purified KAPP protein (KAPP^{-SA}) was incubated with matrix bound [γ^{32}P] ATP phosphorylated AtSERK1^{kin} and aliquots were taken out at different time intervals (Fig. 3B, lanes 2-6). The phosphorylated ASERK1^{kin} was dephosphorylated by KAPP^{-SA} with increased incubation time (Fig. 3B, lanes 2-5). Phosphorylated AtSERK1^{kin} protein was almost completely dephosphorylated by KAPP^{-SA} after 4 hrs (Fig 3B, lane 6). We were also interested in knowing if the AtSERK1^{kin} protein would phosphorylate in presence of KAPP^{-SA} protein. This led us to performing experiments where we incubated affinity purified AtSERK1^{kin} alone (Fig. 3C, lane 1) with either KAPP^{KI} (Fig. 3C, lane 2) or with KAPP^{-SA} (Fig. 3C, lane 3) or with both KAPP^{KI} and KAPP^{-SA} proteins in the presence of [γ^{32}P] ATP. In the presence of the KAPP^{-SA} protein, there was a reduced phosphorylation of AtSERK1^{kin} (Fig. 3C, lane 3) or both AtSERK1^{kin} and KAPP^{KI} (Fig 3C, lane 4). These results demonstrate that KAPP protein not only dephosphorylates AtSERK1 protein but also actively prevents autophosphorylation of the receptor, suggesting that the KAPP protein can maintain the dephosphorylated state of AtSERK1.

Colocalization of AtSERK1 and KAPP proteins in living plant cells

Previously, we showed that AtSERK1 is a plasma membrane-localized protein (Shah et al., 2001b in press). While AtSERK1 and KAPP interact in vitro, it was of interest to determine if the two proteins were co-localized and could also interact in plant cells. For this we used transient expression in plant protoplasts of two different variants of the green fluorescent protein (GFP), the cyan fluorescent protein (CFP) and the yellow fluorescent protein (YFP) that were fused to the C terminal end of AtSERK1 and KAPP protein. The different fusion constructs employed are shown (Fig. 1, 1-L, P-U).

KAPP comprises of an amino terminal hydrophobic segment followed by a number of positively charge residues, which indicate that it may function as a type 1 signal anchor (SAI). An SAI topogenic signal acts as an uncleaved signal peptide resulting in cytoplasmic orientation of the membrane-anchored protein (Stone et al., 1994). To study the localization of the KAPP protein in plant protoplasts we performed transient transfections with KAPP^{KI}-YFP, KAPP^{-SA}-YFP and KAPP-YFP constructs. After transfection and incubation for 12 hrs, total protein was isolated, fractionated by denaturing SDS-PAGE and immunoblotted using an antiserum against GFP. The

results show that all KAPP-YFP/CFP fusion proteins of the correct size are expressed in plant protoplasts (Fig. 4A). The protoplasts expressing the KAPP^{KI}-YFP protein showed that it was mostly localized in the cytoplasm (Fig. 4B). The protoplasts expressing the KAPP-YFP proteins showed the expression of KAPP-YFP protein in the plasma membrane as well as in the cytosol (Fig. 4C). When the protoplasts were transfected with KAPP^{-SA}-YFP, a construct in which the KAPP protein lacking the SAI signal peptide, the KAPP^{-SA}-YFP protein was observed in the cytoplasm compartments (Fig. 4D). This shows the importance of the KAPP-SAI peptide in membrane-anchoring of the KAPP protein.
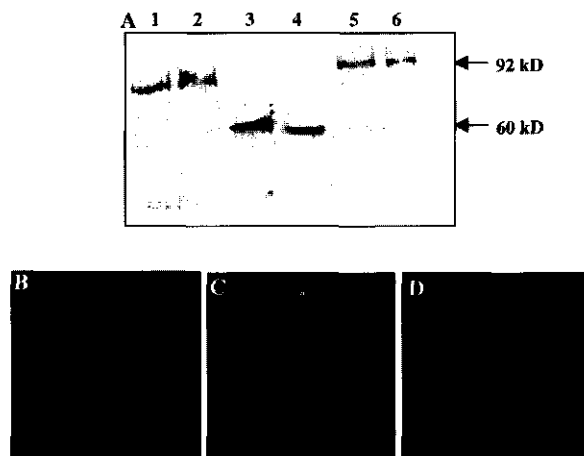
Figure 4. KAPP-CFP/YFP fusion proteins (**A**). Immunoblot of KAPP fusion proteins in cowpea mesophyll protoplasts. The protoplasts were harvested after 12 hrs of transfection with KAPP-CFP/YFP transgenes, lysed and separated on 10% reducing SDS-PAGE. Proteins transferred to nitrocellulose paper were immunoblotted with anti-GFP antibody. KAPP^{-SA}-YFP (lane 1), KAPP^{-SA}-CFP (lane 2), KAPP^{KI}-YFP (lane 3), KAPP^{KI}-CFP (lane 4), KAPP -YFP (lane 5) and KAPP-CFP (lane 6). (**B-D**) Localization of KAPP proteins in cowpea mesophyll protoplasts. Chlorophyll autofluorescence is shown in red and the YFP fluorescence is shown in green. Confocal fluorescence images of protoplasts transfected with KAPP^{KI}-YFP (**B**) KAPP-YFP (**C**) and KAPP-^{SA}-YFP (**D**).

In our previous studies with AtSERK1-YFP fusion protein in plant protoplasts we have shown that most of the AtSERK1-YFP protein is found in the plasma membrane whereas a very small amount of protein was also found in the cytoplasm (Shah et al, 2001b in press). In Fig. 5A, a protoplast expressing the AtSERK-CFP is shown. CFP is represented by the cyan colour and confirms the previously noted location of the AtSERK1 protein. To study the colocalization of

the KAPP and AtSERK1 proteins, protoplasts were cotransfected with AtSERK1-CFP and KAPP-YFP constructs. For comparison the chlorophyll autofluorescence is shown in red (Fig. 5B), while the same protoplast was cotransfected an ER marker (ER-YFP) that contains a C-terminal HDEL-ER retention signal fused to YFP (Fig. 5C).
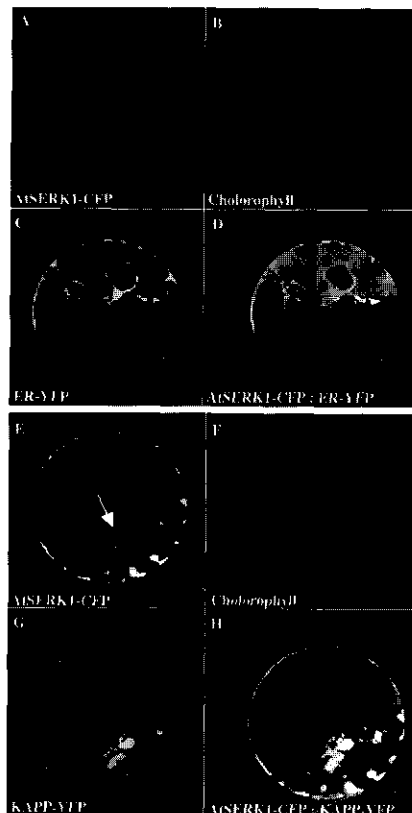
Figure 5. Co-localization of AtSERK1 and KAPP proteins in cowpea mesophyll protoplasts. **(A-D)** Confocal images of the protoplasts cotransfected with AtSERK1-CFP and ER-YFP. The two fluorophores, CFP and YFP were simultaneously visualized. Chlorophyll autofluorescence is shown in red (B) AtSERK1-CFP localization is shown in cyan (A). ER-YFP localization is shown in green (C). The superimposed images of A and C result in D. **(E-H)** Confocal images of the protoplasts cotransfected with and KAPP-YFP and AtSERK1-CFP. The two fluorophores, CFP and YFP were simultaneously visualized. Chlorophyll autofluorescence is shown in red (F) Colocalization is shown in bluish green fluorescence (H) resulting from the overlap of cyan (AtSERK1-CFP) (E) and green (KAPP-YFP) (G).

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From the combined image (Fig. 5D), it is clear that ER-YFP and AtSERK1-CFP have a different localization in the protoplast. AtSERK1-CFP is seen mainly in the plasma membrane while the ER at this stage (12 hrs after transfection) is completely depleted of the AtSERK1 protein. The ER-YFP localization is visible as a punctate pattern (Fig. 5C) that covers most of the cytoplasm-containing area and extends right up to the plasma membrane. It is notably extended from the choloroplasts and the vacuole. However a striking difference was noted when KAPP and AtSERK1 proteins were coexpressed (Fig. 5E-H). Substantial amounts of both proteins now appear internally. While AtSERK1 is found in the areas that coincide with the presence of ER membranes at the periphery, there is also substantial CFP fluorescence in a somewhat diffuse pattern almost in the centre of the cell (arrow in Fig 5E). The KAPP protein appears to be found mainly in this diffuse pattern and less in the peripheral areas. Comparing Fig. 5C with fig. 5G suggests that the diffuse punctate pattern observed represents an intracellular vesicle such as found in the endosomal compartment that is quite different from ER membrane system. Including location of the choloroplasts (Fig. 5F) in the superimposed image (Fig. 5H), it is evident that KAPP and AtSERK1 signals only substantially overlap in the diffuse central area and in the membrane. This suggests that the colocalized AtSERK1-CFP/KAPP-YFP proteins present intracellularly might represent an internalized AtSERK1-KAPP protein complex.

We conclude that in plant cells, KAPP and AtSERK1 are indeed found in the same location, suggesting that they may be inserted into the same signalling complex.

Fluorescence Energy Resonance Transfer studies

To directly study the physical interaction between AtSERK1 and KAPP proteins in living plant cells, the CFP and YFP fusion proteins were used as a donor-acceptor pair in FRET studies. All constructs used in this study are described in Fig. 1. Our experiments were based on fluorescence spectral imaging microscopy (FSPIM) as a detection system. Spectral images were taken from small regions of the protoplasts coexpressing YFP and CFP fusion proteins and a fluorescence emission spectrum corrected for autofluorescence was then generated. In case of FRET, the CFP fluorescence will be quenched and the YFP fluorescence will be increased (sensitized).

For FRET to be detected reliably using FSPIM, certain prerequisites must be satisfied. These are that donor and acceptor fluorophores must be present in a ratio of 1:1, the absorption of the CFP fluorescence by chlorophyll should be minimized, fusion proteins should be properly targeted and finally the fluorophores must be oriented so as to allow close proximity when the target protein fused to the CFP/YFP interact. We performed FRET experiments with the AtSERK1, AtSERK1^{kin}, KAPP and KAPP^{KI} fusion proteins fused either to the donor (CFP) or the acceptor (YFP) molecules. In all experiments where FRET was observed, similar changes in the fluorescence intensity ratios were observed regardless of the donor CFP being fused to AtSERK1 or to the KAPP (Fig 6 and Fig. 7). This indicates that the observed increased donor/acceptor ratio is due the energy transfer between the CFP and the YFP molecules fused to AtSERK1 or KAPP proteins and not due to an unbalanced expression ratio. To avoid interference by chlorophyll absorption, FRET measurements were performed exclusively over the regions lacking chloroplasts. As the fluorophore orientation factor cannot be corrected for, any results showing absence of FRET may not completely guarantee that no interaction occurred between those proteins.

Protoplasts were first cotransfected with either AtSERK1^{kin}-CFP and KAPP^{KI}-YFP or AtSERK1^{kin}-YFP and KAPP^{KI}-CFP constructs. The equal expression of the CFP and YFP fusion proteins in protoplasts was demonstrated by immunoblotting solubilised proteins using anti GFP antisera (Fig. 6A). The spectral images were recorded from the different regions of the protoplasts coexpressing AtSERK1^{kin}-CFP and KAPP^{KI}-YFP fusion proteins. The YFP/CFP fluorescence intensity ratio deduced from these spectra is over 1.5 in every 4 out of 5 measurements (Fig. 6B). The YFP/CFP intensity ratio obtained from AtSERK1^{kin}-YFP and KAPP^{KI}-CFP cotransfected protoplasts was similar to the YFP/CFP intensity ratio shown by AtSERK1^{kin}-CFP and KAPP^{KI}-YFP coexpressing protoplasts (Fig. 6C). When the spectra were recorded from 30 protoplasts each co-expressing AtSERK1^{kin}-CFP and KAPP^{KI}-YFP or AtSERK1^{kin}-YFP and KAPP^{KI}-CFP, the increased YFP/CFP fluorescence intensity ratio of more than 1.5. was seen in about 80% of the measurements (data not shown). These results show that when the AtSERK1^{kin} and KAPP^{KI} proteins are in the cytoplasm, they interact physically, supporting the *in vitro* experiments performed on bacterially expressed proteins (see Fig. 2A)
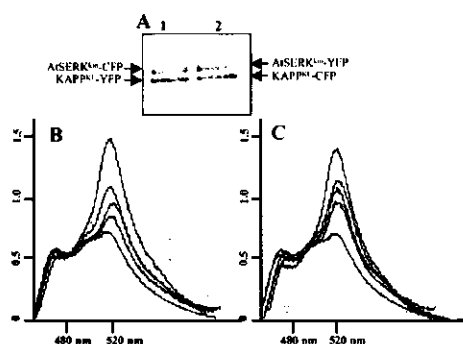
Figure 6. Immunoblot and FSPIM analysis of fluorescent AtSERK1^{kin}-CFP/YFP and KAPP^{KI}-CFP/YFP fusion proteins. **(A)** Immunoblot of proteins extracted from the protoplasts cotransfected with AtSERK1^{kin}-CFP and KAPP^{KI}-YFP (lane 1) and AtSERK1^{kin}-YFP and KAPP^{KI}-CFP (lane 2) **(B)** Emission spectra of the AtSERK1^{kin}-CFP and KAPP^{KI}-YFP proteins coexpressed in protoplasts. The spectra were recorded at a position where CFP/YFP colocalization was observed in the cytoplasm. The X-axis represents the wavelengths of the CFP and YFP fluorophores and the Y-axis represents their intensities. The emission wavelengths of both CFP and YFP are shown below the graph. **(C)** the same as B, for AtSERK1^{kin}-YFP and KAPP^{KI}-CFP expressing protoplasts.

To determine whether physical interaction occurs between, KAPP-CFP/YFP and the AtSERK1-CFP/YFP proteins, we cotransfected protoplasts with either KAPP-CFP and AtSERK1-YFP or KAPP -YFP and AtSERK1-CFP constructs. The immunoblot of the coexpressed proteins using anti-GFP antisera is shown in Fig. 7A. The near identical size of the AtSERK1-CFP/YFP and KAPP-CFP/YFP fusion proteins prevents clear distinction between two proteins on the blot. However, no gross unbalance in expression ratios is evident. We separated the FRET experiments performed on the proteins that were coexpressed at the plasma membrane and the proteins that were present intracellularly. An example of an area that was selected for recording is shown in Fig. 7B where AtSERK1 and KAPP are colocalized in the plasma membrane (shown in Fig. 7B by an arrowhead) and in vesicular structures (shown in Fig. 7B by an arrow). These vesicular structures are present in the cytoplasm and are distinct from the ER as shown in Fig. 5F and may represent endosomes.
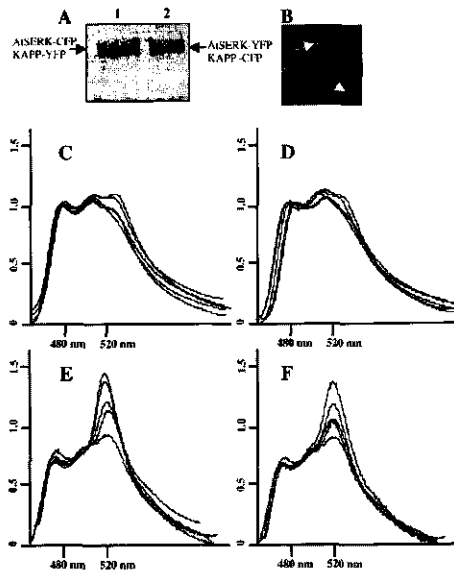
Figure 7. Immunoblot and FSPIM analysis of fluorescent AtSERK1-CFP/YFP and KAPP-CFP/YFP fusion proteins. (A) Immunoblot of proteins extracted from the protoplasts cotransfected with AtSERK1-CFP and KAPP-YFP (lane 1) and AtSERK1-YFP and KAPP-CFP (lane 2) (B) Confocal image of a protoplast cotransfected with AtSERK1-CFP and KAPP-YFP. The two fluorophores, CFP and YFP were simultaneously visualized and the superimposed images are shown. The arrow points to vesicular structures where AtSERK1-YFP and KAPP-CFP are colocalized. The arrowhead points to the plasma membrane where AtSERK1-YFP and KAPP-CFP are colocalized. (C) Emission spectra of the AtSERK1-CFP and KAPP-YFP proteins obtained from the plasma membrane of coexpressed protoplasts. The spectra were recorded at a position where CFP/YFP colocalization was observed in the plasma membrane. The X-axis represents the wavelengths of the CFP and YFP fluorophores and the Y-axis represents their intensities. The emission wavelengths of both CFP and YFP are shown below the graph. (D) the same as C, for AtSERK1-YFP and KAPP-CFP expressing protoplasts. (E) Emission spectra of the AtSERK1-CFP and KAPP-YFP proteins obtained from the coexpressed proteins in the intracellular vesicles of protoplasts. The spectra were recorded at a position where CFP/YFP colocalization was observed intracellularly (F) the same as E, for AtSERK1-YFP and KAPP -CFP expressing protoplasts.

In Fig. 7C, spectra are shown that were obtained from protoplasts coexpressing KAPP-YFP and AtSERK1-CFP at the plasma membrane. The YFP/CFP fluorescence intensity ratio deduced from these spectra was close to 1.0 in all of the measurements suggesting that no interaction

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occurs between AtSERK1 and KAPP at the plasma membrane. The same fluorescence intensity ratios of 1.0 were obtained when protoplasts were cotransfected with KAPP-CFP and AtSERK1-YFP (Fig. 7D). In about 95% of the protoplasts, the YFP/CFP fluorescence intensity ratio was close to 1.0 when the spectra were recorded at the plasma membrane from 30 protoplasts coexpressing either AtSERK1-CFP and KAPP-YFP proteins or AtSERK1-YFP and KAPP-CFP proteins (data not shown). However when the spectra were recorded on the AtSERK1-CFP and KAPP-YFP proteins (Fig. 7E) and AtSERK1-YFP and KAPP-CFP proteins (Fig. 7F) present intracellularly, there was a clear shift in the YFP/CFP fluorescence intensity ratio between 1.3 and 1.5 in 4 out of 5 measurements. About 80% of the protoplasts showed an increased YFP/CFP fluorescence intensity ratio of about 1.4 when the spectra were recorded from 30 protoplasts coexpressing either AtSERK1-CFP and KAPP-YFP proteins or AtSERK1-YFP and KAPP-CFP proteins intracellularly (data not shown).

Taken together, we conclude from these results that the kinase domain of AtSERK1 and kinase interaction domain of KAPP protein can physically interact in the cytoplasm but also do so when they are attached to their respective complete proteins as present in the internalized vesicles. Notably, no interaction appears to take place when both KAPP and AtSERK1 are present in the plasma membrane.

Discussion

AtSERK1 encodes a receptor kinase that is expressed during ovule development and early embryogenesis (Schmidt et al., 1997; Hecht et al, 2001 submitted) and acts as a marker for the transition of non-embryogenic to embryogenic cells in culture (Somleva et al., 2000). The striking similarity of the AtSERK1 intracellular domain with other known RLKs that interact with KAPP (Stone et al., 1994; Braun et al., 1997; Trotochaud et al., 1999) served as the basis of this study.

The major goals of this work were to determine the interaction between the AtSERK1 and KAPP using both an *in vitro* and *in vivo* approaches. The results show that the kinase domain of AtSERK1 and the kinase interaction domain of KAPP interact in a phosphorylation dependent manner. Furthermore it appears that the KAPP and AtSERK1 proteins are colocalized in plant

cells at the plasma membrane and in the intracellular vesicles, but only interact physically intracellularly.

In animals it has been shown that a common feature of protein-protein interactions is the binding to consensus motifs containing phosphorylated residues (Yaffe et al., 1997). Phosphorylation/dephosphorylation events on plant RLKs are likely to be an important element in determining physical interaction. The KAPP protein was originally found by screening *Arabidopsis* cDNA expression library for proteins interacting with the protein kinase catalytic domain of RLK5 (HAESA) (Stone et al., 1994). Subsequently it was postulated to function as a negative regulator of the CLV1 signal transduction pathway (Williams et al., 1995). KAPP is expressed throughout the shoot apical meristem (SAM) and in stage 1-2 flowers but not in stage 3 flowers, thus encompassing the region of CLV1 expression in the central cells of the SAM and young floral meristems (Williams et al., 1997). KAPP has been shown to interact with many, but not all phosphorylated RLKs (Braun and Walker, 1997), which implies that it recognizes a particular sequence on the target kinases. In our previous studies with the AtSERK1 protein, we have shown that AtSERK1 activation requires intermolecular autophosphorylation. This presumably results in movement of the AtSERK1 A-loop followed by release of transphosphorylation activity and protein substrate binding. In this model, A-loop phosphorylation of Thr-462 and Thr-468 of one AtSERK1 monomer is also essential for releasing catalytic activity of that same monomer. The results show that the phosphorylation status of Thr-468 is essential for AtSERK1 transphosphorylation activity on casein and MBP (Shah et al., 2001a submitted). The failure of the KAPP[KI] protein to bind to an A-loop mutant version of AtSERK1, that has all three A loop threonine residues, Thr-462, Thr-463 and Thr-468 replaced by glutamic acid residues shows that the conformational change of the AtSERK1 A-loop is also a pre-requisite for KAPP[KI] binding and subsequent transphosphorylation. When the individual threonine residues in the AtSERK1 A-loop are mutated, KAPP binding and subsequent transphosphorylation is affected differently than the transphosphorylation of common substrates such as casein and MBP. In contrast to the studies on casein and MBP, Thr-459 and Thr-463 also influence KAPP binding and transphosphorylation suggests that the A-loop is actively involved itself in the interaction with protein substrates. It is clear however that the Thr-468 is the most important residue that influences not only auto- and transphosphorylation activities but also interaction with KAPP.

In our studies with the KAPP^{-SA} protein we have shown that when both KAPP^{-SA} and AtSERK1^{kin} proteins are incubated together, AtSERK1 phosphorylation is inhibited to a large extent. This implies that *in vivo* KAPP may function by directly dephosphorylating AtSERK1, thus preventing other downstream components from receiving the AtSERK1-mediated signal. As KAPP protein can be phosphorylated by other RLKs and also by AtSERK1, it is possible that KAPP may be modulated in response to an activated AtSERK1 receptor. However, the role of PP2C type phosphatases and their interaction with RLKs in plants is not fully understood. The observation that binding of RLKs with KAPP is very stable and is only weakened when dephosphorylation of the RLKs by the phosphatase is completed (Li et al 1999) suggests an intimate interaction in controlling receptor phosphorylation status.

KAPP has been shown to interact with many but not all phosphorylated RLKs (Braun and Walker., 1997). *In situ* hybridisation and co-immunoprecipitation studies of CLV1 and KAPP have shown that they can interact physically and biochemically. Our studies using AtSERK1 and KAPP-CFP/YFP fusion proteins have refined these observations and may have more general applications as well. The coexpressed KAPP-CFP/YFP and AtSERK1-YFP/CFP proteins were clearly colocalized in the intracellular vesicles and in the plasma membrane. However only the KAPP-CFP/YFP and AtSERK1-YFP/CFP protein in the intracellular vesicles interacted, whereas they did not in the plasma membrane.

In animals, receptor kinases have been shown to oligomerize, activate, autophosphorylate and rapidly endocytose (Ullrich and Schlessinger, 1990). Receptor internalization has been shown to play an important role in signalling. For example, after activation of epidermal growth factor receptor (EGFR), the receptor is internalized into acidified endosomal compartments where most of the EGFR receptors are degraded and the excess are recycled back to the plasma membrane (Wells, 1999). The EGFR kinase domain remains highly active during endocytosis (Lai et al., 1989) and certain substrates are phosphorylated following internalization. Another example is of the insulin receptor kinase (IRK). Insulin binding to IRK is rapidly followed by internalization of insulin-IRK into the endosomal apparatus (EN). EN has been revealed to be a principle site of tyrosine phosphorylation and PI3K activation. Furthermore, IRK dephosphorylation is effected in the ENs by an intimately associated dephosphorylation activity (Di Guglielmo, 1998). To date, receptor endocytosis has not been described for plant RLKs but our studies on AtSERK1 and KAPP proteins might give some insight into the plant receptor internalization mechanism. Given

that the KI domain of KAPP can only bind to the phosphorylated AtSERK1 protein, the observed FRET in the intracellular vesicles must derive from an interaction with the phosphorylated receptor. Notably no interaction is seen when KAPP and AtSERK1 proteins are present at the plasma membrane of the protoplasts suggesting that the majority of the receptor molecules is in the non-phosphorylated state. At any one time, a minority of AtSERK1 protein is in the dimer state (Shah et al., 2001b, in press). This may equate to a small number of active (phosphorylated) receptors. The phosphorylated AtSERK1 receptor may then get internalized in intracellular vesicles reminiscent of endosomes in animal cells, a process that is clearly very much enhanced by the simultaneous presence of KAPP. Most of the AtSERK1-KAPP complex may then be degraded while some of the AtSERK1 molecules, dephosphorylated by KAPP can be recycled to the plasma membrane. Thus it seems plausible that internalization might play a role in extending and/or initiating signalling events at cellular location other than the plasma membrane in plant cells as well. Studies on the phosphorylation and dephosphorylation of the receptors in living cells such as carried out for the EGF receptor (Verveer et al., 2001) are now required to reveal possible spatial selectivity in the signal transduction cascade mediated by AtSERK1.

Materials and Methods

GST- fusion proteins and their expression in *E. coli*

The cDNA sequence encoding the AtSERK1 kinase catalytic domain, corresponding to nucleotides 1000-2068 (accession no: A67827) was amplified by PCR using the 2.1 kb cDNA cloned in vector pBluescriptII SK⁻ as a template. All the primers used for PCR have tailored restriction sites added. Primer *Sma*I1000 (5' TCCCCCGGGTATTTTCTTC GATGTCCCTG 3') and primer *Not*I2068 (5'ATAAGAATGCGGCCGCCCTTG GACCAGATA 3') amplified a PCR fragment of 1.1 kb that was cloned into a *Sma*I and *Not*I cleaved pGEX-4T1 (Pharmacia) resulting in AtSERK1^{kin} construct. Site-directed mutagenesis of the highly conserved Lys-330 essential for phosphotransfer in protein kinases, to glutamic acid was performed according to manufacturer's instructions (Stratagene), resulting in the AtSERK1^{K330E} construct. Site directed mutagenesis of three threonine residues in the AtSERK1 A-loop, Thr-462, Thr-463 and Thr-468 to glutamic acid residues resulted in an AtSERK1$^{3T \rightarrow E}$ construct. Single amino acid mutations of Thr-459, Thr-462, Thr-463 and Thr-468 to alanine residues resulted in

AtSERK1^{T459A}, AtSERK1^{T462A}, AtSERK1^{T463A} and AtSERK1^{T468A} constructs. The cDNA sequence encoding the *Arabidopsis thaliana* KI domain of KAPP and the KAPP cDNA missing only the N-terminal signal anchor was amplified by PCR using the KAPP cDNA in pBluescriptII SK⁻ provided kindly by John C. Walker. A forward primer (5' GGAAGATCTGGGATTT GCAGAGACCA 3') and the reverse primer (5' GGAAGATCTATTAGCCCCAGGAAGC GGCCA 3') amplified a PCR fragment corresponding to the KI domain of KAPP that was cloned into the *BamH*1 and *Xho*I sites of the pGEX-4T1 vector resulting in the KAPP^{KI} construct. To amplify the entire KAPP cDNA missing only the N-terminal signal anchor a forward primer (5' GGAAGATCTGGGATTTGCAGAGACCA 3') and the reverse primer (5' GGAAGATCT CAGGGAAGTATCGAAATCTAAG 3') were used and the 1.6 kb PCR fragment was cloned into the *BamH*1 and *Xho*I sites of the pGEX-4T1 vector resulting in the KAPP^{SA} construct. All plasmids were verified by restriction endonuclease digestion and DNA sequencing before being transformed into the *E. coli* Bl21 strain for protein expression. A 2 ml overnight culture was transferred to 200 ml of LB medium and the cells were grown at 37°C to an OD_{600} of 0.6 and then induced with 0.1 mM isopropyl-D-thiogalactopyranoside (IPTG) for 3-4 hrs at 30° C. The cells were collected by centrifugation, resuspended in 6 ml of phosphate buffered saline (PBS) lysis buffer containing protease inhibitor cocktail (Mini^{Tm}, Boehringer Mannheim), sonicated and cleared by centrifugation at 12,000 g. The soluble GST-fusion proteins were purified from the supernatant by Glutathione Sepharose 4B (Pharmacia) chromatography according to the manufacturer's instructions.

Phosphorylation and dephosphorylation assays

Transphosphorylation assays were performed by incubating 500 ng (100 µg ml⁻¹) of either AtSERK^{kin} or AtSERK^{kin} mutant proteins with 500 ng of KAPP^{KI} protein in 20 mM Tris (pH 7.5), 50 mM NaCl, 0.01% Triton X-100, 10 mM MgCl$_2$, 1 mM dithiothreitol (DTT), 50 µM unlabeled ATP and 10 µCi of [γ-^{32}P] ATP in a final volume of 30 µl. After incubation for 30 minutes at 30°C, the reaction was quenched by adding Laemmli SDSPAGE sample buffer, boiled at 95° C for 5 minutes and separated by 10% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue (CBB) to verify equal loading and then dried. For dephosphorylation assays, 5 µg of AtSERK1^{kin} was phosphorylated on Glutathione Sepharose 4B (matrix) and the unbound label was washed away. The matrix bound phosphorylated AtSERK1^{kin} was treated with

5 μg of KAPP^{-SA} and incubated at 30˚C. The aliquots were taken at time intervals of 30, 60, 90 120 and 240 min. and the reaction was quenched, proteins separated and stained and gels were dried as described above. The radioactivity was quantified with a PhosphoImager using the ImageQuant program (Molecular Dynamics).

In vitro-binding assays

Immobilized KAPP^{KI} was phosphorylated with 10 units of bovine heart muscle kinase (Sigma) in 20 mM Tris (pH 7.5), 50 mM NaCl, 10 mM MgCl$_2$ with 10 μCi of [γ^{-32}P] ATP and then eluted as described above. The resulting eluent was used to probe the nitrocellulose filter prepared as described below. AtSERK1^{kin}, AtSERK^{kin} mutants and AtSERK1^{kin} treated with 25 units of alkaline phosphatase (AP) with and without AP inhibitor (Sigma) were incubated in 20 mM Tris (pH 7.5), 50 mM NaCl, 10 mM MgCl$_2$ and 50 μM unlabeled ATP in a final volume of 30 μl. After incubation for 2 hrs at 37˚C, the reaction was quenched by adding Laemmli SDS-PAGE sample buffer, boiled at 95˚C for 5 minutes and separated by 10% SDS-PAGE. The proteins were transferred to a nitrocellulose filter, blocked in HBB buffer (25 mM Hepes pH 7.5, 5 mM MgCl$_2$, 1 mM KCl and 5% non fat dry milk) at 4˚C for 4 hrs and probed overnight with [γ^{-32}P] ATP labelled KAPP^{KI} probe in BB buffer (25 mM Hepes pH 7.5, 2.5 mM MgCl$_2$, 7.5 mM KCl, 0.1 mM EDTA and 1% non fat dry milk). After 3 washes in BB buffer, the radioactivity of the filter was quantified with a PhosphoImager using the ImageQuant program (Molecular Dynamics).

Phosphoaminoacid analysis

KAPP^{KI} was phosphorylated by AtSERK^{kin} in the presence of [γ^{-32}P] ATP and separated by SDS-PAGE as described above. The CBB stained phosphorylated bands of KAPP^{KI} were excised from the gel, rehydrated in 50 mM NH$_4$CO$_3$, 0.1% SDS and 0.25% ß-mercaptoethanol (2-ME), ground with a small pestle, boiled for 5 min and extracted by agitation at 37°C for 90 min. The proteins were precipitated by adding 20 μg of BSA and 20% (w/v) solid Tri chloro acetic acid (TCA) and incubated overnight at -20°C. The precipitate was collected by centrifugation and the TCA was removed by lyophilization for 30 min. The sample was hydrolysed in 50 μl 6M HCl for 1 h in at 120°C. The HCl was evaporated, and the pellet was resuspended in the ascending solvent of acetic acid, ethanol and water mixed with phosphoaminoacid standards and applied to a 10x10 cellulose TLC plate (Merck) as described by Boyle et al. (1991) using an ascending solvent for 1

h. The first dimension fractionation was followed by chromatography in the second dimension in a phosphopeptide buffer containing iso-butyl alcohal, glacial acetic acid and water 8:4:2. Phosphoaminoacid standards were visualized by spraying the plate with 0.25% ninhydrin in acetone, and heating it at 65°C for 30 min. The radiographic film was exposed to the TLC plate for 12 h and the radioactivity quantified with ImageQuant program (Molecular Dynamics).

Construction of YFP/CFP tagged vectors

The entire open reading frame of the *AtSERK1* cDNA was amplified by polymerase chain reaction (PCR) from a *AtSERK1* full length cDNA and cloned downstream of the 35S promoter into the *NcoI* site of pMON999-CFP and pMON999-YFP using primers Ncol215f (5' CATGCCATGGTGGAGTCGAGTTATGTGG 3') and Ncol2068r (5' CATGCCATGG ACCTTGGACCAGATAACTC 3'). This resulted in the AtSERK1-CFP and AtSERK1-YFP constructs. The AtSERK1 intracellular kinase domain was similarly cloned into the *NcoI* site of pMON999-CFP and pMON999-YFP using primers nAtSERKC (5' CCATCCGATGG GCCCACTAGATATTTTCTT 3') and Ncol2068r. This resulted in the AtSERK1^{kin}-CFP and AtSERK1^{kin}-YFP constructs.

The entire open reading frame of KAPP cDNA was amplified by PCR using primers BglKAP39f (5' GGAAGATCTATGGCGATGATAGGGATGAAC 3') and BglTKAPr (5' GGAAGATCTC AGGGAAGTATCGAAATCTAAG 3') and cloned into the *BglII* site of pMON999-CFP and pMON999-YFP. This resulted in the KAPP-CFP and KAPP-YFP constructs. A 1.6 kb cDNA fragment corresponding to the entire KAPP cDNA missing only the N-terminal type 1 signal anchor was amplified by PCR using primers BglKIKAPnf and BglTKAPr (5' GGAAGATCTCAGGGAAGTATCGAAATCTAAG 3') and was similarly cloned into the *BglII* site of pMON999-CFP and pMON999-YFP. This resulted in the KAPP^{-SA}-CFP and KAPP^{-SA}-YFP constructs. Finally a 0.8 kb fragment corresponding to the KI domain of KAPP cDNA was amplified by PCR using primers BglKIKAPnf (5' GGAAGATCTATGGGGG ATTTGCAGAGACCAC 3') and BglKIKAPr (5' GGAAGATCTATTAGCCCCAGGAAGC GGC 3') and cloned into the *BglII* site of pMON999-CFP and pMON999-YFP vectors. This resulted in the KAPP^{KI}-CFP and KAPP^{KI}-YFP. ER-YFP construct provided kindly by Drs. Jan Carette and Dr. Joan Wellink contains an N-terminal *Arabidopsis thaliana* basic chitinase signal

sequence and a C-terminal HDEL ER retention signal cloned downstream of the 35S promoter of pMON999-YFP vector.

Transient expression in protoplasts

Cowpea mesophyll protoplasts were prepared essentially as described previously (Rezelman et al., 1989) except that 10mM $CaCl_2$ was added to the enzyme solution (0.5 M mannitol, 10 mM $CaCl_2$, 0.1% cellulase and 0.05% pectinase). Ten to fifteen µg of each purified plasmid DNA in 20-75 µl of water was added to $0.5-1x10^6$ protoplasts in 75-150 µl of ice cold solution C (0.6 M mannitol, 10 mM $CaCl_2$, pH 5.3-5.8), mixed by gentle shaking and followed immediately by the addition of 0.5 ml of solution H (40% PEG Mw 6000, 0.6 M mannitol, 0.1 M $Ca(NO_3)_2$). The protoplast suspension was incubated for 5-25 sec with gentle shaking followed by the addition of 4.5 ml of solution M (0.5 M mannitol, 15 mM $MgCl_2$ and 0.1% MES) (pH 5.3-5.7) and incubated at room temperature for 20 min. Protoplasts were then washed 3 times with solution C and incubated for 8-24 hrs as described previously (Rotter et al.,1979).

SDS PAGE and Western Blotting

Either one or two days after transfection, the cowpea protoplasts were lysed in ice-cold HB buffer (50 mM Tris-HCl pH 7.4, 10 mM KAc, 1mM EDTA. 0.05% Triton X-100) supplemented with single strength proteinase inhibitor cocktail (Boehringer Mannheim, Germany). The lysates were passed through a 25 µm needle and centrifuged at 40,000 g for 30 min at 4°C. Ten µl of 2x SDS sample buffer (100 mM Tris HCl, pH 6.8, 4% SDS, 0.2 % bromophenol blue and 20% glycerol) and dithiothreitol (DTT) was added to lysates. After boiling for 3 min, the proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane (IMMOBILON) for 3 hrs at 50V. The membranes were washed in TBS-T buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2% Tween-20) and blocked with 5% non-fat dried milk in TBS-T for 3 hrs at room temperature, washed with TBS-T and incubated with the anti-rabbit GFP antibody (Clontech; 1:100 final dilution) for 1.5 hrs at room temperature. After 3 washes in TBS-T, membranes were incubated with alkaline phosphatase conjugated anti-rabbit antibody (New England Biolabs; 1:1000 final dilution). Membranes were washed in TBS-T, incubated in Western blue substrate for Alkaline Phosphatase detection (Promega) until staining was visible.

Colocalization studies using confocal microscopy

The intracellular and the membrane fluorescence was analysed by CSLM 510 (Confocol Laser Scanning Microscope) (Carl-Zeiss, GMBH Germany). The protoplasts were excited by a 458 nm laser (Zeiss) sequentially. For CFP, HFT458 dichroic mirror and BP470-500 emission filter and for YFP, HFT514 dichroic mirror and BP535-590 IR emission filter was used. In both cases the cholorophyll fluorescence was filtered out with LP650 emission filter. A 40x oil immersion objective (numerical aperture 1.3) was used for scanning protoplasts with step (pixel) size of 101 μm in the x plane and 7.4 μm in the y plane. The pinhole setting was 60 μm, which yielded a theoretical thickness (full width at half maximum) of approximately 1μm. Images and data captures were analyzed with Zeiss LSM510 software.

Resonance energy transfer between AtSERK1 and KAPP

Fluorescence resonance energy transfer (FRET) between the fluorescently labelled AtSERK1 receptors and the KAPP protein was measured by fluorescence spectral imaging microscopy (FSPIM). Spectral imaging was done using a Leica DMR epifluorescence microscope equipped with a chromex (Albuquerque, NM, USA) 250IS imaging spectrograph coupled to a Photometrics (Tucson, AZ, USA) CH250 CCD camera. The excitation light source was a 100 W-mercury arc lamp coupled to an excitation wheel. Spectral images were acquired using a 20x Plan Neofluar objective (NA 0.5), an omega 435 df10m bandpass excitation filter, an omega 430 DCLP dichroic mirror and a 455 ff longpass emission filter (Schott, Mainz, Germany). Spectral images were acquired using a 150 groove/mm grating, set at a central wavelength of 500 nm and a slit width of 200 μm corresponding to 10 μm in the object plane. Typical exposure and CCD integration time was 2-5 secs. Data processing and background autofluorescence subtraction was performed as described (Gadella et al., 1997).

Acknowledgements

References

1. Cohen, P. 1989. Structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* **103:** 211-225.

2. Cohen, P.T.W 1997. Novel protein serine/threonine phosphatases: variety is the spice of life. *Trends Biochem. Sci.* **22:** 245-251.

3. Torii, K.U. 2000. Receptor kinase activation and signal transduction in plants: an emerging picture. *Curr. Opin. Plant Biol.* **3:** 361-367.

4. Clarke, S.E., Williams, R.W. and Meyerowitz, E.M. 1997. The *CLAVATA*1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in *Arabidopsis*. *Cell* **89:** 575-585.

5. Torii, K.U., Mitsukawa, N., Oosumi, T., Matsuura, Y., Yokoyama, R., Whittier, R.F. and Komeda, Y., 1996. The Arabidopsis ERECTA gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. *Plant Cell* **8:** 735-746.

6. Li, J. and Chory, J. 1997. A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. *Cell* **90:** 929-938.

7. Stone, J.M, Collinge, M.A., Smith, R.D., Horn, M.A. and Walker, J.C. 1994. Interaction of a protein phosphatase with an Arabidopsis serine-threonine receptor kinase. *Science* **266:** 793-795.

8. Shenolikar, S. and Nairn, A. C. 1991. Protein phosphatases: recent progress. *Adv. Sec. Messenger Phosphoprotein Res.* **23:** 1-121.

9. Li. J., Smith, G.P. and Walker, J.C. 1999. Kinase interaction domain of kinase-associated protein phosphatase, a phosphoprotein-binding domain. *Proc. Natl. Acad. Sci.* U.S.A. **96:**7821-7826.

10. Williams, R.W., Wilson, J.M. and Meyerowitz, E.M. 1997. A possible role for Kinase associated protein phosphatase in the Arabidopsis CLAVATA1 signalling pathway. *Proceed. Natl. Acad. Sci.* U.S.A. **94:** 10467-10472.

11. Stone, J.M., Trotochaud, A.E., Walker, J.C. and Clark, S.E. 1998. Control of meristem development by CLAVATA1 receptor kinase and kinase-associated protein phosphatase interactions. *Plant Physiol.* **117:** 1217-1225.

12. Trotochaud, A.E., Hao, T., Wu, G., Yang, Z. and Clark, S.E. 1999. The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signalling complex that includes KAPP and a Rho-related protein. *Plant Cell* **11:** 393-406.

13. Braun, D.M, Stone, J.M. and Walker, J.C. 1997. Interaction of the maize and Arabidopsis kinase interaction domains with a subset of receptor-like protein kinases: implications for transmembrane signalling in plants. *Plant J.* **12:** 83-95.

14. Schmidt, E.D.L., Guzzo, F., Toonen, M.A.J. and de Vries, S.C., 1997 A leucine rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos, *Development* **124** 2049-2062.

15. Hecht, V., Vielle-Calzada, J.P., von Recklinghausen, I., Hartog, M.V., Zwartjes, C.G.M., Schmidt, Ed. D. L., Boutilier, K., van Lammeren A. and de Vries S. C. 2000. The *Arabidopsis somatic embryogenesis receptor kinase* 1 gene during ovule and embryo development (submitted).

16. Shah, K., Vervoort, J. and de Vries S. C. 2001a. Role of threonines in the AtSERK1 activation loop in auto- and transphosphorylation. (submitted).

17. Rizzuto, R., Brini, M., De Giorgi, F., Rossi, R., Heim, R., Tsien, R.Y. and Pozzan, T. 1996. Double labelling of subcellular structures with organelle-targeted GFP mutants in vivo. *Curr Biol.* **1**: 183-188.

18. Gerdes, H.H. and Kaether, C. 1996. Green fluorescent protein: applications in cell biology. *FEBS Lett.* **389**: 44-47.

19. Ludin, B., Doll, T., Meili, R., Kaech, S. and Matus, A. 1996. Application of novel vectors for GFP-tagging of proteins to study microtubule-associated proteins. *Gene* **173**: 107-111.

20. Carey, K.L., Richards, S.A., Lounsbury, K.M. and Macara, I.G., 1996. Evidence using a green fluorescent protein-glucocorticoid receptor chimera that the Ran/TC4 GTPase mediates anessential function independent of nuclear protein import. *J. Cell Biol.* **133**: 985-96.

21. Sorkin, A., McClure, M., Huang, F. and Carter, R. 2000. Interaction of EGF receptor and grb2 in living cells visualized by fluorescence resonance energy transfer FRET microscopy *Curr. Biol.* **10**: 1395-1398.

22. Jovin, T.M. and Arndt-Jovin D.J. 1989. Luminescence digital imaging microscopy. *Annu. Rev. Biophys. Chem.* **18**: 271-308.

23. Shah, K., Gadella, T. W. J., van Erp, H., Hecht, V., and de Vries S.C. 2001b. Subcellular localization and dimerization of the *A. thaliana* Somatic Embryogenesis Receptor Kinase 1 protein. (*J. Mol. Biol.* in press).

24. Somleva, M.N., Schmidt, E.D.L. and de Vries, S.C. 2000. Embryogenic cells in *Dactylis glomerata* L. Poaceae explants identified by cell tracking and by SERK expressions *Plant Cell Rep.* **19**: 718-726.

25. Yaffe, M.B., Rittinger, K., Volinia, S., Caron, P.R., Aitken, A., Leffers, H., Gamblin, S.J., Smerdon, S.J. and Cantley, L.C. 1997. The structural basis for 14-3-3:phosphopeptide binding specificity, *Cell* **91**: 961-971.

26. Ullrich and Schlessinger 1990. Signal transduction by receptors with tyrosine kinase activity *Cell* **61**: 203-212.

27. Wells, A. 1999. EGF receptor. *Int J Biochem Cell Biol.* **31**: 637-643.

28. Lai, W.H., Cameron, P.H., Wada, I., Doherty, J.J., Kay, D.G., Posner, B.I. and Bergeror., J.J. 1989. Ligand-mediated internalization, recycling, and downregulation of the epidermal growth factor receptor in vivo. *J. Cell Biol.* **109**: 2741-2749.

29. Di guglielmo, G.M., Drake, P.G., Baass, P.C., Authier, F., Posner, B.I. and Bergeron, J.J. 1998 Insulin receptor internalization and signalling. *Mol. Cell. Biochem.* **182(1-2)** : 59-63.

30. Verveer, P.J., Wouters, F.S., Reynolds, A.R. and Bastiaens, P.I. (2000) Quantitative imaging of lateral ErbB1 receptor signal propagation in the plasma membrane. *Science* **290**, 1567-1570.

31. Boyle, W.J., Geer, P. van der and Hunter, T. 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol.* **201**: 10-49.

32. Rezelman, G., van Kammen, A. and Wellink, J. 1989. Expression of cowpea mosaic virus mRNA in cowpea protoplasts. *J. Gen. Virol.* **70**: 3043-3050.

33. Rottier, P.J, Rezelman, G. and van Kammen, A. 1979. The inhibition of cowpea mosaic virus replication by actinomycin D. *Virology* **92**: 299-30958.

34. Gadella, T.W. Jr., Vereb, G. Jr., Hadri, A.E., Rohrig, H., Schmidt, J., John, M., Schell, J. and Bisseling, T. 1997. Microspectroscopic imaging of nodulation factor-binding sites on living *Vicia sativa* roots using a novel bioactive fluorescent nodulation factor. *Biophys. J.* **72**: 1986-96.

6

Summarizing Discussion

In plants, many transmembrane receptor kinases called receptor like kinases (RLKs) have been identified and most of them are serine/threonine kinases. Based on the structure of their extracellular domains, RLKs fall into several classes (Torii, 2000): the S domain class; the lectin class; the EGF class; the TNFR class; the PR class and the LRR class of RLKs. To date LRR-RLKs comprise the largest class of plant RLKs and several of them have been to shown to play a critical role in development (Torii, 2000). However only few of them have so far given insight into the signal transduction machinery employed by plants (Lease et al., 1998). Somatic embryogenesis is defined as a process where somatic or asexual cells develop into plantlets through stages morphologically analogous to zygotic embryogenesis. A search for marker genes to monitor the transition of somatic into competent and embryogenic cells in established carrot cell cultures resulted in the identification of the *SERK* gene that is transiently expressed up to the 100 cell globular stage during somatic embryogenesis (Schmidt et al., 1997). In plants *SERK* mRNA can only be detected transiently in the zygotic embryo up to early globular stage and not in later stages (Schmidt et al., 1997). The most closely related *SERK* gene from *Arabidopsis*, is expressed during ovule development and early embryogenesis and like *SERK, AtSERK1* marks cells competent to form embryos in culture (Hecht et al., 2001 submitted). The predicted AtSERK1 protein contains an N-terminal leucine zipper (LZ), 5 leucine rich repeats (LRRs) and a proline-rich region in its predicted extracellular domain. This is followed by a single transmembrane domain that is attached to an intracellular kinase domain with all eleven conserved sub-domains found in serine/threonine kinases.

The subject of the research described in this thesis is the biochemical characterization and localization of the AtSERK1 protein in addition to elucidating the early mechanisms involved in the AtSERK1 mediated signalling pathway. Based on the cDNA sequence, the AtSERK1 protein was predicted to be a membrane associated serine/threonine receptor protein kinase (Hecht et al, 2001). Confocal microscopy on transiently expressed AtSERK1-YFP protein in plant protoplasts showed the presence of the fusion proteins in the plasma membrane in a pattern comparable with that of EGFR, a well-known plasma membrane receptor. To confirm the plasma membrane localization, the AtSERK1-YFP protein was also expressed in insect cells. The expression of AtSERK1-YFP in insect cells also showed the presence of AtSERK1 at the plasma membrane.

The extracellular domains of some transmembrane receptors have been shown to be involved in their correct targeting. For instance, the correct folding, assembly and expression of the human insulin receptor depends on glycosylation of the extracellular domains (Ellemen et al, 2000). The LRRs of AtSERK1 contain 5 putative N-glycosylation sites. Two of these are present in the second LRR, two in the fourth and one in the fifth LRR, starting from the N-terminus of the AtSERK1 protein. Removal of at least two of the LRRs resulted in considerable reduction in the number of AtSERK1 protein molecules being targeted to the plasma membrane. In line with the role of N-linked glycosylation in insulin receptor targeting, AtSERK1 mistargeting could be due to the lack of glycosylation of the extracellular LRRs. Support for this hypothesis comes from tunicamycin studies, suggesting a role for N-linked glycosylation in AtSERK1 protein targeting to the plasma membrane. N-linked oligosaccharides play a role in the "quality control" system of the ER that ensures selective transportation of the properly folded proteins destined for the Golgi complex (High et al., 2000). Removal of the N-linked oligosaccharides either by chemical means or through removal of LRRs may thus result in incorrectly folded, non-glycosylated AtSERK1 proteins and these may be trapped in the ER. This suggests that recognition of properly glycosylated proteins by an ER based sorting machinery is an essential element of the assembly and localization process of AtSERK1.

Most animal receptor kinases have tyrosine kinase activity, only a few are serine/threonine kinases (Ullrich and Schlessinger, 1990; Lin et al., 1992) or can phosphorylate serine/threonine in addition to tyrosine (Douville et al., 1992). Studies on affinity purified plant RLKs expressed in different expression systems have shown that they autophosphorylate on serine and/or threonine residues. An exception is the *Petunia PRK1* gene that autophosphorylates on serine and tyrosine residues (Mu et al., 1995). Biochemical studies with affinity purified AtSERK1-GST fusion proteins showed that AtSERK1 kinase domain exhibits autophosphorylation activity on serine and threonine. Autophosphorylation activity and activation of transmembrane receptor protein kinases is generally mediated by ligand-induced dimerization (Heldin, 1995). Dimerization and intermolecular autophosphorylation of a nuclear serine/threonine kinase from *A. thaliana* has been shown (Roe et al., 1997). AtSERK1^{K330E}, a catalytically inactive AtSERK1 mutant protein is phosphorylated by AtSERK1^{kin} protein revealing the intermolecular mechanism of phosphorylation and is thus the suggestive of dimerization of AtSERK1 monomers.

Receptor dimerization is employed as a mechanism for both receptor activation and autophosphorylation (Schlessinger, 1988; Ullrich and Schlessinger, 1990). The extracellular domains of most of the characterized transmembrane receptors have been shown to be involved in ligand binding, oligomerization and the subsequent activation of the intracellular kinase domain and the initiation of the downstream signal transduction cascade (Heldin, 1995). Well-known examples are the epidermal growth factor receptor (EGFR) and platelet derived growth factor receptor (PDGFR) that undergo dimerization in response to binding of their respective ligands. However, the sensitivity of the PDGFR (Li and Schlessinger, 1991) and some other receptors like calcium sensing receptor (CaR) (Bai et al., 1991) to reducing agents suggests that one type of intermolecular interaction mediating dimerization is *via* intermolecular disulfide bonds. The mechanism by which plant receptors transduce signals across the cell surface is largely unknown. There is evidence to suggest that plant receptors like CLV1 and CLV2 also dimerize (Williams et al., 1997) and are assembled into a heterodimeric complex to transduce extracellular signals (Jeong et al., 1999). Recent studies have shown that a small secreted protein, CLV3 binds to the CLV1 and CLV2 heterodimer to form a multimeric protein complex (Trotochaud et al., 2000).

AtSERK1 dimerization was investigated by the yeast two hybrid protein interaction system and making use of fluorescence resonance energy transfer (FRET), employing spectroscopic variants of GFP in combination with transient expression in cowpea mesophyll protoplasts. FRET between CFP and YFP fused AtSERK1 proteins would only be detected when the proximity of the fluorophores is less than 10 nm. Both interaction-based studies reveal that the AtSERK1 kinase domain is not involved in AtSERK1 receptor dimerization. The FRET studies further provide evidence that only a minority of the molecules in certain regions at the plasma membrane dimerize. In FRET studies, one potential problem may be that AtSERK1 expression is raised far beyond normal levels due to the use of the strong constitutive 35S promoter. This could cause physiologically non-relevant AtSERK1 oligomerization. However, because the majority of the protoplasts expressing AtSERK1 fusion proteins do not show FRET and therefore exist as monomers in the membrane, this does not appear to be a widespread problem. The presence of the small number of AtSERK1 dimers at the plasma membrane is in line with EGF receptor dimerization in mammalian cells. It was estimated that up to 12% of the high affinity EGF receptors dimerize highly in some regions of the plasma membrane. Based on the quantitative

determination of FRET on the EGF receptor in mammalian cells, it was suggested that the high affinity subclass of receptors are present in a predimerized state in the absence of the ligand (Gadella et al., 1995). However, the predimerized AtSERK1 receptor molecules on the plasma membrane appear to be far less abundant.

Many extracellular protein domains of transmembrane receptors have been shown to be involved in protein- protein interactions. The extracellular domain of AtSERK1 consists of two such domains, the LRRs and the LZ domain. In most cases, LRRs have been speculated to be involved in protein-protein interactions (Kobe and Deisenhofer, 1994). However, there are also recent reports that show the involvement of the other extracellular protein domains in receptor dimerization (Behncken et al., 2000). In plant LRR-RLKs, for example, the extracellular domain of the BRI receptor, involved in brassinosteroid signalling, consists of 25 LRRs interrupted by a 70 amino acids loop-out island domain. Mutations in this domain confer a strong phenotype (Li and Chory, 1997) and thus it is speculated that this loop-out island domain might be the binding site for BRs. Since BRs are small molecules, it is quite unlikely to bind a large binding surface like LRRs. LZ domains have been shown to play a role in dimerization of transmembrane receptors (Behncken et al., 2000). The small population of predimerized AtSERK1 receptors is not detected any more in the absence of the LZ domain, which reveals the importance of this domain in AtSERK1 dimerization. Thus, while LRRs of AtSERK1 are involved in correct targeting of the receptor, the LZ domain is involved in the formation of the minority of AtSERK1 dimers at the plasma membrane.

Cysteines in the N-terminal extracellular domains of transmembrane receptors have been shown to play a role in receptor dimerization by the formation of disulfide bridges (Romano et al., 1996). Since it is not known whether ligand/s that bind to the AtSERK1 receptor are present in the protoplast culture medium, we cannot answer the question whether the AtSERK1 dimers detected by FRET represent activated dimers or preassociated dimers. The presence of conserved cysteine pairs in the extracellular domains of plant RLKs may suggest the existence of a population of predimerized receptors on the cell surface (Trotochaud et al, 1999). Among plant RLKs, CLVl and CLV2 are known to form a disulfide-linked heterodimeric receptor complex (Jeong et al., 1999) and also the ethylene receptor ETRI is a disulfide-linked dimer (Schaller et al., 1995). The SRG protein involved in pollen recognition in *Brassica* stigma is also found as a disulfide-linked dimer (Doughty et al, 1998). The AtSERK1 protein has four cysteines in the

extracellular domain so the small amount of AtSERK1 dimers found might arise due to the formation of disulfide bridges between the cysteines in the LZ domain. While identification of the ligand for AtSERK1 is clearly essential for determining its precise mode of action, we propose that AtSERK1 exhibits ligand-induced homodimerization, which would place it in the EGF class of receptors. Owing to the presence of the cell wall that sets apart the plasma membranes of two adjacent cells, ligands for AtSERK1 are presumed to be extracellular proteins rather than membrane bound cell surface proteins.

Employing computer assisted molecular modelling in combination with functional studies of various kinase domains of transmembrane receptors, insight has been obtained into the conformational changes that take place once the receptors are activated (Hubbard et al., 1998). We used the X-ray crystal structure of the unphosphorylated (Hubbard et al., 1994) and phosphorylated (Hubbard, 1997) insulin receptor kinase (IRK) as templates for our modelling experiments. In IRK, the A-loop that is formed from sub-domains VII and VIII, contains three tyrosine residues that can be phosphorylated and that are of importance for receptor kinase signalling activity (Hubbard, 1997). The superimposition of the AtSERK1 kinase model and the IRK model reveals the presence of the same kind of A-loop in the AtSERK1 kinase domain. The major difference is the presence of threonine residues in the AtSERK1 A-loop instead of tyrosine residues in the IRK A-loop. The activation of many protein kinases occurs by autophosphorylation of one to three residues within the activation loop of sub-domain VIII (Johnson, 1996). Among plant RLKs, the AtSERK1 resembles CrRLK1, RLK4, KIK1, TMK1 and OsTMK plant RLKs that have a preference for threonine autophosphorylation. Only two previous studies have identified specific target site for autophosphorylation in plant RLKs and only one of them, the studies on BRI1 receptor, has revealed the importance of phosphorylated serine/threonine residues in sub-domain VII and VIII (Oh et al, 2000). All three phosphorylation sites in sub-domain VIII of BRI1 lie within the predicted A-loop region. In addition, the genetic analysis points to the functional importance of the proposed A-loop region of RLKs. Numerous mutations resulting in the weak phenotype fall within this region (Lease et al., 1998) including bri1-104 and bri1-115 (Li and Chory., 1997). Based on the prediction that the threonine residues in AtSERK1 might fulfill the same role as tyrosine residues in IRK, mutant proteins were constructed in which Thr-459, Thr-462, Thr-463 and Thr-468 were individually mutated. The importance of A-loop phosphorylation is supported by our finding that upon mutation of Thr-468

within the A-loop (conserved in most of the plant RLKs), AtSERK1 autophosphorylation activity is lost completely. The Thr-462 mutation also has a minor influence on AtSERK1 autophosphorylation whereas Thr-459 and Thr-463 do not influence the AtSERK1 autophosphorylation.

AtSERK1 can phosphorylate both serine/threonine and tyrosine-containing artificial substrates, which widens the spectrum of downstream proteins it might interact with. This classifies AtSERK1 as a dual specificity-type receptor kinase with respect to its transphosphorylation activity. So far tyrosine kinase activity has not been widely demonstrated in plants, although the identification of the rolB protein as a tyrosine phosphatase (Fillipini et al., 1996) suggests that it does occur. AtSERK1 transphosphorylation activity is abolished in the threonine mutants. It is known from the studies of animal receptors that the phosphorylation of the A-loop shifts the equilibrium towards a conformation that accommodates protein substrate binding. Point mutations in the A-loop of various receptor kinases are presumed to alter this equilibrium (Hubbard, et al., 1998) and thus influence protein substrate binding and subsequent transphosphorylation. In the AtSERK1 threonine mutant proteins, AtSERK1^{T462A} and AtSERK1^{T468A}, transphosphorylation activity was abolished suggesting that the AtSERK1 A-loop may be involved in protein substrate binding. In light of the above discussion, we propose that, either by the presence of a minor population of catalytically active AtSERK1 molecules *in vitro*, or by ligand induced conformational changes *in vivo*, AtSERK1 activation requires intermolecular autophosphorylation. This would involve movement of the AtSERK1 threonine containing A-loop followed by improved accessibility of the catalytic domain to commence transphosphorylation activity and protein substrate binding. In this model, A-loop phosphorylation of Thr-468 of one AtSERK1 monomer is also essential for intermolecular autophosphorylation activity of that monomer.

Although the molecular basis of the interaction between the RLKs and downstream proteins is still unknown, phosphorylation/dephosphorylation events are most likely involved, because a common feature of the association of receptor kinase with target proteins involves the interaction with consensus sequences containing a phosphorylated amino acid. Many known plant RLKs have been shown to interact with a type 2C protein phosphatase called the kinase associated protein phosphatase (KAPP) (Braun et al, 1997). KAPP is one of the very few proteins identified which may mediate in downstream signalling pathways originating from RLKs. KAPP comprises

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of an amino terminal type one signal anchor, a kinase interaction (KI) domain and a carboxy terminal type 2C protein phosphatase catalytic domain. KAPP is expressed throughout the shoot apical meristem (SAM) and in stage 1-2 flowers but not in stage 3 flowers, thus potentially overlapping the region and timing of the AtSERK1 gene expression, which is prior to fertilization in ovules transiently during embryo development and in the vascular tissues of the seedlings (Hecht et al, 2001 submitted).

KAPP has been shown to interact with many but not all phosphorylated RLKs, which implies that it does not merely bind to phosphorylated amino acids but recognizes a particular sequence on the target kinase. The physical interaction between KAPP and the RLKs is thought to be important for their roles in signal transduction (Li et al., 1999). Both biochemical and genetic approaches have shown that KAPP interacts *in vivo* with CLAVATA1 suggesting that KAPP negatively regulates CLAVATA1 signalling (Williams et al., 1997; Stone et al., 1998 and Trotochaud et al., 1998). KAPP^{KI} binds to AtSERK1^{kin} in a phosphorylation dependent manner, which is shown by our studies on both affinity purified GST fusion proteins, and by CFP/YFP fusion protein based-FRET studies. The failure of KAPP^{KI} protein to bind the threonine substituted AtSERK1 A-loop mutant version of AtSERK1 shows that the conformational change of the AtSERK1 A-loop might be a pre-requisite for the KAPP^{KI} binding and subsequent transphosphorylation.

In our studies with the affinity purified KAPP^{-SA} protein we have shown that when both KAPP^{-SA} and AtSERK1^{kin} proteins are incubated together, AtSERK1 phosphorylation is inhibited to a large extent. This implies that *in vivo* KAPP may function by directly dephosphorylating AtSERK1, thus preventing other downstream components from receiving the AtSERK1 signal. As the KAPP protein is phosphorylated in the presence of AtSERK1, it is suggestive that the KAPP protein is modulated in response to the activated AtSERK1 receptor. The observation that binding of RLKs with KAPP is very stable and the speculation that KAPP binds RLKs until the dephosphorylation by the phosphatase is completed might give some insight into the dephosphorylation mechanism of KAPP (Li et al 1999). If true for AtSERK1 and KAPP interaction, this might have an important consequence for the downstream signalling initiated by AtSERK1.

The KAPP-YFP and AtSERK1-CFP protein were colocalized at the plasma membrane in plant protoplasts. However, our FRET studies show that these proteins interact intracellularly and not at the plasma membrane. The complete KAPP-YFP and AtSERK1-CFP proteins that show

interaction intracellularly might represent the AtSERK1 receptor complex that is undergoing endocytosis. Receptor internalization has been shown to play a major role in signalling. For example, after activation of EGFR, the receptor is internalized and remains highly active during endocytosis (Lai et al., 1989) and certain substrates are transphosphorylated after internalization. Another example is of that of the insulin receptor kinase (IRK). Insulin binding to IRK is rapidly followed by internalization of insulin-IRK into the endosomal apparatus, which is the predominant site of tyrosine phosphorylation and PI3K activation. Furthermore, IRK dephosphorylation is affected in the endosomal apparatus by an intimately associated phosphatase (Di Guglielmo, 1998). To date receptor endocytosis has not been described for plant RLKs but our studies on AtSERK1 and KAPP proteins might give some insight into the receptor internalization mechanism. The model of receptor activation, internalization and recycling might explain AtSERK1-KAPP interactions. AtSERK1 molecules, which are present mainly as monomers on the plasma membrane, are in a non-phosphorylated form. Either by ligand binding, which might be present in the protoplast medium or by some other constitutive receptor activation mechanism, AtSERK1 gets phosphorylated. The phosphorylated AtSERK1 receptor gets internalized and binds to the KAPP protein, which gets phosphorylated in turn. The associated AtSERK1-KAPP complex is then detected by FRET. Some of the AtSERK1-KAPP complex may then be subsequently degraded, whereas the AtSERK1 molecules, dephosphorylated by KAPP may be recycled back to the plasma membrane. Because phosphorylation-induced conformational changes in proteins have been shown to influence their protein-protein interaction capabilities (this work and others), it is possible that the CFP and YFP moieties of AtSERK1-CFP and KAPP-YFP fusion proteins are in different orientation at the plasma membrane and in the intracellular vesicles. The studies on the phosphorylation and dephosphorylation of these fusion proteins in live plant cells would therefore give more insight into the internalization mechanisms of the AtSERK1 protein.

The research described in this thesis provides ample information regarding the molecular nature of the AtSERK1 mediated signal transduction. It has formed the basis for *in vivo* experiments to study receptor dimerization and activation, and subsequent binding of downstream partners of AtSERK1. The identification of ligand(s) for AtSERK1 is now of crucial importance for future studies on AtSERK1 signal transduction.

References

1.Torii, K.U. (2000) Receptor kinase activation and signal transduction in plants: an emerging picture. *Curr. Opin. Plant Biol.* **3**, 361-367.

2. Lease, K., Ingham, E. and Walker, J.C. (1998) Challenges in understanding RLK function. *Curr. Opin. Plant Biol.* **1**, 388-92.

3. Schmidt, E.D.L., Guzzo, F., Toonen, M.A.J. and de Vries, S.C., (1997) A leucine rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos, *Development* **124** 2049-2062.

4. Hecht, V., Vielle-Calzada, J.P., von Recklinghausen, I., Hartog, M.V., Zwartjes, C.G.M., Schmidt, Ed. D. L., Boutilier, K., van Lammeren A. and de Vries S. C. (2000). The *Arabidopsis somatic embryogenesis receptor kinase* 1 gene during ovule and embryo development (submitted)

5. Elleman,T.C., Frenkel, M.J., Hoyne, P.A., McKern, N.M., Cosgrove, L., Hewish, D.R., Jachno, K.M., Bentley, J.D., Sankovich, S.E. and Ward, C.W. (2000) Mutational analysis of the N-linked glycosylation sites of the human insulin receptor. *Biochem J.* **347**, 771-79.

6. High, S., Lecomte, F.J., Russell, S.J., Abell, B.M. and Oliver, J.D. (2000) Glycoprotein folding in the endoplasmic reticulum: a tale of three chaperones? *FEBS Lett.* **476**, 38-41.

7. Schlessinger, J. Signal transduction by allosteric receptor oligomerization. (1988) *Trends Biochem. Sci.* **13**, 443-47.

8. Ullrich, A. and Schlessinger, J. (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203-12.

9. Heldin, C.H. (1995) Dimerization of cell surface receptors in signal transduction.
Cell **80**, 213-223.

10. Li, W. and Schlessinger, J. (1991) Platlet derived growth factor receptor (PDGFR)-Induced Dimerization of PDGF Receptor in living cells. *Mol. Cell Biol.,* **11**, 3756-3761

11. Bai, M., Trivedi, S. and Brown, E.M. (1998) Dimerization of the extracellular calcium-sensing receptor (CaR) on the cell surface of CaR-transfected HEK293 cells. *J. Biol. Chem.* **273**, 23605-10.

12. Williams, R.W., Wilson, J.M., and Meyerowitz, E.M. (1997) A possible role of kinase-associated protein phosphatase in the *Arabidopsis* CLAVATA1 signalling pathway. *proc. Natl Acad. Sci.,* USA **94**, 10467-72

13. Jeong, S., Trotochaud, A.E. and Clark, S.E. (1999) The *Arabidopsis* CLAVATA2 gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase. *Plant Cell* **11**, 1925-34.

14. Trotochaud, A.E., Jeong, S. and Clark, S.E. (2000) CLAVATA3, a multimeric ligand for the CLAVATA1 receptor-kinase. *Science* **289**, 613-17.

15. Gadella, T.W. Jr. and Jovin, T.M. (1995) Oligomerization of epidermal growth factor receptors on A431 cells studied by time-resolved fluorescence imaging microscopy. A stereochemical model for tyrosine kinase receptor activation. *J. Cell Biol.* **129**, 1543-58

16. Kobe, B. and Deisenhofer, J. (1995) A structural basis of the interactions between leucine-rich repeats and protein ligands. *Nature* **374**, 183-186.

17. Behncken, S.N., Billestrup, N., Brown, R., Amstrup, J., Conway-Campbell, B. and Waters M.J. (2000) Growth hormone (GH)-independent dimerization of GH receptor by a leucine zipper results in constitutive activation. *J. Biol. Chem.* **275**, 17000-07.

18. Li, J. and Chory, J. (1997) A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. *Cell* **90**, 929-938.

19. Romano, C., Yang, W.L. and O'Malley, K.L. (1996) Metabotropic glutamate receptor 5 is a disulfide-linked dimer. *J Biol. Chem.* **271**, 28612-6. *Biophys. Chem.* **18**, 271-308.

20. Trotochaud, A.E., Hao, T., Wu, G., Yang, Z. and Clark, S.E. (1999) The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signalling complex that includes KAPP and a Rho-related protein. *Plant Cell* **11**, 393-406.

22. Schaller, G.E., Ladd, A.N., Lanahan, M.B., Spanbauer, J.M. and Bleecker, A.B. (1995) The ethylene response mediator ETR1 from Arabidopsis forms a disulfide-linked dimer. *J. Biol. Chem.* **270**, 12526-30.

23. Doughty, J., Dixon, S., Hiscock, S.J., Willis, A.C., Parkin, I.A., and Dickinson, H.G. (1998) PCP-A1, a defensin-like Brassica pollen coat protein that binds the S locus glycoprotein, is the product of gametophytic gene expression. *Plant Cell* **10**, 1333-47.

24. Ullrich, A. and Schlessinger, J., (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203-212.

25. Lin, H.Y., Wang, X.F., Ng-Eaton, E., Weinberg, R.A. and Lodish, H.F. (1992) Expression cloning of the TGF-beta type II receptor, a functional transmembrane serine/threonine kinase. *Cell* **68**, 775-785.

26. Douville, E.M., Afar, D.E., Howell, B.W., Letwin, K., Tannock, L., Ben-David, Y., Pawson, T. and Bell, J.C. (1992) Multiple cDNAs encoding the esk kinase predict transmembrane and intracellular enzyme isoforms. *Mol. Cell Biol.* **12**, 2681-2689.

27. Mu, J.H., Lee H.S. and Kao, T.H., (1994) Characterization of a pollen-expressed receptor-like kinase gene of *Petunia inflata* and the activity of its encoded kinase. *Plant Cell* **6**, 709-721

28. Roe, J.L, Durfee, T., Zupan, J.R., Repetti, P.P., McLean, B.G. and Zambryski, P.C. (1997) TOUSLED is a nuclear serine/threonine protein kinase that requires a coiled-coil region for oligomerization and catalytic activity. *J. Biol. Chem.* **272**, 5838-5845.

29. Hubbard, S.R., Mohammadi, M. and Schlessinger, J. (1998) Autoregulatory mechanisms in protein tyrosine kinases. *J. Biol. Chem.* **273**, 11987-11990

30. Hubbard, S.R., Wei, L., Ellis, L. and Hendrickson, W.A. (1994) Crystal structure of the tyrosine kinase domain of the human insulin receptor. *Nature* **372**,746-754.

31. Hubbard, S.R. (1997) Crystal structure of the activated insulin receptor tyrosine kinase in complex with peptide substrate and ATP analog. *EMBO J.* **16**, 5572-5581.

32. Johnson, L.N., Noble, M.E., and Owen, D.J. (1996) Active and inactive protein kinases: structural basis for regulation. *Cell* **85**, 149-158.

33. Oh, M.H., Ray, W.K., Huber, S.C., Asara, J.M., Gage, D.A. and Clouse S.D. (2000) Recombinant brassinosteroid insensitive 1 receptor-like kinase autophosphorylates on serine and threonine residues andphosphorylates a conserved peptide motif in vitro. *Plant Physiol.* 124, 751-766.

34. Filippini, F., Rossi, V., Marin, O., Trovato, M., Costantino, P., Downey, P.M., Lo Schiavo F. and Terzi, M.A. (1996) A plant oncogene as a phosphatase. *Nature* 379, 499-500.

35. Braun, D.M, Stone, J.M. and Walker, J.C. (1997) Interaction of the maize and Arabidopsis kinase interaction domains with a subset of receptor-like protein kinases: implications for transmembrane signalling in plants. *Plant J.* 12, 83-95.

36. Li, J., Smith, G.P. and Walker, J.C. (1999) Kinase interaction domain of kinase-associated protein phosphatase, a phosphoprotein-binding domain. *Proc. Natl. Acad. Sci.* U S A. 96,7821-7826

37. Williams, R.W., Wilson, J.M. and Meyerowitz, E.M. (1997) A possible role for Kinase associated protein phosphatase in the Arabidopsis CLAVATA1 signalling pathway. *Proceed. Natl. Acad. Sci.* U.S.A. 94, 10467-10472

38. Stone, J.M., Trotochaud, A.E., Walker, J.C. and Clark, S.E. (1998) Control of meristem development by CLAVATA1 receptor kinase and kinase-associated protein phosphatase interactions. *Plant Physiol.* 117, 1217-1225.

39. Di guglielmo, G.M., Drake, P.G., Baass, P.C., Authier, F., Posner, B.I. and Bergeron, J.J. (1998) Insulin receptor internalization and signalling. *Mol. Cell. Biochem.* 182(1-2), 59-63.

Summary

Transmembrane receptors are prime components of cellular signalling pathways and thus help determine cell fate, growth, differentiation, migration and death. Somatic embryogenesis is the process where by somatic cells develop into plants via the same morphological stages. The *Somatic Embryogenesis Receptor Kinase (SERK)* gene was isolated from *Daucus carota* somatic cells as a marker to monitor the transition of somatic into embryogenic cells. The *Arabidopsis thaliana Somatic Embryogenesis Receptor Kinase* 1 *(AtSERK1)* gene is the most closely related *Arabidopsis SERK* gene and is expressed in developing ovules, early embryos and in the vascular tissues of seedlings and adult plants. The predicted AtSERK1 protein contains an extracellular domain with a leucine zipper (LZ) motif followed by five leucine-rich repeats (LRRs), a proline-rich region, a single transmembrane region and an intracellular kinase domain. The major goal of the study presented in this thesis is the biochemical characterization of the AtSERK1 protein in order to reveal some of the early steps in the signal transduction cascade mediated by AtSERK1.

The experiments to reveal the biochemical properties were performed employing the comparative modelleing of the AtSERK1 kinase domain using the insulin receptor kinase domain as a template and the phosphorylation assays of affinity purified AtSERK1 and AtSERK1 mutant proteins produced *in vitro*. Based on the computer modeling studies, threonine residues in the AtSERK1 activation loop of catalytic sub-domain VIII were postulated to be targets for autophosphorylation activity. Single amino acid mutations replacing threonine residues in the AtSERK1 A-loop reveal the importance of Thr-468 in both AtSERK1 auto- and transphosphorylation. The ability of the AtSERK1 protein to transphosphorylate a catalytically inactive AtSERK1 protein shows that AtSERK1 catalyzes an intermolecular mechanism of autophosphorylation. Based on these observations, similar to the mechanism employed by animal receptors, the presence of a minor population of catalytically active AtSERK1 molecules *in vitro*, or ligand induced conformational changes *in vivo*, the activation of AtSERK1 requires intermolecular autophosphorylation. Activation of the receptor may require the movement of the AtSERK1 threonine containing A-loop followed by release of transphosphorylation activity and protein substrate binding. In this model, phosphorylation of Thr-468 in the A-loop of one AtSERK1 monomer is also essential for releasing catalytic activity of that same monomer.

The experiments to reveal the sub-cellular localization and dimerization of the AtSERK1 protein were performed using transient transfections in cowpea mesophyll protoplasts. In addition, the potential for dimerization of the AtSERK1 protein was determined by fluorescence resonance energy transfer (FRET) between GFP-derivative-AtSERK1 fusion in plant cells and in the yeast two hybrid based protein interaction system. The AtSERK1 protein is targeted to plasma membranes of plant cells. The extracellular LRRs, and in particular the N-linked oligosaccharides that are present on them are essential for correct localization of the AtSERK1 protein. The AtSERK1 protein exists as a monomer in the membrane. Only a minority of the AtSERK1 receptor molecules on the plasma membrane is in a predimerized state. In the absence of the LZ domain, the small population of predimerized AtSERK1 receptors is not detectable anymore, which shows the importance of the LZ domain in dimerization. While identification of the ligand for AtSERK1 is clearly essential for determining its precise mode of action, it is proposed that AtSERK1 exhibits ligand-induced homodimerization.

To demonstrate the interaction of the AtSERK1 protein with one of the potential downstream proteins, the *Arabidopsis* kinase associated protein phosphatase (KAPP) *in vitro*, phosphorylation experiments and transient transfections in cowpea mesophyll protoplasts were performed. It was shown that the kinase domain of AtSERK1 interacts with the kinase interaction domain of KAPP in a phosphorylation-dependant manner. The kinase interaction domain of KAPP does not interact with the catalytically inactive kinase mutant or the mutants lacking the essential threonines in the AtSERK1 A-loop for phosphorylation of AtSERK1 kinase. Using GFP-derivative fusions, we also show that the KAPP and AtSERK1 proteins are colocalized in plant cells at the plasma membrane and in intracellular vesicles, but only interact physically intracellularly. It is proposed that KAPP plays an essential role in internalization and inactivation of the AtSERK1 protein. The studies on the phosphorylation and dephosphorylation of these fusion proteins in live plant cells would therefore give more insight into the internalization mechanisms of the AtSERK1 protein.

The research described in this thesis provides ample information regarding the molecular nature of the AtSERK1 mediated signal transduction. It has formed the basis for *in vivo* experiments to study receptor dimerization and activation, and subsequent binding of downstream partners of AtSERK1. The identification of ligand(s) for AtSERK1 is now of crucial importance for future studies on AtSERK1 signal transduction.

Kashmiri summary

Science kis duniyahas manz akh baud baar challenge chu ki aakis jaandar sinz cell kithpaet chi celli nebrimis mahaul siet raebti kayam karan. Celli hindis sathas peth chi keinh molecule aasan yim aath manz madad chi karaan. Yiman moleculan chi vanan receptors, yim chi celli sarei nebrim khabar divan ti tath mutabiq chi cell andre badalaan. Ya chi akis celli zi banaan, ya chi celli badal taan banaan , ya chi cell maran. Yimwie manz chu aak receptor *AtSERK1* gene, yeth peth mei vase vanje kam kar. Ye receptor chu celli manz varyahas kamas kalas zahir gachaan, yotaan akh cell 100 cell chi banaan. Darasal chu yi sciencedaanan hinz rai ki ye receptor chu akis celli madad karan pauchar dinas manz taki ye cell heki braunt kun pakit akh poore jaan banit.

Yeth kaami manz dit mei varya karnaama anjaam. Aakis celli hindis sathas peth chi keinh receptor yim celli nebre peth chi signal ander anaan. *AtSERK1* gene chu banavaan akh poore protein. Ath proteinas chi paanch hise, zi hise yim celli nebar chi rozan (LRR ti LZ), zi hise yim celli ander chi rozan (kinase ti protein binding domain) ti akh hise yus celli hindis sathas pet chu aasan (transmembrane domain). Yemi kaami huind aham maqsad aus AtSERK1 proteineik khasoosi cheez vechnavin ti bayie vuchun ki yi receptor kithpat chu celli nebri path ander signale sauzaan.

Aaz kal chi science researchi manz cloning, sabaz ranguk gene, cell line ti bacteria aham role apanavan, meti heut yuhand varya madad. Aat alau kare mei zi aham technique istaimaal, protein phosphorylation ti beyi fluoresence resonance transfer (FRET). FRET technique chu vuchaan dun proteinan darmiaan kuta mael chu. *AtSERK1* genekis kinase hisas duit me aakis beis geneas (GST) seit vaat. Ami vathe seit banau mei aak protein bacteria cellan manz yus mei pata bakaya bacterial proteiniv nish saaf kor. Ami saaf protein seit kari mei varya tehkikaat. Yimav tehkikatav hove mei zi AtSERK1 protein chu serine/threonine kinase ti beyi threoonine 468 chu akh amino acid yus zaroori chu AtSERK1 proteinas active thavnas manz ti bei bakyan proteins seit milaap karnas manz.

AtSERK1 genas duit mei sabaz rang kis genas, GFPs seit vaat ti ami clonuk DNA banavit trovum cellan manz. 12 gantie pate vuch mein sabaz rang cell hindis sathas peth. Ami tehkikaat howe mei zi AtSERK1protein chu celli hindis sathaas peth muqam karan. AtSERK1 protein yemi manz mein LRR aus kodmit aus ni celli hindis sathas peth keinh balki aus celli underi akh yeri duing hui. FRET technique seit hau mei zi AtSERK1 chu aksar monomer aasan. Variyahen kam vaqti

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chui yi dimer aasan. AtSERK1 proteinuk LZ hisa chu aat dimer banas manz madad karan. Receptor chi aksar varya dimer banavaan yeli timan celli naeber kani akh lauqut protein vitish di. Yeuth protein chhuna wini aasi laubmut kein.

Saani labi manz aus bei aakh gene yus ma AtSERK1as seit milit kaam kare ha, ath chu naav kinase associated protein phosphate (KAPP). Asi aus sakh shokh ki aes vichav AtSERK1 cha KAPPas seit ralith kaem karan. Phosphorylation tehkikaat techniqav hauv asi ki AtSERK1 chu KAPPas seit milit kaam karan ti beyi AtSERK1 uk theronine-468 chu zaroori aath milaapas manz. FRET tehkikaat ti hauv asi zi KAPP ti AtSERK1 chi panavaan milan. Umeid chi mein pate yim lokh AtSERK1 peth kaem karan tim cchandan AtSERK1as vitish dina voel protein. Ami seit lagi AtSERK1 protein kaami saakh paucher. Ye kaam chi madad divan AtSERK1 proteinik taur tariqe samajnas manz.

Samenvatting

Een van de meest spectaculaire ontwikkelingen binnen de moleculaire celbiologie betreft de ontrafeling van signaaltransductie cascades, die ervoor zorgen dat signalen van buiten de cel naar hun 'effectoren' binnenin de cel overgedragen worden. Transmembraan receptoren zijn belangrijke componenten in veel van deze cascades, en zijn zo betrokken bij het bepalen van cel-identiteit, -groei, -differentiatie, -migratie en celdood. Somatische embryogenese is het proces waarin somatische plantencellen zich in cultuurmedium ontwikkelen tot kiemplantjes, via een reeks ontwikkelingsstadia vergelijkbaar aan die in de geslachtelijke of zygotische embryogenese. Het *Somatische Embryogenese Receptor Kinase (SERK)* gen uit de bospeen (*Daucus carota*) is geïsoleerd als merker voor cellen in de overgang van somatische naar embryogene staat. In *Arabidopsis* (zandraket) komt het gen dat het meest op het *SERK* gen lijkt, het *Arabidopsis thaliana SERK 1 (AtSERK1)* gen, tot expressie in ontwikkelende ovules, jonge embryos en in het vaatweefsel van zaailingen en volwassen planten. Het AtSERK1 eiwit bevat een extracellulair domein dat bestaat uit een leucine-zipper (LZ) motief gevolgd door vijf leucine-rijke 'repeats' (LRRs) en een proline-rijke regio. Daarnaast bevat het eiwit een enkele transmembraan regio en een intracellulair kinase domein. Het hoofddoel van het onderzoek dat in dit proefschrift wordt beschreven is de biochemische karakterisering van het AtSERK1 eiwit, om zo bij te dragen aan het ophelderen van de eerste stappen van de AtSERK1 signaaltransductie cascade.

Als basis voor de experimenten die uitgevoerd zijn om de biochemische eigenschappen van het AtSERK1 eiwit te bepalen is het kinase domein van het AtSERK1 eiwit gemodelleerd met als vergelijking de bekende structuur van het kinasedomein van de insuline receptor. Bovendien zijn fosforylerings-studies uitgevoerd met affiniteits-gezuiverde AtSERK1 en mutant AtSERK1 eiwitten, die *in vitro* geproduceerd werden. Op basis van computer-modellering werd voorspeld dat threonine-residuen in de activatie-lus ('A-loop') van het catalytische sub-domein VIII van AtSERK1 ten prooi konden vallen aan autofosforylering. Mutaties waarin individuele threonine residuen in de AtSERK1 A-loop vervangen werden wezen op het belang van Thr-468 voor zowel auto- als trans-fosforylering van AtSERK1. De observatie dat het AtSERK1 eiwit in staat is een katalytisch inactieve vorm van AtSERK1 te trans-fosforyleren toont aan dat AtSERK1 auto-fosforylatie kataliseert via een inter-moleculair mechanisme. De activatie van AtSERK1 lijkt dus afhankelijk te zijn van inter-moleculaire auto-fosforylering, die, naar analogie met mechanismen van dierlijke receptoren, in gang gezet zou kunnen worden door een kleine populatie katalytisch

actieve AtSERK1 moleculen *in vitro*, of door ligand-geïnduceerde conformatie veranderingen *in vivo*. Activatie van de receptor zou afhankelijk kunnen zijn van de beweging van de threonine-bevattende 'A-loop', gevolgd door trans-fosforylerings activiteit en tenslotte de binding van het substraat. In dit model is fosforylering van het Thr-468 in de 'A-loop' van een AtSERK1 monomeer essentieel voor de katalytische activiteit van datzelfde monomeer.

De experimenten ter bepaling van de sub-cellulaire localisatie en de mogelijke dimerisatie van het AtSERK1 eiwit zijn uitgevoerd met behulp van transiente transfecties van kousenband mesofiel protoplasten. De mogelijke dimerisatie van AtSERK1 werd bepaald door middel van Fluorescence Resonance Energy Transfer (FRET) tussen GFP-afgeleide AtSERK1 fusie-eiwitten in planten protoplasten, en met behulp van het gist 'two-hybrid' eiwit interactie systeem. In plantencellen wordt het AtSERK1 eiwit getransporteerd naar de celmembraan. De extracellulaire leucine-rijke-repeats (LRRs), en in het bijzonder hun N-gekoppelde oligosacchariden, zijn essentieel voor de correcte localisatie van AtSERK1. Het AtSERK1 eiwit bevindt zich voornamelijk als monomeer in de celmembraan. Slechts een klein gedeelte van de AtSERK1 moleculen bevindt zich in een 'ge-pre-dimeriseerde' vorm. Deze laatste vorm wordt niet aangetroffen in afwezigheid van het leucine zipper domein. Dit domein blijkt dus essentieel voor de dimerisatie van AtSERK1. Waarschijnlijk induceert binding van het ligand normaal geproken de homo-dimerisatie van het AtSERK1 eiwit, alhoewel de identificatie van dit ligand essentiëel blijft voor het ophelderen van het precieze mechanisme van dimerisatie.

Met behulp van fosforylerings experimenten en transiente transfectie van kousenband mesofiel protoplasten werd de *in vitro* interactie tussen AtSERK1 en het Kinase Associated Protein Phosphatase KAPP, een van de mogelijke *downstream* eiwitten van AtSERK1, aangetoond. In deze experimenten werd aangetoond dat het kinase domein van AtSERK1 fosforylerings-afhankelijke interactie vertoont met het 'kinase interactie domein' van KAPP. Deze interactie vindt niet plaats bij de catalytisch inactieve kinase mutant van AtSERK1, en ook niet bij de AtSERK1 mutanten die 'A-loop' threonine residuen missen die essentieel zijn voor de AtSERK1 fosforylering. Met behulp van GFP-afgeleide fusie-eiwitten werd bovendien aangetoond dat de KAPP en AtSERK1 eiwitten in plantencellen gecolocaliseerd zijn in de plasmamembraan en in intracellulaire *vesicles*, maar alleen in deze laatste fysieke interactie vertonen. Op grond hiervan wordt voorgesteld dat KAPP een belangrijke rol speelt bij de internalisatie en de inactivatie van de AtSERK1 receptor. Het bestuderen van de fosforylering en defosforylering van de AtSERK1

en KAPP fusie-eiwitten in levende plantencellen zou meer inzicht kunnen opleveren in de precieze mechanismen van AtSERK1 internalisatie.

Het in dit proefschrift beschreven onderzoek heeft uitgebreide informatie opgeleverd over de moleculaire aspecten van de signaaltransductie door AtSERK1. Het vormt de basis voor *in vivo* experimenten ter bestudering van receptor dimerisatie en activatie, en de daarop volgende binding van *downstream* partners van AtSERK1. Voor verdere bestudering van de AtSERK1 signaaltransductie is de identificatie van ligand(en) van AtSERK1 van cruciaal belang.

Acknowledgements

Curriculum Vitae

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B.Sc. in Biology and Agritechnology:

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Presentations

1. 9th Arabidopsis meeting in Wisconsin, Madison, USA, 1998
2. 2nd European embryogenesis network meeting, Barcelona, Spain, 1998
3. EDBC 99,"From life's design to molecular medicine", Oslo, Norway, 1999
4. 10th Arabidopsis meeting in Melbourne, Australia, 1999
5. European conference on signal transduction pathways and regulation of gene expression as therapeutic targets, Luxembourg, 2000

Manuscripts

1. Shah, K., Schmidt, E.D.L., Vlak, J.M., de Vries, S.C. (2001) Expression of Daucus carota Somatic Embryogenesis Receptor Kinase (DcSERK) protein in insect cells. Biochimie 83, 1-7.

2. Shah, K. and de Vries, S.C. (2001) Signal transduction mediated by single membrane spanning serine/threonine kinase receptors. (Manuscript in preparation for submission to Trends in Plant Sciences).

3. Shah, K., Vervoort, J., de Vries, S.C. (2001) Role of threonines in the AtSERK1 activation loop in auto- and transphosphorylation . Journal of Biological Chemistry (submitted).

4. Shah, K., Gadella, T.W.J., van Erp, H., Hecht, V. and de Vries, S.C. (2001) Subcellular localization and oligomerization of the Arabidopsis thaliana Somatic Embryogenesis Receptor-like Kinase 1 protein. Journal of Molecular Biology (in press).

5. Shah, K., Gadella, T.W.J. and de Vries, S.C. Physical and functional interactions between AtSERK1 and KAPP proteins. (Manuscript in preparation for submission to Genes and Development).

6. Shah, K. and de Vries, S.C. (2001) Leucine rich repeats of transmembrane proteins: roles in signal transduction and development (Manuscript in preparation).

Patent

1. Shah, K., Sijmons, P., de Vries, S.C. (2001) Improving the efficiency of transient transfection and expression of fluorescently labeled proteins in plant cells.