Biochemical investigations into the regulation and specificity of **SERK co-receptors**

Marije aan den Toorn

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Biochemical investigations into the regulation and specificity of SERK co-receptors

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Chapter 1

The SERK protein family

Structure and function of multi-tasking co-receptors in plants

M. aan den Toorn

The cell is the smallest biological unit that still exhibits all the hallmark properties of life. No cell can exist in isolation, for its survival it needs to interact with and respond correctly to environmental changes or developmental cues. This is true whether it is a unicellular organism, or part of a specialised tissue or organ in a multicellular organism. All cells possess a distinct physical barrier, the plasma membrane, between their internal components and the external environment. For extracellular signals, or ligands, to elicit intracellular responses, the plasma membrane contains many membrane spanning receptor molecules. These proteins contain an extracellular domain capable of perceiving signals and an intracellular domain capable of transducing that signal into the cell. The signal transduction cascade can be seen as a canonical reaction, where ligand binding to a receptor protein causes a conformational change leading to activation of intracellular signalling proteins (e.g. second messengers) and ending with the generation of transcriptional and non-transcriptional effects. During the cascade the signal is often amplified; more downstream proteins are activated than the number of ligand-bound-receptors initially present (Berg et al. 2002). Signalling cascades do not function as separate pathways but are part of intricate signalling networks, which are essential to be able to integrate the multitude of signals that a single cell perceives at any given moment. The precise composition and connections in these signalling networks differ for cells of different tissues, organs or developmental stages, providing plasticity in responses to a signal (Hynes et al. 2013). Cells achieve their specific signalling networks by altering the organisation of signalling proteins in location, time and concentration (Bhattacharyya et al. 2006, Kholodenko 2006, Scott et al. 2009; Good et al. 2011).

Formation of receptor complexes is essential in most signalling pathways. Many of the well-known eukaryotic receptor proteins, such as the receptor tyrosine kinases (RTKs) (van der Geer et al. 1994) and class I cytokine receptors (Bazan 1990) in animals and the receptor like kinases (RLKs) in plants (Shiu et al. 2001), are single pass transmembrane receptors that either have an intracellular kinase domain or associate with intracellular kinases. Since the intracellular kinase and extracellular ligand-binding domain are only linked via a single pass transmembrane helix, activation of these receptors occurs via dimeric or oligomeric complexes (Heldin 1995; Zhang et al. 2006; Ehrlich et al. 2011). Initial models stated that the inactive receptors are monomers and ligand-induced recruitment of interaction partners caused activation (Schlessinger 2000). In contrast, recent studies show that at least some receptors are already present in preformed oligomeric complexes in absence of activating ligand, such as epidermal growth factor receptor EGFR (Gadella et al. 1995; Clayton et al. 2005), cytokine receptor gp130 (Tenhumberg et al. 2006) and brassinosteroid receptor BRI1 (Bücherl et al. 2013). Whether the inactive receptor is monomeric or present in preformed complexes, activation still requires a structural change to an “active” dimeric or oligomeric conformation, induced by ligand binding. This brings the kinase domains of the receptors in close

enough proximity to activate each other via transphosphorylation events (Pang et al. 2012) With the growing amount of structural data available on receptor proteins (with and without ligand, in complex with interaction partners or inhibitors) we are now moving towards an atomic-level understanding of the mechanisms of signal transduction (as of July 2013, 3336 entries with the GO ID *cell-surface receptor signalling pathway* can be found in the RCSB Protein Data Bank; Berman et al. 2000). It is becoming increasingly clear that conformational changes in receptor complex structure are essential for ligand binding, complex formation and subsequent enzymatic catalysis (Grant et al. 2010).

When more than one type of receptor is part of the signalling complex required to mediate a cellular response, we can distinguish main receptors and co-receptors. The main receptor is defined here as the protein that both perceives a specific signal and induces intracellular changes in response to that signal. A co-receptor, or accessory/auxiliary receptor, is defined as a receptor that, on its own, cannot perceive the signal or transduce the signal across the membrane but still has a function in the signal transduction pathway. Many co-receptors are found to be promiscuous, being able to form complexes with several different main receptors, which allows them to function in multiple signalling pathways (Kirkbride et al. 2005). The Somatic Embryogenesis Receptor-like Kinase (SERK) protein family in plants is a good example of such multi-tasking co-receptors (Chinchilla et al. 2009). This family of receptor-like kinases functions in several diverse signalling pathways, and its members have partially overlapping functions (explained in more detail below). They are essential for proper signal transduction in several signalling cascades. This raises the questions why such additional receptors are needed, what kind of additional layer of modulation and regulation they provide, and how they confer their specific functions in the different signalling complexes.

In this chapter I will discuss current knowledge on signalling functions of the SERK co- receptor family and how this relates to other co-receptors. Next I will discuss the recent structural data on SERK proteins and their signalling complexes and the implications this new data provides for plant signalling complexes.

The multi-tasking functions of the SERK co-receptor family

The SERKs are a family of leucine-rich-repeat receptor like kinases (LRR-RLKs). After initially being implicated in the pluripotent potential of somatic cells (Schmidt et al. 1997), the SERK proteins were later identified to function in several different, seemingly unrelated, signalling pathways and show partial redundancy (Albrecht et al. 2005; Albrecht et al. 2008; Roux et al. 2011). The family consists of five highly homologous members in *A. thaliana*, arisen through gene duplication events (Hecht et al. 2001). Since the high homology between the SERK proteins

and their overlapping expression pattern would suggest redundancy, the precise biochemical mechanism for specificity in this protein family remains unclear. As of yet, autonomous ligand binding has not been established for the SERKs, and the emerging picture is that they form higher order complexes with main receptors to modulate signal transduction as co-receptors, which are essential for proper signal transduction (Gou et al. 2012) (see Figure 1). The SERK co-receptors are promiscuous, implicated in more than one signalling pathway including somatic embryogenesis (SERK1; Schmidt et al. 1997), abscission (SERK1;Lewis et al. 2010), male

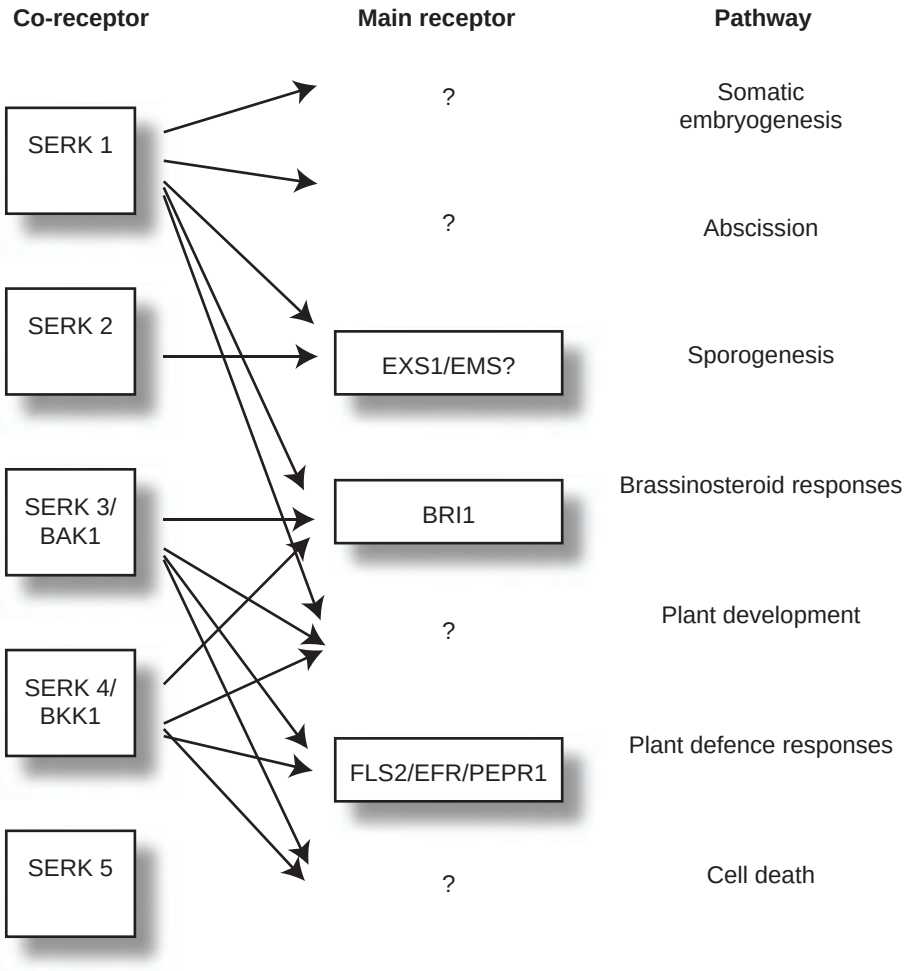

Figure 1 The different signalling pathways that make use of SERK co-receptors
The SERK co-receptors function in divers pathways, and only show partial functional redundancy. Their precise mode of action and necessity in the different pathways is not completely clear, and might differ for the different signalling pathways.

sporogenesis (SERK1 and SERK2; Albrecht et al. 2005), brassinosteroid signalling (SERK1, SERK3/BRI1 Associated Kinase 1 (BAK1) and SERK4/BAK1-like Kinase 1 (BKK1); Li et al. 2002; Nam et al. 2002; Karlova et al. 2006; He et al. 2007; Albrecht et al. 2008), plant development (SERK1, SERK3/BAK1 and SERK4/BKK1; Du et al. 2012), plant defence (SERK3/BAK1 and SERK4/BKK1; Chinchilla et al. 2007; Heese et al. 2007; Postel et al. 2010) and cell death responses (SERK3/BAK1 and SERK4/BKK1; He et al. 2007; Kemmerling et al. 2007). They form complexes with main receptors such as Brassinosteroid Insensitive 1 (BRI11; Russinova et al. 2004; Wang et al. 2008), Flagellin Sensitive 2 (FLS2; the main receptor for bacterial flagellin; Chinchilla et al. 2007; Heese et al. 2007) , Elongation Factor Receptor (EFR; the main receptor for the bacterial effector elongation factor Tu; Zipfel et al. 2006; Roux et al. 2011) and interaction has been proposed for Excess Microsporocytes 1 (EMS1)/Extra Sporogenous Cells (EXS) (involved in sporogenesis; Albrecht et al. 2005)) (see Figure 1). How the highly homologous SERK proteins are able to convey their specificity both in function and complex formation is not clear (see Chapter 2). Differences have been reported in the manner in which different signalling pathways use the SERK co-receptors. For example, BRI1 kinase activity is essential for the formation of SERK3/ BAK1 - BRI1 complexes (Wang et al. 2008), while neither FLS2 nor SERK3/ BAK1 kinase activity is necessary for the FLS2-SERK3/BAK1 complex formation (Schulze et al. 2010; Schwessinger et al. 2011). Also, a C-terminal tagged SERK3/BAK1 was found to be impaired in its function in flagellin signalling, while such a fusion protein was still completely functional in the brassinosteroid pathway (Ntoukakis et al. 2011).

The SERK proteins have been implicated in the regulation of main receptors via several modes of action. Many of these have also been described for other co-receptors in the animal and plant field, and will be discussed below in more detail.

Co-receptor function in receptor activation and signal amplification

Co-receptors can regulate receptor complex activity in different manners, one of which is by activating the main receptor via transphosphorylation events. An example of this can be found for SERK3/BAK1 co-receptor and main receptor BRI1 (Li et al. 2002). The main receptor BRI1 first activates its co-receptor by specific transphosphorylation of activation loop residues of SERK3/ BAK1. Activated SERK3/ BAK1 then phosphorylates the main receptor on residues in the juxtamembrane and C-terminal domain, events essential to achieve full downstream signalling activity (Wang et al. 2008).

A similar mechanism can be seen in the EGFR pathway, where transphosphorylation between main and co–receptors is also important for activation of the receptor kinase. Here the kinase domains form so-called asymmetrical dimers where one receptor kinase domain acts as a donor, ‘donating’ a phosphor group to the ‘receiver’ kinase that activates downstream signalling (Zhang et al. 2006). Of the

four EGFR family members (EGFR, ERBB2, 3 and 4) only EGFR and ERBB4 are main receptors. The ERBB2 receptor has an active kinase domain, but is not known to bind any ligand. ERBB2 can form heterodimers with the other three EGF receptors (EGFR, ERBB3 and ERBB4) when they are bound to ligands and act as a 'donor' kinase (Liu et al. 2012). ERBB2 is the preferred heterodimerization partner for the other EGFRs and increases their binding affinity for ligand (Karunagaran et al. 1996) resulting in prolonged signalling. In addition, heterodimers including ERBB2 are more stable and less prone to endocytosis and degradation indicating that presence of ERBB2 strengthens the cellular response to EGF (Haslekas et al. 2005; Offterdinger et al. 2008). In contrast to ERBB2, ERBB3 can bind ligand, but possesses an inactive kinase domain. ERBB3 can also form heterodimers with the other EGFRs (including ERBB2) and acts as an 'receiver' kinase to initiate downstream signalling (Campbell et al. 2010 and Figure 2).

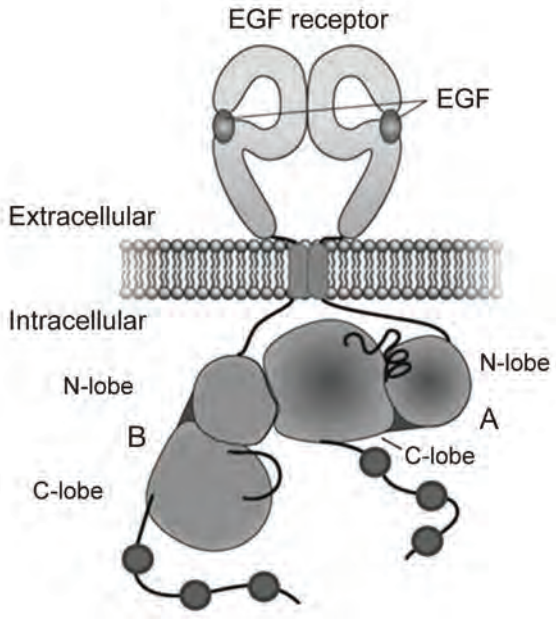

Figure 2 Mode of action of the EGFR signalling complexes with main and co-receptors
The EGFR protein family consists of four members, two of which can be seen as proper main receptors, while the other two either miss the ligand binding capacity (ERBB2) or intracellular enzymatic activity (ERBB3). The active complex is an asymmetric homo- or heterodimer, where one kinase acts as a donor (B) and one as a receiver (A) moiety. The co-receptors are either always donor (ERBB2) or receiver (ERBB3), while the main receptors (EGFR and ERBB4) can fulfil both roles. Picture from Gerbin 2010.

Co-receptor function in signal modulation via ligand interactions

In contrast to the SERK and ERBB co-receptors, many co-receptors do not possess an intracellular kinase domain. This type of co-receptors can either be transmembrane proteins with short intracellular domains without any enzymatic activity, or extracellular proteins anchored to the membrane by glycosylphosphatidylinositol (GPI). They function in ligand binding and mediating cellular adhesion (Kirkbride et al. 2005). In some signalling complexes, the co-receptors contribute so much to the affinity for the signalling molecule that ligand binding in absence of the co-receptor is negligible. Such is for instance the case in the neurophilin (NRP) co-receptors and semaphorin binding. NRPs have a high binding affinity for two structurally unrelated classes of ligands, the class 3 semaphorins (secreted polypeptides with key roles in axonal guidance) and certain members of the vascular endothelial growth factors (VEGF). Most of the class 3 semaphorins (but not VEGF) require NRP1 or 2 as obligate co-receptors to the plexin main receptors (Pellet-Many et al. 2008), which do not bind semaphorins on their own. The semaphorins seem to share a binding interface with both the plexin receptor and the NRP1, but only the plexins mediate the signal into the cell (Antipenko et al. 2003). In VEGF signalling, NRP1 was found to enhance (i) VEGF binding to VEGF receptor2 (VEGFR2), (ii) VEGFR2 phosphorylation and (iii) VEGF-induced signalling (Whitaker et al. 2001; Soker et al. 2002; Mac Gabhann et al. 2005). NRP1 is not an obligate co-receptor in VEGF-signalling, and VEGFR2 activation can occur in absence of NRP1 (Pan et al. 2007), however NRP1 is required for optimal signalling in certain biological processes (Pellet-Many et al. 2008). The precise manner in which NRP1 modulates VEGR2 signalling is not known, presence of NRP1 does not appear to change the affinity of VEGR2 for ligand (Whitaker et al. 2001), but it is hypothesised that NRP1 stabilises the signalling complex thereby lengthening the signal propagation (Jia et al. 2006).

In Fibroblast Growth Factor (FGF) signalling, the FGF-receptor depends on the Klotho co-receptors for endocrine signalling, but not for paracrine signalling. The main FGF receptor (FGFR) has a much lower binding affinity for endocrine FGF compared to paracrine FGFs. The Klotho co-receptors increase the affinity of the FGFR for the endocrine FGFs and lower it for paracrine FGFs in specific tissues (Goetz et al. 2012). In this manner, the FGF receptor complex can be converted in a specific endocrine or paracrine receptor complex based on the presence or absence of the co-receptor. In plants, the co-receptor Too Many Mouths (TMM) interacts with main receptors of the ERECTA-family functioning stomatal patterning (Shpak et al. 2005). TMM modulates the signal by differing in affinity for the two ERECTA-ligands (EPF1 and EPF2) and forming heterodimers with the different ERECTA main receptors (Lee et al. 2012). The incredibly complex WNT signalling pathway is involved in a multitude of developmental processes, such as embryonic axis formation and segmentation, and involves more than 15 different receptors and co-receptors (Niehrs 2012). The main receptor, Frizzled, uses different co-receptors

(such as LRP5, LRP6, ROR1 and ROR2) as a means to direct signal transduction through distinct signalling pathways.

In the brassinosteroid pathway, the main receptor BRI1 has roughly the same binding affinity for its ligand with or without the presence of its co-receptor SERK3/ BAK1, which led to the hypothesis that SERK3/ BAK1 did not contribute to brassinosteroid binding (Kinoshita et al. 2005). However, recent crystallographic studies of the extracellular domain of BRI1 showed that binding of ligand created a small hydrophobic patch, which might serve as an interaction platform for co-receptors. In this scenario, co-receptors would interact with the ligand and could have a function in binding affinity of the main receptor BRI1 to its ligand (Hothorn et al. 2011; She et al. 2011). This notion was shown to be correct in a recent study where the extracellular domains of BRI1 and SERK1 were co-crystallised in the presence of the BL ligand (Santiago et al. 2013), designating the SERKs as true co-receptors. The structure of the FLS2 extracellular domain with the SERK3/ BAK1 extracellular domain in presence of the ligand flg22 (a 22 amino acid peptide which was shown to bind to FLS2 and elicit immune responses (Gómez-Gómez et al. 2000) has shown that also in this signalling pathway the SERK co-receptor has interactions with both the ligand and the main receptor (Sun et al. 2013). It is thus unlikely that the SERK co-receptors do not contribute to the binding affinity of the signalling complex. Most likely the unchanged binding affinity in absence of SERK3/ BAK1 reported by Kinoshita et al. is due to redundancy between the SERK family members.

Co-receptor function in receptor localisation

After receptor activation, signal propagation is often controlled by means of receptor downregulation. This downregulation of receptors involves endocytosis of signalling complexes and subsequent intracellular degradation of both receptor and ligand, or the recycling of receptor back to the plasma membrane. Endosomal trafficking affects receptor concentration at the plasma membrane and can thus profoundly affect signalling (Chen et al. 2005). In addition, some receptors continue to signal from endosomal compartments, as was seen for the platelet-derived growth factor receptor (Wang et al. 2004) and the receptor tyrosine kinase c-Met (the receptor of hepatocyte growth factor; Kermorgant et al. 2008). The Ephrin receptors even require internalisation for their signalling functions (Sadowski et al. 2009). Co-receptors can affect signalling by regulating the endocytosis of (parts of) the receptor complex. An example is the TβRIII co-receptor, which is phosphorylated by the TβRII main receptor in response to TGF-β (Chen et al. 2003). The phosphorylation of TβRIII causes recruitment of β-arrestin 2, followed by internalisation of the complex and downregulation of signalling. For VEGFR2, interaction with its co-receptor NRP1 causes the main receptor to follow a different endocytic

route, leading to reduced degradation and more recycling of the receptor, increasing signal attenuation (Ballmer-Hofer et al. 2011).

In protoplasts, SERK3/ BAK1 accelerates endocytosis of BRI1, changing the ratio of endocytic to PM localised BRI1 receptor (Russinova et al. 2004). The precise function of endocytosis in BRI1-mediate signalling (downregulation or attenuation) is not yet completely clear (Geldner et al. 2007; Irani et al. 2012). Geldner et al. 2007 reported an increase in brassinosteroid signalling activity upon addition of the drug BFA (Brefeldin A), which they attributed to the increased endosomal localisation of BRI1. This led to the conclusion that a substantial amount of brassinosteroid signalling takes place in endosomal compartments. However, recent studies indicate that the PM is the major site for brassinosteroid signalling (Irani et al. 2012) and the increased signalling upon BFA treatment is caused by an increase of PM localised BRI1- SERK3/BAK1 hetero-oligomers (Bücherl et al. 2013). These new studies suggest that endocytosis of the BRI1-SERK3/BAK1 complex most likely is involved in downregulation of signalling. The same co-receptor SERK3/ BAK1 was found to impair endocytosis of a different main receptor in flagellin signalling (FLS2) (Chinchilla et al. 2007), indicating that co-receptors can have opposing functions in different signalling pathways.

Another means by which co-receptors can influence signal transduction is by sequestering the signalling complex to specific locations on the plasma membrane, such as microdomains or membrane rafts enriched in sphingolipids and cholesterol. An example of this is the glycosyl phosphatidylinositol (GPI)-anchored co-receptor GFR α1, which recruits the main receptor c-RET (binding a specific class of neurotrophic factors) to membrane rafts, which is necessary for efficient downstream signalling (Tansey et al. 2000). Whether the SERKs also function in recruiting main receptors to specific membrane locations is a question that remains to be answered (see Chapter 5).

Co-receptors as mediators of cross-talk

At any given moment, a cell is bombarded by a multitude of signals. All cells have to integrate these signals into a comprehensive response or developmental change. For this reason, cross-talk between different signal transduction cascades is essential. Cross-talk can exist on multiple levels within a signalling cascade, one of which can be co-receptors shared between different signalling routes. This cross-talk can manifest itself as competition, two signalling cascades competing for a limited pool of shared co-receptors, or synergistic, where the co-receptor activates a common downstream signalling partner. In animal systems, the insulin and WNT pathway show cross talk at several levels in the signalling cascade, one of which is at the level of co-receptors. The co-receptor LRP5 is used both by the insulin receptor and the WNT pathway, and here the cross-talk seems to be synergistic, LRP5 providing a bridge between the two different signalling pathways (Palsgaard et al. 2012).

The SERK3/BAK1 co-receptor is an essential player in both the brassinosteroid and flagellin (immunity) pathway in *A. thaliana*. Cross-talk between these two pathways is evident, and is proposed to be at the level of the co-receptor (Belkhadir et al. 2012). However, investigations proved that SERK3/BAK1 could not be the limiting factor (Albrecht et al. 2012) and recent studies have identified at least one position of cross talk between the innate immunity and brassinosteroid signalling pathway further downstream (Shi et al. 2013). Whether this downstream cross-talk is mediated via SERK3/BAK1 is not clear.

The structure of the SERK co-receptors

As has been explained above, one of the hallmarks of co-receptors is that they modulate the signal transduction across the membrane, either by extracellular interactions, intracellular interactions, or both. Here, we will review the current knowledge on the structure of the SERK proteins and their interaction with main receptors, and discuss functional domains of the co-receptors.

Domain structure of the SERK proteins

SERK-protein homologues were identified in a number of plant species, including monocots, dicots and nonvascular plants (Baudino et al. 2001; Hecht et al. 2001; Sasaki et al. 2007; Mantelin et al. 2011; Nolan et al. 2011; Park et al. 2011; Ma et al. 2012), where they all follow the same basic domain structure (see Figure 3). The extracellular domain of SERKs consists of a leucine zipper, sometimes also revered to as the N-capping residues, followed by ~5 leucine-rich-repeats (LRRs) and a serine-proline-proline (SPP) motif containing domain, a combination that is unique for the SERK proteins (Hecht et al. 2001). A single transmembrane helix connects the extracellular domain to the intracellular domain, which consists of a juxtamembrane domain, a kinase domain and a C-terminal tail.

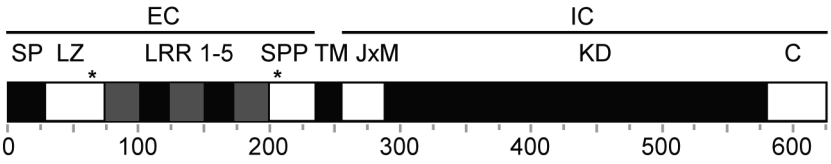

Figure 3 Overview of the typical domain structure of SERK proteins
Numbers below the graphic depict approximate amino acid numbers (based on SERK1 domain structure and sequence). EC= extracellular domain, IC= intracellular domain. SP= signal peptide. LZ= leucine zipper, LRR= leucine rich repeats, SPP= serine-proline-proline motif containing domain, TM= transmembrane domain, JxM= juxtamembrane domain, KD= kinase domain, C = C-terminal tail domain.

Three dimensional structure of SERK intracellular domain

In 2011 and 2012 two crystal structures of the SERK3/ BAK1 intracellular domain have been elucidated (Cheng et al. 2011; Yan et al. 2012). Besides the complete kinase, both structures contain part of the JxM domain and the C-terminal tail, but neither of these domains is well-resolved. The structure of the SERK3/ BAK1 kinase domain follows the basic structure found for Ser/Thr kinases, with a two-lobed structure consisting of a small N-terminal lobe (mostly consisting of anti-parallel β-sheets) and the larger C-terminal lobe (consisting mostly of α-helices) as well as a catalytic cleft between the two lobes (Cheng et al. 2011; Yan et al. 2012). Both crystal structures of SERK3/ BAK1 are of the active state of the protein, as similarity to other active kinases indicates. Phosphorylation of residues in the catalytic loop, and especially T450 and T455, stabilises the active state (see Figure 4). T450 phosphorylation was found to be essential for transphosphorylation activity of SERK3/ BAK1 on FLS2 (Wang et al. 2008; Yan et al. 2012), however, not for BRI1 transphosphorylation in yeast (Yun et al. 2009) indicating that differential phosphorylation of the activation loop probably changes the conformation and specificity of the SERK kinase domains. *In vivo* experiments do indicate that T450 is an important residue in the brassinosteroid pathway (Wang et al. 2008). In brassinosteroid signalling, SERK3/ BAK1 is first phosphorylated by BRI1, followed by the reciprocal reaction which leads to a fully activation signalling complex (Wang et al. 2008). So although mutating T450 does not influence basal transphorhorylation activity of SERK3/ BAK1 onto BRI1, it does influence the sequential activation of receptors. Further investigations into the function of different phosphorylation sites on the activity and more importantly the specificity of the SERK proteins in different pathways is necessary to increase our knowledge on these plant-signalling pathways.

The SERK kinase domains, as well as several other kinase domains of receptor kinases show intrinsic activity *in vitro* (Shah et al. 2001; Wang et al. 2005; Karlova et al. 2009; Aan den Toorn et al. 2012), without the need of complex formation or other cellular components to activate its phosphorylation activity. For receptor kinases, this means that their cytoplasmic kinase domain needs to be inhibited by some means *in vivo* to avoid aberrant, premature phosphorylation. The biochemical mechanisms with which receptor kinase domains are kept inactive are poorly understood, mechanisms that have been proposed are inhibitor binding (Jaillais et al. 2011b), auto-inhibitory sequences (Wang et al. 2005) or dephosphorylation of essential phosphorylation sites (Ostman et al. 2001). In addition, protein intrinsic disorder has been implicated in regulation of signalling events, especially for receptors that use multiple protein chains to confer signal (Sigalov et al. 2011). How the kinase domains of the SERK co-receptors are kept inactive remains to be elucidated (see Chapter 4).

In the structure reported by Cheng et al. the SERK3/ BAK1 kinase domain is in complex with the BAK1 Interacting Domain (BID) of the bacterial effector AvrPtoB (Cheng et al. 2011). This bacterial effector was shown to interact with the kinase

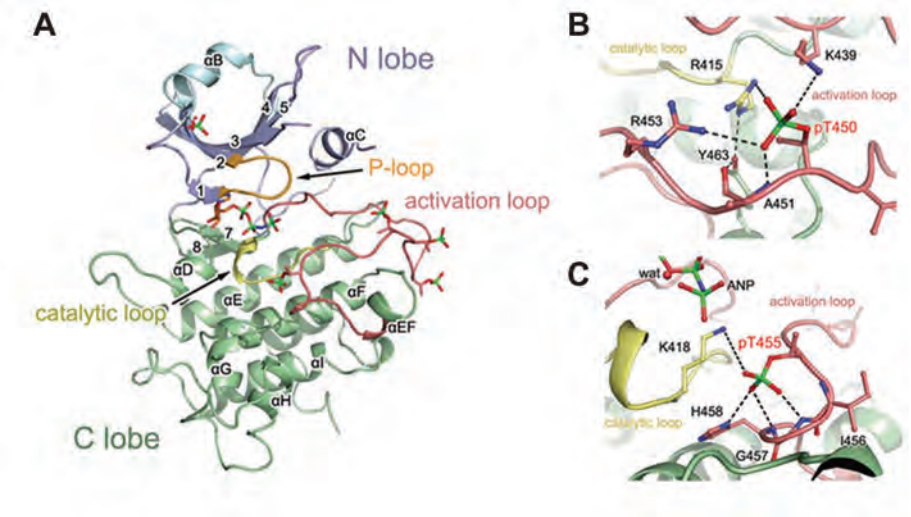

Figure 4 Phosphorylation of the BAK1 activation loop stabilises the kinase active state. A) Ribbon model of the BAK kinase domain structure in complex with the ATP analogue AMP-PNP (coloured stick). B&C) phosphorylated residue T450 (B) and T455 (C) contribute to the stabilisation of the activation loop conformation. Dashed lines indicate hydrogen-bonding interactions. Picture adapted from Yan et al. 2012.

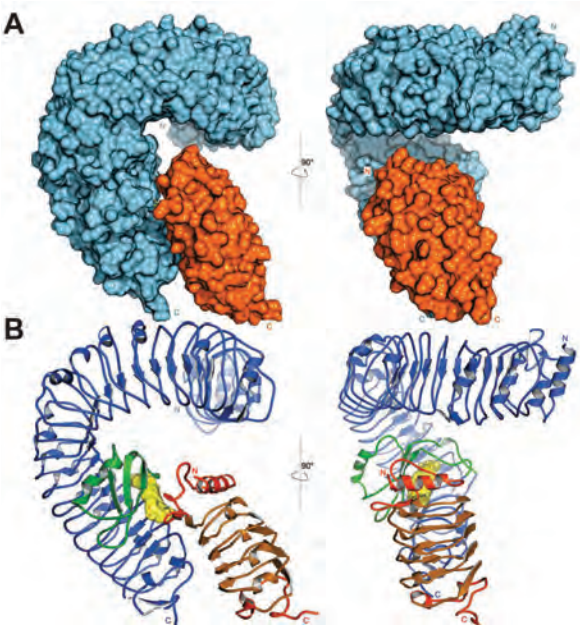

Figure 5
Crystal structure of BRI1 extracellular domain in complex with SERK1 extracellular domain
A) Surface representation of the BRI1 extracellular domain (light blue) in complex with SERK1 co-receptor extracellular domain (red). B) Ribbon representation of the BRI1- SERK1 extracellular domain complex, with the brassinosteroid ligand depicted in yellow, BRI1 LRRs in blue, BRI1 island domain in green, SERK1 N-capping residues in red and SERK1 LRRs in brown. No structural rearrangements were apparent upon complex formation; the binding of extracellular domains seems to function mainly in bringing the intracellular kinase domains in close proximity. The SERK1 N-capping residues fold onto the steroid ligand, and seem to have a function in brassinosteroid binding. Picture from Santiago et al. 2013.

domains of SERK3/ BAK1 and FLS2, interfering with PAMP-triggered immunity (PTI) (Xing et al. 2007). The crystal structure shows that the method by which AvrPtoB inhibits SERK3/ BAK1 function is by interaction with the catalytic cleft of SERK3/ BAK1, via contacts with the P+1-loop (in the substrate-binding region) of the SERK3/ BAK1 kinase domain. The presence of AvrPto in the catalytic cleft of SERK3/ BAK1 would make complex formation with interaction partners impossible and thus inhibit formation of the active signalling complex FLS2-SERK3/ BAK1. Since SERK3/ BAK1 also makes other complexes where kinase interactions are essential (Wang et al. 2008), it could be that the presence of AvrPtoB influences more than just PTI.

Structural implications of complex formation of SERK with main receptors

Currently, two crystal structures have been reported of SERK extracellular domains in complex with main receptor extracellular domains. These are the SERK1 -BRI1 extracellular domain complex (Santiago et al. 2013) and the SERK3/ BAK1- FLS2 extracellular domain complex (Sun et al. 2013). These structures provide the first observations on the SERK co-receptors in the signalling complexes

The BRI1 extracellular domain was first elucidated (with and without ligand binding) in absence of SERK co-receptors (Hothorn et al. 2011; She et al. 2011). The 25 LRRs of BRI1 curve to form a 'super-helix', where the island domain, involved in ligand binding, folds back into the interior of the curved structure. The super-helical curvature was also observed for the FLS2 extracellular domain (Sun et al. 2013) and is clearly distinct from animal LRR-domains which fold to a horseshoe like shape without the extra curvature of the super-helix (Kobe et al. 2001). Both the FLS2 and BRI1 extracellular domains were found as a monomer irrespective of ligand presence, both in crystals as well as in solution (Hothorn et al. 2011; She et al. 2011; Sun et al. 2013). This, and the fact that homodimerization seemed to create some impossible steric clashes for BRI1, indicates that ligand induced homodimerization of the main receptor extracellular domain is unlikely (Hothorn et al. 2011). Ligand binding also does not induce large conformational changes to the main receptor extracellular domain; besides some structuring of the island domain and connecting loops no significant changes are observed in the BRI1 structure (Hothorn et al. 2011) and also the FLS2 extracellular domain does not change substantially after ligand binding (Sun et al. 2013). This is in contrast to the previous hypothesis that ligand binding would induce large conformational changes in the signalling complex, resulting in activation (Wang et al. 2008; Jaillais et al. 2011a). These observations led to the hypothesis that the main receptor and ligand alone are not sufficient for initial activation of the main receptor; an additional player (a co-receptor) is essential to induce conformational changes in the complex upon ligand binding. The crystal structures of the BRI1-SERK1 and FLS2-SERK3/ BAK1

extracellular domain complexes corroborate this hypothesis. For both complexes, ligand binding is essential for interaction between the co-receptor and main receptor extracellular domains. The SERK1- BRI1 complex showed that the SERK1 N-capping residues (sometimes also referred to as a leucine zipper domain, see Figure 3, although this crystal structure seems to indicate that this is not the correct term) fold onto the island domain of BRI1, making contacts with both the ligand binding domain of the main receptor and the steroid hormone, establishing the SERKs as true co-receptors (see Figure 5). The structure of the FLS2- SERK3/ BAK1 complex shows similar observations. The SERK3/ BAK1 extracellular domain makes interactions with both the ligand (flg22) and the main receptor, indicating that this seems the common mode in which SERK co-receptor function in signalling complexes.

The observation that ligand binding is essential for interaction between main and co-receptor, and that homodimerization of the main receptor seems unlikely, is in contrast to some other reports that show the presence of preformed complexes of BRI1 and SERK3/ BAK1 *in planta* (Bücherl et al. 2013) and homodimerization of BRI1 (Wang et al. 2005; Hink et al. 2008) and FLS2 (Sun et al. 2012). It is important to realise that the current structures only include the extracellular domains and not the complete receptors. Interactions between transmembrane domains or kinase domains might very well contribute to interactions in the absence of ligand. Ligand binding could then induce extracellular domain interactions that cause a change in overall receptor complex formation, which might lead to signalling activation.

Concluding remarks

Co-receptors are important players in signalling, due to their promiscuous nature they create more flexibility and provide a place of confluence between different signalling pathways. Often, co-receptors have either extracellular interactions with the main ligand functioning in ligand binding and specificity, or an intracellular interaction functioning in signalling complex activation or downstream signalling. The SERK protein family in plants seems to have a dual co-receptor function, having both interactions with the ligand as well as transphosphorylation activity on the main receptor. Most likely, different interaction sites and different substrate specificity of the kinases are important for their functions as multi-tasking co-receptors.

The current crystal structures of plant LRR-RLKs provide us with a first atomic scale map of interactions between the SERK co-receptors and their partners. These structures and others will be essential for our understanding of the different functions and complexes of the SERK co-receptor family.

Outline of this thesis

In this thesis I will report the work done on the SERK co-receptor family to further our knowledge on their specific functions in the different signalling cascades. I try to take a more structural look at the proteins to identify domains and regulatory elements that could provide these proteins with their multi-tasking yet specific functions in plant development and immunity.

SERK co-receptors are found in all monocots and dicots, but also in non-flowering plants such as the moss *Physcomitrella patens*. In **Chapter 2** I provide a thorough sequence analysis of SERKs in different plant species, to try and identify which motifs or domains in the sequence are important for the observed functional differences between the SERK family members in the *A. thaliana* . Using chimeric SERK constructs, we attempt to couple specific signalling functions to either the intracellular or extracellular SERK domains.

Additional high resolution crystal structures of the SERK proteins can greatly increase our knowledge on the function and complex formation of these proteins. In **Chapter 3** I discuss our attempts to elucidate the crystal structure of different domains of the SERK proteins.

Receptor activation needs to be tightly controlled to avoid aberrant signalling. Although many investigations have been focused on how receptors can be activated, a perhaps even more important question is how receptors are kept in their "non-responsive" states. For EGF, BRI1, SERK1, 2, 3 and 4 as well as many more, their *in-vitro* produced kinase domains have intrinsic phosphorylation activities. Thus additional regulatory elements have to be present in the cell to keep the receptors from signalling in the absence of ligand. In **Chapter 4** I investigate one possible manner to regulate protein activity, namely intrinsic disorder.

Proper localisation is essential for proper function of proteins. As was previously discussed, some co-receptors influence signalling complex activity by changing the subcellular location. In **Chapter 5**, I discuss the use of a microscopic technique called Variable Angle Epifluorescence Microscopy (VAEM), a variant of Total Internal Reflection Fluorescence (TIRF) microscopy to investigate the subcellular and membrane location of proteins *in planta*, and use this technique to visualise the membrane localisation of co-receptor SERK3/BAK1 and main receptor BRI1.

The final chapter of this thesis is the general discussion, where I will review all the new observations discribed in this thesis and discuss them in light of current literature.

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Chapter 2

On the Origin of SERKs

Bioinformatics analysis of the Somatic Embryogenesis Receptor Kinase

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Abstract

SERKs (somatic embryogenesis receptor like kinases) are leucine -rich- repeat receptor-like-kinases involved in several, seemingly unrelated, plant-signalling pathways. In *A. thaliana*, functional and genetic analysis on four SERK proteins have indicated that they are only partly redundant; their functions overlap but each member performs a specific subset of signalling roles. The molecular basis for the functional specificity within this highly homologous protein family is currently not known.

Sequence analysis of SERK proteins from different plant species indicates that the SERKs are a highly conserved protein family present in monocots, dicots and non-vascular plants. Residues in the extracellular domain that are important for interaction with other receptor kinases are highly conserved, even amongst SERK members without function in the corresponding pathways. SERK2, for instance, is conserved in its interaction domain for BRI1, while SERK2 does not function in the brassinosteroid pathway. Further sequence analysis indicates that SERK3/BAK1 and SERK4/BKK1 have diverged from the original SERK protein sequence in both their extracellular domain and cytoplasmic domain, which could account for the functional divergence. Functional analysis of chimeric SERK proteins shows that different domains provide the SERK proteins with different functional specificity. The SERK1 or SERK2 extracellular domains are essential for SERK function in male sporogenesis, while both the SERK3 extracellular domain and cytoplasmic domain are essential for SERK3 specific brassinosteroid signalling and flagellin signalling functions.

The emerging picture is that SERKs are ancient genes, which have been recruited to newly evolved signalling pathways. The interaction domains in the extracellular SERK domain are conserved, allowing all SERKs to form complexes. However, specific functional residues must have been altered, both in the extracellular and intracellular domain, which allow for differences in functionality.

Introduction

In plants, the majority of cell-surface receptors belong to the very large protein family of RLKs (Shiu and Bleecker 2001b), which in turn is part of the monophyletic RLK/Pelle gene family that shares a common ancestor with the animal receptor tyrosine and receptor serine/threonine kinases (RTKs and RSKs) (Shiu and Bleecker 2001b). After the divergence of plant and animal lineages, a significant expansion of the plant RLK family has occurred, resulting in a gene family of more than 600 members in *Arabidopsis*, representing 2.5% of the protein-coding genes (Shiu and Bleecker 2001a). Such an extensive number of different receptors provide plants with impressive possibilities to respond rapidly and precisely to a large number of external signals. The RLK family expansion seems to be lineage-specific, and caused both by whole genome duplications and tandem duplications (Shiu and Bleecker 2001a). Gene duplicates were most likely retained because they either developed new functions (as can be seen for instance for the symbiosis receptors), or because a partitioning of ancestral functions or expression patterns has occurred between duplicates (Nadeau and Sankoff 1997). The many duplicates of plant RLKs have made functional studies on specific receptors difficult, as single mutants often do not show clear phenotypes due to (partial) redundancy.

A typical RLK follows the same basic domain structure as animal RTKs (van der Geer et al. 1994, Walker 1994) with an N-terminal signal sequence, an extracellular, ligand binding domain (extracellular domain) followed by a single pass transmembrane domain (TM) and an intracellular kinase domain (cytoplasmic domain). The motifs making up the extracellular part of RLKs vary greatly among members, the most common being leucine rich repeats (LRR) (235 of the ~610 RLKs contain 1 to 32 LRRs in their extracellular domain; Shiu and Bleecker 2001a). LRR-RLKs are involved in many different signalling processes such as hormone signalling (Li and Chory 1997), immunity (Gómez-Gómez and Boller 2000) and growth and development (Clark et al. 1997). The family of LRR-RLKs can be divided into 13 subfamilies (LRR-RLK I to LRR-RLK XIII) (Shiu and Bleecker 2001b). This subdivision was based on kinase phylogeny, but for the most part follows a division based on the extracellular domain structure. This division does not appear to be coupled to functionality, as a great diversity of transcriptional responses and expression patterns was found within the different subfamilies (Chae et al. 2009). The SERKs, or Somatic Embryogenesis Receptor Kinases, belong to the LRR-RLK II group of receptor-like kinases. Besides the SERK genes, the LRR-RLK II group consists of two other distinct branches in *A. thaliana*; a group of functionally unassigned proteins and the NIKs (NSP-interacting kinases), which are virulence targets of the bipartite geminivirus nuclear shuttle protein (NSP) (Fontes et al. 2004). The SERK proteins have a small extracellular domain consisting of 4.5-5 LRRs. They distinguish themselves from other LRR-RLK II members by the presence of a proline-rich domain (SPP-domain) in the extracellular domain, just preceding

the transmembrane domain (Hecht et al. 2001). This unique domain of unknown function was hypothesised to provide a flexible hinge to the SERK extracellular domain (Schmidt et al. 1997, Hecht et al. 2001). Genes encoding SERKs have been identified in all higher plants genomes, both monocots and dicots, and Sasaki et al. reportedly identified the presence of SERK homologues in the lower plant liverwort *Marchantia polymorpha* and even in the unicellular green algae *Closterium ehrenbergii* (Sasaki et al. 2007). SERK was first identified in *Daucus carota* cell cultures, where it marked single somatic cells competent to form embryos (Schmidt et al. 1997). In *A. thaliana*, the SERK protein family consists of five close homologues (SERK1-5) (Hecht et al. 2001) that have arisen through gene duplications. An initial duplication event that gave rise to ancestral precursors of SERK1, 2 and SERK3, 4, 5 was followed by further duplications to form the current protein family (Hecht et al. 2001, He et al. 2007). The five *At*SERK proteins were found to function in multiple and diverse signalling pathways, which are seemingly unrelated (Albrecht et al. 2005, Albrecht et al. 2008, Roux et al. 2011). These signalling pathways include e.g. male sporogenesis, separation of floral organs and Mi-1-mediated resistance to potato aphids (SERK1 and SERK2) (Albrecht et al. 2005, Lewis et al. 2010, Mantelin et al. 2011), brassinosteroid (BR) signalling (SERK1, SERK3/BRI-associated kinase 1,BAK1, and SERK4/BAK1-like kinase 1, BKK1) (Li et al. 2002, Nam and Li 2002, Karlova et al. 2006, He et al. 2007, Albrecht et al. 2008), flagellin signalling (SERK3/BAK1 and SERK4/BKK1) (Chinchilla et al. 2007, Heese et al. 2007) and cell death responses (SERK3/BAK1 and SERK4/BKK1) (He et al. 2007, Kemmerling et al. 2007). SERK5 has no recorded function and might be a pseudogene (He et al. 2007). Interaction has been established between SERK3/BAK1 and the main ligand-binding receptors Brassinosteroid Insensitive 1 (BRI1) (Li and Chory 1997, Li et al. 2002, Nam and Li 2002, Russinova et al. 2004, Wang et al. 2008), BRI-like 3 (BRL3) (Fabregas et al. 2013) Flagellin Sensing 2 (FLS2) (Chinchilla et al. 2007, Heese et al. 2007), and EF-tu Receptor (EFR) (Roux et al. 2011), while Excess Microsporocytes 1 (EMS1)/Extra Sporogenous Cells (EXS) has been proposed as interaction partner of SERK1 and SERK2 (Albrecht et al. 2005). These main ligand binding receptors are also LRR-RLKs, BRI1 and BRL3 (both 24 LRRs, involved in brassinosteroid signalling; Li and Chory 1997, Fabregas et al. 2013) and EMS1/EXS (30 LRRs, involved in sporogenesis; Canales et al. 2002, Zhao et al. 2002) are both part of the LRR-RLK X subfamily, while FLS2 (28 LRRs, involved in flagellin sensing; Gómez-Gómez and Boller 2000) and EFR (21 LRRs, binding bacterial elicitor EF-tu; Zipfel et al. 2006) belong to the LRR-RLK XII subfamily. SERK1 and SERK3 act as a true co-receptor involved in binding the ligand together with the main receptor BRI1 (Santiago et al. 2013) and FLS2 (Sun et al. 2013) respectively. The current model for the interaction between BRI1 and SERK3 / BAK1 assumes reciprocal transphosphorylation events to arrive at a fully functional signalling unit (Wang et al. 2005a, Wang et al. 2008) and in the absence of several of the SERK members BR signalling is fully impaired (Gou et al. 2012). For clarity, we will refer hereafter to SERK3 without mentioning its BAK1 nomenclature.

Current genetic data indicate that the SERKs are critical regulators of the plant developmental pathways served by the corresponding main receptors (Gou et al. 2012). However, it is not clear which precise cellular functions are under control of the SERKs. The genetic data also shows that the diverse SERK genes have partially non-overlapping functions, and isoform-specific signalling contributes to the diversity of SERK activities. Potential divergence of cis-regulatory sequences (Mazet and Shimeld 2002, Prince and Pickett 2002) is not the mechanism used to obtain the functional divergence, because the *serk1 serk2* male sterile phenotype cannot be restored by the SERK3 protein while expression of SERK2 or SERK1 under control of the SERK3 promoter is sufficient (Albrecht et al. 2005). This observation is also supported by the fact that SERK3 functions in different signalling pathways at the same time, in the same cell (Robatzek et al. 2006, Savaldi-Goldstein et al. 2007). At the moment, the protein domains that contribute to SERK specificity and redundancy are not very well defined.

This chapter describes attempts to map SERK specificity to certain structural domains, motifs or residues. Sequence analysis shows that both extracellular domains and cytoplasmic domains contain divergent sequences that could account for the different functionality. Domain-swap experiments corroborate the conclusion that evolutionary changes to both extracellular domain and cytoplasmic domain are important for SERK specificity. Remarkably, some SERK domains are interchangeable between family members in one pathway but not in another. In particular, whereas the SERK3 protein cannot replace SERK1 or SERK2 in male fertility, its kinase domain can replace the ones of both SERK1 and SERK2. In contrast, neither the SERK2 extracellular nor the kinase domain can replace SERK3 domains in BR signalling. Interestingly this last observation can also be predicted from the bioinformatics analysis while the finding that the SERK3 kinase domain restores fertility is rather unexpected. These data suggest that the SERK proteins have a phosphorylation dependent role as well as a more structural function in the different pathways.

Materials and Methods

Plant materials and growth

Arabidopsis thaliana ecotype Columbia (Col-0) was used as wild-type control. For *Arabidopsis* sterile seedlings, seeds were surface sterilised and germinated on ½ strength Murashige and Skoog (MS) medium including vitamins (DUCHEFA) supplemented with 1% sucrose and 0.8% agar. Plants were grown at 22°C under fluorescent light with 16h light/8h dark photoperiods, unless otherwise specified. The mutant lines used in this study are *serk1-1*, *serk1-3, serk2-2, serk3-2, bri1-119, fls2.*

Constructs and plant transformation

Most manipulations involved Gateway-mediated cloning following the manufacturer instructions. Primers used for each step are described in Supplemental Tables 1 and 2.

Coding regions and promoter sequences were amplified from genomic DNA using Phusion high fidelity DNA polymerase (New England Biolabs). The 2 kb promoter regions were directionally cloned with *Xho*I-*Sac*II in a modified pDONRP4-P1r. The amplified coding regions were directionally cloned into pENTR-D-TOPO (Invitrogen). These promoter and TOPO constructs together with pDONRstop or pDONRP2r-P3-GFP and pK7m34GW were recombined using LR clonase II PLUS (Invitrogen), creating untagged or C-terminal *GFP* gene fusions respectively (Karimi et al. 2007). The constructs were verified by sequencing. These binary vectors containing the different constructs tagged with GFP or untagged were used to transform Arabidopsis Col-0 plants.

Hypocotyl and root growth assays

Freshly harvested seeds were surface sterilised and placed on either ½ MS plates without hormones or ½ MS plates containing different concentrations of brassinolide (Sigma). The plates were kept at 4°C for 2 days and then placed at 22°C either in dark or grown under 16h light/8h dark photoperiods. The hypocotyl length was measured after 4 days incubation in dark and the root length with and without BRs was determined after 7 days growth in light. Every experiment was performed in duplicate and repeated twice.

Seedling growth inhibition

Seedlings were germinated on MS agar for 5 days and then transferred to 96-well plates containing liquid MS with flg22 at 25 or 50 nM concentrations (one seedling per well and 12 wells per concentration). Seedlings were incubated with peptides for 10 days before determination of fresh weight.

Protein extraction and immunoprecipitation in Arabidopsis

Seedlings were ground in liquid nitrogen and extraction buffer (50 mM Tris-HCl pH 7.5; 150 mM NaCl; 1% NP-40; 0.5% sodium deoxycholate; 1% (v/v) protease inhibitor cocktail (Sigma)) added at 2ml/g tissue powder. Samples were clarified by two steps of centrifugation, 20 minutes centrifugation at 4°C 5,000g followed by a second centrifugation of the supernatant at 18,000g at 4°C. Supernatants were adjusted to 4 mg/ml protein and incubated 4 hours at 4°C with 30 μl GFP Trap-A beads (Chromotek, Germany). Following incubation, beads were then collected

by centrifugation at 2000g at 4°C and washed 4 times with PBS containing 0.5 % (v/v) Nonidet P-40, before adding 2x loading buffer and heating at 70°C for 15 min.

SDS-PAGE and immunoblotting

Proteins were separated by SDS/PAGE 8.5% before electroblotting onto PVDF membrane (BioRad) at 30V overnight. Membranes were rinsed in PBS and blocked for 4 hour in 5 % nonfat milk in PBS-Tween (0.1% (v/v)). Antibodies were diluted in blocking to the following dilutions: anti-GFP-HRP (Miltenyi Biotec) 1:5000; rabbit polyclonal anti-BRI1 1:2000 (Cyril Zipfel, The Sainsbury Laboratories, Norwich, UK); rabbit polyclonal anti-phosphothreonine 1:1000 (Zymed-Invitrogen) 1: 5000. Membranes were incubated with primary antibodies overnight. Membranes were washed 3 x 10 minutes in PBS-Tween (0.1%) and then incubated 1 hour with the secondary antibodies, anti-rabbit-HRP (Sigma). Chemiluminescent substrate (Lumigen ECL, GE Healthcare) was applied before reading on Chemidoc system (BioRad).

Fluorescence Microscopy

Anthers and root apices from transgenic plants harbouring the 35S::*bes-1D*-GFP construct were used for confocal analyses. Transgenic roots were analysed using a ZEISS confocal microscope (ZEISS AXIOVERT 100M equipped with a LSM510, Argon LASER with 488nm laserline). The settings for the GFP were as follow, 488-nm LASER → HFT488/543 → Sample → HFT488/543 → mirror → NFT545 → BP505-550 → detector. Autofluorescence spectral bleed-through was assessed by imaging at the same time with the YFP/GFP channel a channel that detects red fluorescence: 514 nm LASER → HFT458/514 → Sample → NFT635vis → LP650 → detector. Pinhole was adjusted for each channel in such a way that Z-resolution is equal (typically 2 micrometers). Amplifier gain for YFP/GFP and autofluorescence/ spectral bleed-through channels is always the same between experiments.

Data set

Amino acid sequence and CDS (coding DNA sequence) of genes annotated as SERKs either in literature or in database entries were collected from the NCBI database (www.ncbi.nlm.nih.gov). Additional sequences were found with a protein Blast (Altschul et al. 1990) against the non-redundant protein sequences from fully sequenced plant genomes, using NCBI website tools. The *A. thaliana* SERK1 protein sequence was used as query sequence. Homologous sequences with the specific SERK domain structure (similar amount of LRRs, an SPP domain and a kinase domain) were taken along in the analysis. A total of 67 putative SERK sequences

were used for further analysis (see Table 1). As an outgroup the *At*NIK1 and *At*NIK2 CDS and protein sequences (protein id Q9LFS4 and Q8RY65 respectively) and the CDS and protein sequences of LRR-II genes AT5G63710 and AT5G65240 (protein id Q8W4S5 and C0LGX1 respectively) were taken along in the analysis.

Sequence analysis

Multiple sequence alignment (MSA) was performed in MEGA5.1 (Tamura et al. 2011) using ClustalW and Protein Weight Matrix Gonnet. Parameters for the MSA were; gap opening penalty: 30 (pairwise alignment) and 5 (multiple alignment), gap extension penalty: 0.2 (pairwise alignment) and 0.1 (multiple alignment).

The different SERK protein domains were defined from the MSA compared to the *A. thaliana* SERK1 sequence (Aan den Toorn et al. 2012). These domains are: The signal sequence (aa 1- 29) the leucine zipper (or N-capping residues) (aa 30 - 73), the LRRs (aa 74 - 198), the SPP domain (aa 199 - 235), the transmembrane domain (aa 236 - 258), the juxta-membrane domain (aa 259 – 295) the kinase domain (aa 296 - 577) and the C-terminal tail (aa 578 - 625).

Divergence between the different protein sequences was calculated by the number of amino acid substitutions per site from averaging over all sequence pairs. Analyses were conducted using the JTT matrix-based model (Jones et al. 1992). All ambiguous positions were removed for each sequence pair. Analyses were conducted in MEGA5.1 (Tamura et al. 2011).

Selection pressure on the sequences was evaluated by calculating non synonymous (Ka) and synonymous (Ks) substitution rates for SERK coding sequences as reported in the NCBI database (Table 1). Ka/Ks ratio was calculated using the KaKs_Calculator with model averaging (Zhang et al. 2006b).

Phylogenetic analysis

Phylogenetic analysis of SERK MSA was performed using the Neighbour-Joining (NJ) method (Saitou and Nei 1987) with a bootstrap test of 1000 replicates. Distances are computed using the Dayhoff method (Schwartz and Dayhoff 1978) and are in the units of the number of amino acid substitutions per site. Analysis was performed in MEGA 5.1 (Tamura et al. 2011). The tree was either rooted with the non-SERK LRRII outgroup or using the more distantly related LRR-RLK BRI1 protein sequence (protein ID O22476).

Protein Structure analysis

Conservation score as determined by the CONSURF webserver (Glaser et al. 2005, Landau et al. 2005, Ashkenazy et al. 2010) was plotted onto the reported structure of the SERK1 extracellular domain (pdb 4LSC) (Santiago et al. 2013) and

the SERK3/ BAK1 kinase domain (pdb 3TL8) (Cheng et al. 2011). Input was a MSA of SERK protein sequences (Table 1), produced similarly as described above, only the sequences of non-SERKs (protein ids Q67X31, Q6K4T4, G4XGX3, Q659J0, Q659J1, A7VM46) and sequences with large gaps in the alignment (protein id C6FF61) were removed. The remaining 57 sequences were used in the analysis. Molecular graphics and analyses were performed with the UCSF Chimera 1.8.1 (Pettersen et al. 2004), which was also used to plot hydrophobicity.

Results

Phylogenetic analyses of the SERK protein family in plants

To identify the residues or domains that could account for the functional divergence in the SERK family, SERK protein sequences from different plants species were analysed (Table 1). SERK genes are characterised by the presence of 11 exons with conserved splicing boundaries (Baudino et al. 2001, Hecht et al. 2001). To study the phylogenetic relationship between SERK proteins from different plant species, a multiple sequence analysis was performed on 67 protein sequences that were either annotated as SERK in literature, or were identified in database searches using the characteristic domain structure (4.5-5 LRRs and the SPP motifs) as criterion. All known plant genomes contain multiple SERK homologues, including the moss *Physcomitrella patens* (3 SERK homologues) and club moss *Selaginella moellendorffii* (4 SERK homologues). For phylogenetic analysis, two *A. thaliana* protein sequences from each of the two other LRR-RLK II clades (NIK proteins and functionally unassigned proteins) were taken along as outliers (see Table 1).

The phylogenetic relationship between the SERK proteins was investigated by constructing a neighbour-joining tree (see Figure 1). The results revealed an evolutionary partitioning of the SERKs in four major clusters; non-vascular SERK proteins, monocot SERK proteins, and two distinct clusters of dicot SERK proteins. Only one rice protein (Uniprot id: Q67X31) does not follow this division as it is placed outside the monocot SERK proteins. This protein does not contain a true SPP domain and differs from the other SERKs at several positions across the protein sequence, indicating that it is not a true SERK protein. Taking a more distantly related protein sequence as outlier (the LRR-RLK BRI1), does not change the shape of the tree (data not shown). Our finding that the SERK protein family branches are based on plant lineages, in contrast to clusters of SERK orthologues from different plant lineages, seems to suggest that parallel lineage specific expansion is common in this protein family. As a result, true orthologues of specific SERK proteins are difficult to distinguish between e.g. monocot and dicot plants based on sequence comparison only. This problem has already surfaced in literature in the search for SERK3 proteins in other plant species (Park et al. 2011).

Figure 1

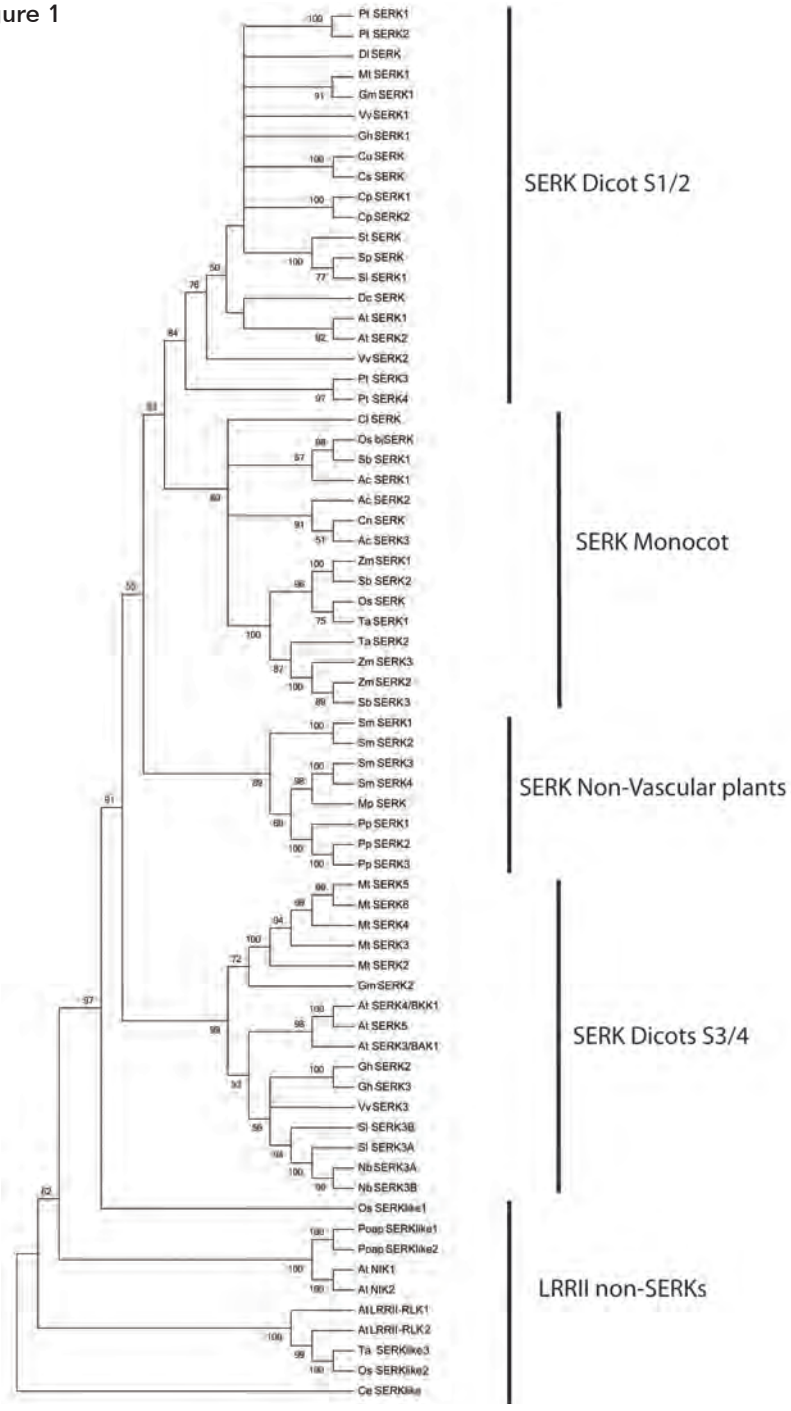

Pt SERK1
Pt SERK2
Dl SERK
Mt SERK1
Gm SERK1
Vv SERK1
Gh SERK1
Cu SERK
Cs SERK
Cp SERK1
Cp SERK2
St SERK
Sp SERK
Sl SERK1
Dc SERK
At SERK1
At SERK2
Vv SERK2
Pt SERK3
Pt SERK4
Cl SERK
Os bi SERK
Sb SERK1
Ac SERK1
Ac SERK2
Cn SERK
Ac SERK3
Zm SERK1
Sb SERK2
Os SERK
Ta SERK1
Ta SERK2
Zm SERK3
Zm SERK2
Sb SERK3
Sm SERK1
Sm SERK2
Sm SERK3
Sm SERK4
Mp SERK
Pp SERK1
Pp SERK2
Pp SERK3
Mt SERK5
Mt SERK6
Mt SERK4
Mt SERK3
Mt SERK2
Gm SERK2
At SERK4/BKK1
At SERK5
At SERK3/BAK1
Gh SERK2
Gh SERK3
Vv SERK3
Sl SERK3B
Sl SERK3A
Nb SERK3A
Nb SERK3B
Os SERKlike1
Poap SERKlike1
Poap SERKlike2
At NIK1
At NIK2
At LRRII-RLK1
At LRRII-RLK2
Ta SERKlike3
Os SERKlike2
Ce SERKlike
SERK Dicot S1/2
SERK Monocot
SERK Non-Vascular plants
SERK Dicots S3/4
LRRII non-SERKs

Figure 1 Neighbour-joining tree SERK protein family
NJ joining tree based on a multiple sequence alignment of the 71 proteins sequence from Table 1. These include 67 putative SERK proteins sequences and four *A. thaliana* LRR-II non-SERK proteins (taken along as out-group). Bootstrap values (in percentages) from 1000 replicates are shown next to the branches. Branches with a replicate percentage below 50% were collapsed. The evolutionary distances were computed using the Dayhoff matrix based method. Four distinct groups can be inferred from this NJ tree; LRRII non-SERKs are proteins which group together with the *A. thaliana* non-SERK proteins and are thus not true SERKs. SERK Dicot S3/4 proteins are sequences solely from dicotyledons that group together with *A. thaliana* SERK3, 4 and 5, while the SERK Dicots S1./2 proteins are dicot SERKs which group with *A. thaliana* S1/2. All SERK sequences from non-vascular plants group together as do the sequences from monocotyledons (Monocot).

In addition, our analysis identified two proteins in *Poa pratensis* (Uniprot id: Q659J0 and Uniprot id: Q659J1) that are annotated as SERKs (Albertini et al. 2005), but are actually more closely related to the *A. thaliana* NIK-proteins. Also, we identified one rice protein (Uniprot id: Q6K4T4) and one wheat protein (Uniprot id: G4XGX3) that place within the third (unassigned) group of the LRR-RLK II subfamily (Singla et al. 2008). The receptor-like kinase from *Closterium ehrenbergii* (Uniprot id: A7VM46), a unicellular alga, was reported as a SERK homologue (Sasaki et al. 2007), however, our analysis indicates that this *C. ehrenbergii* protein is not a SERK, but is placed in another clade within the LRR-RLK II-subfamily.

Divergence in the dicot SERK proteins

The neighbour-joining (NJ) tree in Figure 1 indicates a bifurcation in the dicot SERK proteins, one group containing *At*SERK1 and 2 (hereafter dicot SERK1/2) and one containing *At*SERK3, 4 and 5 (hereafter called dicot SERK3/4). Of all dicot genomes analysed, only poplar contained no SERK3/4 protein. The monocot SERK proteins do not show a similar bifurcation. Based on sequence analysis and on their place within the NJ tree, the monocot SERKs are more similar to dicot SERK1/2 proteins than dicot SERK3/4 proteins (see Figure 1 and 2). Thus, it appears that monocot genomes do not contain a gene closely related to *A. thaliana* SERK3 or SERK4/BKK1. Similarly, the non-vascular SERKs analysed in this study form one cluster and do not split into two groups. Based on sequence alignments, the non-vascular SERKs resemble the dicot SERK1/2 proteins more closely than the dicot SERK3/4 protein (see Figure 2). This indicates that the duplication event giving rise to *At*SERK1, 2 and *At*SERK3, 4 is dicot specific and occurred after the split between monocot and dicot plants.

To investigate how tightly these groups can be defined, the evolutionary divergence within the four groups (dicot SERK1/2, dicot SERK3/4, monocot and nonvascular SERKs) was estimated by calculating the number of amino acid substitutions per site in the extracellular domain and cytoplasmic domain within each group (see Table 2, '*intra*'). In addition, the number of amino acid substitutions between each group and the other three groups was calculated to see which group

Table 1 Overview of SERK sequences used in analysis

Italic protein and CDS ids refer to sequences that, according to the phylogenetic analysis in Figure 1, do not belong to the SERK protein family but were annotated as such previously. These sequences were left out in further analyses. If no reference is given, sequence was either annotated as SERK in the NCBI database, or retrieved via a protein BLAST using the *At*SERK1 protein sequence as query. The column "name" provides the denominator under which the protein is depicted in Figure 1.Protein id is according to Uniprot.

Species	Common species name	Group		Name	Protein id	CDS id	Ref
Populus trichocarpa	Black cottonwood	Eudicots	Rosids	Pp SERK1	B9MW41	XM_002327180.1	-
				Pp SERK2	B9IQM9	XM_002325978.1	-
				Pp SERK3	B9HFX1	XM_002310563.1	-
				Pp SERK4	B9H599	XM_002307088.1	-
Dimocarpus longan	Longan	Eudicots	Rosids	Dl SERK	B5TTV0	FJ013227.3	-
Medicago truncatula	Barrel clover	Eudicots	Rosids	Mt SERK1	Q8GRK2	AY162176.1	(Nolan et al. 2009)
				Mt SERK2	E2IXG1	HM640001.1	(Nolan et al. 2011)
				Mt SERK3	E2IXG8	HM640008.1	(Nolan et al. 2011)
				Mt SERK4	E2IXG2	HM640002.1	(Nolan et al. 2011)
				Mt SERK5	E2IXG3	HM640003.1	(Nolan et al. 2011)
				Mt SERK6	E2IXG4	HM640004.1	(Nolan et al. 2011)
Glycine max	Soy bean	Eudicots	Rosids	Gm SERK1	C6ZGA8	EU869193.1	(Yang et al. 2011)
				Gm SERK2	C6FF61	EU888313.1	-
Citrus unshiu	Tangerine	Eudicots	Rosids	Cu SERK	Q6BE26	AB115767.1	-
Citrus sinensis	Orange	Eudicots	Rosids	Cs SERK	C3V9W0	FJ851422.1	(Ge et al. 2010)
Vitis vinifera	Grape	Eudicots	Rosids	Vv SERK1	D7TXV2	XM_002270811.1	-
				Vv SERK2	A5BIY4	XM_002276378.2	-
				Vv SERK3	D7STF6	XM_002262662.2	-
Cyclamen persicum	Persian cyclamen	Eudicots	Asterids	Cp SERK1	A7L5U3	EF672247.2	-
				Cp SERK2	E5D6S9	GU189408.1	-
Gossypium hirsutum	Mexican cotton	Eudicots	Rosids	Gh SERK1	E5Q8K6	HQ621831.1	-
				Gh SERK2	F5BZU9	JF430801.1	-

Table 1 Continued

Species	Common species name	Group		Name	Protein id	CDS id	Ref
				Gh SERK3	F6MF11	JF800909.1	-
Solanum tubersosum	Potato	Eudicots	Asterids	St SERK	A3R789	EF175215.1	(Sharma et al. 2008)
Solanum peruvianum	Wild tomato	Eudicots	Asterids	Sp SERK	A6N8J2	EF623824.1	-
Solanum lycopersicum	Tomato	Eudicots	Asterids	Sl SERK1	G0XZA3	HQ438097.1	(Mantelin et al. 2011)
				Sl SERK3A	G0XZA5	NM_001247697.1	(Mantelin et al. 2011)
				Sl SERK3B	G0XZA6	HQ438099.1	(Mantelin et al. 2011)
Daucus carota	Wild carrot	Eudicots	Asterids	Dc SERK	O23921	U93048.1	(Schmidt et al. 1997)
Arabidopsis thaliana	Thale cress	Eudicots	Rosids	At SERK1	Q94AG2	NM_105841.4	(Hecht et al. 2001)
				At SERK2	Q9XIC7	NM_103144.4	(Hecht et al. 2001)
				At SERK3	Q94F62	NM_119497.4	(Hecht et al. 2001)
				At SERK4	Q9SKG5	NM_126955.4	(Hecht et al. 2001)
				At SERK5	Q8LPS5	NM_126956.3	(Hecht et al. 2001)
Nicotiana benthamiana	Tabacco	Eudicots	Asterids	Nb SERK3A	E3VXE6	HQ332144.1	(Mantelin et al. 2011)
				Nb SERK3B	E3VXE7	HQ332145.1	(Mantelin et al. 2011)
Oryza sativa	Rice	Monocotelydons	Poales	Os biSERK	Q6S7F1	AY463361.1	(Song et al. 2008)
				Os SERK	Q5Y8C8	AY652735.1	(Hu et al. 2005)
				Os SERKlike1	*Q67X31*	*NM_001063731.1*	-
				Os SERKlike2	*Q6K4T4*	*HB796725.1*	-
Sorghum bicolor	Sorghum	Monocotelydons	Poales	Sb SERK1	C5YHV3	XM_002443894.1	-
				Sb SERK2	C5Y9S6	XM_002447912.1	-
				Sb SERK3	C5XVP5	XM_002454009.1	-
Ananas comosus	Pineapple	Monocotelydons	Poales	Ac SERK1	H6SU43	HM236375.2	(Ma et al. 2012a)
				Ac SERK2	H6UP78	HM236376.1	(Ma et al. 2012b)
				Ac SERK3	H6UP79	HM236377.1	-

Table 1 Continued

Species	Common species name	Group		Name	Protein id	CDS id	Ref
Cyrtochilum loxense		Monocotelydons	Asparagales	Cl SERK	G2XLB1	FN994192.1	(Cueva et al. 2012)
Cocos nucifera	Cocos palm	Monocotelydons	Arecales	Cn SERK	Q5S1N9	AY791293.2	(Perez-Nunez et al. 2009)
Zea mays	Maize	Monocotelydons	Poales	Zm SERK1	Q93W70	AJ277702.1	(Baudino et al. 2001)
				Zm SERK2	Q94IJ5	AJ277703.1	(Baudino et al. 2001)
				Zm SERK3	B4G007	BT042695.1	-
Triticum aestivum	Common wheat	Monocotelydons	Poales	Ta SERK1	G4XGX1	JF808017.1	(Singla et al. 2008)
				Ta SERK2	G4XGX2	JF808018.1	(Singla et al. 2008)
				Ta SERKlike3	G4XGX3	JF808019.1	(Singla et al. 2008)
Selaginella moellen-dorffii	Club moss	Lycopodiophyta		Sm SERK1	D8SBB8	XM_002980668.1	-
				Sm SERK2	D8S0N3	XM_002976930.1	-
				Sm SERK3	D8S4M4	XM_002978263.1	-
				Sm SERK4	D8R6C9	XM_002966218.1	-
Marchantia polymorpha	Common liverwort	Bryophytes	Marchantiophyta	Mp SERK	A7VM18	AB306524.1	(Sasaki et al. 2007)
Physcomitrella patens	Moss	Bryophytes	Bryophyta	Pp SERK1	A9STU8	XM_001769715.1	-
				Pp SERK2	A9SMW5	XM_001767626.1	-
				Pp SERK3	A9RY79	XM_001759070.1	-
Poa pratensis	Common meadow grass	Monocotelydons	Poales	Poap SERKlike1	*Q659J0*	*HB796747.1*	(Albertini et al. 2005)
				Poap SERKlike2	*Q659J1*	*HB465085.1*	(Albertini et al. 2005)
Closterium ehrenbergii		Algea		Ce SERK	A7VM46	AB306552.1	(Sasaki et al. 2007)
Arabidopsis thaliana	Thale cress	Eudicots	Rosids	At NIK1	Q9LFS4	NM_121605.2	
				At NIK2	Q8RY65	NM_113453.2	
				At LRRII- RLK1	Q8W4S5	NM_125766.2	
				At LRRII-RLK2	C0LGX1	NM_125922.4	

Table 2 Estimates of Average Evolutionary Divergence of SERK complete protein sequence, extracellular domain and intracellular domain
The number of amino acid substitutions per site from averaging over all sequence pairs is shown, either within a clade (intra) or for sequences in a clade compared to all other sequences (inter). The complete amino acid sequence minus the signal sequence (complete), the extracellular part of the protein (ectodomain) or the intracellular part (cytoplasmic) was used for analysis.

	Complete		Ectodomain		Cytoplasmic	
	intra	inter	intra	inter	intra	inter
Dicot S1/2	0.082	0.197	0.142	0.317	0.053	0.144
Monocot	0.118	0.195	0.165	0.302	0.091	0.145
Non-Vasc	0.179	0.235	0.304	0.371	0.121	0.176
Dicot S3/4	0.226	0.253	0.356	0.388	0.173	0.197
All	0.195		0.305		0.145	

Table 3 Estimates of Average Evolutionary Divergence of different Domains of SERK
The number of amino acid substitutions per site from averaging over all sequence pairs is shown, either within a clade (intra) or for sequences in a clade compared to all other sequences (inter). The different domains analysed are leucine zipper/ N-capping residues (LZ-Ncap), leucine rich repeats (LRR), serine-proline rich domain (SPP), transmembrane domain (TM), juxtamembrane domain (JxM), kinase domain (KD) and C-terminal tail (C-tail).

	LZ-Ncap		LRR		SPP		TM		JxM		KD		C-tail	
	intra	inter	intra	inter	intra	inter	intra	inter	intra	inter	intra	inter	intra	inter
Dicot S1/2	0.21	0.30	0.13	0.25	0.32	0.92	0.05	0.11	0.07	0.15	0.04	0.10	0.15	0.49
Monocot	0.11	0.25	0.14	0.24	0.32	0.86	0.14	0.14	0.09	0.15	0.07	0.11	0.29	0.48
Non-Vasc	0.35	0.36	0.20	0.28	0.69	0.10	0.10	0.11	0.12	0.20	0.11	0.14	0.19	0.41
Dicot S3/4	0.34	0.39	0.27	0.30	1.30	1.33	0.11	0.11	0.18	0.22	0.14	0.14	0.40	0.67
All	0.29		0.24		0.90		0.11		0.16		0.11		0.45	

had diverged most from the others (see Table 2 '*inter*'). The dicot SERK1/2 shows the least variation within the group, while the dicot SERK3/4 group is most heterogeneous. For all groups, the cytoplasmic domain shows much less variation than the extracellular domain. This is most likely due to the kinase domain, which has to retain its biochemical properties and is therefore more restricted in its variation. This analysis was repeated for each of the SERK domains separately (see Table 3). The TM domain and the kinase domain are the most conserved regions of the SERKs while the C-terminal tail and the SPP-domain vary the most. All SERK proteins contain the RD motif (an arginine residue immediately preceding the conserved catalytic aspartate) in the catalytic loop (except for *At*SERK5, which might be a pseudogene) indicating that all are activated by phosphorylation of the activation loop (Nolen et al. 2004). The dicot SERK3/4 group shows most amino acid substitutions in every domain, but is especially different from other SERKs in the SPP domain and the C-terminal tail (see Table 3). The multiple sequence alignment

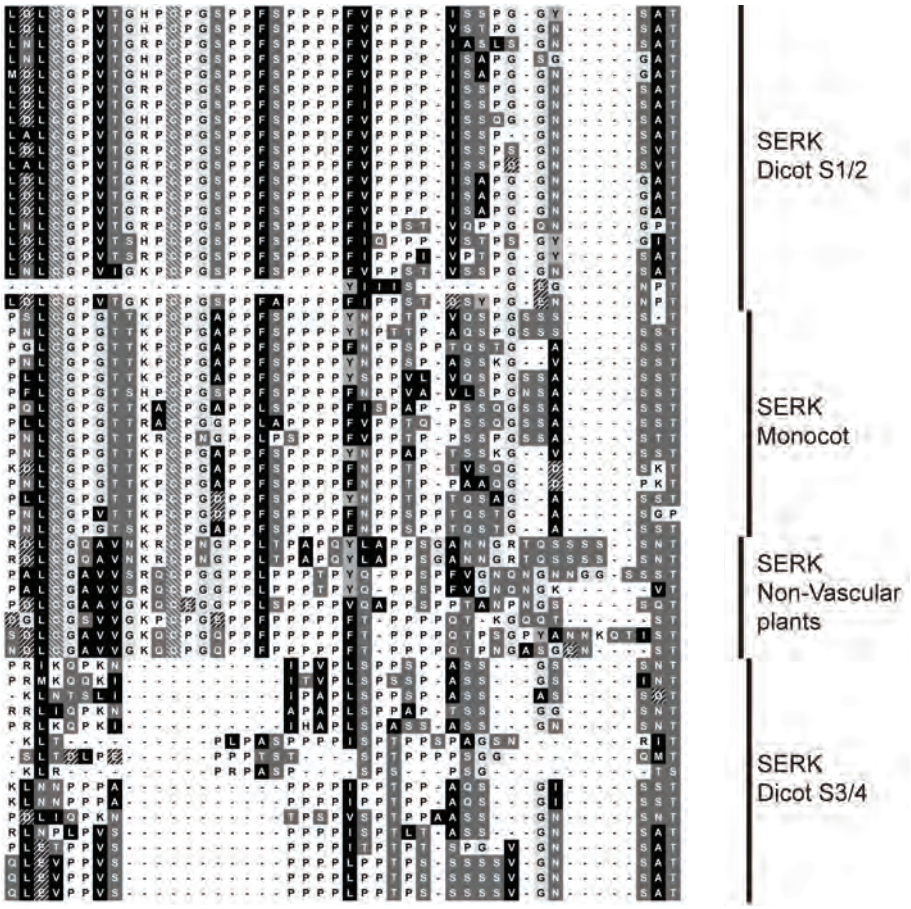


Figure 2A Multiple sequence alignment of SPP domain
Detail of the SERK MSA, focused on the SPP domain. The dicot SERK3/4 protein sequences are clearly distinct from the other SERK proteins sequences by missing a, otherwise conserved, cysteine pair preceding the SPP domain, and having a shorter more heterogeneous SPP domain.

shows that the dicot SERK3/4 proteins have lost an otherwise conserved cysteine pair placed just before the SPP domain while this domain is more variable and often shorter (see Figure 2A). In the C-terminal tail, tyrosine residues are more abundant in the SERK3/4 proteins (see Figure 2B). The juxtamembrane domain, a domain that was found to be important in the regulation of other LRR-RLKs (Wang et al. 2005b), is well conserved in the entire protein family.

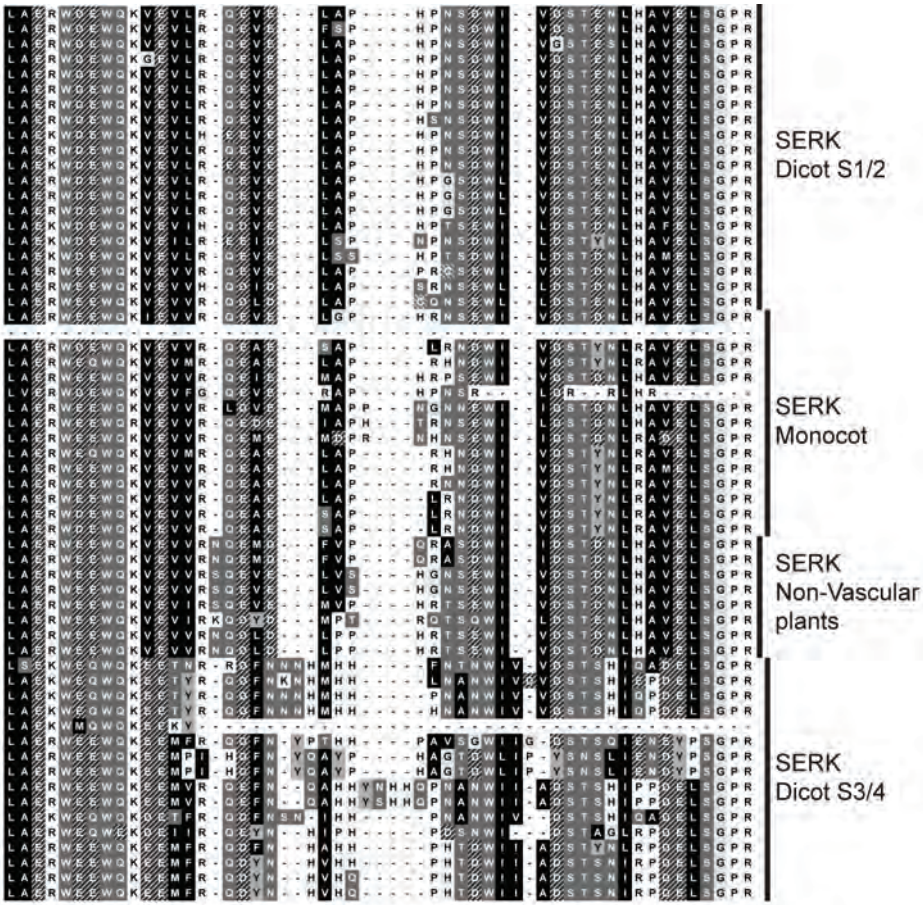


Figure 2B Multiple sequence alignment of C-terminal tail
Detail of the SERK MSA, focused on the C-terminal tail domain. Although highly homologous amongst the monocot, nonvascular and dicot SERK1/2 protein sequences, the dicot SERK3/4 show more divergence in this domain, and especially a higher occurrence of tyrosine residues across the domain.

The SERK genes are under purifying selection

Genes that obtain a new function are often found to be under positive selection (selection to maximise change), meaning that most retained mutations cause changes in the amino-acid sequence. On the other hand, genes that perform vital functions in a cell are usually under purifying selection (selection to minimise change), where most mutations are silent on protein level. To infer the direction of natural selection for the SERK genes, the Ka/Ks ratio (nonsynonymous/ synonymous substitution rates) for each of the SERK protein coding sequences was calculated. A Ka/Ks ratio of 1 is indicative of neutral selection (no specific direction), lower than

1 indicates purifying selection (selection to minimise change) and higher than 1 indicates positive selection (selection to maximise change). Ka/Ks was estimated using the KaKs calculator software (Zhang et al. 2006b), with the method of model averaging. The SERK genes that had a significant deviation from the 0-hypothesis (Ka/Ks = 1, or neutral selection) all showed purifying evolution (see Table 4). Because this might be due to pressure on the kinase domain to maintain its phosphorylation activity, the same analysis was performed on the extracellular domain, cytoplasmic domain and TM domain separately. This analysis shows that indeed the extracellular domain is more often under positive selection compared to the cytoplasmic domain, and the average Ka/Ks ratio for the extracellular domain of the dicot SERK3/4 group is slightly higher compared to the other groups. This might indicate that some proteins, and more so in the dicot SERK3/4 group, have diverged from the original SERK function by changes in their extracellular domain. However, the majority of SERKs are under selective pressure to minimise variation on protein sequence. An interesting observation is the high occurrence of positive selection found on the transmembrane domain (see Table 4). There is evidence that TMs in animal receptors are crucial for dimerization and play an important role in 'strengthening' receptor complexes (Li and Hristova 2010). Thus, the positive selection pressure on the TM could be involved in accommodating different oligomerization partners. However, since no changes in TM are observed between, for instance, SERK1 and SERK3, this cannot be the reason for the observed functional differences between SERK family members.

SERKs show lower conservation in specific structural domains

The conservation score of each amino acid calculated for 57 SERK protein sequences (see Table1, all identified SERK proteins but excluding C6FF61 due to a large gap in the LRR domain), was plotted onto the published crystal structure of SERK1-extracellular domain (PDB 4LSC) (Santiago et al. 2013) with use of the ConSurf algorithm (see Figure 3A and B, where cyan corresponds to low conservation scores and red to higher conservation scores). The solvent exposed convex side (see Figure 3A) is more variable compared to the concave side of the LRR domain (see Figure 3B). Figure 3C and 3D display the hydrophobicity plots of the convex and the concave side respectively. On the solvent exposed side (convex), there is a conserved hydrophobic groove (coloured red) between variable, more hydrophilic, regions (coloured blue) (see Figure 3A and 3C). The concave side, which was found to interact with the extracellular domain of BRI1, displays two patches of high conservation. One of these is implicated in the BRI1-SERK1 interaction (Santiago et al. 2013) which for the most part overlaps with the reported interaction side of SERK3-FLS2 (Sun et al. 2013; and Table 5). All the residues that were reported as having interaction with the BRI1 and FLS2 extracellular domains and the corresponding BR and flg22 ligand (flg22 constitutes the 22 amino acids

Table 4 Ka/Ks ratio for SERK coding sequence, extracellular domain, cytoplasmic domain and TM

Evolutionary pressure on SERK coding sequences as evaluated by Ka/Ks ratio. Ratios with a p-value < 0.05 (0-hypothesize is Ka/Ks=1) are coloured, red depicting a ratio below 1 (purifying selection), blue above 1 (positive selection). Analysis is done on the complete coding sequence (Tot), the extracellular domain (Ecto), the cytoplasmic domain (Cyto) or the transmembrane domain (TM).

Dicot S1/2	Ka/Ks				Dicot S3/4	Ka/Ks			
	Tot.	Ecto	Cyto	TM		Tot.	Ecto	Cyto	TM
PtSERK1	0.90	0.89	0.91	1.71	MtSERK2	0.86	1.25	0.79	1.26
PtSERK2	0.89	0.73	0.73	1.62	MtSERK3	0.82	1.21	0.90	1.27
PtSERK3	0.89	1.15	0.95	1.9	MtSERK4	0.98	0.94	0.73	1.64
PtSERK4	0.84	1.29	0.96	2.94	MtSERK5	0.96	0.95	0.8	2.21
DlSERK	0.92	0.82	0.99	1.43	MtSERK6	1.07	1.75	0.71	1.22
MtSERK1	0.96	0.92	0.88	2.31	GmSERK2	0.93	1.79	0.8	2.21
GmSERK1	0.90	0.85	1.05	1.67	GhSERK2	1.03	0.97	1.23	0.92
CuSERK	0.94	0.95	0.75	1.97	GhSERK3	1.05	0.94	1.3	0.98
CsSERK	0.98	0.89	0.76	1.97	VvSERK3	0.88	1.34	0.91	1.4
VvSERK1	0.81	1.01	0.98	1.03	SlSERK3A	0.84	1.04	0.79	1.16
VvSERK2	0.88	1.17	0.91	1.38	SlSERK3B	1.01	1.1	0.97	1.35
GhSERK1	0.76	0.95	0.9	1.67	AtSERK3	0.91	0.82	0.97	1.06
CpSERK1	0.98	1.24	0.85	0.97	AtSERK4	1.07	1.09	1	0.91
CpSERK2	1.05	1.24	0.84	1.1	AtSERK5	0.96	0.88	0.93	nd
StSERK	0.82	1.21	0.98	1.1	NbSERK3A	0.90	1.01	0.92	1.14
SpSERK	0.83	1.29	0.96	1.2	NbSERK3B	0.91	0.92	0.91	1.14
SlSERK1	0.84	1.26	0.87	1.1					
DcSERK	1.07	1.13	1.02	1.39					
AtSERK1	1.02	0.87	1.54	1.43					
AtSERK2	0.90	0.79	0.95	1.12					
average	**0.91**	**1.03**	**0.94**	**1.55**	**average**	**0.95**	**1.13**	**0.92**	**1.33**

Non-vascular	Ka/Ks				Monocots	Ka/Ks			
	Total	Ecto	Cyto	TM		Total	Ecto	Cyto	TM
SmSERK1	0.96	1.18	0.94	0.44	OsSERK1	0.96	0.97	0.89	1.53
SmSERK2	0.96	1.15	0.94	0.61	OsbiSERK	1	0.92	0.96	0.99
SmSERK3	1.01	0.95	1.12	3.81	SbSERK1	1.02	0.91	0.84	1.12
SmSERK4	0.98	1	1.14	3.81	SbSERK2	1.03	1.23	0.83	1.28
MpSERK	1.08	1.29	1.09	1.71	SbSERK3	0.92	0.99	1.07	1.8
PpSERK1	0.9	0.91	1.03	2.27	AcSERK1	0.85	1.04	0.89	1.13
PpSERK2	1.07	0.95	1.05	1.4	AcSERK2	0.94	0.86	1.21	1.38
PpSERK3	0.97	0.91	1.23	1.76	AcSERK3	0.91	1.05	0.91	1.55
					ClSERK	1.01	1.02	1.04	1.13
					CnSERK	0.89	0.9	1.27	0.99
					ZmSERK1	0.87	1.16	0.63	1.43
					ZmSERK2	0.98	0.93	1.07	1.62
					ZmSERK3	1.03	0.92	1.11	1.36
					TaSERK1	1.01	1.49	0.82	0.99
average	**0.99**	**1.04**	**1.07**	**1.97**	**average**	**0.94**	**1.01**	**0.96**	**1.3**

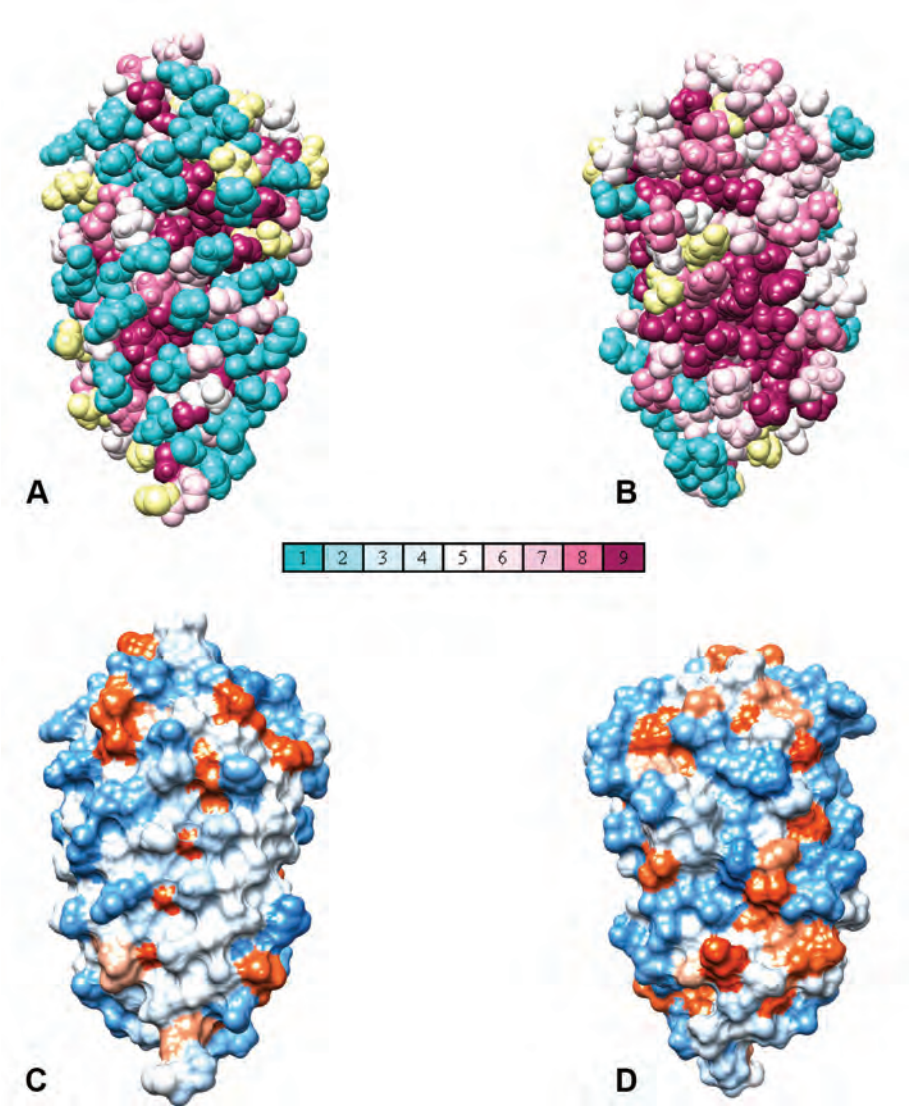


Figure 3 Conservation in the SERK LRR domain
Conservation score for each amino acid as determined using the ConSurf algorithm (Ashkenazy et al. 2010) was plotted onto the reported structure of the SERK1 extracellular domain (pdb 4LSC; Santiago et al. 2013). Higher scores, plotted as red onto the structure, represent residues of higher conservation, whereas lower scores, indicated by cyan, depict more variable residues. Input was the MSA of 57 SERK protein sequences. In (A) the convex or solvent exposed side of the SERK LRR is depicted, in (B) the concave side is shown, which has interactions with the main ligand receptors. In (C) and (D) the hydrophobicity plots (hydrophobic regions are depicted by red, hydrophilic regions by blue) of the convex and concave sides are depicted respectively.

Figure 4 Conservation in the SERK kinase domain
The ConSurf algorithm (Ashkenazy et al. 2010) was used to plot the conservation score per amino acid onto the crystal structure of AtSERK3 kinase domain (PDB 3TL8; Cheng et al 2011), using the MSA of 57 SERK kinase sequences as input. Most of the kinase domain is conserved between the family members, all non-conserved residues (depicted in cyan space-fill) are found on one side of the kinase domain, away from the catalytic cleft of the kinase.

of flagellin competent to elicit a biological response) are well conserved among the SERK family members, but are much less so in the non-SERK LRR-RLK II subfamily proteins that were included in the NJ tree (see Table 5). Therefore, these interaction domains appear to be SERK-specific. The conserved groove on the solvent exposed site and the additional conserved region on the concave site seem to indicate that other important interaction domains are present on the extracellular domain of SERKs.

The kinase domain in the SERK protein family contains many completely conserved amino acids. Again, the ConSurf algorithm was used to plot the conservation score of this domain for the 57 sequences onto the crystal structure of SERK3 kinase domain (PDB 8TL) (see Figure 4). The activation loop, the catalytic loop and catalytic cleft between the N-lobe and C-lobe, are almost completely conserved. All

variability in the kinase domain of the SERKs can be found on one site of the kinase domain, away from the catalytic side. Within this variable region is the KID, kinase insert domain, which is a known variable region in kinases, thought to function in protein-protein interactions (Hubbard et al. 1994, Shewchuk et al. 2000).

Table 5 Conservation of SERK interacting residues
Interacting residues of SERK1 and SERK3 (numbering according to SERK1 sequence) with main receptors BRI1 and FLS2 respectively were assessed for their conservation amongst SERKs (Cons. SERKs), amongst the non-SERK LRR-II proteins depicted in Figure 1 (Cons. Non-SERKs) and specifically in the non-vascular plant *Physcomitrella patens* (P. patens). If this final column is empty, residues in *P. patens* are similar to those of SERK1. Non-conserved residues were only found in one of the three P. patens SERK proteins (protein id A9STU8). Conservation score assessed via Jalview 2.8, score of 11 indicates 100% conservation

Residue	Main receptor interaction	Ligand interaction	Cons. SERK	Cons. non-SERK	*P. Patens*
Thr 53		flg22	10	4	Pro
Leu 54		flg22	10	4	
Val 55		flg22	10	6	
Cys 58	BRI1		10	11	
Phe 61	FLS2	BL	10	5	
His 62		BL	10	3	Tyr
Thr 64	BRI1		10	6	
Cys 65	BRI1		10	11	
Asn 66	BRI1		7	3	
Arg 73	BRI &FLS2		5	4	
Asp 75	BRI1		10	4	
Gly 77	BRI1		4	6	
Tyr97	BRI &FLS2		10	0	
Glu 99	BRI1		10	0	Val
Tyr 101	BRI &FLS2		5	0	
Ser 121	BRI1		11	7	
Asp 123	BRI1		11	8	
Tyr 125	BRI1		10	4	
Arg 144	FLS2		9	5	
Phe 145	BRI &FLS2		11	3	
Arg 147	BRI &FLS2		11	3	

SERKs show different affinities for the main receptors BRI1 and FLS2

The residues implicated in interaction with the ligand and main receptor are well conserved amongst the SERK family, suggesting all SERKs should be able to form these complexes. However, genetic studies neither provide evidence for a role of

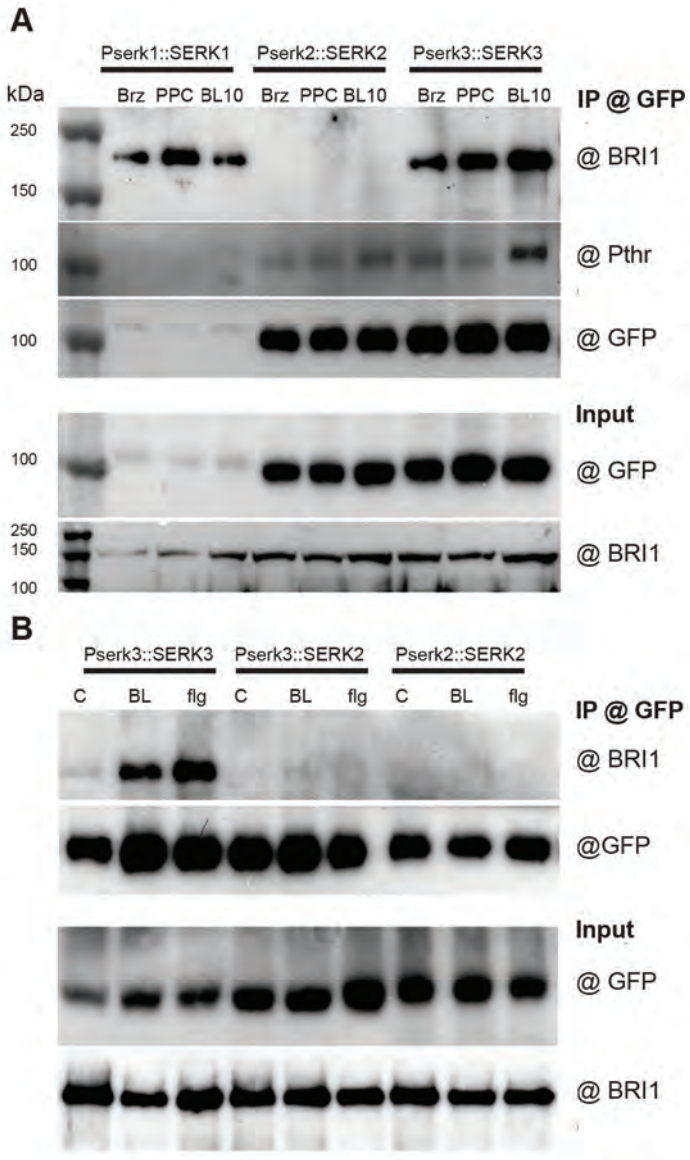


Figure 5 Co-immunoprecipitation of SERKs-GFP and BRI1 proteins.
A: Transgenic Arabidopsis seedlings expressing SERK1, SERK2 and SERK3 driven by their own promoter and tagged with GFP were treated with brassinazole (Brz, 2.5 μM for 3 days), propiconazole (PPC, 1μM for 3 days) or epibrassinosteroid (BL, 1μM for 10 minutes). Total proteins (input) were subjected to immunoprecipitation with anti-GFP beads followed by immunoblot analysis with anti-GFP antibodies to detect SERK-GFP and anti-BRI1 antibodies to detect BRI1. BRI1 is only co-immunoprecipitated in SERK1 (left columns) and SERK3 (right columns) but not SERK2 (middle). Molecular weight of detected proteins indicated in kDa.
B: SERK2 under the SERK3 promotor (middle columns) behaves as SERK2 under its own promotor (right), and not like SERK3 (left).

SERK2 in brassinosteroid signalling nor for SERK1 and SERK2 in PTI (PAMP triggered immunity)/flagellin signalling (supplemental Figure 1). SERK2 is expressed in the right cells to potentially interact with BRI1 (supplemental data 2) while SERK1 and SERK2 have been shown to form a ligand-induced complex with FLS2 *in vivo* but with much lower affinity compared to SERK3 (Roux et al. 2011). To determine whether SERK1 and SERK2 interact with BRI1, extracts of seedlings expressing SERK1, SERK2 or, as a control, SERK3 tagged with GFP were subjected to co-immunoprecipitation experiments using native anti-BRI1 antibodies. SERK1 expressed under its own promoter shows a much lower expression than SERK2 and SERK3 driven by their respective promoters. Heterooligomer formation in the absence of the BR ligand was observed between SERK1, SERK3 and BRI1, while a clear ligand-dependent increase was noted only for SERK3. No interaction was found between SERK2 and BRI1 (see Figure 5). The amount of BRI1 being immunoprecipitated by SERK1 is more or less similar to that of SERK3, while the SERK1 input is much lower. Apparently there are marked differences in terms of stoichiometry or affinity in the main/co-receptor complexes (see Figure 5). Thus, the SERK proteins, although used redundantly in some pathways, are functionally distinct; they show different phenotypes and affinities for the main receptors BRI1 and FLS2 (Roux et al., 2011).

Both extracellular and intracellular SERK domains convey specificity in different pathways

To determine whether the observed differences in protein-protein interactions and functionality can be attributed to either the extracellular or the cytoplasmic domain of the SERK proteins a number of chimeric proteins were tested in three different

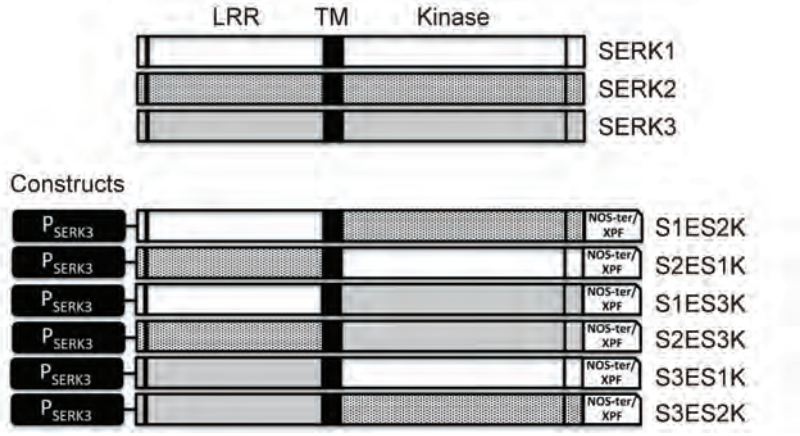


Figure 6 Overview of chimeric SERK proteins

receptor mutant backgrounds, i.e. *serk1-1 serk2 serk3-2, serk1-3 serk3-2, serk3-2 serk4*. All the constructs employed the SERK3 promoter because the phenotypes of interest can be rescued with a SERK member expressed under control of the SERK3 promoter fragment. A schematic representation of all chimeric constructs using a SxESyK terminology in which x is the SERK member donating the E(extracellular

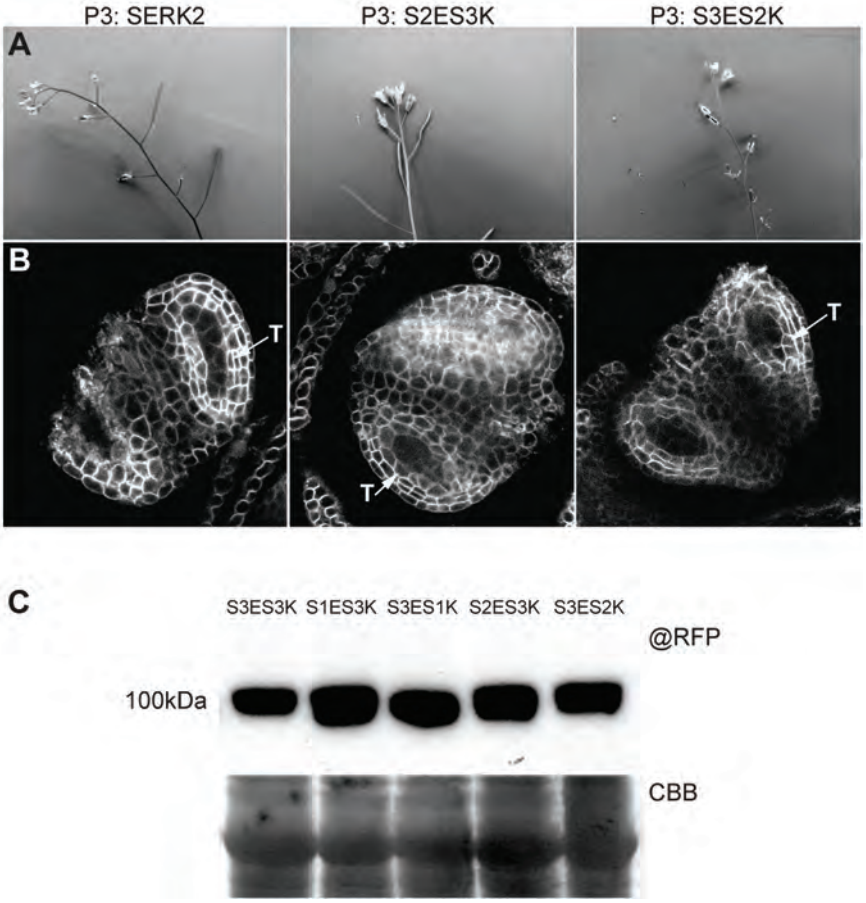


Figure 7 SERK1 or SERK2 extracellular domain is required to restore male sporogenesis. **A.** Inflorescence of a *serk1 serk2* double mutant, expressing the SERK3 gene driven by the SERK2 promoter (P_{SERK2}:SERK3-YFP), the S2ES3K and S3ES2K chimers under the SERK3 promoter. Plants expressing P_{SERK2}:SERK3-YFP and P_{SERK3}:S2ES3K show normal seedpods, plants expressing P_{SERK3}:S3ES2K are sterile. **B.** The chimeric proteins are visualised in the anther tissue by confocal microscopy, in double mutant background for P_{SERK2}:SERK3-YFP and P_{SERK3}:S2ES3K and in heterozygote *serk1serk1 serk2*SERK2 background for P_{SERK3}:S3ES2K. T is tapetum. **C.** Western blot shows the expression of the chimeric proteins and the corresponding Coomassie blue staining (CBB).

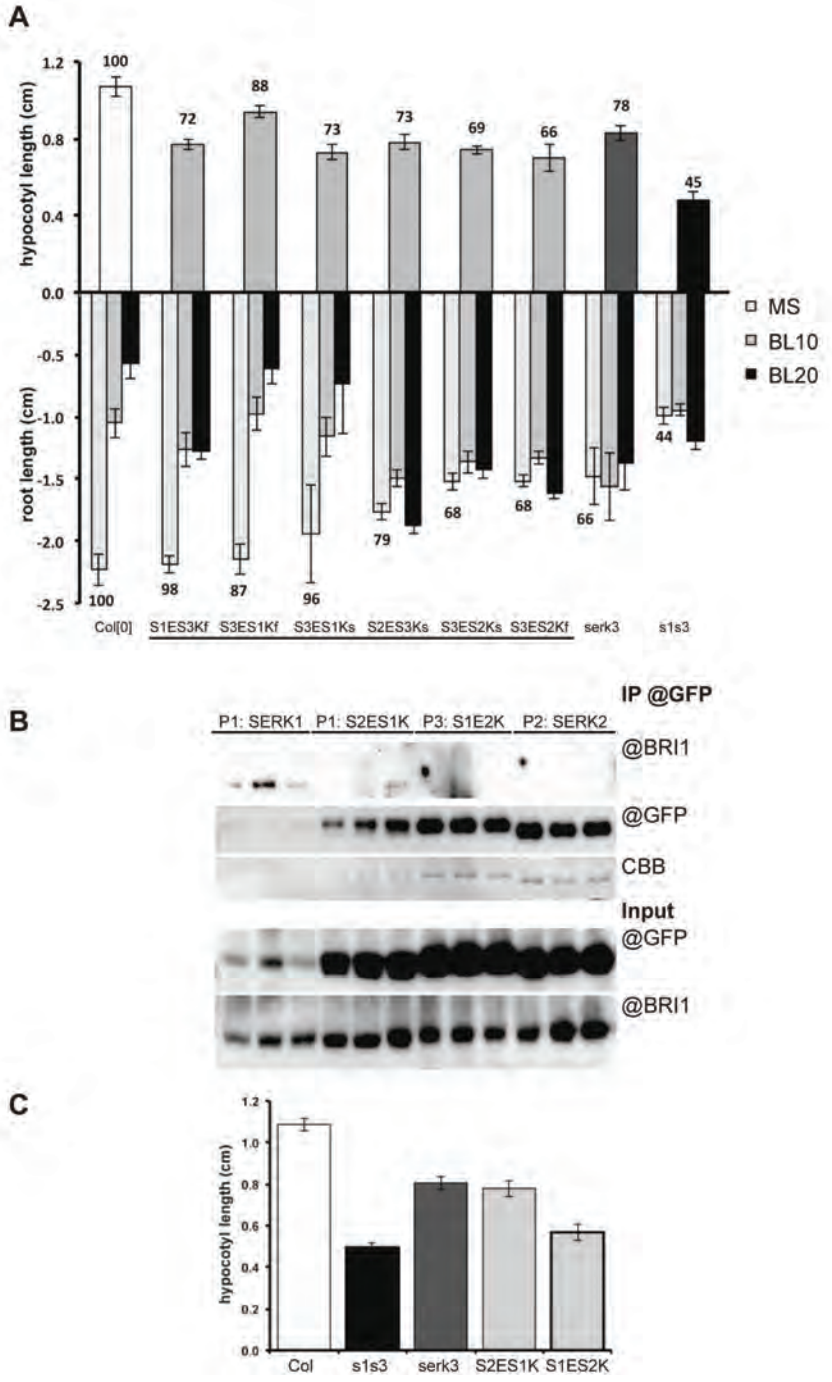
domain) and y the member from which the cytoplasmic K(inase) domain is derived is presented in Figure 6.

The sterility phenotype of the *serk1 serk2* double mutant can be fully complemented by S1ES3K and S2ES3K chimeric proteins using 4 and 6 independent transgenic lines respectively. All show full complementation as shown by the presence of pollen and the restoration of the silique size. In contrast, no rescue of the sterility phenotype can be observed with any other chimeric protein, such as S3ES1K or S3ES2K, 4 and 9 independent transgenic lines respectively have been tested, all showing full sterility phenotype (see Figure 7A). There is no difference in the level of expression of the transgenes that could account for the failure of the rescue (see Figure 7C) and the transgenes are expressed in the proper cell layer (i.e the tapetum layer) to allow full complementation (see Figure 7B). Apparently, the SERK1 and SERK2 extracellular domains, but not the kinase moieties, are critical for sporogenesis and mediate the specificity of the SERK1 and SERK2 genes within that pathway.

In the brassinosteroid pathway, *serk3* mutant plants show a weak BR-deficient phenotype. Neither *serk1* nor *serk2* single mutants show any defects in BR-signalling. The double mutant *serk1 serk3* (but not *serk2 serk3*) shows a more severe BR-deficiency than the single *serk3* (although still not as severe as the "cabbage" phenotype of a *bri1* strong allele) (Li et al. 2002, Nam and Li 2002, Albrecht et al. 2008). SERK1 thus has an overlapping but not completely redundant function to SERK3 in the BR-signalling pathway. In order to study the relevance of the different receptor domains to mediate specificity in brassinosteroid signalling, a series of chimeric proteins were introduced in the double mutant *serk1-3 serk3-2* and in the triple mutant *serk1-1 serk2-2 serk3-2*. Due to fertility problems of the homozygous double and triple mutants, the constructs were transformed in heterozygote backgrounds and further characterised by genotyping to identify double or triple

Figure 8 BR-phenotype complementation by chimeric SERK proteins
A. Hypocotyl assay and BR-induced root growth inhibition of complemented serk1 serk3 mutant with chimeric SERK proteins. Absolute growth of hypocotyl was measured after 4 day in the dark. Root growth of 8 days old seedlings grown on medium containing increasing amount of brassinolide. Bars represent SE (n=20). These experiments were repeated tree times with similar results.
B. Complex formation between SERK2 and BRI1 is restored when exchanging the cytoplasmic domain of SERK2 with the cytoplasmic domain of SERK1. Transgenic Arabidopsis seedlings expressing SERK1, SERK2, S1ES2K and S2ES1K driven by their own promoter (SERK1 and SERK2) or by SERK3 promoter (S1ES2K and S2ES1K) and tagged with GFP were treated with brassinazole (BZR, 2.5μM for 3 days), propiconazole (PPC, 1μM for 3 days) or epibrassinosteroid (BL, 1μM for 10 minutes). Total proteins (input) were subjected to immunoprecipitation with anti-GFP immunoaffinity beads followed by immunoblot analysis with anti-GFP antibodies to detect SERK-GFP, anti-BRI1 antibodies to detect BRI1. Molecular weight of detected proteins indicated in kDa.
C. Hypocotyl assay of complemented serk1 serk3 mutant with chimeric SERK proteins. Absolute growth of hypocotyl was measured after 4 day in the dark. Bars represent SE (n=20). These experiments were repeated tree times with similar results.

Figure 8


A
hypocotyl length (cm)
root length (cm)
MS
BL10
BL20
Col[0]
S1ES3Kf
S3ES1Kf
S3ES1Ks
S2ES3Ks
S3ES2Ks
S3ES2Kf
serk3
s1s3
B
IP @GFP
P1: SERK1
P1: S2ES1K
P3: S1E2K
P2: SERK2
@BRI1
@GFP
CBB
Input
@GFP
@BRI1
C
hypocotyl length (cm)
Col
s1s3
serk3
S2ES1K
S1ES2K

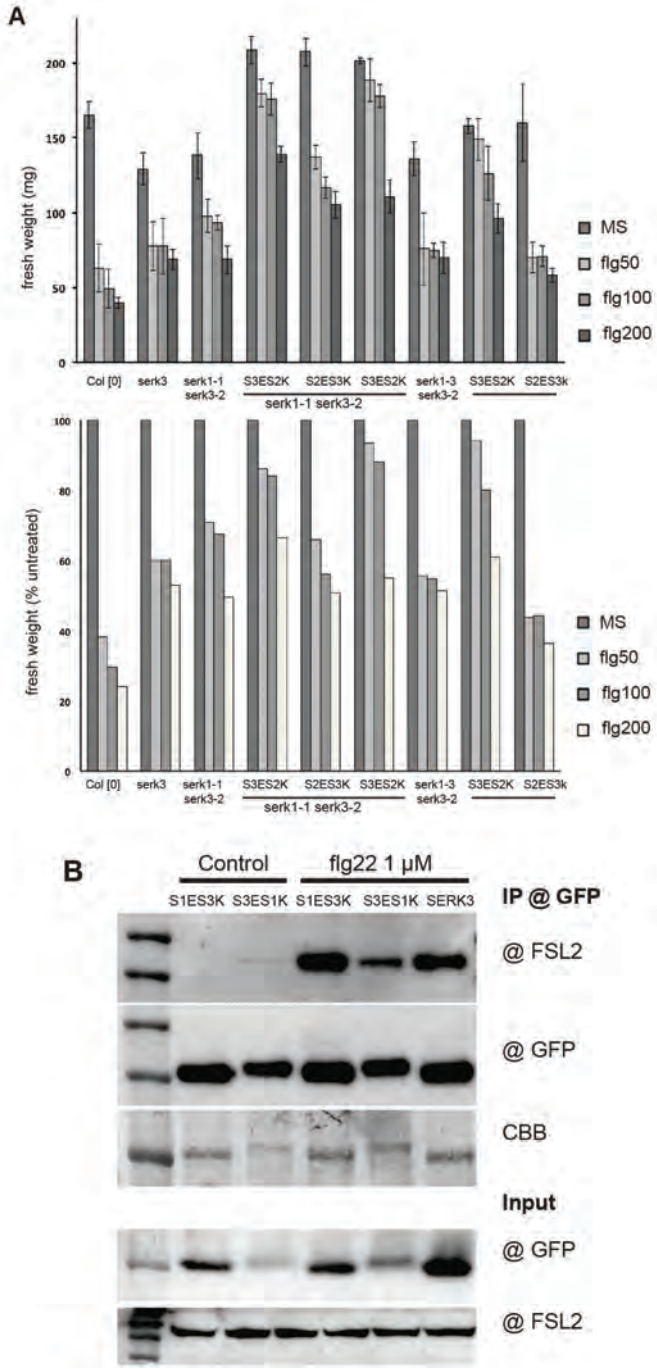
homozygotes carrying the transgene. Rescue was confirmed through quantification of hypocotyl length and root inhibition growth assay (see Figure 8).

The results show that both the SERK1 extracellular (S1ES3K) and cytoplasmic (S3ES1K) domains can revert the *serk1 serk3* phenotype to nearly wild type. The S2ES3K or S3ES2K chimers can revert the *serk1 serk3* phenotype close to the *serk3* phenotype but not to wild type (see Figure 8A). The differences in complementation observed between SERK1-SERK3 and SERK2-SERK3 chimers can be partly attributed to the kinase domain; the SERK2 extracellular domain coupled to a SERK1 kinase regains the ability to interact with BRI1 and can partially rescue the *serk1 serk3* double mutant (see Figure 8B and C).

Null *serk3* mutants are impaired in flagellin signalling indicating that other members of the SERK family cannot fully substitute for SERK3 in this pathway. This is further supported by the fact that *serk1* and *serk2* mutants in combination with *serk3* do not enhance the *serk3* PTI phenotype. Since GFP-tagged versions of SERK3 were found to impair flagellin signalling (Ntoukakis et al. 2011), only untagged chimeric constructs were used for the rescue of the PTI-related phenotypes. The previously described chimeric constructs introduced in *serk1 serk3* mutant background were therefore used to assess which domain is conferring SERK3 specificity in the flagellin pathway. A late response triggered by flg22 is the inhibition of seedling growth. In that assay, *serk3* mutants are impaired in flagellin-induced inhibition of seedling growth and are insensitive up to 50 nM of flg22 (Chinchilla et al. 2007). None of the chimeric proteins in the *serk3* mutants restored responsiveness to flagellin back to wild type. *serk3* seedlings carrying the S3ES1K or S3ES2K chimers are less sensitive to flg22 than untransformed *serk3* (see Figure 9A). Instead, the S3ES1K and S3ES2K chimers even showed a dominant-negative effect on FLS2-mediated signalling, suggesting that both the SERK3 extracellular and cytoplasmic domains are indispensable for the response to flagellin. The failure of the chimeric proteins to replace the SERK3 protein in PTI was not due to a reduced ability to associate with FLS2 (see Figure 9B).

Figure 9 None of the chimeric proteins restores PAMP signalling.
A. PAMP-induced seedling growth inhibition of complemented serk1 serk3 mutant with chimeric SERK proteins. Seedling growth inhibition in response to increasing concentrations of flg22 in Col[0], serk3, serk1 serk3 and complemented serk1 serk3 mutants with different swap domains SERK proteins. Seedlings are weighed 10 days after treatment. Results are given as absolute or relative growth measured as fresh weight in Figure A and B, respectively. Results are average +- SE (n=10). All experiments were repeated at least three times with similar results.
B. The chimeric proteins are not impaired in their ability to interact with FLS2. Transgenic Arabidopsis seedlings expressing SERK3, S1ES3K and S3ES1K driven by SERK3 promoter and tagged with GFP were treated with flagellin (flg22, 1μM for 10 minutes). Total proteins (input) were subjected to immunoprecipitation with anti-GFP immunoaffinity beads followed by immunoblot analysis with anti-GFP antibodies to detect SERK-GFP, anti-FLS2 antibodies to detect FLS2. Molecular weight of detected proteins indicated in kDa.

Figure 9


A
fresh weight (mg)
200
150
100
50
0
MS
flg50
flg100
flg200
Col [0]
serk3
serk1-1 serk3-2
S3ES2K
S2ES3K
S3ES2K
serk1-3 serk3-2
S3ES2K
S2ES3k
serk1-1 serk3-2
fresh weight (% untreated)
100
80
60
40
20
0
B
Control
flg22 1 µM
S1ES3K
S3ES1K
S1ES3K
S3ES1K
SERK3
IP @ GFP
@ FSL2
@ GFP
CBB
Input
@ GFP
@ FSL2

Discussion

The SERK family in *A. thaliana* has arisen through gene duplications followed by functional divergence. The end result is a protein family of five highly homologous members that show only partial functional redundancy (Hecht et al. 2001, Albrecht et al. 2008). The functional divergence between these highly similar proteins, and the multiple and distinct pathways in which they are implicated, raises questions on how the SERKs operate. The functional plasticity of the SERK protein family as found in *A. thaliana* was identified as well in rice (Singla et al. 2009, Park et al. 2011), barrel clover (Nolan et al. 2009, 2011) and tobacco (Chaparro-Garcia et al. 2011, Mantelin et al. 2011), indicating that the ability to function in multiple signalling pathways, via interaction with different main receptors, is probably a common mode of action for SERK proteins of different plant species. Previous analyses on SERK genes and proteins have clearly demonstrated that the differences in SERK functions are mostly caused by differences in protein sequence, rather than different gene expression patterns or levels. Sequence analysis coupled to domain swap experiments show that changes in both SERK extracellular and cytoplasmic domains contribute to the functional divergence of the proteins.

SERKs are ancient, essential genes that have been conserved during speciation

Multiple SERK genes are found in each of the analysed plant genomes, including dicots, monocots and non-vascular plants. This indicates that SERK genes are at least 160 M years old, and were present before the split of non-vascular and vascular plants. The brassinosteroid and flagellin pathways, in which *At*SERK1, 3 and 4 have been implicated, are thought to have arisen after this split (Rensing et al. 2008, Boller and Felix 2009). This hypothesis is supported by our genome analyses of *P. patens* and *S. moelendorffii* using BLAST algorithms with the BRI1 sequence of *A. thaliana*. The analyses did not result in the identification of confident BRI1 orthologues in these species (data not shown). Thus, the SERK co-receptors predate the main ligand binding receptor BRI1 and FLS2. The SERKs present in genomes that diverged earlier during speciation (such as the *P. patens* genome) closely resemble the SERKs from monocot and dicots. The SERKs have thus been conserved throughout evolution. Indeed we found that the SERKs are under purifying evolutionary pressure, i.e. under evolutionary pressure to minimise sequence diversity.

All plant genomes analysed have multiple homologous SERK gene copies, presumably arisen through duplications. These duplicates have been preserved as close homologues in the genome, while classic evolutionary theory predicts that duplicated genes either diverge or are lost after time (Wagner 1998). The presence, and maintenance, of multiple SERK copies in the plant genome, indicates the functional relevance of the simultaneous presence of these receptors. This might be due

to unequal or partial redundancy restricted to a particular signalling process; unequal redundancy is defined as the situation where "the duplicated gene, although dispensable, contributes significantly to the overall activity of the gene pair, as revealed by double mutant analysis" (Briggs et al. 2006). This unequal redundancy has led to sub-functionalisation in non-overlapping processes; SERK1, 2 and 4 are not essential for BR signalling, but are under selective pressure because they are essential for sporogenesis and cell death signalling respectively. It is currently unclear whether the observed unequal redundancy is caused by different heterooligomer configurations with different subcellular localisation and/or differences in the use of phosphorylation sites. Differences in the affinity between SERKs and their respective main receptors (Roux et al. 2011 and this study) and differences in the autophosphorylation status of the SERKs (Karlova et al. 2009, Roux et al. 2011) are observed that could support both ideas.

Evolutionary divergence in the SERK protein family and related functions

SERK3 or BAK1 is the only SERK family member in *A. thaliana* which displays a developmental as well as a PTI phenotype, and thus seems to be the most active signalling member. However, the SERKs from the non-vascular plant genomes and monocot genomes resemble the *A. thaliana* SERK1 and 2 proteins more. Also, the NJ-tree shows that the dicot SERK1 and SERK2 proteins are more closely related to monocot SERKs than to the dicot SERK3 and SERK4 proteins. Thus, it appears that *At*SERK3, SERK4 and SERK5 are evolutionary later expansions of the protein family, and the additional functionality of SERK3 probably also evolved later. This is in line with the observation that SERK1 and 2 are the only SERKs involved in male sporogenesis, a signalling pathway from which the putative main receptor in that pathway, EMS1,has a homologue in the *Physcomitrella* genome, in contrast to BRI1 and FLS2 (Wang and Mao 2013). Members of both the early SERK1-2 clade and the SERK3-4-5 clade have apparently been recruited by BRI1 receptor while only SERK3-4-5 clade members acquired advantageous mutations leading to neo-functionalisation in immune signalling and cell death control (He et al. 2007, Roux et al. 2011). However, although no BRI1 homologue is found in non-vascular plant genomes, brassinosteroids were found to induce growth in *Selaginalla*, and downstream signalling components are present (Cheon et al. 2013). Thus brassinosteroid signalling probably does occur in non-vascular plant, albeit via different mechanisms, and SERKs could still function in brassinosteroid signalling in these evolutionary older genomes.

The inability of SERK1 and 2 to substitute for SERK3 in the immune signalling might be due to the inability to recognise the signal properly and/or to couple to downstream signalling components. Residues involved in flg22 binding are conserved between SERK1, 2 and 3 and the interaction between FLS2 and SERK1-2

is strongly ligand dependent, similar as reported for SERK3. Hence, it is unlikely that perception of the ligand is altered in the heterodimer formed by SERK1-2 and FLS2 receptors. None of the chimeric proteins rescues flagellin-related phenotypes, indicating a high stringency on both SERK3 extracellular domain and cytoplasmic domain for PTI signalling. The data presented here corroborates previous findings suggesting that heterodimerization would be regulated by a double-lock system in which both the extracellular domain and cytoplasmic domain would participate (Jaillais et al. 2011). Furthermore, it indicates that the usage of the SERK receptor is different depending of the pathway in which they are serving; the extracellular domain is essential for sporogenesis, the cytoplasmic domain for BR signalling and both extracellular domain and cytoplasmic domain for the PTI pathway. This is in line with the fact that the main receptors involved in these pathways have different mode of action, RD versus non-RD kinases. For instance, BRI1 kinase activity is necessary for ligand induced SERK3/BAK1-BRI1 oligomerization (Wang et al. 2008) while kinase activity of neither main receptor nor co-receptor is necessary for ligand induced FLS2-SERK3/BAK1 or EFR-SERK3/BAK1 oligomerization (Schwessinger et al. 2011). Also, a C-terminal tag on SERK3/BAK1 was found to impair flagellin signalling, while it has a minor effect on BR signalling (Ntoukakis et al. 2011, Lozano-Duran et al. 2013). The data presented in this manuscript confirm those findings and further highlight major differences in the regulation of the pathways in which the SERKs are serving.

The cytoplasmic domain is not the sole cause of specificity

One attractive mechanism to employ the same SERK protein in different pathways in the same cell would be to slightly alter the auto- and transphosphorylation pattern after activation by different ligand-perceiving main receptors (Wang et al., 2008). However, our results indicate that there is only limited specificity in the cytoplasmic SERK domains, as the SERK3 cytoplasmic domain can fully substitute for the SERK1 and SERK2 cytoplasmic domains in male sporogenesis signalling. Thus, transphosphorylation specificity of the kinase domain cannot be the differential factor between SERK1/SERK2 and SERK3 for this pathway. For the PTI pathway, exchange of the cytoplasmic domains is not sufficient to acquire the capacity to relay the perception event into signalling output; the flagellin-related phenotypes could not be rescued by an exchange of the kinase domain between SERK1 or SERK2 and SERK3. Those studies established that swapping the intracellular kinase domain does not reprogram the cellular response output. This is a strong indication that the kinase activity is not the sole cause for differences in specific downstream signal activation observed between the different SERKs. Similar studies using a chimera strategy have been used in animal and plant signalling to elucidate receptor kinase activation and downstream signalling mechanisms (e.g. Yin et al. 2004). Those studies clearly indicate that receptor kinases employ closely

related or identical mechanisms to transmit the signal across the plasma membrane to trigger cellular responses. They furthermore clearly show that the extracellular domain is conferring perception specificity of the ligand while the intracellular kinase domain defines downstream signalling specificity, receptor regulation and trafficking. In receptor chimera constructed from the major structural domains of the EGF and insulin receptors, the signalling output is defined by the origin of the intracellular kinase domain (Riedel et al., 1986; Riedel et al., 1989). Likewise, a chimeric receptor composed of the extracellular domain of the brassinosteroid receptor BRI1 fused to the intracellular kinase domain of the rice disease resistant receptor, XA21, initiates plant defense responses in rice cells upon treatment with brassinosteroids (He et al., 2000). In contrast, the data presented here with chimera of genes from the same family, indicate that the kinase domain is not solely conferring downstream specificity, i.e they have different requirements for the extracellular and/or intracellular domain depending of the signalling output. Hence, we propose that the formation of SERK-main receptor heterodimers creates specific interaction platforms with combinatorial complement of downstream targets, which lead to distinct outcomes of stimulation.

Due to complex redundancy issues and the resulting lethality phenotypes, it is difficult to clearly assign specific role to the individual SERK members. We show that we can manipulate this specificity and created SERK1SERK3 chimers that split the brassinosteroid and PTI and SERK2 extracellular-SERK3 kinase chimers that split the brassinosteroid and sporogenesis pathways. The chimers will be instrumental to decipher individual SERK signalling pathways in the absence of other interfering SERK, thereby greatly increasing our understanding of specificity within the SERK protein family.

Heteromerization is at the core of SERK activation and signalling

Ligand-binding receptors possess all the necessary domains from perception of the external signal via the LRR region to its transduction into cytoplasmic signalling via the kinase. Regardless, a common mechanistic property associated with the activation of many receptor kinases, whether in the animal or plant kingdom, is their obligate homo- or heterooligomeric activated configuration. Homodimerization of both BRI1 and FLS2 receptors has been documented (Wang et al. 2005b, Hink et al. 2008, Sun et al. 2012). However, it is unclear whether this represents functionally active complexes or whether they are part of a more elaborate structure, as BRI1-SERK3 hetero-oligomers partially exist with a size corresponding to a tetrameric configuration (Karlova et al. 2006). Recent crystal structures of the SERK1-BRI1 (Santiago et al. 2013) and SERK3/ BAK1- FLS2 (Sun et al. 2013) extracellular domain complexes have established the SERKs as true co-receptors that have interactions with both the main ligand binding receptor and the ligand itself. The residues that are involved in the interactions between SERKs and their

main receptor extracellular domains are well conserved among the SERK family members, even in the non-vascular plant genomes, which suggest that all SERKs have the ability to form these complexes. Indeed, we find that SERK2, although genetically unlinked to BR signalling, can interact with BRI1 in systems such as protoplasts (unpublished data). In addition, SERK1 and SERK2, for which no genetic evidence exists for a role in immunity signalling, can interact with FLS2 (Roux et al. 2011). The residues involved in the recognition of the ligand are also well conserved in all the SERK members, including the SERK members present in non-vascular genomes, lacking the BRI1 (Wang and Mao 2013) and the FLS2 receptors (data not shown). Hence, specificity conferred by SERK is not due the inability of some members to appropriately recognise the ligand. The recent structures of the extracellular domains of the main receptors BRI1 and FLS2 with their respective ligands further indicate that, at least for these receptors, ligand binding does not induce homodimerization of the main receptor, or large conformational changes to the extracellular domains (Hothorn et al. 2011, She et al. 2011, Sun et al. 2013). This indicates that for the initial ligand induced receptor activation an additional player, besides the ligand binding receptor, is needed. All current genetic, biochemical, microscopic and structural data indicate that this role is fulfilled by members of the SERK co-receptor family (Albrecht et al. 2012, Gou et al. 2012, Bücherl et al. 2013, Santiago et al. 2013, Sun et al. 2013). As in EGF signalling, co-receptors most likely fulfill a structural role in generating and maintaining a structure to achieve the required transmembrane conformational changes to turn on kinase activity (Zhang et al. 2006a, Campbell et al. 2010, Liu et al. 2012). In addition, they can modulate the signal initiated by ligand binding (van Esse et al. 2013) in such a way that it can precisely target the correct intracellular response.

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Supplemental Material

Supplemental Table 1 Primers used for genotyping

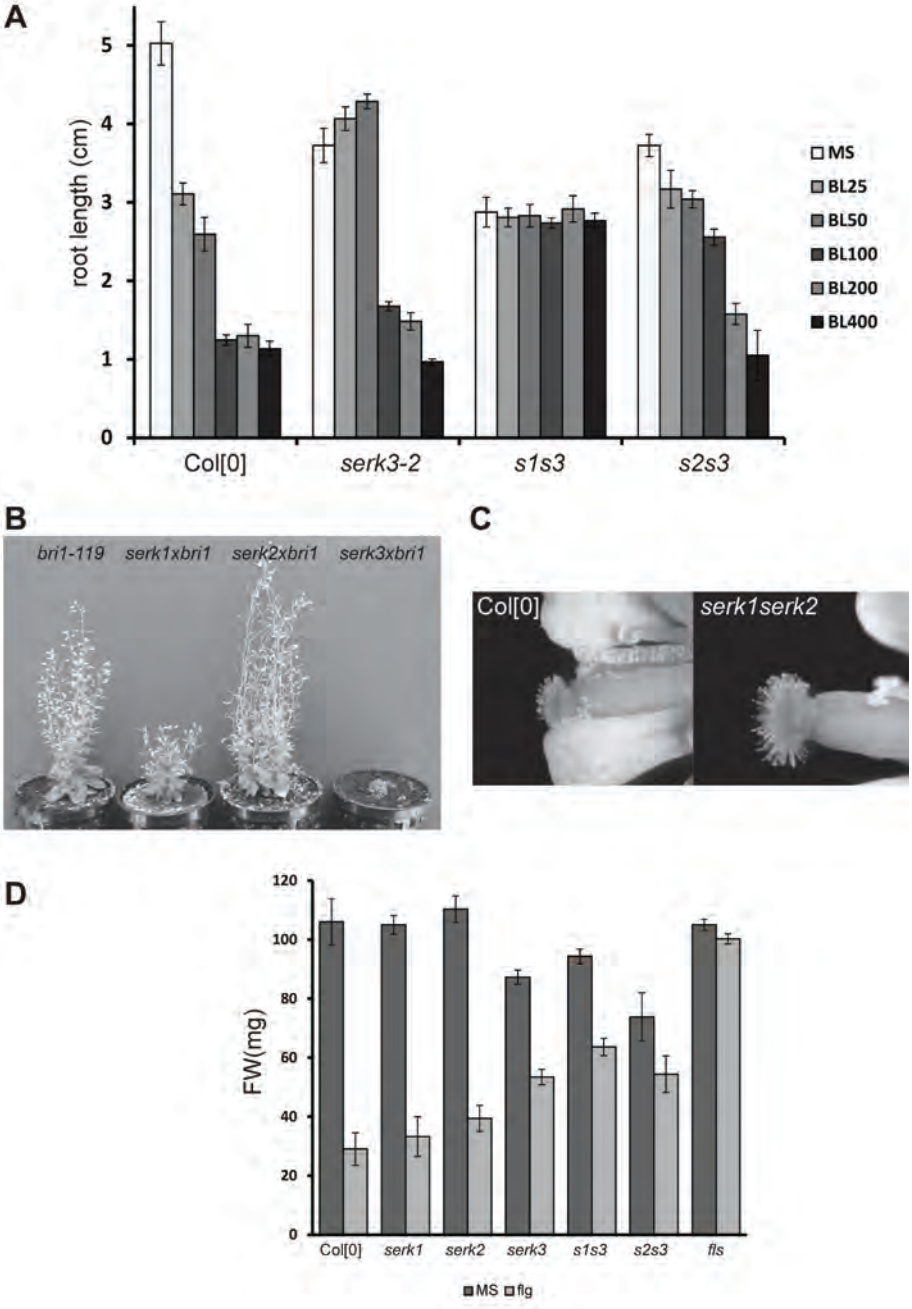
serk1-1	*serk1-1* F	CGTGACAACAGCAGTCCGTGGCACCATCGG
	serk1-1 R	CCCTTTTAATCGAACCATAGCAC
	T-DNA R	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC
serk1-3	*serk1-3* F	AGCAATTTTGTTTTGCAGAAAAGT
	serk1-3 R	AGAGATATTCTGGAGCGATGTGACCGATGG
serk2	T-DNA R	CCCATTTGGACGTGAATGTAGACAC
	serk2 F	CTCTGGTATGGGAAGATGGTAATGTGGTCTGAG
	serk2 R	CGGCTAGTAACTGGGCCGCATAGATCC
	T-DNA R	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC
serk3-2	*serk3-2* F	GCCACTAAAGTACCATCAGC
	serk3-2 R	CAACACCAAGTTGACTCCCCTTCCTGC
	T-DNA R	GCGTGGACCGCTTGCTGCAACT

Supplemental Table 2 Primers used for producing chimers

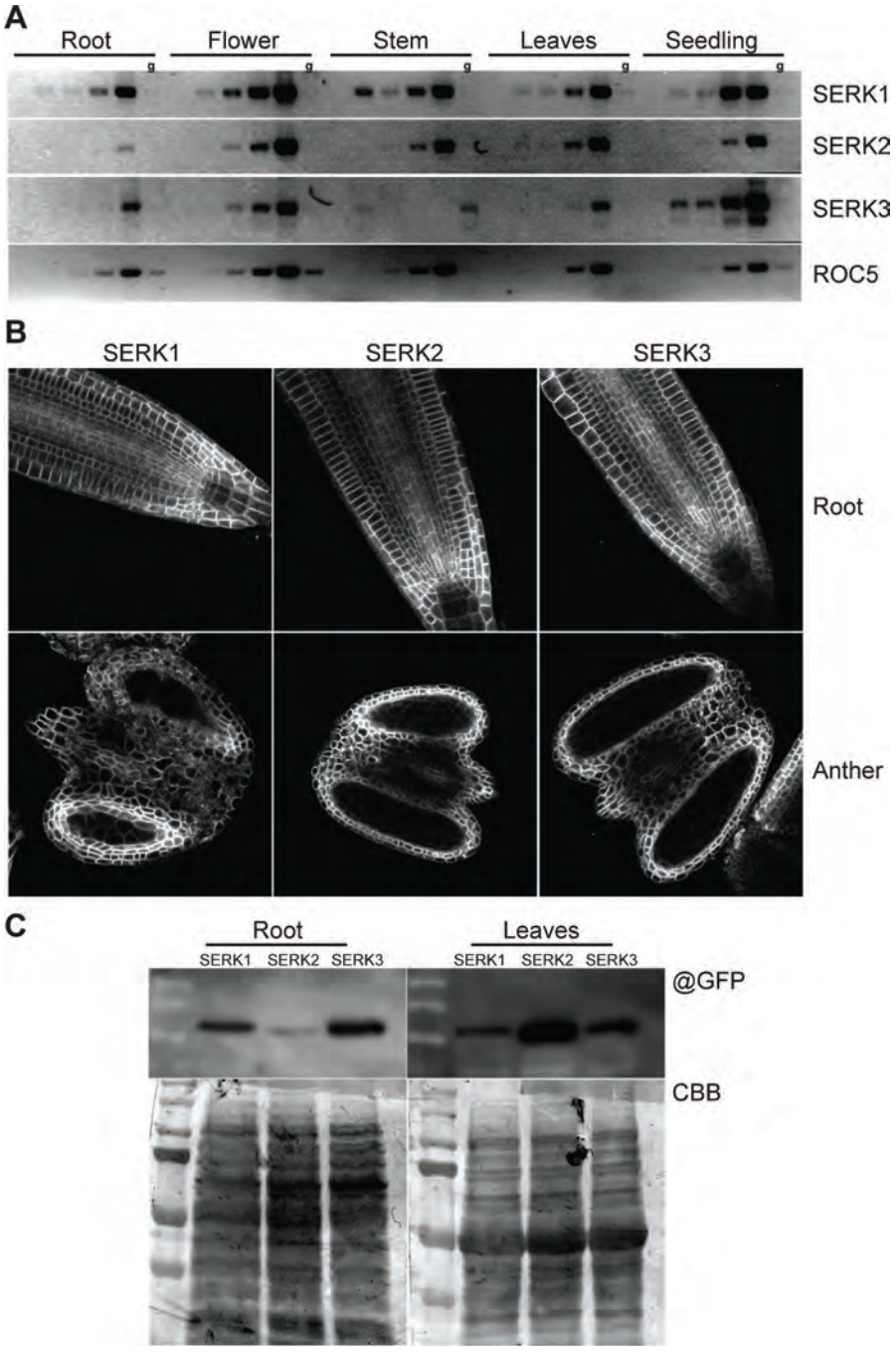
S1ES3K	S1ES3K F	ACTGGAGCAATAGCTGGTGGAGTTGCTGCAGGTGCTGCTCTTC-TATTTGCTGTTCCGGCCATTGCACTAGCTTGGTGG
	S1ES3K R	CCACCAAGCTAGTGCAATGGCCGGAACAGCAAATAGAAGAG-CAGCACCTGCAGCAACTCCACCAGCTATTGCTCCAGT
S2ES3K	S2ES3K F	ACTGGAGCCATTGCGGGAGGAGTTGCTGCTGGTGCTGCTCTTC-TATTTGCTGTTCCGGCCATTGCACTAGCTTGGTGG
	S2ES3K R	CCACCAAGCTAGTGCAATGGCCGGAACAGCAAATAGAAGAG-CAGCACCAGCAGCAACTCCTCCCGCAATGGCTCCAGT
S3ES1K	S3ES1K F	ACTGGAGCGATTGCGGGAGGAGTTGCTGCAGGTGCTG-CATTGCTCTTTGCTGCTCCTGCAATAGCCTTTGCTTGGTGG
	S3ES1K R	CCACCAAGCAAAGGCTATTGCAGGAGCAGCAAAGAGCAATG-CAGCACCTGCAGCAACTCCTCCCGCAATCGCTCCAGT
S3ES2K	S3ES2K F	ACTGGAGCGATTGCGGGAGGAGTTGCTGCAGGTGCTGCATTAC-TATTTGCTGCCCCTGCTTTAGCTTTTGCTTGGTGG
	S3ES2K R	CCACCAAGCAAAAGCTAAAGCAGGGGCAGCAAATAGTAATGCAG-CACCTGCAGCAACTCCTCCCGCAATCGCTCCAGT

Supplemental Figure 1 SERK genes show unequal/only show partial redundancy.
A. Root growth measurements of seedlings grown on media containing different BL concentrations using various mutant combinations. Growth is represented relative to the untreated wild type. Results are average ± SE (n=10). All experiments were repeated at least twice with similar results. *s1* stands for the *serk1-1* allele, *s2* for *serk2-2* and s3 for serk3-2.
B. serk1-1 and serk3 but not serk2-2 mutants enhance the weak phenotype of bri1-119 mutant. Three weeks-old plants are shown.
C. Phenotype of the double serk1-1 serk2 double mutant. The *serk1-1 serk2* double mutant does not produce pollen hence is male sterile. *1*- A wild-type flower showing pollen grains. *2*- A *serk1-1 serk2* double mutant flower with shortened anther filament and no pollen grain.
D. Seedling growth inhibition triggered by flg22 in Col-0, *serk1-3*, *serk2-2*, *serk3-2* and *fls* mutant seedlings and in the double mutants *serk1-3 serk3-2* (*s1s3*), *serk2-2 serk3-2* (*s2s3*). Growth is represented relative to the untreated wild type. Results are average ± SE (n=12). All experiments were repeated at least twice with similar results.

Supplemental Figure 1



Supplemental Figure 2

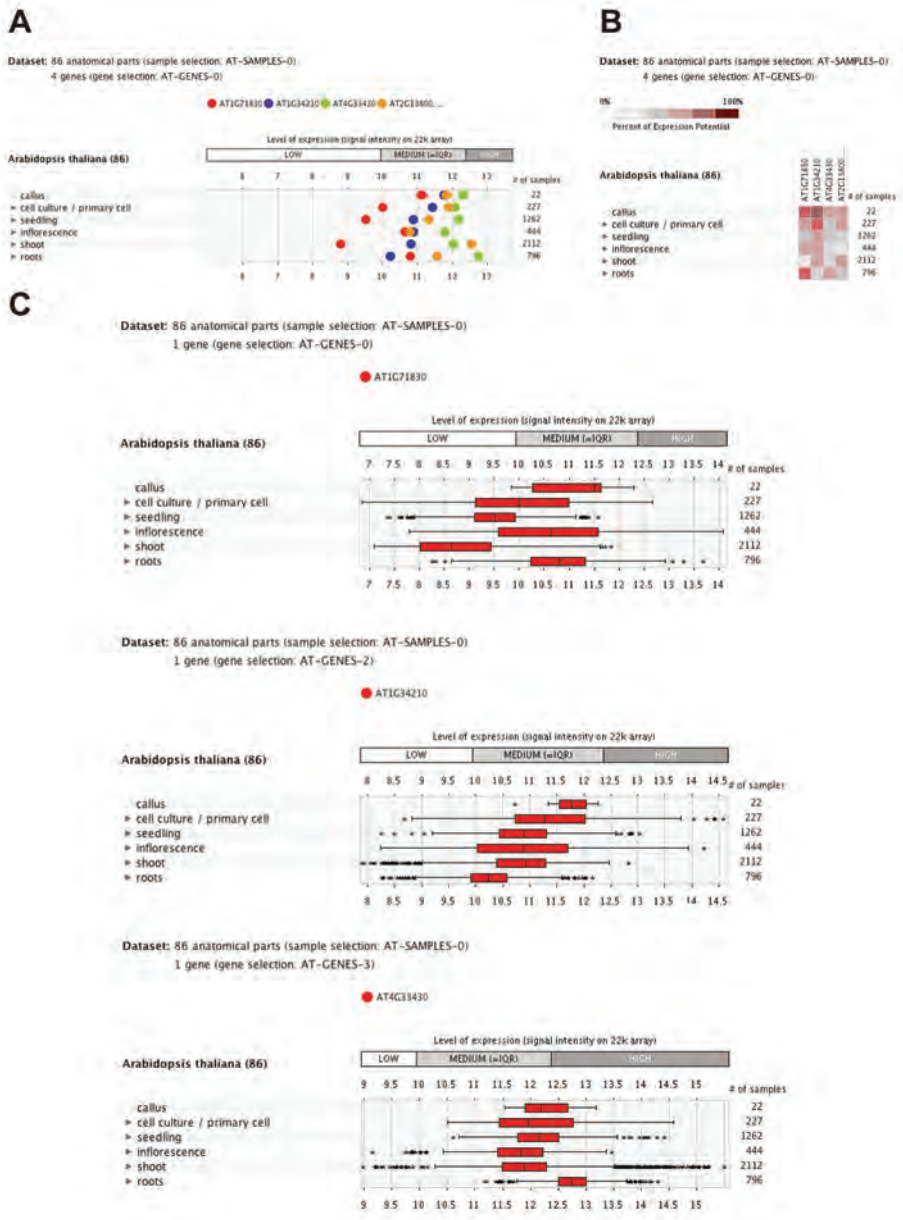


Supplemental Figure 2 SERK1, SERK2 and SERK3 expression patterns are largely overlapping.

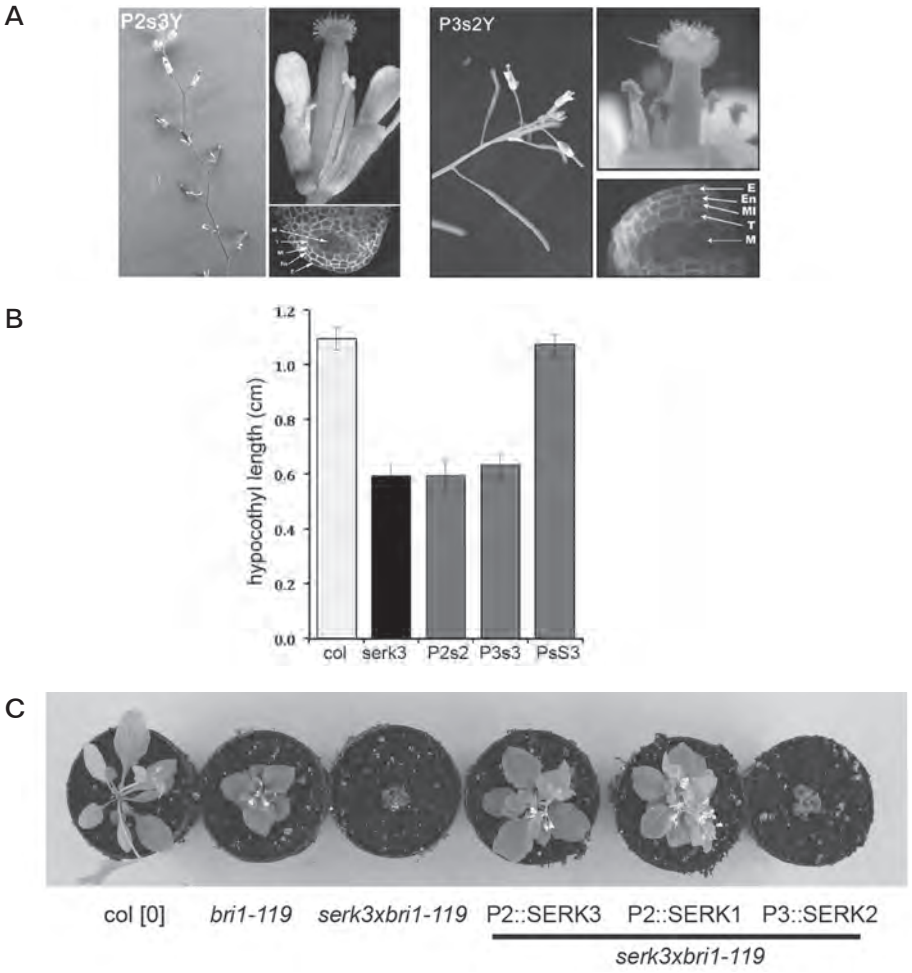
A. Semi-quantitative RT-PCR analysis of SERK1, SERK2 and SERK3 transcripts in different tissues. The constitutively expressed cyclophilin gene, ROC5 (Chou and Gasser, 1997) was amplified simultaneously as a control. PCR products were collected after 28-30-32-34 cycles for SERK1, SERK2 and SERK3 and after 20-22-24-26 cycles for ROC5.

B. SERK1, SERK2 and SERK3 fluorescent fusion proteins localisation during root and anther development.

C. Roots and anthers of transgenic plants harbouring the SERK1, SERK2 and SERK3 cDNAs fused to YFP and driven by their respective promoters are visualised by confocal microscopy. 1- Expression in the root tip. 2- Expression in the anther.



Supplemental Figure 2bis Expression profile of SERKs genes in various organs.
The X axis indicates the level of gene expression. The various organs are indicated in Y axis. The data used in the Figures were retrieved from Genevestigator (https://www.genevestigator.ethz.ch; Zimmermann et al. 2004).



Supplemental Figure 3 Neo and/or sub-functionalisation of SERK genes is not determined by divergence in cis-regulatory sequences.

A. Complementation of the male sterility phenotype.

1- Inflorescence of a *serk1 serk2* double mutant, expressing the SERK3 gene under the SERK2 promoter (P_{SERK2}:SERK3-YFP), plant showing normal seedpods. Close–up on the flowers showing pollen grains. Expression of the transgene in the anther tissue is visualised by confocal microscopy. **2**- Inflorescence of *serk1-1 serk2* double mutant, expressing the SERK2 gene under the SERK3 promoter (P_{SERK3}:-SERK2-YFP), plant with small seedpods and no developing seeds. Close–up on the flowers showing no pollen grains. The transgene is visualised in the anther tissue by confocal microscopy.

B. Complementation of the brassinosteroid-related phenotypes.

Quantitative analysis of the hypocotyl length of *serk3-2* mutants complemented with SERK2 under SERK2 (P2s2) or SERK3 (P3s2) promoters and SERK3 under SERK2 promoter (P2s3) as compared to Col[0] and *serk3-2*. The seedlings are grown in the dark for 4 days. Each measurement represents an average of hypocotyl lengths of 20 seedlings. Error bars indicate standard deviation. All experiments were repeated at least twice with similar results * indicates significant differences from Col-0 wild type ($p \leq 0.05$). ** indicates significant differences from the *serk3-2* mutant ($p \leq 0.05$).

C. Rescue of the dwarf stature of the double mutant *bri1-119 serk3* with various promoter swap constructs, SERK3 and SERK1 under the SERK2 promoter (P_{SERK2}:SERK3 and P_{SERK2}:SERK1 respectively) and SERK2 under the SERK3 promoter (P_{SERK3}:SERK2). The transgenic lines are compared with Col[0], *bri1-119* and *bri1-119 serk3*.

Chapter 3

Purification and Crystallisation attempts on the SERK protein family

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Abstract

Receptor-like kinases are major players in plant signalling pathways. Recent crystal structures of extracellular domains of receptor kinases Brassinosteroid Insensitive 1 and Somatic Embryogenesis Receptor Kinase 1 complex as well as the kinase domain of receptor BRI1-associated kinase 1 have provided the first insights in receptor structure-function relationships. To increase this knowledge it is important to obtain more structures of these proteins, in different conformational and functional states. For this reason, we attempted to elucidate the three dimensional structure of the five members of the Somatic Embryogenesis Receptor-like kinases from *Arabidopsis thaliana*. We were successful in producing crystals of the SERK1 kinase domain, but were unable to determine its protein structure.

Introduction

The elucidation of the atomic structures of proteins and other macromolecules has provided great insight into the molecular mechanisms by which cellular functions are achieved. One interesting question that these structures have started to answer is the manner in which extracellular signals are translated to cellular responses across the plasma membrane.

Often, signal transduction is initiated at the plasma membrane by receptor protein kinases that perceive the extracellular ligand, which leads to intracellular transphosphorylation events via conformational changes in receptor or signalling complex. Examples of such receptor kinases are the well-known animal receptor tyrosine kinases (RTKs), and the related plant receptor-like kinases (RLKs) (van der Geer et al. 1994; Shiu et al. 2001). The vast amount of RTK structures, in different conformational stages and with or without ligand, has provided a structural basis for understanding receptor activation, complex formation and specificity (Hubbard et al. 2000), with the EGFR (epidermal growth factor receptor) as prime example (Ferguson et al. 2008; Bublil et al. 2010; Liu et al. 2012). In contrast, very little structural data is available on the plant counterparts of the RTKs, the RLKs. The largest group within the plant RLKs are those of the leucine-rich-repeat RLKs (LRR-RLKs), whose extracellular domain contains the LRR-motif (Torii 2004). In recent years, the first few crystal structures of LRR-RLK domains have been reported, increasing our understanding of the molecular mechanisms of plant signalling. These crystal structures include the extracellular domain and cytoplasmic domain of members of an LRR-RLK subfamily known as the SERKs in complex with interaction partners.

The Arabidopsis SERKs (somatic embryogenesis receptor kinases) are LRR-RLKs, containing five extracellular leucine rich repeats and an intracellular kinase domain (Hecht et al. 2001; Shah et al. 2001). They are a family of five RLKs in *A. thaliana* that function in several different, seemingly unrelated, plant signalling pathways, such as hormone signalling, somatic embryogenesis, male sporogenesis, immune responses and cell death (Schmidt et al. 1997; Li et al. 2002; Albrecht et al. 2005; Chinchilla et al. 2007; He et al. 2007; Albrecht et al. 2008; Chinchilla et al. 2009). Although the SERK family members are highly homologous in sequence, they are not completely interchangeable *in planta* (see chapter 2 and (Albrecht et al. 2008)). SERKs function in these different pathways by forming specific higher order complexes with ligand binding LRR-RLKs, such as BRI1 (main ligand-binding receptor for the hormone brassinosteroid), FLS2 (main ligand binding receptor for bacterial flagellin) and EFR (main ligand binding receptor for the bacterial effector elongation factor Tu) (Nam et al. 2002; Russinova et al. 2004; Chinchilla et al. 2007; Roux et al. 2011) and thus act as co-receptors. Transphosphorylation events between main receptor and co-receptor are important for proper signal transduction (Wang et al. 2008; Schulze et al. 2010; Gou et al. 2012).

The cytoplasmic domain of SERK3 (also called BAK1 for BRI1-associated kinase 1) has been elucidated in its apo-form, and in complex with the SERK3/ BAK1 interacting domain (BID) of the bacterial effector AvrPtoB (Cheng et al. 2011; Yan et al. 2012). AvrPtoB was shown to interact with the kinase domains of SERK3/ BAK1 and FLS2, interfering with PAMP-triggered immunity (PTI) (Xing et al. 2007). The structure of the SERK3/ BAK1 kinase domain follows the basic structure found for eukaryotic protein kinases (ePK), which share a common catalytic core structure consisting of 12 subdomains and catalyse the transfer of the γ-phosphate of ATP to the hydroxyl group of serine, threonine, or tyrosine residues (Hanks et al. 1995; Stone et al. 1995). The ePK family can be divided in two groups, based on the ability to either transfer a phosphate to the phenol group of tyrosine residues (the protein tyrosine kinases) or to the alcohol group of serine and threonine residues (the serine/threonine kinases). Based on phylogenetic analysis and certain sequence motifs, the plant RLKs were grouped in the serine/threonine class of kinases, together with the animal Pelle and IRAK kinases (Shiu and Bleecker 2001). However, some RLKs were found to phosphorylate both serine/threonine and tyrosine residues. These kinases are termed 'dual specificity kinases'. Examples of these include the brassinosteroid insensitive 1 receptor (BRI1) and at least two members of the somatic embryogenesis receptor-like kinases (SERK1 and SERK3/ BAK1) (Shah et al. 2001; Oh et al. 2009; Oh et al. 2010). The SERK3/ BAK1 kinase domain shows the common two-lobed kinase structure consisting of a small N-terminal lobe (mostly consisting of anti-parallel β-sheets) and the larger C-terminal lobe (consisting mostly of α-helices) and a catalytic cleft between the two lobes. The complex of SERK3/ BAK1 kinase with the BID domain of AvrPtoB shows that the bacterial effector probably inhibits SERK3/ BAK1 kinase function by interaction with the catalytic cleft, via contacts with the P+1-loop (in the substrate-binding region) (Cheng et al. 2011). Both the apo SERK3/ BAK1 kinase domain structure as well as the kinase in complex with AvrPtoB are in the active state, as similarities to other active kinase structures indicate (Cheng et al. 2011; Yan et al. 2012). This active state is stabilised by two phosphorylated threonine residues, T455 and T450 (Yan et al. 2012). Although both these residues were found to be important for signalling activity of SERK3/ BAK1 in flagellin signalling, and (to lesser extent for T450) also for brassinosteroid signalling (Wang et al. 2008; Yan et al. 2012) most likely T455 is more important for inducing the active state of the SERK3/ BAK1 kinase domain, while T450 phosphorylation has a more complex influence on transphosphorylation specificity. T455A or T455D SERK3/BAK1 has lost almost all phosphorylation activity, while mutating T450 of SERK3/ BAK1 has only minor influence on SERK3/BAK1 autophosphorylation activity (Wang et al. 2008). SERK3/ BAK1 T450 found to be an essential residue for transphosphorylation on FLS2 (Wang et al. 2008; Yan et al. 2012), however, not for transphosphorylation on BRI1 in yeast (Yun et al. 2009). This seems to indicate that differential phosphorylation of the activation loop of SERK3/ BAK1 changes kinase substrate specificity.

In recent years the first crystal structures of LRR-RLK extracellular domains (ectodomains) have been resolved. In 2011 crystal structures of the BRI1 extracellular domain, both in presence and absence of ligand were published (Hothorn et al. 2011; She et al. 2011). BRI1 contains 25 LRRs that, besides the normal LRR curvature into a horse-shoe like structure, show a unique additional curvature to form a 'super-helix', different from animal LRR domains. The island domain, involved in ligand binding, folds back into the interior of the 'super-helix'. Binding of ligand to the island domain causes some structural changes to this domain and connecting loops, however the rest of the extracellular domain does not undergo any conformational changes upon ligand binding. Also, the BRI1 extracellular domain was found as a monomer in crystals and in solution, independent of ligand binding, thus ligand binding does not induce homodimerization of BRI1 extracellular domains (Hothorn et al. 2011; She et al. 2011). Hothorn et al. hypothesised that ligand binding could induce the formation of a hydrophobic interaction platform, causing heterodimerization with co-receptors such as the SERKs, which in turn would create an active signalling complex. This hypothesis was proven by the elucidation of the BRI1-SERK1 extracellular domain complex (Santiago et al. 2013). Heterodimerization between the SERK1 and BRI1 extracellular domains is ligand dependent, and the N-capping residues of SERK1 extracellular domain fold over the steroid ligand and dock onto the hydrophobic patch created by ligand binding. The finding that a ligand acts as a 'molecular glue' between main receptor and co-receptor, was further established by the elucidation of another SERK complex, consisting of the SERK3/BAK1 extracellular domain, the FLS2 extracellular domain and the flg22 ligand (a small peptide capable of inducing flagellin signalling (Sun et al.). Similar as reported for the SERK1-BRI1 complex, ligand binding to FLS2 does not induce large conformational changes or FLS2 extracellular domain homodimerization, but is essential for complex formation with the SERK3/ BAK1 extracellular domain. These reports, indicating that SERKs interacts not only with the main receptor but with the ligand as well, establish the SERKs as true co-receptors.

To understand the intricate ways in which plant signalling pathways are specified and intertwine, it is essential to gain a better insight in the atomic structures of the major players of the signalling cascades. The extracellular domain of BRI1, FLS2, SERK1, and SERK3/ BAK1, and the intracellular domain of SERK3/ BAK1 begin to give us the important insights in the ways these signalling complexes are build up. To further this knowledge and to better understand how highly homologous co-receptors can be involved in such different signalling pathways, more three-dimensional structure of the different SERK family members need to be elucidated. This chapter will discuss the attempts made to obtain crystal structures of different domains of the SERK proteins. The results indicate that these proteins do not crystallise readily, and are difficult to produce and purify. Although crystals diffracting to +/- 2.5 Å were obtained for the kinase domain of SERK1, structure elucidation was not possible at this time.

Materials & Methods

Constructs

Initial constructs for SERK1 were based on secondary sequence predictions of the amino acid sequence. Domain overview of these constructs can be seen in Figure 1. Primers and number of amino acids of the different constructs can be found in

Table 1 Initial SERK1 constructs

Construct	Aa	Primers
SERK1-A1	(26-198)	- CAC CAA TTT GGA AGG TGA TGC TTT GCA T - TCA GTTATTAGCAAAACTGATGGGTGTGA
SERK1-A2	(55-198)	- CAC CGT GAA TCC TTG CAC ATG GTT C - TCA GTTATTAGCAAAACTGATGGGTGTGA
SERK1-B1	(26-237)	- CAC CAA TTT GGA AGG TGA TGC TTT GCA T - TCA AGT TAT ACC ATA CCC ACT CGG GGT
SERK1-B2	(55-237)	- CAC CGT GAA TCC TTG CAC ATG GTT C - TCA AGT TAT ACC ATA CCC ACT CGG GGT
SERK1-C1	(26-262)	- CAC CAA TTT GGA AGG TGA TGC TTT GCA T - TCA GCC ACC AAG CAA AGG CTA TTG CA
SERK1-C2	(55-262)	- CAC CGT GAA TCC TTG CAC ATG GTT C - TCA GCC ACC AAG CAA AGG CTA TTG CA
SERK1-D1	(285-580)	- CAC CGG ACA GCT CAA GAG GTT TTC T - TCA CCC ATC TCC TTC CAG CAT CCT TAC A
SERK1-D2	(290-580)	- CAC CTC TTT GCG GGA GCT ACA AGT - TCA CCC ATC TCC TTC CAG CAT CCT TAC A
SERK1-D3	(285-end)	- CAC CGG ACA GCT CAA GAG GTT TTC T - TTA CCT TGG ACC AGA TAA CTC AAC GGC
SERK1-D4	(290-end)	- CAC CTC TTT GCG GGA GCT ACA AGT - TTA CCT TGG ACC AGA TAA CTC AAC GGC
SERK1-G1	(26-end)	- CAC CAA TTT GGA AGG TGA TGC TTT GCA T - TTA CCT TGG ACC AGA TAA CTC AAC GGC
SERK1-G2	(55-end)	- CAC CGT GAA TCC TTG CAC ATG GTT C - TTA CCT TGG ACC AGA TAA CTC AAC GGC
SERK1-G3	(26-580)	- CAC CAA TTT GGA AGG TGA TGC TTT GCA T - TCA CCC ATC TCC TTC CAG CAT CCT TAC A
SERK1-G4	(55-580)	- CAC CGT GAA TCC TTG CAC ATG GTT C - TCA CCC ATC TCC TTC CAG CAT CCT TAC A
SERK1-BL	(263-605)	- CAC CCG ACG AAG AAA GCC AC - CCA ATC TTA GTT AGG ATT AGG ACT CAA ATC
SERK3-BL	(250-591)	- CAC CCG AAG GAA AAA GCC G - TTA ATG GTG GGT TGG GTA GTT G
SERK4-BL	(255-597)	- CAC CCT CAG AAG AAA ACC ACA GG - CAG TCA GTG CCT TAA TGA GGA TAG G
SERK5-BL	(236-578)	- CAC CCT GAG AAG AAA ACT GCA GG - CAG TGC CTT AAT GAG GAT AGG CTT G

Table 1. The kinase domain constructs D1 and D3 were also produced as inactive variants by mutating the essential lysine 330 to a glutamic acid as was described before (Shah et al. 2001). Constructs A1-G4, and the constructs containing the inactive D1k and D3k sequences were cloned into a pET151/D-TOPO vector (Invitrogen). The fusion protein of NUS - D3 was obtained by cloning the D3 construct into a pET43.1b (Novagen). Additional constructs were designed based on the crystallised BAK1-KD in complex with AvrPtoB. These constructs are termed SERK1-5 BL (for BAK1-Like). The fusion proteins SUMO- SERK-BL were obtained by cloning the SERK-BL constructs into the pOPINS vector (OPPF).

Protein production and purification

All plasmids were transformed in *E.coli* strain BL21 (DE3), SERK4-BL and SERK5-BL were additionally transformed in strain Rosetta 2. Bacterial cultures containing plasmid were grown in LB at 37 °C until OD600=0.8, after which the culture was cooled to 20 °C and protein production was started by addition of IPTG. After 20 h cells were harvested. Cells were broken using a French Press or by passing them through a cell disruptor (Constant Systems Ltd) at 20 kpsi at 4 °C. The cell free extract was run over a Histrap ^{tm} HP (5ml, GE Healthcare) while present in 20 mM Tris (pH 7.8), 300 mM NaCl, 10 mM imidazole, 10 mM $MgCl_2$. Protein was eluted in the same buffer supplemented with 300 mM imidazole. Subsequently, the protein sample was desalted, 1 mM DTT was added and the sample was run over a HiTrapQ XL (5ml, GE Healthcare). Protein was eluted of the HiTrap using a salt gradient (NaCl, 0-1 M). Final purification of the sample was done via gel filtration using a superdex 75 26/60 column, in 20 mM Hepes (pH 7.4) 150 mM NaCl, 10 mM $MgCl_2$ 1 mM DTT. Protein samples were concentrated using Vivaspin 5000 MWO PES membrane (Satorius).

Dephosphorylation experiments were performed using Antartic Phosphatase (New England Biolabs) and PP1 (New England Bioloabs). The reactions were performed in the provided reaction buffers at 30 °C for one hour.

Protein bands visible on SDS were analysed by nanoHPLC-MS/MS analysis performed by the Plataforma de Proteòmica of the Parc Científic de Barcelona (Barcelona).

Crystal screens

Protein constructs D1, D3, D1k, D3k, NUS-D3, S1-Bl and S4-BL were used for crystal screening, using screens depicted in Table 2. Protein concentration was varied between 5 mg/ml and 20 mg/ml. Crystallisation screens were performed using the sitting-drop-vapour-diffusion technique on 96-wells two-drop-MRC crystallisation plates (MRC laboratory of molecular biology). Plates were set up using the Phoenix System (Art Robbins, USA) and the CARTESION Microsys 400 XL (Bruker

Nonius). Screenings were performed by combining 100 nl protein sample with 100 nl crystallisation buffer and equilibrating this against 90 μl crystallisation buffer. Optimisations were set up by adding 200 nl protein sample to 200 nl crystallisation buffer, which was equilibrated against 80 μl crystallisation buffer. Plates typically were stored at 20 °C, although some initial screens were performed at 4°C, and monitored by Crystalfarm (Bruker Nonius) and manually.

Table 2 Crystal screens
The different crystal screens used for SERK protein constructs. A cross depicts that this screen was used for that protein sample, a circle that this screen was used in the presence of 10 mM AT-PgS (an ATP analogue) and a minus sign indicates that the screen was set up in the presence of a phosphatase.

Crystal screen	D1	D3	D1k	D3k	NUS-D3	S1-BL	S4-BL
Crystal screen I&II (Hampton Research)	x	x°-	x	x	x		X
Wizard I&II (Emerald BioSystems)		x°		x	x	x	
Index (Hampton Research)		x-	x	x		x	
Salt RX (Hampton Research)	x	x°-	x	x	x		X
A/S Ion & $AmSo_4$ & Quick phosph. (inhouse screen)	x	x				x	
PEG 6000 & 6000/LiCl, PEG400 & 400/ LiCl (inhouse screen)						x	
Crystal screen Lite & Crystal screen Cryo (Hamptons Research)	x	x				x	
PEG-ion & MPD & NaCl (inhouse screen)	x	x				x	
Protein-DNA screen (KeraFAST)	x	x				x	
Membfac & MemPlus (Molecular Dimensions)	x	x°-		x	x		X
Memstart & Memsys (Molecular Dimenstions)					x		
Cryo Wizard I &II (Emerald BioSystems)		x					
JBS 1-4 (Jena Bioscience)		x°					
Citrat screen & Mc Pherson screen & Clear strategy I&II (Molecular Dimensions)		-				x	X
Pact Premier HT-96 (Molecular Dimensions)	x	x		x			
Pi-PEG screen (Jena Bioscience)	x	x	x				
Pi-minimal screen (Jena Bioscience)	x	x	x			x	
Kinase screen (Jena Bioscience)		x	x				

Crystal growth and diffraction

The protein construct D3 grew to crystals in 100 mM Bis-Tris propane pH7, 1.2 M DL-malic acid. The crystals were frozen in liquid nitrogen after addition of 15% glycerol to the crystallisation buffer for cryoprotection. The X-ray diffraction pattern was measured at beamline ID14-1 at the ESRF (Grenoble, France), with $\Delta\varphi$ = 1° at a temperature of 100K. 180 images were recorded.

Suitable crystals of SERK1-BL grew in 2M ammonium sulphate, 100 mM Bis-Tris pH 5.5, (3) 1M tri-sodium citrate/ citric acid pH 7.0. The crystals were frozen in liquid nitrogen after moving to crystal buffer supplemented with 15% glycerol. The X-ray diffraction pattern was obtained at beamline ID23-2 at the ESRF with the following settings; $\Delta\varphi = 0.35°$, temp: 100K, 160 images were collected.

Data analysis

The program MOSFLM 7.04 (Leslie et al. 2007) and scaling program SCALA 3.3.18 (Evans 2006) were used for integration of the diffraction data and initial assignment of space group (see Table 3). Molecular replacement for structure elucidation was done using PHASER 2.5.5 (Mccoy et al. 2007) from the CCP4 software suite (Winn et al. 2011). Inspection of the resulting density maps was done with COOT (Emsley et al. 2010).

Table 3 XDATA. Data statistics of reasonably diffracting crystals of SERK constructs.

	S1-D3	S1-BL (C2)*	S1-BL ($H3_2$)*
Space group	$P6_n22$	C2	$H3_2$
Collected at	ID14-1(ESRF,Grenoble)	ID23-2 (ESRF, Grenoble)	
Cell dimensions	a=b=61.49 Å c=168.57 Å	a=154.1 Å, b=233.2 Å c=111.1Å	a=b=233.2 Å c=111.1 Å
	90°, 90°, 120°	90°, 118.7.°, 120°	90°, 90°, 120°
Resolution (Å)	30.0-2.35 (2.48-2.35)	58.3-3.3 (3.48-3.3)	58.3-3.1 (3.3-3.1)
R_{merge}	0.28 (0.93)	0.19 (0.57)	0.23 (0.80)
R_{meas}	0.29 (0.96)	0.27 (0.81)	0.32 (1.1)
$\langle I / \sigma(I) \rangle$	9.8 (3.7)	3.5 (1.4)	3.5 (1.1)
Completeness (%)	100 (100)	61.5 (65.7)	97.1 (98.3)
Multiplicity	20 (21)	1.5 (1.4)	(1.8)

* These datasets are from the same diffraction experiment, but processed in different space groups

Results

Production of SERK1-A - G

Initially, 14 constructs of different parts of the SERK1 protein were designed based on secondary structure features predicted from the amino acid sequence (see Figure 1 A-G and Table 1 A-G).

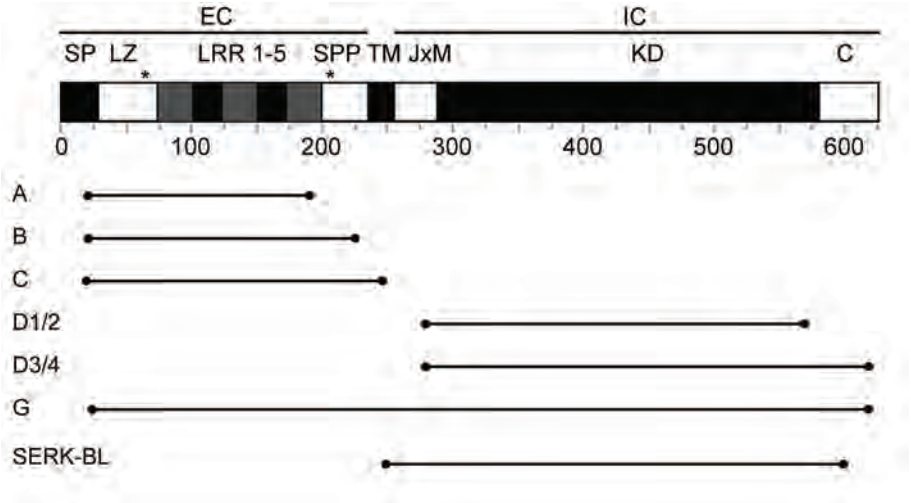

Figure 1 Domain overview of SERK and construct design
Graphical representation of domain structure of a typical SERK protein. Numbers under the diagram indicate approximate amino acid residues as found in SERK1. EC= extracellular domain, IC= intracellular domain. SP= signal peptide, LZ= leucine zipper/N-capping residues, LRR= leucine-rich-repeats, TM= transmembrane helix, JxM = juxtamembrane domain, KD= kinase domain, C= C-terminal tail domain. See Table 1 for precise amino acid numbers included in the different constructs.

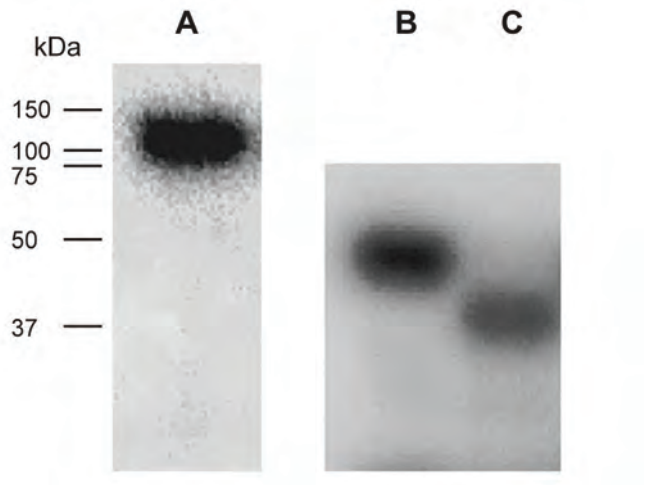

Figure 2 Phosphorylation activity of NUS-D3, SERK-D3 and SERK-D1
Autoradiograph of Nus-D3 (A), SERK1-D3 (B) and SERK1-D1 (C) after 30 min. incubation in activity buffer containing 5 μCi [γ-^{32}P] ATP to assess autophosphorylation activity. Al constructs show autophosphorylation activity and thus contain active kinase domain. Marker positions were assessed from corresponding SDS-PAGE.

When bacterial cultures were grown at 37°C with 5 hour induction, all recombinant protein was found in the pellet fraction of the cell free extract, most probably as part of inclusion bodies. Optimisation included varying the temperature, induction time and media composition. A 20 hour induction at 20°C in LB-media resulted in soluble protein for the constructs B1, D1, D3 and D4. For the kinase domain constructs (D-constructs) approximately 50% of the recombinant protein was found in the soluble fraction, while only a small amount (+/- 25%) of the recombinant extracellular domain (B1) was found in the soluble fraction. The D4 construct is similar to D3, except the D4 construct is 5 amino acids shorter in the N-terminus. For this reason we decided to continue with the D1 (complete kinase domain) and D3 (complete kinase domain with C-terminal tail) constructs for purification. To increase protein yield, an additional fusion construct was created of D3 fused N-terminally to NUS, a bacterial protein that is highly soluble in *E. coli* (Davis et al. 1999). The D1, D3 and NUS-D3 constructs produced active kinase domains with autophosphorylating activity (see Figure 2).

Purification of D1, D3 and NUS-D3

Purification of the kinase domain constructs started with a metal affinity column, followed by ion exchange chromatography and as a final step a gel filtration column. Initial tests excluded the ion exchange chromatography step, but this resulted in a sample that was relatively pure on protein level, but contained high amounts of DNA/RNA. Ion exchange chromatography was successful in removing the DNA/RNA from the sample, although it did not increase the purity of the sample greatly in respect to protein contaminants. The final sample resulting from this purification was approximately 90-95% pure (see Figure 3A). The protein eluted from the size exclusion column as a monomer, however at a slightly smaller elution volume than could be expected based on theoretical size. Consistent co-purification of impurities was observed; most prominently two bands at approximately 30 kD and one band at approximately 64 kD (see Figure 3C). Removal of these impurities varied greatly in its successfulness. The impurities were identified by MS/MS analysis as *E. coli* Chain A of the Catabolite-gene-activator protein (CAP)-DNA-complex (the 30 kD bands) and *E. coli* transcription termination factor Rho (the 64 kD band).

The kinase constructs eluted as multiple peaks from the ion exchange column and were often detected as multiple bands on SDS-PAGE (see Figure 3C). This is probably due to differential phosphorylation statuses of the kinase, which contains 24 possible phosphorylation sites. This heterogeneity of the sample could interfere with further crystallisation attempts. For this reason inactive variants of the D1 and D3 constructs were constructed, where an essential lysine was changed to a glutamic acid (K330E), referred to as D1k and D3k. Although these constructs did not show the multiple protein bands on SDS-page characteristic for phosphorylated kinase, the yield or purity (in respect to other proteins) of the inactive variants

was not higher compared to the active constructs. Attempts to remove phosphate groups from the active D1 and D3 constructs with Antarctic phosphatase and PP1 resulted in very low yield. This could be due to destabilisation of the protein sample, caused by the relatively high temperature (30°C) of the dephosphorylation reaction.

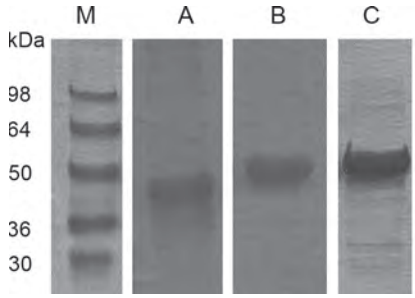

Figure 3 Purified SERK1-D1 and D3
SERK1-D1 and D3 were purified with a three-step protocol, starting with a metal affinity column, followed by ion exchange chromatography and finally gel filtration chromatography. The resulting sample was 90-95% pure, this varied slightly between different purifications. Examples of typical end results of purification are depicted above, with in lane **(A)** SERK1-D1 and in **(B)** and **(C)** SERK1-D3. Lane **C** depicts the multiple bands often observed for a kinase construct, and the impurities at +/- 30 kDa and 64 kDa. (M)= marker lane with to the left the weight in kDa of the different marker lines.

Crystallisation screens and crystal evaluation of D1 (k), D3 (k) and NUS-D3

Crystallisation screens of the initial SERK1 kinase domain constructs were set up at varying protein concentrations (5-20 mg /ml) and at 4 and 20°C. The samples were present in 20 mM Hepes, 150 mM NaCl, 10 mM $MgCl_2$ and 1 mM DTT and this was mixed 1:1 with crystallisation buffer. All constructs were screened using commercial crystal screens (see Table 1 for an overview of crystal screens used for the different protein samples) using the sitting-drop-vapour-evaporation technique. Promising conditions were further optimised by varying protein concentration, buffer composition, pH and additives. The S1-D3 construct was also screened with the addition of 10 mM ATPγS (an ATP analog) and in the presence of phosphatase. However, addition of these compounds did not induce crystal growth.

Of the inactive kinase constructs D1k and D3k no conditions were found that yielded suitable crystals for further analysis. Nus-D3 showed crystals in two conditions; (1) 0.2 M CaAc, 100 mM MES 6.5, 10% PEG 8K and (2) 0.2M M MgCl2, 100 mM Tris 7.0, 14% PEG 8K. The best diffracting crystal was grown in the latter condition, which was slowly mixed with cryobuffer (30% glycerol added to the crystalbuffer) before freezing in liquid nitrogen. The crystal diffracted to a resolution of approximately 10 Å, which was insufficient to solve the protein structure.

The D1 construct yielded crystals in three different conditions; (1) 100 mM imidazole pH 8.0, 11% ethyleneglycol, 5% PEG 5K MME, 10 mM Cl3Si, (2) 100 mM Hepes pH 7.0, 7% ethyleneglycol, 5% PEG 5K MME and (3) 100 mM Hepes pH 7.5, 14% ethyleneglycol, 5% PEG5K MME. The crystals from (2) did not show diffraction. The crystals from (1) and (3) gave crystals which diffracted but only to low resolution (less than 20 Å). The manner of introducing the crystals to cryo buffer was varied for these crystals (slow and fast introduction to cryo buffer, and flash freezing after addition of 25% glycerol) but this was not found to improve diffraction.

The D3 construct grew to crystals in one condition; 100 mM Bis-Tris propane pH7, 1.2 M DL-malic acid (see Figure 4). The crystals were slowly introduced to cryo buffer (crystallisation buffer +15% glycerol) and flash frozen in liquid nitrogen. This crystal diffracted to approximately 2.5 Å and a full dataset was collected at beam line ID14-1 at the ESRF, with $\Delta\varphi = 1°$ at a temperature of 100K.

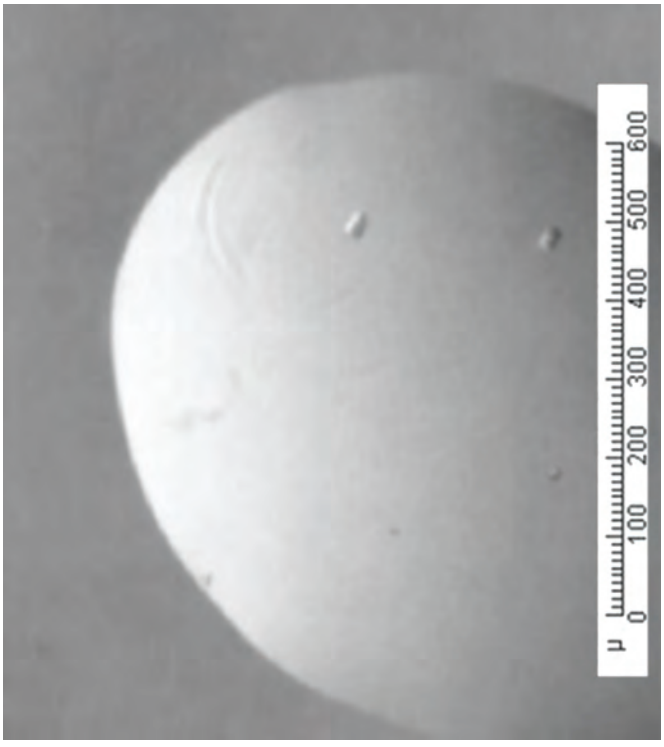

Figure 4 Crystals of SERK1-D3
SERK1-D3 grew to crystals in 100 mM Bis-Tris propane pH7, 1.2 M DL-malic acid after 1 month at 20 °C, using the sitting-drop-vapour-evaporation method. The crystals diffracted to approximately 2.5 Å resolution.

Data-analysis of SERK1-D3 diffraction data

With use of MOSFLM (Leslie and Powell 2007) and scaling program SCALA (Evans 2006), the dataset of D3 was analysed to belong to one of the $P6_n22$ space groups, with cell parameters: (a), 61.49 Å (b) 61.49 Å (c) 168.57 Å, (α) 90° (β) 90° (γ) 120° (see Table 3). Cell content analysis indicated that the cell dimensions were not big enough to contain the protein structure. Attempts to solve the structure using molecular replacement making use of the program PHASER (Mccoy et al. 2007) and the published structures of BAK1 (pdb: 3TL8, 3UIM), IRAK4 (pdb: 2O8Y) and Pto kinase (2QKW) failed.

Production of SERK#-BL

Since the initial kinase structures of SERK1 did not result in the elucidation of a protein structure, we decided to mimic the constructs from Yan et al. 2012 (Yan et al. 2012), which yielded a 2.2 Å structure of BAK1 (SERK3) kinase domain. The constructs were designed using the alignment of amino acid sequence of SERK1-5. This resulted in the constructs SERK1-BL (SERK1 BAK-like), SERK1-BLk (containing the K330E inactivating mutation), SERK3-BL, SERK4-BL and SERK5-BL. For exact amino acid composition of the constructs see Table 1. Cloning of the SERK2-BL construct failed. Protein production was performed similar to the D1/3 constructs. Since SERK4-BL and SERK5-BL showed no protein production under these conditions in the *E. coli* BL21 (DE3) strain, these constructs were introduced in the Rosetta 2 strain. The Rosetta 2 strain produced soluble SERK4-B protein, but no SERK5-BL. In addition, to improve protein production and aid purification, the SERK1-BL construct was cloned into the pOPINS plasmid, introducing a cleavable N-terminal HIS-SUMO tag.

Purification of SERK#-BL

The SERK#-BL constructs were purified following the same protocol as the D-constructs, with a Histrap™ HP column for metal affinity purification, a Hitrap™ column for ion exchange chromatography and gelfiltration using a superdex 75 GL (see Figure 5). Similar to the initial constructs, a multiple peaked elution profile during ion exchange chromatography, and often multiple bands on SDS-gel were observed (see Figure 5). The 6xHIS-tag was removed using a TEV protease, and the HIS-SUMO-tag using an inhouse-made SUMO protease treatment. The HIS-SUMO tag was removed o/n at 4 °C, followed by removal of uncleaved protein using a Histrap™ HP column.

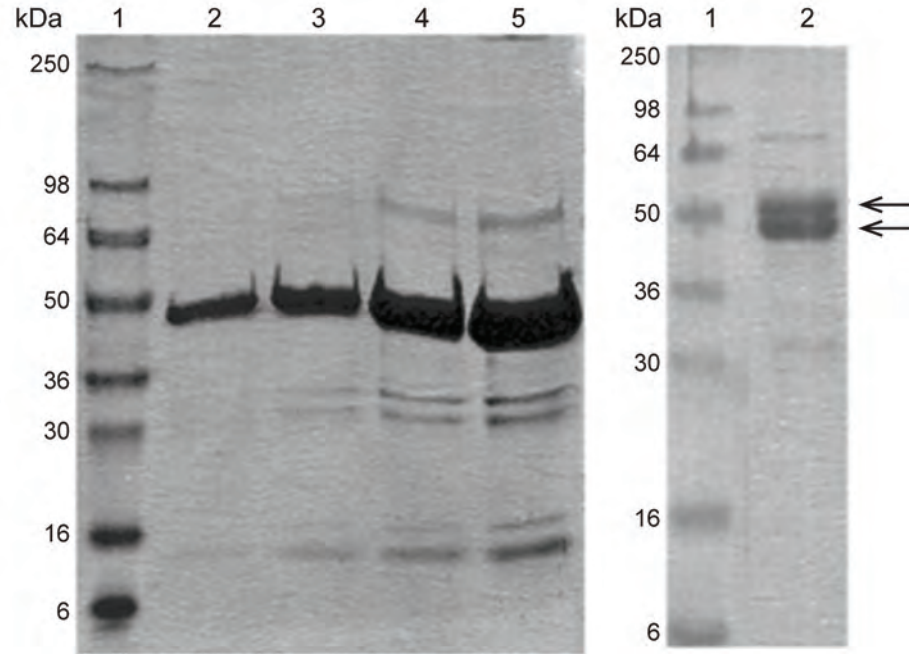

Figure 5 Purified samples of SERK-BL
SDS-PAGE of final samples of different SERK1-BL purifications. In **(A)** four samples of increasing protein concentrations are loaded onto the gel. At higher concentrations the consistent copurified impurities at 30 and 64 kDa are visible, which were identified by MS/MS as *E. coli* proteins. The impurity at 16 kDa was not identified. In **(B)** the double protein band, which was often observed for the kinase domain samples, is depicted.

Crystallisation screens and crystal evaluation of SERK#-BL

After purification, protein samples were present in 20 mM Hepes, 150 mM NaCl, 10 mM MgCl2 and 1 mM DTT. Samples were mixed 1:1 with crystallisation buffer. Of all SERK#-BL constructs, only SERK1-BL crystallised. In an attempt to recreate the results from Yan et al. 2012, SERK3-BL protein sample was added to the conditions described previously, however in our hands this did not result in crystal growth. In addition, an optimisation screen was designed based on the crystallisation conditions described (Yan et al. 2012), which included an experiment in which the sample was supplemented with 0.5 mM of ATPγS. None of the optimisation plates showed crystal formation.

The three buffer conditions that resulted in crystals for SERK1-BL were (1) 2M ammonium sulphate, 100 mM tri sodium citrate pH 5.5, (2) 2M ammonium sulphate, 100 mM Bis-Tris pH 5.5, (3) 1M tri-sodium citrate/ citric acid pH 7.0 (see Figure 6). Removal of the HIS-tag produced an additional condition in which crystal growth was observed; (4) 200 mM di ammonium tartrate, 20% PEG 3350

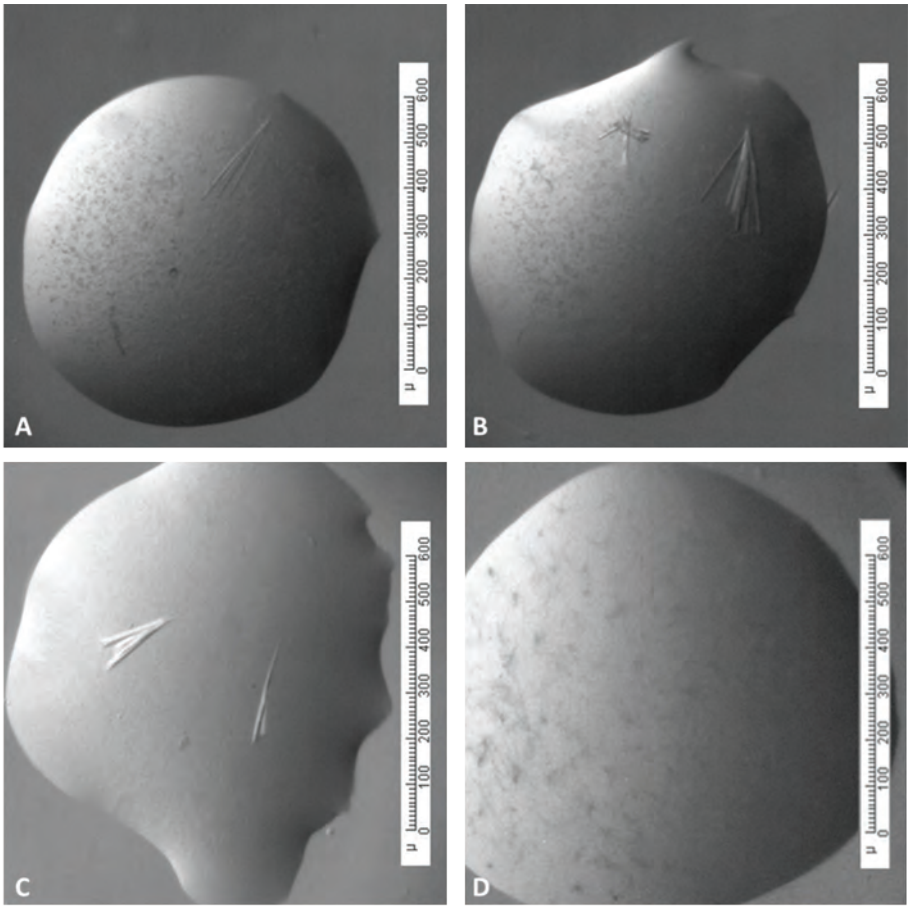

Figure 6 Crystals of SERK1-BL
SERK1-BL grew to crystals in conditions **(A)** 2M ammonium sulphate, 100 mM tri-sodium citrate pH 5.5 after sixteen days, **(B)** 2M ammonium sulphate, 100 mM Bis-Tris pH 5.5 after 5 days, **(C)** 1M tri-sodium citrate/ citric acid pH 7.0 after 9 days and **(D)** 200 mM di ammonium tartrate, 20% PEG 3350 after 5 days. All crystals grew at 20°C using the sitting-drop-vapour-evaporation method. Crystals in condition **(D)** grew from a sample which had had its 6xHIS-tag removed. Only crystals from **(B)** diffracted to a reasonable resolution, namely 2.5-3 Å.

(see Figure 6). Of these four crystals, only the crystals from (2) diffracted to a reasonable resolution (2.5-3 Å) (frozen in liquid nitrogen after moving to crystal buffer supplemented with 15% glycerol) and a full data set was obtained at beam line ID23-2 at the ESRF with the following settings; $\Delta\varphi = 0.35°$, temp: 100K (see Table 3 and the discussion below). Optimisation screens were performed on all four SERK1-BL crystal conditions (see Figure 7 for optimisation conditions).

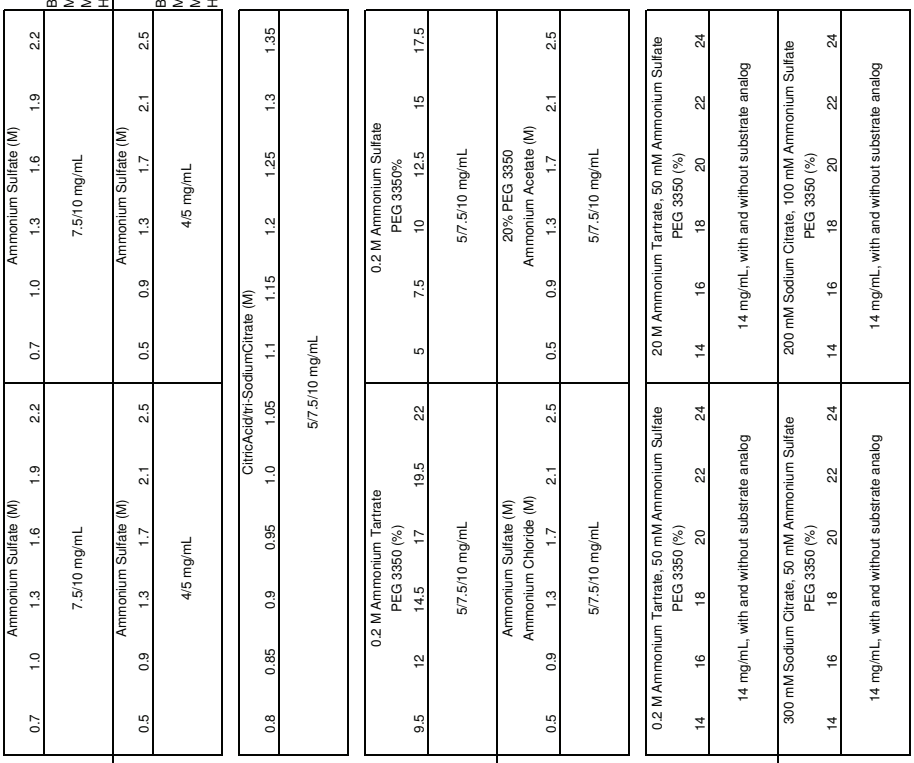

Figure 7 Optimisation plate designs of SERK1-BL and SERK3-BL

Plates designed to optimise crystallisation conditions based on SERK1-BL crystals grown in 2M ammonium sulphate, 100 mM tri-sodium citrate pH 5.5 and 2M ammonium sulphate, 100 mM Bis-Tris pH 5.5 **(A)**, 1M tri-sodium citrate/ citric acid pH 7.0 **(B)**, 200 mM di ammonium tartrate, 20% PEG 3350 **(C)**. The plate depicted in **(D)** was based on the crystallisation conditions described by Yan et al, 2012, for the SERK3/ BAK1 kinase domain (Yan et al. 2012).

Data-analysis

Due to loss of diffraction resolution during the data acquisition of the SERK1-BL diffraction pattern, only the first 120 images (corresponding to an accumulated crystal rotation of 42°) were of sufficient quality for analysis. Using the MOSFLM (Leslie and Powell 2007) and scaling program SCALA (Evans 2006), the dataset was analysed to belong to two possible space groups (see Table 3); either a monoclinic C2-cel with parameters (a) 154.1 Å, (b) 233.2 Å (c) 111.1 Å (α) 90° (β) 119° (γ) 90°, or a hexagonal H32 cell with parameters (a) 233.2 Å, (b) 233.2 Å, (c) 111.1 Å (α) 90° (β) 90° (γ) 120°. Structure elucidation in the C2 space group was hampered due to the fact that for this space group the data corresponded to only 60% completeness of the structure. There was sufficient data to attempt structure elucidation based on a H32 cell. To solve the structure a similar approach as for the SERK-D3 crystal was used, with molecular replacement making use of the program PHASER and the published structures of BAK1 (pdb: 3TL8, 3UIM), IRAK4 (pdb: 2O8Y) and Pto kinase (2QKW). However, this did not yield any reliable solution, which might be an indication that this is not the correct space group for this crystal. Unfortunately there was not enough data to solve the structure based on the monoclinic C2-cell. Attempts to recreate the SERK1-BL crystals grown in 2M ammonium sulphate, 100 mM Bis-Tris pH 5.5 failed.

Discussion & Conclusion

Atomic level understanding of receptor structure has proven invaluable for the understanding of signalling pathways. An example of this is epidermal growth factor signalling, where crystal structures of numerous constructs and conformations have given a detailed impression of receptor activation (Ferguson 2008; Bublil et al. 2010; Endres et al. 2011; Liu et al. 2012). The recent structures on plant LRR-RLKs give a first impression of receptor function and regulation in plant signalling. The crystal structure of the brassinosteroid receptor BRI1 indicates that ligand binding on its own does not induce clear conformational changes in the extracellular domain (Hothorn et al. 2011; She et al. 2011) and that, in contrast to many animal receptors, ligand induced homo-dimerization is unlikely (Hothorn et al. 2011). Activation of the receptor needs additional players in the form of SERK co-receptors (Gou et al. 2012) which form complexes with the main receptor (Russinova et al. 2004; Karlova et al. 2006; Santiago et al. 2013). For the BRI1-SERK1 and FLS2-SERK3/ BAK1 extracellular domain complexes, it has now been proven that the co-receptors also interact with the ligand (Santiago et al. 2013). Further investigations of other co-receptor and main receptor complexes would provide us with the answer whether this is really a common mode of action, and whether these extracellular domain interactions are the cause of the observed

SERK specificity and whether the co-receptors adopt different conformations in the different complexes.

In addition to the extracellular domains of the co-receptors, elucidating additional structures of the kinase domains could also greatly help with our understanding of the specificity of these proteins. Protein phosphorylation and dephosphorylation are key regulatory elements to cellular signal transduction. Almost every known signalling pathway includes a protein kinase or phosphatase at some point in the cascade (Graves et al. 1999). The structure of kinase domains and their regulatory flanking regions can help in our understanding of their signalling properties. For the SERK3/ BAK1 kinase domain it is known that different phosphorylation sites are important for different signalling pathways (Wang et al. 2008; Yan et al. 2012), and the pT450 changes the activation loop conformation (Yan et al. 2012). Specific phosphorylation sites are thus closely related to structural changes, which link to kinase function and specificity. Our attempts to crystallise different kinase constructs for the SERK proteins were hampered by low protein yield in *E. coli*. In addition approximately 50% of produced protein was present in the insoluble fraction of cell free extract (probably due to inclusion bodies). One way to overcome such problems is by fusing the problem protein to a soluble tag, which often increases the solubility of the fusion protein. We made attempts of this by fusing the kinase domain and C-terminal tail (residues 285-625) of SERK1 to the NUS protein (Davis et al. 1999) and the kinase domains of SERK 1,3,4,5 (residues 263-605 of SERK1 and corresponding residues from SERK3, 4, 5) to the SUMO protein. The fusion proteins were produced and the NUS fusion protein was catalytically active, before and after removal of the tag. However, the yield of the fusion proteins was not substantially higher than upon using the construct without fusion tag, and removal of the tag often was problematic resulting in massive loss of protein. There are several possible reasons for the low yield of these constructs. First of all, the kinase constructs code for an active protein with auto- and trans-phosphorylation activity (Aan den Toorn et al. 2012) which may create toxicity problems for the bacterial host. To circumvent this problem, inactive variants of the kinase constructs were designed and produced. This, however, did not increase protein yield. It is possible that codon usage in the constructs is not suitable for *E. coli*, and codon optimisation for *E. coli* or changing the host organism might improve protein production. Purification of the kinase domains was also not straightforward. The kinase domain of SERK1 did not elute as a single peak from the anion exchange column, but showed a broad, three peaked elution profile. Most likely this is due to the differential phosphorylation states of the protein sample, which was also evident by the multiple bands visible on protein gel. SERK1 has 24 possible phosphorylation sites, three of which can also be phosphorylated by *E. coli* kinases (Karlova et al. 2009), which is probably why even the inactive kinase constructs showed this behavior.

We obtained crystals of two different SERK1 kinase domain constructs, both diffracting to roughly 2.5 Å. The conditions under which the proteins crystalise are rather different from each other, and from the reported conditions for the SERK3/ BAK1 apo-structure (Yan et al. 2012). Our attempts to recreate the reported SERK3/ BAK1 crystals, by incubating the SERK3-BL protein in the same conditions, did not induce crystal growth. In our hands, these conditions resulted in heavy precipitation of the protein, indicating that protein instability and denaturation is the most likely cause for the failure to crystallise.

The crystal cell parameters that were found for the SERK1-D3 crystal corresponded to a crystal cell too small to contain the SERK1 kinase domain. This indicates that either the selected space group was not correct, which might indicate twinning of the crystal, or the protein has been degraded and only part of the protein is present in the crystal. Attempts were made to search for a solution with molecular replacement using only part of a kinase domain as template, however this also did not yield any reliable solution. Finally, the too small crystal cell might indicate that the crystal was grown from a contaminant in the protein sample instead of the actual SERK1-D3 protein itself. The second construct that grew to suitable crystals was the SERK1-BL construct. This protein construct crystallised under more conditions than any of the other constructs, and four diffracting crystals were found during the screens. Only one of these diffracted to a sufficient resolution for structure elucidation. Calculations on the diffraction data indicated that the crystal cell could contain up to nine kinase domains. However, molecular replacement again yielded no suitable solution. To circumvent this problem in the future, SeMet incorporation could be used for structure elucidation without the need for molecular replacement. Unfortunately, the diffraction data obtained for SERK1-BL deteriorated during data collection. These crystals were relatively small and crystals of this size are difficult to focus in the beam-line, generally show little diffraction and are substantially more damaged by the X-ray beam, which could be an explanation for the deterioration of the diffraction. Another possibility could be anisotropic diffraction of the crystal. Anisotropic diffraction is seen when the diffraction quality is orientation dependent. Some crystals show intrinsic differences in order (and thus diffraction) in different directions throughout the crystal caused by e.g. tighter contacts in certain directions.

The reproducibility of both the purifications and the crystallisation is low; often crystal growth could not be duplicated with different batches of purified protein. This might be due to the heterogeneity of the sample caused by differential phosphorylation status. To circumvent this problem, phosphatase was added to the crystal drops, in the hopes that dephosphorylation would increase the homogeneity of the sample and at a certain point induce crystal growth. The drops containing phosphatase showed a lot of denaturation and no crystal growth.

Concluding remarks

Unfortunately, although many attempts were made, and crystals were obtained, structure elucidation of the SERK kinase domains was not possible at this time. Likely reasons for this are the heterogeneity of the sample, as also evident from the ion exchange chromatography elution pattern, and intrinsic instability of the protein, as also evident from the precipitation often observed in the crystallisation drops. Chances of successful crystallisation could be increased by increasing protein stability, for instance via co-crystallisation with an interaction partner (where complex formation would increase stability). Sample homogeneity could be increased by targeted mutation of highly phosphorylated sites. In addition, gene optimisation for production in E. coli, or the choice for a different host for protein production (e.g. *Pichia pastoris*) could increase protein yield, which would greatly benefit the screening and optimisation of many crystallisation conditions. Finally, improving the purification protocol could increase the purity of the final sample. For instance, the purity could increase by adding ATP in the lysis buffer, or changing salt concentration or pH during chromatography.

Although this study did not result in a high resolution structure of a SERK kinase domain, the purification protocol and crystallisation conditions described in this Chapter can serve as leads for future structural studies on the SERK proteins.

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Chapter 4

The *Arabidopsis* thaliana SERK1 Kinase Domain Spontaneously Refolds to an Active State *In Vitro*

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Abstract

Auto-phosphorylating kinase activity of plant leucine-rich-repeat receptor-like kinases (LRR-RLK's) needs to be under tight negative control to avoid unscheduled activation. One way to achieve this would be to keep these kinase domains as intrinsically disordered protein (IDP) during synthesis and transport to its final location. Subsequent folding, which may depend on chaperone activity or presence of interaction partners, is then required for full activation of the kinase domain. Bacterially produced SERK1 kinase domain was previously shown to be an active Ser/Thr kinase. SERK1 is predicted to contain a disordered region in kinase domains X and XI. Here, we show that loss of structure of the SERK1 kinase domain during unfolding is intimately linked to loss of activity. Phosphorylation of the SERK1 kinase domain neither changes its structure nor its stability. Unfolded SERK1 kinase has no autophosphorylation activity and upon removal of denaturant about one half of the protein population spontaneously refolds to an active protein *in vitro*. Thus, neither chaperones nor interaction partners are required during folding of this protein to its catalytically active state.

Introduction

Plant Receptor-like Kinases (RLK's) belong to a large gene family (Shiu and Bleecker 2001b) involved in perceiving developmental cues and environmental changes. Activation of receptor-like kinases is thought to occur upon ligand induced complex formation, followed by activation of downstream associated proteins (Shiu and Bleecker 2001a). In inactive receptors, activity of the kinase domain is often regulated by several methods of control, such as (release of) inhibitors (Jaillais et al. 2011), dimerization (Bublil et al. 2010), activation segment phosphorylation (Nolen et al. 2004), and changes in sub-cellular localisation (Rey et al. 2004). Although a huge variety in modes of regulation exist among RLK's, activation of protein kinases is usually coupled to conformational changes in protein structure (Huse and Kuriyan 2002).

Here, we focus on an RLK from *Arabidopsis thaliana*, the Somatic Embryogenesis Receptor-like Kinase 1 (SERK1). SERK1 is part of a protein family consisting of five highly conserved leucine-rich-repeat receptor-like kinases that function in plant signalling pathways (Hecht et al. 2001, Albrecht et al. 2008 , Chinchilla et al. 2009). SERK proteins are co-receptors, which are required for transmission of signal (Gou et al. 2012). The SERK1 domain overview is shown in Figure 1.

Previous studies show that the kinase domain of SERK1 (SERK1-KD) is active *in vitro* (Shah et al. 2001), and has the highest autophosphorylation activity of the SERK protein family (Karlova et al. 2009). SERK1 has been implicated to function in somatic embryogenesis, male sporogenesis and brassinosteroid (BR) signalling in *Arabidopsis thaliana* (Hecht et al. 2001, Albrecht et al. 2008 , Albrecht et al. 2005, Kwaaitaal et al. 2005). The three-dimensional structure of the kinase domain of a close paralog of SERK1, BAK1 (also called SERK3) in complex with AvrPtoB has been elucidated (Cheng et al. 2011). This structure is similar to the one adopted by several tyrosine kinases. BAK1 seems to belong to the IRAK family of kinases, because it contains a tyrosine as "gatekeeper" residue (Cheng et al. 2011). The "gatekeeper" controls access to a hydrophobic pocket behind the ATP-binding site,

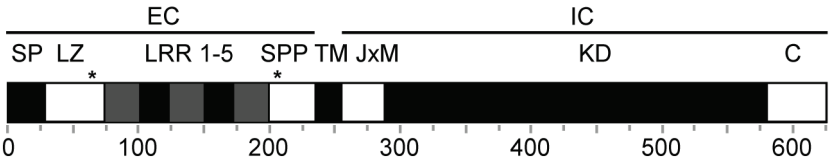


Figure 1 Domain architecture of SERK1.
SP = signal peptide, LZ = leucine zipper, LRR = leucine-rich-repeats, SPP = serine-proline rich domain, TM = single pass transmembrane domain, JxM = juxtamembrane domain, KD = kinase domain, C = C-terminal tail, EC = extracellular domain, and IC = intracellular domain. Two cysteine pairs in the extracellular domain are depicted with *, and numbers below the graph indicate amino acid residues.

and probably plays a role in selectivity of a kinase with regard to small-molecule, ATP-competitive inhibitors (Wang et al. 2006). The tyrosine gatekeeper identified in BAK1 (i.e., Y363) is conserved in the SERK1 sequence (i.e., Y376), indicating that SERK1-KD is a member of the IRAK family of protein kinases.

SERK1 functions in an oligomeric complex with other RLK's such as BRI1, the main ligand binding receptor for BRs (Li and Chory 1997), and transphosphorylation occurs between these receptors (Wang et al. 2008, Karlova et al. 2009). For the BR signalling pathway, SERK co-receptors are required for BRI1-mediated signal transduction, and whole seedlings lacking three of five family members completely lack dephosphorylation of the transcriptional regulator BES1 as a read-out of BR signalling (Gou et al. 2012). Interaction between BRI1 and SERK proteins involves the extracellular domains of both proteins, and is hypothesised to be induced upon ligand binding to the extracellular domain of BRI1 (Hothorn et al. 2011). Fluorescence Correlation Spectroscopy (FCS) shows that in protoplasts that have not been treated with BRs, about 15 % of SERK1 proteins exist as dimers (Hink et al. 2008). SERK1 interacts with PP2C type phosphatase KAPP, CDC 48 and 14-3-3 protein GF14λ (Shah et al. 2002, Rienties et al. 2005).

Since RLK activation is often the first response to a signal, RLK's need to be kept in the non-responsive or "off"-state in absence of activating ligands. One way to achieve this would be to keep these RLK's disordered during synthesis and transport to their final location. Many proteins are intrinsically disordered and fold, in whole or in part, upon binding to their physiological targets (Dyson and Wright 2005, Wright and Dyson 2009, Uversky and Dunker 2010). Protein phosphorylation often occurs within intrinsically disordered protein regions (Iakoucheva et al. 2004). Even in protein complexes significant regions of intrinsic disorder can exist (Fong et al. 2009). Especially in eukaryotes many proteins seem to contain unstructured regions of significant size (>50 residues) or are completely disordered (Dunker et al. 2002, Uversky 2002, Uversky and Dunker 2010). IDP's function in transcription, translation, signal transduction and protein assembly (Uversky 2011,Iakoucheva et al 2002.). These unstructured proteins do not fold until their activity is required and folding is triggered by, for instance, binding of a substrate or a dimerization partner, or a change in subcellular localisation (Dyson and Wright 2005). For the *Arabidopsis thaliana* proteome, it has been predicted that 29 % of its proteins contain intrinsically disordered regions, and 8 % of its proteome consists of completely disordered proteins (Dunker et al. 2000).

An example of IDP's in plants is the GRAS-protein family of transcriptional regulators (Sun et al. 2011). This protein family functions in several pathways in plant development and signal transduction, including the brassinosteroid pathway (Sun et al. 2011). Disordered regions of these proteins are thought to enable a particular protein to interact with different partners and as a result it can function in different signalling pathways (Sun et al. 2012). Also, plant specific plasma membrane-located remorin proteins, part of signal transduction cascades, have intrinsically

disordered regions (Marin and Ott 2012). For both GRAS proteins and remorins, phosphorylation is an important component during their modulation (Sun et al. 2011),(Marin and Ott 2012).

In this study we show that use of a disorder analysis computer program predicts that the kinase domain of SERK1 (SERK1-KD) possibly contains a disordered region. We report experimental data of folding, stability and folding-induced phosphorylation activity of SERK1-KD *in vitro*. These data show that SERK1-KD predominantly is a structured protein at ambient condition. Loss of this structure is intimately linked to loss of activity of SERK1-KD. *In vitro*, unfolded SERK1-KD spontaneously refolds to an active state in absence of transphosphorylating partners or chaperones.

Materials and Methods

Disorder analysis

Kinase domains and C-terminal tails of SERK1 (AT1G71830, residues 295-625), SERK3/BAK1 (AT4G33430, residues 282- 615), FLS2 (AT5G46330, residues 863-1173), BRI1 (AT4G39400, residues 877-1196), MPK3 (AT3G45640, residues 30-370), EFR (AT5G20480 residues 705-1031) and BSK1 (At4G35230, residues 66-413) were defined by sequence alignment using Clustal Omega (version 1.1.0) from the EBI web server (www.ebi.ac.uk/Tools/msa/clustalo/). Potential presence of disordered regions within these protein sequences was analysed using PONDR® VL-XT (www.pondr.com) (Romero et al. 1997, Li et al. 1999, Romero et al. 2001), VSL2B (www.dabi.temple.edu/disprot/) (Obradovic et al. 2005, Peng et al. 2006) and IUPred (iupred.enzim.hu/) (Dosztanyi et al. 2005).

Protein purification and sample preparation

SERK1-KD (residues 285-625) was cloned into the pET151 bacterial expression vector and contains an N-terminal His-tag (Invitrogen). Primers used for cloning were CAC CGG ACA GCT CAA GAG GTT TTC T and TTA CCT TGG ACC AGA TAA CTC AAC GGC. *Escherichia coli* BL21* (Invitrogen) cells containing plasmid were grown in LB at 37 °C until OD_{600}=0.8, after which the culture was cooled to 20 °C and protein production was started by addition of IPTG. After 20 h cells were harvested. Cells were broken using a French Press. The protein was purified by running cell free extract over a HIS-pure cobalt column (Pierce Biotech, bed volume of 8ml). Subsequently, protein was further purified through use of a SourceQ-15 column. Final purification was achieved by analytical gel filtration on a Superdex 75 10/300 GL (see supplementary Figure S1). Purification of SERK1-KD was followed by standard sodium dodecyl sulfate polyacrylamide gel electrophoresis

(SDS-PAGE). Purified protein was suspended in 20 mM Hepes, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.4. All measurements were performed in this buffer, unless stated otherwise. A total of about 5 mg of pure fusion protein was obtained from 6 L of culture. Figure S2 shows that SERK1-KD produced in *E. coli* has the ability to autophosphorylate itself, and to transphosphorylate artificial substrate casein (Sigma).

Phosphorylated SERK1-KD was obtained by incubating 24 μM SERK1-KD with 3 mM ATP for 45 min at 30 °C. ATP was subsequently removed using a P-10 desalting column (Amersham Biosciences). For determination of phosphorylation status, SERK1-KD after autophosphorylation was subjected to immunoblotting with anti-Phosphoserine, anti-Phosphothreonine and anti-Phosphotyrosine antibodies (BD biosciences).

Fluorescence spectroscopy

Fluorescence was measured using a Varian Cary Eclipse Fluorescence Spectrophotometer.

For denaturant induced unfolding, 0.24 μM SERK1-KD was incubated for 6 h at room temperature at various concentrations of urea, after which fluorescence spectra were recorded. For refolding, 12 μM SERK1-KD was first unfolded in 4.5 M urea for a period of 6 h. Subsequently, the sample was diluted 50 fold with buffer containing no urea and refolding took place during a period of 30 min at room temperature. Fluorescence emission was acquired from 290 to 600 nm, using an excitation wavelength of 280 nm with emission and excitation slits set to 10 nm. For each measurement, 5 scans were averaged and every nm a data point was collected. All spectra were recorded at 20 °C in 10 mm quartz cuvettes.

Fluorescence emission of SERK1-KD was also recorded during its temperature induced unfolding, which was achieved by increasing temperature from 15 °C to 70 °C at a rate of 1 °C/min. Data points were collected every 0.5 °C. Protein concentration of both phosphorylated and native SERK1-KD was approximately 0.24 μM. Excitation was at 280 nm, and fluorescence emission was recorded at 340 nm. Emission and excitation slits were set to 10 nm. The thermal midpoint of unfolding as reported by fluorescence spectroscopy was determined by using a two state model of unfolding (Nabuurs et al. 2009).

Circular dichroism (CD)

Far-UV CD data were acquired on a Jasco J-715 Spectropolarimeter. Spectra were recorded at 20 °C using 1 mm quartz cuvettes. All spectra were corrected by subtracting spectra of corresponding blank solutions. Buffer is 10 mM NaPi, pH 7.4, unless stated otherwise.

For acquisition of far-UV CD spectra of native and phosphorylated SERK1-KD, samples had a protein concentration of 5 μM and 5.5 μM respectively. Spectra were obtained by averaging 20 wavelength scans acquired from 185 to 260 nm. A data point was collected every 0.2 nm.

For acquisition of far-UV CD spectra of native SERK1-KD in 0.45 M urea, 1.5 μM protein was incubated with 0.45 M urea. For spectra of unfolded SERK1-KD, 2.4 μM protein was incubated with 4.5 M urea. Both samples were kept at room temperature for 6 h before measurements commenced. For spectra of refolded SERK1-KD, native SERK1-KD at a concentration of 17 μM was first unfolded by incubation with 4.5 M urea for 6 h at room temperature. Subsequently, the sample was diluted 10 fold with buffer containing no urea and refolding took place during a period of 30 min at room temperature. Far-UV CD spectra were obtained by averaging 20 wavelength scans acquired from 210 to 260 nm. Below 210 nm the presence of urea causes considerable scatter of the CD signal. A data point was collected every 0.2 nm.

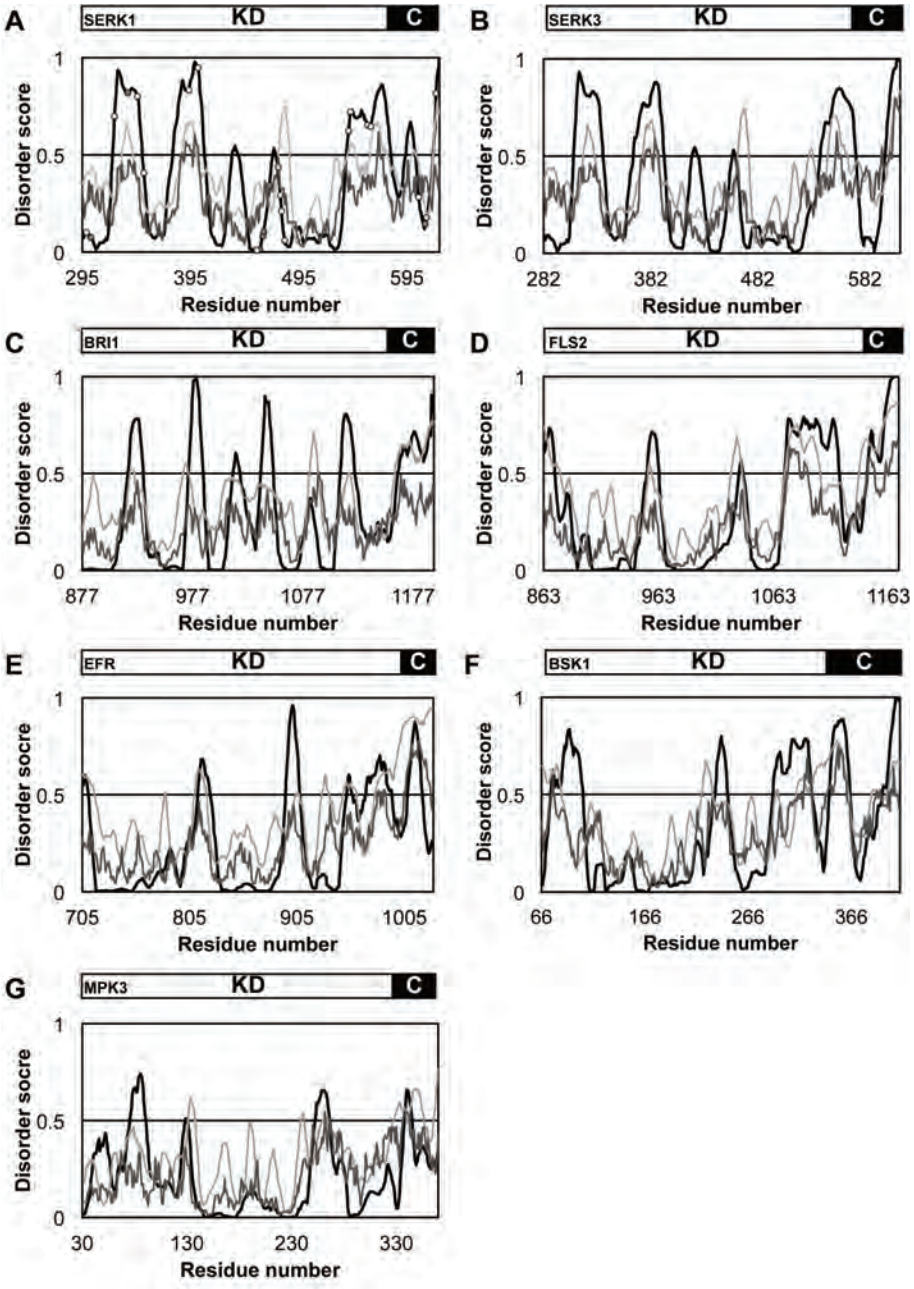
Thermal unfolding of 5 μM native and 5.5 μM phosphorylated SERK1-KD was followed at 210 nm. Temperature was increased from 15 °C to 80 °C at a rate of 1 °C/min and a data point was collected every 0.5 °C. The thermal midpoint of unfolding as reported by far-UV CD spectroscopy was determined by using a two state model of unfolding (Nabuurs et al. 2009).

Kinase phosphorylation assay

For each measurement of SERK1-KD autophosphorylation, 0.5 μM SERK1-KD was incubated at a particular concentration of urea (ranging from 0 M to 3 M urea). All samples were kept at room temperature for 6 h before the assay was started. For refolding, 8 μM SERK1-KD was first unfolded in 3 M urea for 6 h at room temperature. Subsequently this sample was diluted with buffer containing no urea to a final concentration of 0.18 M urea and 0.5 μM SERK1-KD. Refolding took place during a period of 30 min at room temperature.

To measure kinase phosphorylation activity, cold ATP and 500 μCi ^{32}P-γATP (Perkin Elmer) was added to the samples. Final concentration of ATP in each sample was 50 μM. Phosphorylation took place for 30 min at 30 °C and was stopped by addition of SDS-loading buffer. Samples were subsequently boiled for 3 min at 100 °C and separated by SDS-PAGE. Incorporated radioactive phosphate was determined using a PhosphorImager. Relative intensities of radioactive bands were analysed using ImageJ software (ImageJ 1.45s). Background intensity was subtracted and intensity at 0 M urea was set to 100 %.

Figure 2


A
SERK1
KD
C
Disorder score
1
0.5
0
295
395
495
595
Residue number
B
SERK3
KD
C
Disorder score
1
0.5
0
282
382
482
582
Residue number
C
BRI1
KD
C
Disorder score
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130
230
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Residue number

Figure 2 Predicted disorder within plant kinases using various disorder analyses programs. A disorder score lower than 0.5 indicates that a region is predicted to be ordered, whereas scores above 0.5 predict disordered protein sequences. Schematic above the graph indicates which part of the primary sequence belongs to the catalytic kinase domain (KD) or to the C-terminal tail (C). Disorder prediction of PONDR® VL-XT is depicted in black, VSL2B disorder analysis is depicted in light grey and IUPred disorder analysis in dark grey. **(A)** Disorder prediction for SERK1-KD. White dots indicate phosphorylation sites identified *in vitro*, grey dots indicate phosphorylation sites identified *in planta* (Karlova et al. 2009). Disorder profiles are also shown for kinase domains of **(B)** BAK1 (SERK3), a close paralog of SERK1, **(C)** BRI1, **(D)** FLS2 and **(E)** EFR, three main ligand binding plant RLK's, and for **(F)** BSK1 and **(G)** MPK3, two cytoplasmic kinases.

Results

SERK1-KD potentially contains disordered segments

To identify possible regions of disorder within SERK1-KD, we use disorder predictors PONDR® VL-XT, VSL2B and IUPred. SERK1-KD consists of the catalytic kinase domain (residues 295- 581) and a C-terminal region (the C-terminal tail) of 45 amino acids. Figure 2a shows that although SERK1-KD seems to be largely structured several short segments of its primary sequence are potentially disordered, as suggested by PONDR® VL-XT. These segments include its final C-terminal residues and a segment of more than 40 amino acids in the C-terminal part of the catalytic domain of SERK1-KD (residues 540-580).

As a comparison, disorder predicted for several other plant kinases is also analysed. Sequence alignment identified the catalytic kinase domain and the C-terminal tail of these proteins. BAK1 (SERK3) is a close paralog of SERK1. Its disorder profile (see Figure 2B) strongly resembles the one of SERK1.

BRI1, FLS2 and EFR are the main ligand binding LRR-RLK's for brassinosteroid, flagellin and EF-Tu respectively (Li and Chory 1997, Gómez-Gómez and Boller 2000, Zipfel et al. 2006). All three receptors potentially contain a disordered C-terminal tail (see Figure. 2C to E). PONDR® VL-XT suggests that only FLS2 has a disordered segment of more than 40 amino acids in the C-terminal part of its catalytic domain, just as SERK1.

BSK1 and MPK3 are cytoplasmic kinases functional in brassinosteroid and flagellin signalling, respectively (Asai et al. 2002, Tang et al. 2008). Both proteins apparently have no disordered segments of more than 40 amino acids (see Figure 2F and G). BSK1 does contain several short segments of predicted disorder, including disorder of its final C-terminal residues, whereas MPK3 is much more ordered (see Figure 2F and G).

Protein phosphorylation is thought to occur predominantly within disordered regions of a protein (Iakoucheva et al. 2004). Figure 2a shows that SERK1-KD contains many phosphorylation sites (Karlova et al. 2009), however, no bias towards disordered regions exists.

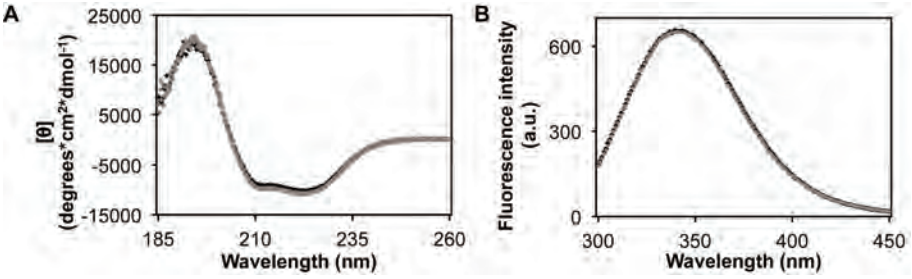

Figure 3 Phosphorylation hardly affects the structure of SERK1-KD.
(A) Far-UV CD spectra of native SERK1-KD (black) and phosphorylated SERK1-KD (grey) at 15 °C. **(B)** Fluorescence emission spectra of 22 μM native SERK1-KD (black) and of 25 μM of phosphorylated SERK1-KD (gray), both acquired at 20 °C. Overlay of both spectra is obtained upon correcting for protein concentration difference.

Phosphorylation hardly affects the structure of SERK1-KD

Phosphorylation of the activation loop is a major event in kinase activation and involves extensive structural movements (Huse and Kuriyan 2002). SERK1-KD purified from *E. coli* is referred to here as native SERK1-KD. To assess whether phosphorylation of the kinase domain of SERK1 alters protein structure, and to obtain insight into potential control mechanisms of kinase activity *in planta*, we acquired far-UV CD spectra of native SERK1-KD and of *in vitro* autophosphorylated SERK1-KD. Phosphorylation status of SERK1-KD is assessed with antiphospho Ser/Thr/Tyr-antibodies. Native SERK1-KD turns out to be only marginally phosphorylated compared to *in vitro* autophosphorylated proteins (see supplementary Figure S3).

Far-UV CD (see Figure 3A) shows that SERK1-KD purified from *E. coli* is well structured, because it has a spectrum that is typical for $\alpha+\beta$ proteins and is similar to the far-UV CD spectrum of BAK1-KD (Cheng et al. 2011). *In vitro* autophosphorylation of SERK1-KD is maximal after 35 min of incubation (Shah et al. 2001). After 45 min of autophosphorylation activity, little change in secondary structure of SERK1-KD is observed (see Figure 3A). SERK1-KD has five tryptophan residues, which are distributed across the protein. Fluorescence emission reports the local microenvironment of tryptophan. Figure 3B shows that phosphorylation does not result in detectable change in tertiary structure of the protein, as is shown by the unaltered fluorescence emission maximum at 340 nm and the constant shape of the fluorescence emission spectrum. In interpreting these data, it is important to note that after auto-phosphorylation both phosphorylated and non-phosphorylated residues are present for most SERK1 phospho-sites (Karlova et al. 2009).

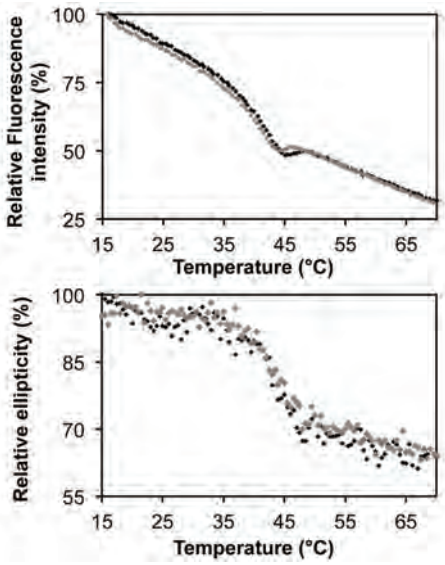

Figure 4 Effect of phosphorylation on stability of SERK1-KD against thermal unfolding. Top: Thermal unfolding monitored by the change in fluorescence emission at 340 nm, upon excitation at 280 nm. Bottom: Thermal unfolding monitored by the change in ellipticity at 210 nm. Data of native SERK1-KD are shown in black, and data of phosphorylated SERK1-KD are shown in grey.

Phosphorylation hardly alters the stability of SERK1-KD against unfolding

Although phosphorylation *in vitro* does not affect the structure of SERK1-KD, phosphorylation might alter SERK1 kinase stability against unfolding and thereby influence its function *in planta*. To probe this phenomenon, thermal unfolding of native and autophosphorylated SERK1-KD is followed by fluorescence spectroscopy (see Figure 4A). This method probes the tertiary microenvironment of tryptophan and tyrosine residues within a protein, and loss of fluorescence intensity at 340 nm upon excitation at 280 nm indicates loss of tertiary structure. In addition, far UV-CD spectroscopy is used to probe loss of secondary structure upon thermal unfolding of SERK1-KD (see Figure 4B). Fluorescence emission of SERK1-KD drops in the 15 to 35 °C temperature range (see Figure 4A), whereas ellipticity is hardly altered in the native baseline part of the thermal unfolding curve, as indicated by far-UV CD (see Figure 4B). Global protein unfolding occurs in the 35 to 45 °C temperature range, as cooperative unfolding transitions in both far-UV CD and fluorescence spectroscopy data show. Fluorescence of free tryptophan is rather sensitive to temperature, with fluorescence decreasing upon increasing temperature (Schmid 1989). In case of SERK1-KD, sensitivity of tryptophan fluorescence to temperature results in the slope of the native baseline of its thermal unfolding curve being negative. Possibly,

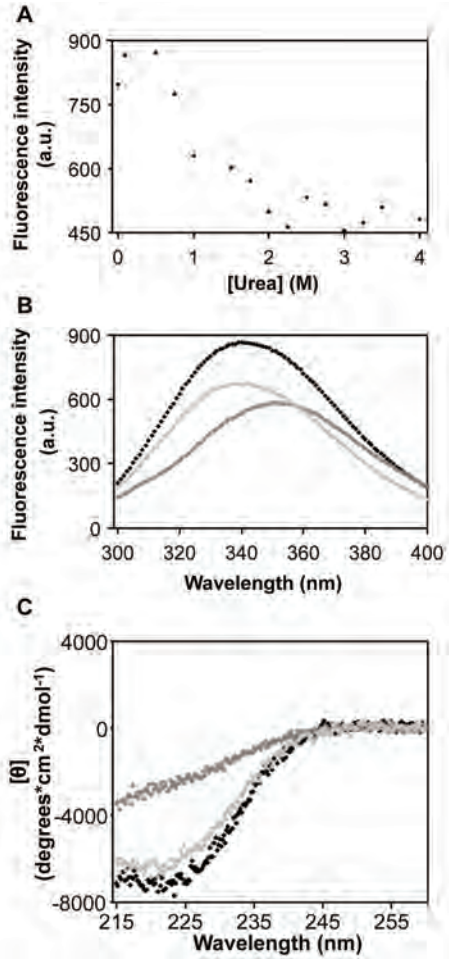

Figure 5 Denaturant-dependent folding of SERK1-KD.
(A) Urea induced unfolding of SERK1-KD followed by fluorescence spectroscopy. Fluorescence intensity of 0.24 μM of SERK1-KD is measured at 340 nm, upon excitation at 280 nm. The native baseline ranges from 0 to about 0.5 M urea. Above 2 M urea, the unfolded baseline commences. The transition region of urea-induced protein unfolding ranges from about 0.5 to 2.0 M. **(B)** Fluorescence emission of native, unfolded and of refolded SERK1-KD. Black: native SERK1-KD in 0.09 M urea. Dark grey: unfolded SERK1-KD in 6 M urea. Light grey: refolded SERK1-KD, obtained by unfolding the protein for a period of 6 hours in 4.5 M urea, and subsequent dilution of denaturant to 0.09 M urea. Refolding took place during a period of 30 min at room temperature. Refolded SER1-KD has a fluorescence maximum at 340 nm, indicative of folded protein. Approximately 80 % of unfolded SERK1-KD properly refolds, according to the difference in fluorescence intensity between native and refolded protein. **(C)** Far-UV CD spectra of native, unfolded and of refolded SERK1-KD. Black: native SERK1-KD in 0.45 M urea. Dark grey: unfolded SERK1-KD in 4.5 M urea. Light grey: refolded SERK1-KD, obtained by unfolding the protein for a period of 6 hours in 4.5 M urea, and subsequent dilution of denaturant to 0.45 M urea. Refolding took place during a period of 30 min at room temperature.

increased conformational flexibility of native protein upon increasing temperature may cause this decrease in fluorescence.
Figure 4 shows that autophosphorylation hardly affects the stability of SERK1-KD against thermal unfolding. Midpoints of unfolding of native and phosphorylated SERK1-KD as determined by fluorescence spectroscopy are 40.2 °C and 39.4 °C, respectively. The corresponding midpoints of unfolding as determined by far-UV CD are 42.7 °C and 43.7 °C, respectively. Upon cooling, thermally unfolded SERK1-KD protein is not able to regain its native structure or its catalytic activity (see Figure S4 and data not shown).

Unfolding of SERK1-KD is intimately linked to loss of its phosphorylation activity

Unfolding of SERK1-KD is also investigated by adding urea and subsequent determination of its spectroscopic and catalytic properties. Before measurements, samples are kept at room temperature for 6 hours to ensure achieving thermodynamic equilibrium.

Upon urea-induced unfolding of SERK1-KD fluorescence at 340 nm decreases (see Figure 5A) and the maximum of fluorescence emission shifts from 340 to 350 nm (see Figure 5B). Up to urea concentrations of about 0.5 M, fluorescence intensity of the sample is large, indicating that this region of the unfolding curve of SERK1-KD is the native baseline (see Figure 5A). In this denaturant range, virtually all SERK1-KD molecules are in the native state. Upon increasing the denaturant concentration, the ratio of molecules in the native state to those in the unfolded state drops, and as a result fluorescence emission decreases. This decrease in fluorescence highlights the transition region of urea-induced SER1-KD unfolding, which ranges from about 0.5 to 2.0 M urea. Above 2 M urea, SERK1-KD molecules are in the unfolded state, giving rise to the unfolded baseline in the fluorescence unfolding curve of Figure 5A.

To assess whether diminishing of the population of the native state during urea induced unfolding is linked to loss of phosphorylation activity of SERK1-KD, protein activity is measured at increasing denaturant concentrations (see Figure 6). Up to 0.5 M urea, autophosphorylation activity only marginally decreases, most likely because all SERK1-KD molecules are in the native state. Upon increasing urea concentration above 0.5 M, loss of kinase activity happens gradually (see Figure 6). This denaturant-dependent loss of activity coincides with the observed diminished population of the native state of SERK1-KD, as reported by fluorescence emission (see Figure 5A). Above 2 M urea no incorporation of radioactive phosphate is observed, because SERK1-KD is unfolded and thus inactive.

The above data show that unfolding of SERK1-KD is intimately linked to loss of its phosphorylation activity.

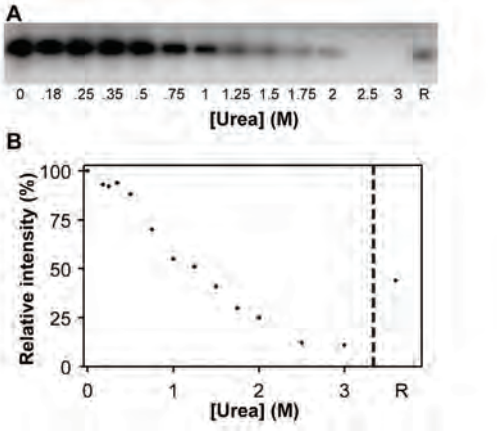

Figure 6 Dependence of SERK1-KD kinase activity on urea concentration.
(A) Autoradiograph of autophosphorylation activity of SERK1-KD at different concentrations of urea. R: Refolded SERK1-KD, obtained by unfolding the protein for a period of 2 hours in 3 M urea and subsequent dilution of denaturant to 0.18 M urea. Refolding took place during a period of 30 min at room temperature. **(B)** Urea-dependent loss of phosphorylation activity of SERK1-KD. The gel presented in (A) is analysed with ImageJ to quantify intensities of the bands observed, which correlates with incorporation of radioactive phosphate. Loss of kinase activity happens gradually upon increasing urea concentration above 0.5 M. Phosphorylation activity is completely lost at concentrations urea above 2 M.

Upon spontaneous refolding in vitro, SERK1-KD regains native structure and catalytic activity

To investigate whether urea-induced unfolded SERK1-KD is able to spontaneously refold *in vitro,* the protein is first unfolded at high concentration denaturant (i.e., above 2 M urea, see Figure 5A). To induce refolding, urea concentration is then lowered to a value at which SERK1-KD molecules are in the native state (i.e., below 0.5 M urea; see Figure 5A). Subsequently, we determine spectroscopic and catalytic properties of refolded SERK1-KD, to probe whether refolded protein has native three-dimensional structure.

Fluorescence emission of refolded SERK1-KD has its maximum at 340 nm, typical for native SERK1-KD (see Figure 5B). Approximately 80 % of unfolded protein refolds properly, as comparison of fluorescence intensity of native and refolded protein indicates. The remaining fraction is most likely aggregated. Far-UV CD confirms that the large majority of unfolded SERK1-KD spontaneously refolds *in vitro* to native protein (see Figure 5C) because CD spectra of native and refolded SERK1-KD are similar. Again, due to some aggregation, the amplitude of the CD signal of refolded protein is slightly less than that of native protein. To evaluate whether refolded SERK1-KD also regains catalytic activity, incorporation of radioactive phosphate is probed. Figure 6 shows that refolded SERK1-KD indeed autophosphorylates, which is the hallmark for properly folded, catalytically active

SERK1-KD. Compared to native SERK1-KD about 50 % of autophosphorylation activity is recovered.

Protein concentration in the initial refolding sample (i.e., before dilution of denaturant) is an important parameter determining refolding efficiency, since aggregation highly depends on protein concentration. This concentration is highest in the refolding sample used for activity determination, explaining the lower refolding efficiency observed (see Figure 6) compared to refolding detected by spectroscopic techniques (see Figure5B and C). Nevertheless, we conclude that fully active SERK1-KD can be obtained in the absence of any cellular context.

Discussion

In this work we addressed whether the catalytic activity of LRR-RLK SERK1 from *Arabidopsis thaliana* is an inherent property of its kinase domain. Our results show that SERK1-KD regains its catalytic activity upon refolding *in vitro*, indicating that the protein has the intrinsic capacity to become active. Because *in planta* SERK1 RLK is a non-ligand binding co-receptor functioning in several signalling pathways, maintaining the inactive status of the kinase domain is necessary to prevent unscheduled signalling. Inactivation of the kinase domain could be achieved by either intra- or intermolecular inhibitory elements. One way by which such an inhibitory element could act is by maintaining parts of the protein disordered until activity is required.

The bioinformatics tool PONDR® VL-XT predicts that SERK1-KD is largely structured, with one extended region of disorder. This disordered region comprises part of its C-terminal tail and 41 amino acids in domains X and XI, regions previously identified as essential for both *in vitro* kinase phosphorylation activity and *in planta* function of SERK1 (Albrecht et al. 2005). Disorder analysis of six other plant kinases indicates that the C-terminal tail of plant kinases functioning in signalling often is disordered. The C-terminal tail of BRI1 has been identified as an auto-inhibitory element for BRI kinase activity (Wang et al. 2005). This could indicate that the C-terminal tail is a more common regulatory element in plant kinases. An extended region of disorder (i.e. more than 40 residues) in the catalytic kinase domain was only predicted for FLS2 and SERK1. Such a region can function as another form of kinase regulation, as was previously identified for epidermal growth factor receptor (EGFR). Molecular dynamics studies identified a region of disorder in the kinase domain of EGFR. This region is shown to be important for dimerization between two kinase domains after which it becomes more ordered (Shan et al. 2012). SERK1 can form homo- and hetero-oligomers *in planta* (Hink et al. 2008). While it has been proven that EGFR only functions as a dimer, it is not clear whether homodimerization plays a role in controlling the catalytic activity of SERK1, because purified SERK1-KD, which is monomeric according to analytical

gelfiltration, is already catalytic active. The predicted disordered regions of SERK1 might have a function in interaction with ligand-perceiving RLK's with which members of the SERK family form hetero-oligomers (Karlova et al. 2006).

Phosphorylation sites are often found in disordered regions of proteins, probably due to easy access of these sites (Iakoucheva et al. 2004). Phosphorylation can induce structuring in disordered regions (Espinoza-Fonseca et al. 2007, 2008, Garza et al. 2010), or hardly influences these regions (Ganguly and Chen 2009, Mittag et al. 2008). In addition, phosphorylation can change the ability of these regions to undergo protein-protein interactions (Mittag et al. 2010). The number of phosphorylation sites differs vastly between SERK1 and its close paralogs SERK2 and BAK1 (SERK3) (Karlova et al. 2009). Comparison of previously identified phosphorylation sites of SERK1 and predicted disorder indicates that there is no bias towards phosphorylating disordered regions. More strikingly, seven of eight phosphorylation sites identified *in planta* are in predicted structured parts of the protein. Four of these sites are found in the activation loop of SERK1-KD (Thr459, Thr462, Thr463 and Thr468), of which phosphorylation is known to be important for kinase activity (Huse and Kuriyan 2002).

Fluorescence, far-UV CD and activity data presented in this paper show that SERK1-KD is largely structured and active *in vitro*. The observed coincidence of loss of activity and three-dimensional protein structure upon denaturant induced unfolding demonstrates that properly folded structure is essential for SERK1-KD to be active. Upon lowering denaturant concentration, unfolded SERK1-KD is able to refold to its native structure and regains catalytic activity. Thus, SERK1-KD folds to active kinase, while its cellular context comprising chaperones, membrane components and interaction partners, is absent. However, to counteract aggregation and make folding more efficient in the hugely crowded cellular context (Gershenson and Gierasch 2011), chaperones most likely assist in folding SERK1-KD molecules *in vivo*.

For organisms it is important to keep RLK's inactive until their signalling is required to properly respond to signals. For plant RLK's this issue has not received much attention. We observe that SERK1-KD autonomously folds to its active state within 30 minutes. This observation suggests that *in planta* SERK1 already contains an active kinase domain before it is transported to its proper location, the plasma membrane (Shah et al. 2002), or incorporated in the correct protein complex required for signalling activity. Thus, mechanisms are likely needed *in planta* to keep kinase activity of SERK1 in check. For other plant RLK's candidate inhibitory proteins have indeed been identified, such as BKI1 or protein phosphate 2A for BRI1 (Jaillais et al. 2011, Wu et al. 2011). Dephosphorylation of specific residues essential for phosphorylation activity has also been proposed to deactivate RLK's (Oh et al. 2012). A possible candidate inhibitor of SERK1 is KAPP, the PP2C protein phosphatase (Shah et al. 2002). Finally, endoplasmatic reticulum associated protein degradation (ERAD) potentially plays a role during incorporation of SERK1-

KD into correctly assembled receptor complexes, because ERAD also is important during biogenesis and quality control of other plant receptor complexes (Li et al. 2009, Nekrasov et al. 2009, Su et al. 2011). The next challenge is to elucidate whether and how the above mechanisms contribute to keeping SERK1-KD inactive *in planta*, until its activity is required.

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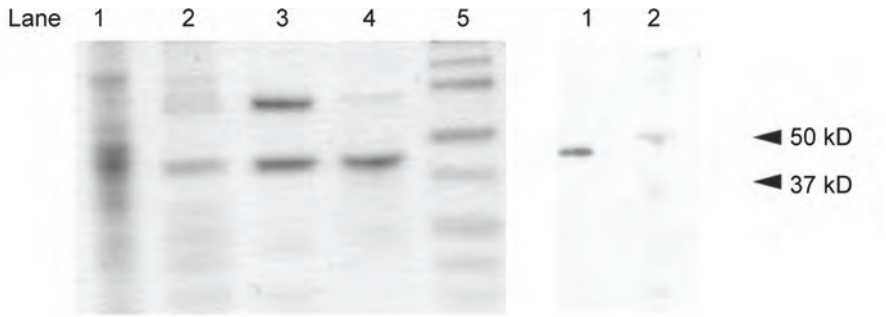
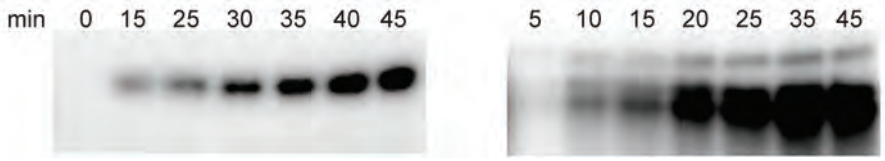
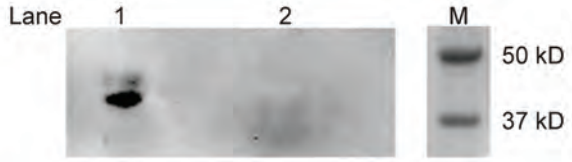
Supplemental material



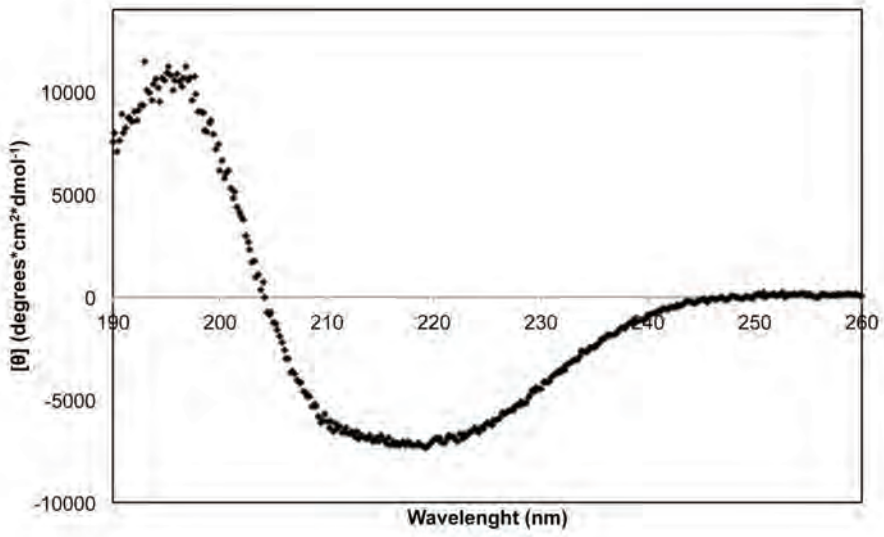
Supplemental Figure S1 SDS-PAGE and Western blot of SERK1-KD.
Left: SDS-PAGE of aliquots taken at different steps during SERK1-KD purification. Lane 1, cell extract from BL21* *E. coli* cells expressing 6xHIS-SERK1-KD; lane 2; eluate from HIS-pure cobalt column; lane 3, eluate from SourceQ-15; lane 4, eluate from Superdex 75 10/300 GL (i.e., final, purified sample); lane 5, Marker. Right: Western blot of final purified protein, using anti- His-tag antibodies. Lane 1, purified SERK1-KD; lane 2, Marker.



Supplemental Figure S2 SERK1-KD phosphorylation properties.
Left: Autophosphorylation of SERK1-KD (1 μg); Right: Transphosphorylation of casein (1 μg) by SERK1-KD (0.2 μg). Aliquots are taken at the time points indicated (in minutes) and subsequently separated by SDS-PAGE. Incorporation of ^{32}P-yATP is visualised using a PhosphoImager.



Supplemental Figure S3 Phosphorylation status of native and autophosphorylated SERK1-KD.
Anti- Phosphoserine, -threonine and -tyrosine antibodies are used to probe the phosphorylation status of SERK1-KD. Lane 1, 0.75 μg of SERK1-KD after 45 min of incubation with ATP; lane 2, 0.75 μg of SERK1-KD as purified from *E. coli*; M, marker.



Supplemental Figure S4 Far-UV CD spectrum of refolded SERK1-KD.
SERK1-KD was first heated to 85 °C and subsequently cooled to 15 °C. The far-UV CD spectrum of this protein differs from the corresponding spectrum of native SERK1-KD shown in Figure 2, and consequently thermally unfolded SERK1-KD does not properly refold upon lowering temperature.

Chapter 5

Visualisation of SERK3 and BRI1 Plasma Membrane Distribution using VAEM

M. aan den Toorn, S.J. Hutten, G. W. van Esse, A. Nolles, J. Hohlbein, S.C. de Vries

Abstract

The brassinosteroid plant hormones (BRs) are perceived by the main ligand binding receptor BRI1. BRI1 forms hetero-oligomeric complexes with co-receptors of the SERK protein family, amongst which SERK3/ BAK1. The plasma membrane (PM) constitutes the main site of BR signalling, as this is where the SERK3-BRI1 hetero-oligomeric complexes are present and ligand binding occurs. The PM is highly organised, and many proteins show a distinct distribution within this lipid bilayer.

In this chapter, the plasma membrane distribution of BRI1 and SERK3 is visualised using Variable Angle Epifluorescence Microscopy (VAEM). This technique, which allows for selective illumination of a thin surface layer, can be used to visualise fluorescent molecules in the plant plasma membrane (PM) and to study intracellular membrane compartments in close proximity to the PM. Both BRI1 and SERK3 show an inhomogeneous distribution on the PM, forming distinct clusters. Surprisingly, the density of these nanoclusters is not influenced by depletion of endogenous ligand or signal activation. On average, each nanocluster contains 6 BRI1-GFP and 2 SERK3-GFP receptor molecules. Overexpression of BRI1 resulted in up to 22 receptor molecules in a nanocluster, but not in more clusters per μm^2 PM. Tyrphostin A23 treatment, which inhibits endocytosis of ligand-bound BRI1, reduces the overall fluorescence intensity on the PM but did not influence the cluster density. Thus, whereas the number of receptor molecules per nanocluster varies, the number of clusters remains constant. We propose that the formation of nanoclusters in the plant PM is subjected to biophysical restraints, while the stoichiometry of receptors inside the clusters is a variable entity affecting signal transduction.

Introduction

Brassinosteroids (BRs) are plant steroid hormones that regulate cellular expansion, differentiation and proliferation (Clouse 2001). The brassinosteroid signalling pathway starts at the plasma membrane (PM), where BRs bind to the island domain in the extracellular part of the leucine-rich-repeat receptor like kinase (LRR-RLK) brassinosteroid insensitive 1 (BRI1). Ligand binding to the extracellular domain of BRI1 induces the phosphorylation and subsequent disassociation of the inhibitor protein BRI1 Kinase Inhibitor 1 (BKI1) from its cytoplasmic kinase domain (Wang et al. 2006; Jaillais et al. 2011). BKI1 prevents BRI1 from interacting with its co-receptor SERK3/ BAK1 (Somatic Embryogenesis Receptor Kinase 3/ BRI1 Associated Kinase 1) (Jaillais et al. 2011). The hetero-oligomerization between BRI1 and its SERK co-receptor causes sequential transphosphorylation events on their cytoplasmic kinase domains (Wang et al. 2008) and is a prerequisite for proper BR signal transduction (Gou et al. 2012). Transphosphorylation events between SERK3/ BAK1 and BRI1 lead to full activation of the kinase domains and subsequent phosphorylation of downstream signalling components (Tang et al. 2008; Kim et al. 2011) which relay the signal further to transcription factors BZR1 and BES1, resulting in regulated expression of BR- responsive genes. Recent investigations suggest that hetero-oligomers between BRI1 and SERK3/ BAK1 are, at least in part, preformed in absence of ligand, as a functional unit to perceive BRs and initiate downstream signalling (Bücherl et al. 2013). Extracellular domain interactions between SERK1 (a highly homologous family member of SERK3/ BAK1) and BRI1 are ligand dependent (Santiago et al. 2013), suggesting that other domains (such as the transmembrane domain or cytoplasmic domains) are essential for the observed ligand independent hetero-oligomers.

Although there are indications of some endosomal BR signalling (Geldner et al. 2007), the initial recognition of BRs and activation of the receptor complex via ligand binding occurs at the PM (Irani et al. 2012). The PM is a highly organised lipid bilayer interspersed with proteins. Most PM located proteins do not diffuse unrestricted through the lipid bilayer and some show clear inhomogeneous patterning across the PM (Jaqaman et al. 2012, and references therein). Proteins in the PM can be restricted in their movement via for example the cortical cytoskeleton, protein 'crowding', interaction between membrane components and inhomogeneity in membrane composition and state (Jaqaman and Grinstein 2012). In *Arabidopsis*, the lateral movement of PM localised proteins is also restricted by the presence of the cell wall, although not necessarily due to direct interactions (Martinière et al. 2012). As a result of these restrictions, protein distribution across the membrane is inhomogeneous and cluster formation can occur. In animal cells, the presence of so-called nanoclusters of receptor proteins in the PM has been established. Organisation of receptors into these clusters would be essential for signal transduction. In addition, relocalisation of receptors upon ligand binding has been

reported, via endocytosis to specific endosomal locations (Di Fiore et al. 2001) but also relocalisation within the PM itself (Wilson et al. 2011). This chapter describes the visualisation of PM distribution of two LRR-RLK's involved in the brassinosteroid-signalling pathway, namely BRI1 and SERK3/BAK1, in high resolution in live *A. thaliana* epidermal root cells. The epidermal cell layer has a fundamental role for executing BR signalling and regulating BR-related developmental processes in root and shoot (Savaldi-Goldstein et al. 2007; Hacham et al. 2011). For the visualisation of receptor distribution on the PM we use Variable Angle Epifluoresence Microscopy (VAEM), a technique closely related to Total Internal Reflection Fluorescence (TIRF) microscopy (Konopka et al. 2008). In objective -type TIRF microscopy, the laser light is focussed into the rim of the backfocal plane of a microscope objective with high numerical aperture instead of the centre, as it is the case for conventional widefield microscopy. As a result, the strong inclination of the passing laser light can lead to the phenomena of total internal reflection at the interface between the cover slide and the sample medium due to the lower refractive index of the sample medium. Even though the light does not pass the interface, a evanescent wave is generated which decays exponentially within a few hundred nanometers. Both in plant and animal cells, TIRF has been used to visualise proteins located on the PM (Webb et al. 2006; Vizcay-Barrena et al. 2011). In plant cells, however, the utilisation of TIRF is hampered due to presence of the plant cell wall (Shaw 2006) whose thickness is comparable to the effective excitation depth of the evanescent wave. In VAEM, the laser light is focussed closer to the centre of the backfocal plane such that not all light is reflected at the cover glass to water interface; instead, a thin band of illuminating light penetrates the sample. By varying the the focus position in the backfocal plane, we can adjust the depth at which the sample is illuminated. Due to the curvature of the plant root, however, only a narrow region of the outer PM of the epidermal root cells in close proximity to the cover slip can be visualised.

In this chapter we explore the possibilities of VAEM in live *A. thaliana* epidermal root cells and investigate wheter this technique can provide us with insights into receptor distribution and behaviour after receptor activation. Our results indicate that VAEM can visualise both the PM of plant roots, and also the endosomal compartments just below the PM. The PM receptor molecules tested in these experiments all show an inhomogeneous distribution, clustered across the membrane. The cluster density is not altered by activating the signalling complex, by over-expression of the receptor or by influencing the endocytosis of the receptor, although the number of receptors per cluster was variable. This indicates that the formation of clusters is not specifically coupled to the signalling activity of the receptor.

Materials & Methods

Growth conditions and plant lines

Arabidopsis thaliana plants of ecotype Columbia (Col-0) were used as wild type. Seeds were surface sterilised and germinated on ½ Murashige and Skoog medium (Duchefa) supplemented with 1% sucrose (Sigma) and 1% Daishin agar (Duchefa). Plants were grown at 22 ˚C under fluorescent light, with 16 h light/8h dark photoperiods. Col-0 plants expressing BRI1 (AT4G39400) fused to GFP under its native promoter, here referred to as BRI1-GFP 1, were provided by N. Geldner (Geldner et al. 2007). BRI1-GFP 2 was a BRI1-GFP line overexpressing the transgene roughly three-fold, and was provided by J. Chory (Friedrichsen et al. 2000). Col-0 plants expressing SERK3-mCherry or SERK3-GFP under control of its native promoter were generated as previously described (Bücherl et al. 2013). BRI1-GFP line 2 was crossed with the SERK3-mCherry line to create a plant harbouring both transgenes .The *serk1 serk3* mutant plant harbouring BRI-GFP was produced by crossing BRI-GFP line 2 with the double mutant *serk1-3* (GABI-KAT line 448E10) *serk3-2* (SALK_116202) resulting in the *serk1serk3* BRI-GFP line. *det*2 seeds were obtained from the Arabidopsis seed stock centre and crossed with BRI-GFP line 2. Col-0 plants containing the transgenes Wave6-mCherry and Wave18-RFP were provided by N. Geldner (Geldner et al. 2009), LT16B-GFP was provided by C. ten Hove (Cutler et al. 2000), VHAa1-mRFP (Dettmer et al. 2006), ARA7/Rab F2B-mRFP and ARA6/ Rab F1-mRFP were provided by K. Schumacher, Heidelberg. KNOLLE-GFP was used as a positive control for the FRAP experiments based on the data of (Boutte et al. 2010). The pBIR3::BIR3-GFP line was constructed by Walter van Dongen (Biochemistry, WU). Protoplast isolation and transfection for the FRAP experiments was performed as described previously (Bücherl et al. 2010).

Hormone and inhibitor treatments

For hormone treatment, six day old seedlings were incubated in 1 mL ½ Murashige and Skoog medium, supplemented with 1% sucrose and 1 μM 24-epi-brassinolide (BL, Sigma). For brassinazole treatment, seeds were first germinated and grown for four days on ½ Murashige and Skoog medium, supplemented with 1% sucrose and 1% Daishin agar. After four days the seedlings were transferred to plates complemented with 5 μM brassinazole (BRZ, TCI Europe) and grown on these plates for an additional two days. For tyrphostin treatment, six day old seedlings were incubated in 1 ml ½ Murashige and Skoog, supplemented with 1% sucrose and 50 μM tyrphostin A23 or tyrphostin A51.

VAEM set-up

Live root imaging was performed on a home- build microscopy setup. Instead of a standard microscope body, we used a RAMM system as a stage holder (ASI) together with a motorised x,y-scanning stage with a z-piezo for controlling precise sample placement along the optical axis of the microscope. We used a fibre-coupled laser engine (Omicron) equipped with four lasers (405 nm, 473 nm, 561 nm, and 642 nm) for excitation. Laser intensities are independently controlled by a home-written LabVIEW program. The single mode fiber generates a Gaussian shaped beam profile and a point source like output. The divergent light is collimated (f = 100 mm, Thorlabs) and then focussed into the backfocal plane of a 100x NA 1.49 TIRF objective (Nikon) by a lens (f = 200 mm, Thorlabs) mounted on a translational stage perpendicular to the laser beam in order to allow adjusting the TIRF angle. A custom-made multicolor polychroic mirror and a multibandpass filter (Chroma, USA) are used to avoid leakage of laser light into the emission path. After the spatial filtering of the fluorescence with a two-lens system consisting of two tube lenses (f = 200 mm) and an adjustable slit (Thorlabs), the light is spectrally split using dichroic mirrors (Chroma) into three beams corresponding to a blue, green, and red fluorescence detection channel. The three beams are then focused (f = 250 mm) on an Ixon Ultra 897 emCCD camera with 512 x 512 pixel (Andor). The total magnification of the optical system is 125 x and we measured a pixel width corresponding to 130 nm. Data was recorded using micromanager (Edelstein et al. 2010).

VAEM imaging, processing and analysing

Live root imaging was performed on the set-up described above. GFP was excited with a 473 nm laser (laser power in front of the polychroic mirror 0.98 mW) and fluorescence emission was detected from 475-550nm. mCherry was excited with a 561 nm laser (laser power set at 0.35 mW) and fluorescence emission was detected from 560-640 nm. Images were recorded every 100 msec, with an exposure time of 100 msec. Movies of 250 or 500 frames were recorded. Each image shown here corresponds to the first frame taken, to minimise bleaching effects. For the image of the ER marker WAVE6-mCherry, the first 40 frames were merged into one picture. A background subtraction (rolling ball radius = 50.0 pixels) was performed on all images of PM localised proteins with use of IMAGEJ (IMAGEJA, 51.45j, Max Planck Society; Scheider et al. 2012).

For particle size analysis a Gaussian blur filter of 2μm (σ) is subtracted from the images. A binary image is constructed from this image, taking a lower intensity threshold of 80 a.u. The binary image is subsequently analysed with the particle analysis plug-in from FIJI software (IMAGEJA, 51.45j, Max Planck Society; Schneider et al. 2012).

For the decay curves a region of interest was defined by an area of 5x5 pixels. This region is analysed using the plot z-axis profile function from IMAGEJ (IMAGEJA, 51.45j, Max Planck Society; Schneider et al. 2012).

Confocal microscopy and FRAP experiments

Roots of Arabidopsis seedlings expressing BRI1-GFP line 1, SERK3-GFP or KNOLLE-GFP, and protoplasts transfected with these constructs were imaged with a CONFOCOR2/LSM510 confocal microscope (Zeiss) equipped with a 40x water objective (numerical aperture 1.2) and an argon ion laser. The argon laser was used for excitation of GFP at 488 nm with an output of 40% (6.1 A). GFP fluorescence emission was detected with a band-pass filter at 505 to 550 nm. The image size was set to 512x512 pixels and four scans were averaged for each picture. The bleaching routine started with two or three pre-bleached scans, followed by a bleach pulse and a follow-up dependent on the bleached protein. For the background-correction, wild type roots underwent the same bleaching procedure. Protoplasts transfected with BRI1-GFP or SERK3-GFP were analysed in an 8-chamber slide, with 300 μl of protoplast solution in one chamber. Confocal microscope settings were adjusted depending on the size of the protoplasts and signal intensity. Zoom was adjusted from 3.9 to 6.9, the scanning time was 786 or 983 ms and the pinhole was 1.82 or 2.64 AU. The bleach pulse was applied in 15 iterations at 50% transmission at 488 nm. The size of bleach regions depended on the sample and settings. Images were recorded up to 53 s after the bleach pulse.

Roots of five day old seedling roots stably expressing BRI1-GFP or SERK3-GFP were immobilised in 1x PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 1 L distilled H_2O) in a 2-chamber slide (pre-incubated with 0.1% poly-L-lysine for a minimum of one hour) with a cover glass and a weight on top. The bleached area differed from the whole cell plate to just a part of the cell plate. The bleach pulse was applied in 20 iterations at 75% transmission at 488 nm. The recovery was followed up to 260 s after the bleach pulse. The excitation laser light was set at 488nm, at a power of 5% at and 9% for BRI1-GFP and SERK3-GFP respectively. The images were taken with a 6x zoom, a scan time of 15.73 s and a cycle delay of 5 s. A fixed rectangular area of 60x15 pixels (4.5x1.1 μm) was bleached on the plasma membrane with 50 iterations at 45% transmission at 488 nm for both proteins. The recovery of the fluorescent signal was followed up to 499 s and 436 s for BRI1-GFP and SERK3-GFP respectively. For FRAP measurements in the elongation zone, the excitation laser power was set to 1% at 488 nm to reduce the scan-bleaching. The images were taken with a 4x zoom and a scan time of 6.29 s. A fixed rectangular area of 60x15 pixels (6.8x1.7 μm) was bleached on the plasma membrane for SERK3-GFP. For BRI1-GFP two different areas were used, 30x20 pixels (3.4x2.3 μm) or 20x15 pixels (2.3x 1.7 μm), but this did not change the recovery curve. The bleach pulse was given with 15 iterations at 50% transmission at 488 nm for both proteins.

FRAP data analysis

FRAP data analysis was performed after background-correction of the data (Supplemental File S1). The data were visualised with Excel (Microsoft Office 2003), which was also used for curve fitting (Curve fitting in Microsoft Excel by William Lee).

Accession numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or EMBL/GenBank data libraries under accession numbers: BRI1 (AT4G39400), SERK3/BAK1 (AT4G22430), WAVE6/NIP1;1 (AT4G19030), WAVE18/Got1p homolog (AT3G03180), ARA6/RABF1 (AT3G54840), ARA7/RABF2B (AT4G19640), VHA-A1 (AT2G28520) LTI6B/RCI2B (AT3G05890), DET2 (AT2G38050) KNOLLE (AT1G08560) and BIR3 (AT1G27190).

Results

Utilising VAEM to visualise endosomal compartments in live A. thaliana epidermal root cells

To investigate whether the adapted TIRF technique called VAEM can be used to visualise endosomal compartments close to the PM, fluorescently tagged marker proteins for different endosomal compartments were employed. In Figure 1A a VAEM image shows the PM located protein LT16B-GFP (Cutler et al. 2000) in live *A. thaliana* epidermal root cells. This small integral membrane protein is visible in the PM, and shows a rather homogenous distribution. The ER is visualised by WAVE6-mCherry (Geldner et al. 2009) (see Figure 1B). In plant cells, the ER forms a net-like basket occupying the cortical space just below the plasma membrane. The structure seen with VAEM shows the network of membrane tubules and sheets, and the movies (supplementary on-line files) show the flowing movement typical for the ER. WAVE18-mRFP (Geldner et al. 2009) is a marker protein for Golgi stacks. The Golgi apparatus is mobile and shows numerous vesicular structures, which can easily be visualised with VAEM (see Figure 1C). The trans-Golgi network (TGN) is visualised with the VHAa1-mRFP marker protein (Dettmer et al.) (see Figure 1D). These mobile vesicular structures are dynamic, and fusions and protrusions from vesicles can be observed (Viotti et al. 2010). In plants, the TGN functions both as a sorting place for newly synthesised proteins and in the retrograde transport of recycled plasma membrane proteins (Dettmer et al. 2006; Lam et al. 2007). The plant TGN is thus equivalent to the early endosome compartment as defined in animal cells, with early endosomes (EE) budding off, which go on to mature to late endosomes or multi-vesicular bodies (LE/MVB). The marker protein ARA7/Rab

F2b (Ueda et al. 2004; Ebine et al. 2011) is a marker for EE and LE/MVB and can thus be used to visualise the retrograde trafficking vesicles. In Figure 1E a VAEM image of ARA7-mRFP fluorescence in epidermal root cells shows the retrograde endosomal vesicles. In Figure 1F ARA6/Rab F1-mRFP fluorescence is visualised; this homologue of ARA7 is more specific for LE/MVB's (Ebine et al. 2011). Thus, as Figure 1 indicates, VAEM can be exploited to obtain high-resolution images of many different intracellular membrane compartments.

All VAEM images as shown in the manuscript are derived from series of movies that can be found in the supplementary on-line files only. Comparative images using full TIRF mode and VAEM are shown in Supplemental Figure S1, indicating the problems associated with full TIRF due to the presence of the cell wall.

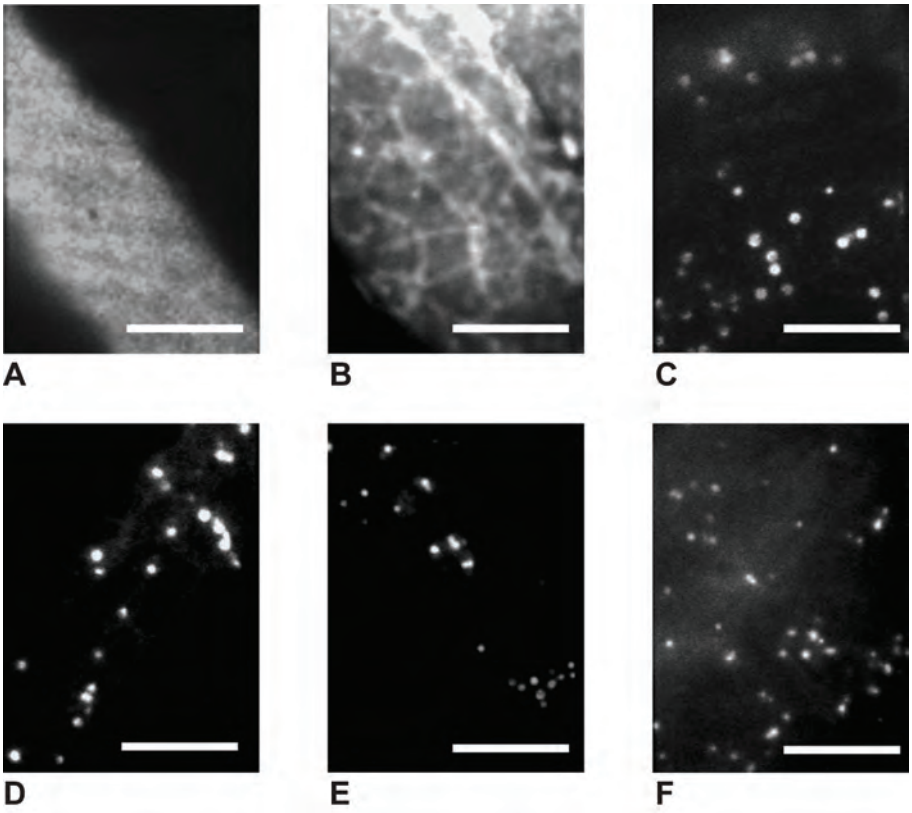


Figure 1 Visualisation of A. thaliana membrane compartments with VAEM
Live-cell VAEM imaging was performed on 6 day old Arabidopsis seedling roots expressing fluorescent markers for different membrane compartments. Fluorescent markers visualised in the different images are **(A)** PM localised LT16B-GFP, **(B)** ER localised WAVE6-mCherry, 40 sequential images of 100 msec exposure each were merged, **(C)** Golgi localised WAVE18-mRFP, **(D)** TGN localised VHAa1-mRFP, **(E)** EE/LE localised ARA7-mRFP and **(F)** LE localised ARA6/Rab F1-mRFP. Exposure time for all images (except (B)) was 100 msec. Scale bars represent 10 μm.

Receptor distribution on the membrane

The PM is composed of a lipid bilayer intermingled by proteins. Studies from animal receptors indicate that proteins do not show free lateral movement through the membrane, but movement is restricted within areas, resulting in an uneven distribution of proteins in the plane of the membrane (Mattila et al. 2013). To investigate whether plant membrane receptor molecules also show inhomogeneous distribution along the membrane, BRI1-GFP and SERK3-mCherry were visualised within the plane of the PM using VAEM.

The results show that both BRI1 and SERK3 are not homogenously distributed across the plasma membrane, but show a clear spotted pattern (see Figure 2), much more so than the PM-marker LT16-B (see Figure 1). In animal literature, such a pattern is referred to as a nanocluster distribution, a term that we will employ here as well. BRI1 nanoclusters with clearly high fluorescence intensity are visible, with a size in the order of 2-5 pixels per cluster, which equates to roughly 300-500 nm in diameter. These clusters are observed for both the main receptor BRI1 and the co-receptor SERK3. The receptors can only be visualised in the epidermal root cells of the elongation zone, an area where BR signalling is reported to be active (González-García et al. 2011; Hacham et al. 2011). The root meristem zone itself cannot be visualised due to curvature of the root, which causes the epidermal cell layer to be outside the critical range for VAEM. Clearly visible are the endosomal vesicles on the cytoplasmic side of the PM. These vesicles can be observed both for SERK3 and BRI1, although a higher number of vesicles are observed for BRI1. Especially the BRI1-line 2 (which shows an approximate three time over-expression of BRI-GFP compared to wild type) shows a high number of fluorescent endosomal compartments, which are reminiscent of LE/EE compartments. This is in line with the observations made using confocal microscopy with the same plant lines (Bücherl et al. 2013).

Brassinosteroid signalling is dependent on both the presence of the main ligand binding receptor BRI1 and the SERK co-receptors (Gou et al. 2012). In the root, the active signalling complex consists of BRI1 with SERK1 and/or SERK3/BAK1 (Li et al. 2002; Karlova et al. 2006). Confocal images have shown that BRI1 and SERK3 co-localise on the membrane, and FRET-FLIM data have shown that a minor amount of the BRI1 and SERK3 receptors are already in preformed complexes before activation of the signalling pathway (Bücherl et al. 2013). Via these interactions, the co-receptors could influence the distribution of the main receptor in the PM. To determine whether the co-receptor affects the main receptor distribution, VAEM was employed of BRI1-GFP in a *serk1 serk3* mutant. The results show that the absence of these co-receptors did not influence the main receptor distribution, and overall fluorescence intensity does not differ from the normal BRI1-GFP line 2 roots (see Figure 2D). The roots of a *serk1serk3* double mutant are almost completely insensitive to the BL ligand, indicating that no active signalling

complexes are made in these roots. This is an indication that the SERK co-receptors do not have a role in maintaining the PM distribution of BRI1.

By combining the BRI1 and SERK3 PM receptor density data from van Esse et al. (2011) with the number of nanoclusters per μm^2 PM, an estimate of the number of receptors present in the clusters can be obtained (see Table 1). For BRI1, it appears that at near endogenous receptor level there are approximately 6 fluorescent receptors in each nanocluster. Intriguingly, upon overexpressing the BRI1 receptor approximately three times, each nanocluster now accommodates more BRI1 receptors whereas the nanocluster density appears to be somewhat lower. In the SERK3-GFP line there are about 50 percent more nanoclusters but these contain only two receptors each. A similar calculation for the SERK3-mCherry line results in about 1 ± 1 receptors per nanocluster, but these measurements appear less accurate compared to the GFP-tagged versions. The number of receptors per cluster is in line with what can be estimated from the fluorescence decay curves of the clusters, that show only few typical single molecule step-wise decay events and mostly a mixture (see Supplemental File S2).

Table 1 quantification of receptors in nanoclusters

Given are average number of receptors (column one, (van Esse et al. 2011) or nanoclusters (column two) per μm^2. From these numbers, the average number of receptor per nanocluster is calculated. Numbers are given with their respective standard error of the mean (SEM). For each experiment, at least three movies were recorded of three different roots (n=9)

Plant line	receptor . μm^{-2} PM	nanoclusters . μm^{-2} PM	no. of receptors per nanocluster
BRI- GFP line 1	12 ± 1	2 ± 0.4 (n=9)	6 ± 1
BRI-GFP line 2	34 ± 3	1.5 ± 0.4 (n=9)	22 ± 4
SERK3-GFP	5 ± 1	3 ± 0.4 (n=11)	2 ± 1

n= number of individual measurements
Values are given ±SEM

To rule out that the observed nanocluster distribution pattern was an inherent property of only these two receptors, a third plant receptor, BIR3, was investigated as well as an unrelated membrane protein, PIN2 (Friml 2010). BIR3 is an abundant PM receptor-like kinase for which the related members BIR1 (Gao et al. 2009) and BIR2 (Halter et al. 2014) have been implicated as stabilising components of PM receptor complexes involved in PTI. Also BIR3 is distributed in an inhomogenous fashion, similar to BRI1 and SERK3, suggesting that a distribution into nanoclusters is a common configuration for plant membrane receptors (see Supplemental Figure S3).

When viewing recordings of the VAEM images it appeared that different patterns of movement were present ranging from almost static to rapid directional and seemingly more random patterns (see Supplemental files online). Comparing

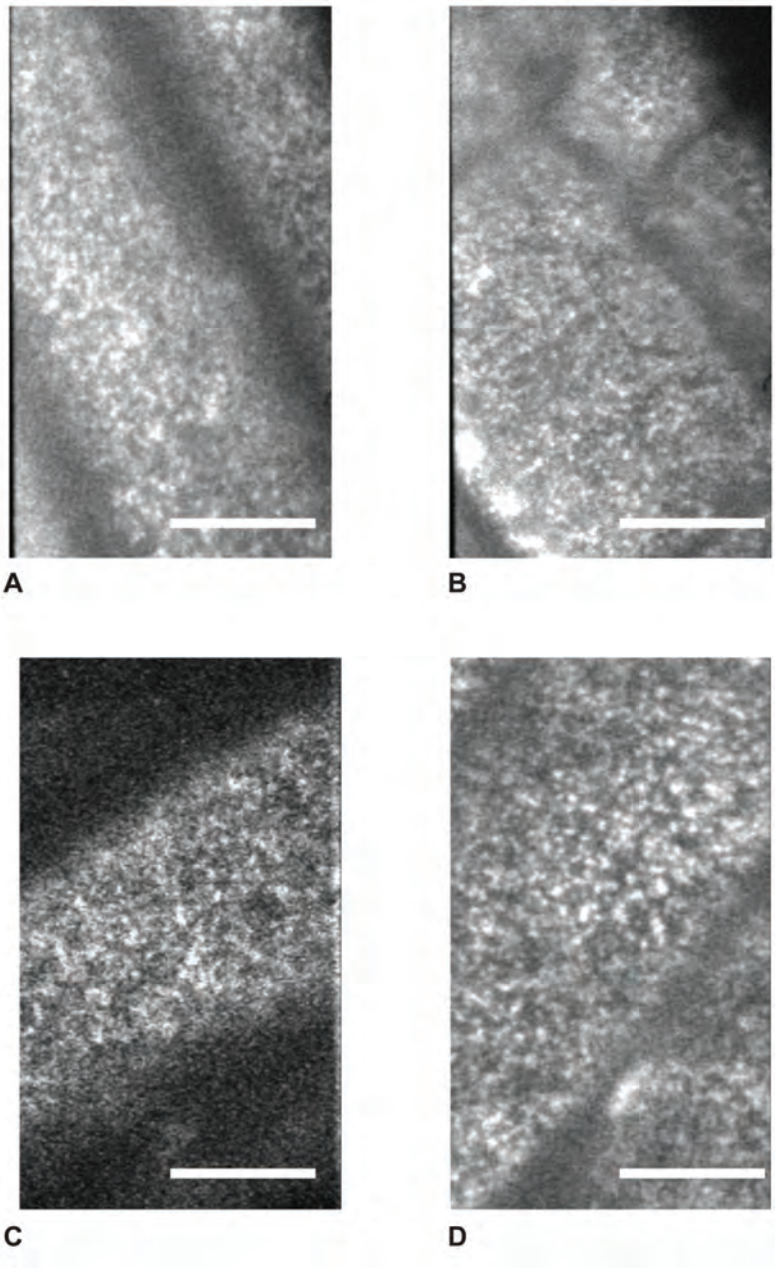


Figure 2 PM distribution of BRI1-GFP and SERK3-mCherry
VAEM images of live root cell in 6 day old A. thaliana seedlings showing PM distribution of **(A)** BRI-GFP line1, **(B)** BRI1-GFP line 2 and **(C)** SERK3-mCherry. **(D)** BRI1-GFP in a *serk1serk3* mutant plant. Images taken are of epidermal root cells in the early elongation zone. Scale bars represent 10 μm.

these patterns of movement with those of the markers employed above, it appeared that rapid movements were associated with intracellular membrane compartments rather than with the PM. Because VAEM as used here with fluorescent dyes is not reliable for comparing fast-moving objects differing in fluorescent intensity, Fluorescence Recovery after Photobleaching (FRAP) was employed. For BRI1-GFP and SERK3-GFP diffusion coefficients were noted of approximately 0.1 $\mu m^2 * s^{-1}$ and a mobile fraction between 35 and 60 %. These values are far below the value of 10 $\mu m^2 * s^{-1}$, determined for free lateral diffusion of a PM receptor (Meissner et al. 2003; Bacia et al. 2006) and of the KNOLLE-GFP control (see Figure 3). In the meristem cells the diffusion coefficients are even lower, rendering both receptors virtually immobile when compared to protoplasts (see Table 2). Further analysis

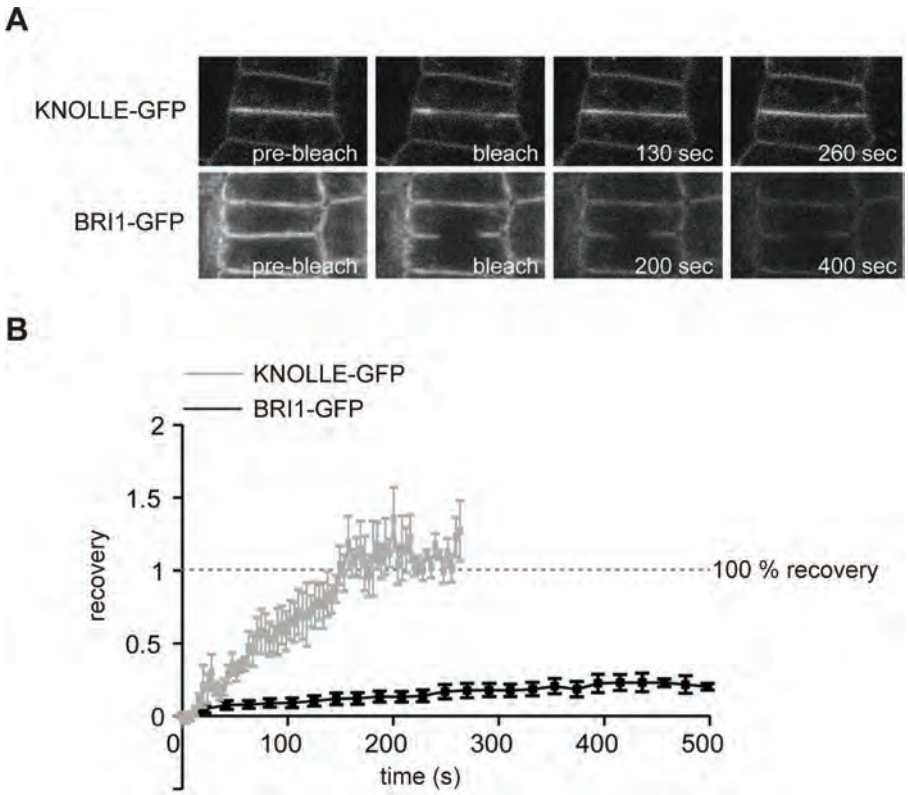


Figure 3 FRAP analysis of BRI-GFP and KNOLLE-GFP in epidermal root meristem
(A) 260 seconds after bleaching, KNOLLE-GFP fluorescence intensity at the PM is restored. No such recovery is observed for BRI1-GFP, which was imaged up to 400 seconds after bleaching the PM (only the bleached area is analysed,) **(B)** Recovery-curves of KNOLLE-GFP (blue line) and BRI1-GFP (black line) in epidermal cells in the root meristem. For KNOLLE-GFP n=7, for BRI1-GFP n=15, measured in independent replicas, error bars indicate standard error of means (SEM). Error bars ± SEM.

employing Gaussian fits suggest that most of the observed mobility in the elongation zones is due to replenishment from internal receptor pools (see Supplemental File 3). Taken together, VAEM and FRAP data suggest that both BRI1 and SERK3 receptors are distributed in PM nanoclusters that are largely immobile and contain a small number of individual receptors.

Table 2 Mobile fractions and diffusion coefficients of BRI1 and SERK3

	Tissue	Mf (%)	DC μm^2*s-1	N	Reference
BRI1-GFP	Protoplasts	70 ± 17	2.2 ± 0.7		Kwaaitaal et al. (2011)
	Meristem	28 ± 2	0.009 ± 0.002	15	van Esse, 2013
	Elongation zone	35 ± 3	0.11 ± 0.04	15	van Esse, 2013
SERK3-GFP	Protoplasts	78 ± 5	0.55 ± 0.07	4	van Esse, 2013
	Meristem	78 ± 3	0.0009 ± 0.002	5	van Esse, 2013
	Elongation zone	58 ± 2	0.08 ± 0.02	5	van Esse, 2013

N= number of individual measurements.
Mf= mobile fraction
DC-= diffusion coefficients
Values are given ±SEM

BRI1 PM-distribution is not altered upon signal activation or absence of endogenous ligand

To investigate whether receptor distribution across the PM is influenced by activation of the signalling pathway, seedlings were first depleted of endogenous ligand by incubation with brassinazole (BRZ), a brassinosteroid synthesis inhibitor (Asami et al. 2000). Prior to VAEM, seedlings were incubated with 1 μM 24 epi-brassinolide (BL), a biologically active brassinosteroid, for 1 h. This treatment is routinely used to fully activate the brassinosteroid signalling pathway and calculated to achieve a near maximal receptor-ligand occupancy (van Esse et al. 2012). Interestingly, under these conditions of full activation, only a minor amount of BRI1 and SERK3 was found to interact (Bücherl et al. 2013). It was therefore of great interest to apply this mode of full activation to see whether any change was visible in nanocluster distribution or amount using VAEM. Surprisingly, for both BRI1-GFP and SERK3-mCherry no significant change in the amount of clusters per μm^2 PM or cluster size could be detected between the BRZ treated roots and the BRZ +BL treated roots (see Table 3 and Supplemental Figure S2). The SERK3-mCherry expressing roots showed a slight decrease in the total number of clusters, presumably due to BRZ treatment. BRI1-GFP distribution was also not affected in the brassinosteroid synthesis mutant *det*2 (see Supplemental Figure S2), which contains less than 10 % of the normal WT levels of brassinosteroid (Fujioka et al. 1997). Taken together these data indicate that compared to the normal wild-type situation of untreated roots, neither the absence nor the exogenous application of BRs substantially affects the BRI1 and SERK3 PM nanocluster distribution.

Table 3 Quantification of clusters/ μm2 and cluster size of BRI1 and SERK3 upon ligand application

Given are average number of clusters per μm^2 and average cluster size in μm^2 upon different treatments. 5 μM BRZ was added to the growth media for 3 days prior to ligand stimulation. Ligand stimulation was accomplished by incubation of seedlings with 1 μM 24- epi-brassinolide (BL) for 1h. All experiments were performed twice, three images were collected for three seedlings per experiment (n=9). Numbers are given with their respective pooled standard error of the mean (SEM).

		BRZ	BL after BRZ pre-treatment	No treatment
BRI1-GFP line 1	clusters/ μm^2	2.4 ± 0.2	2.2 ± 0.1	2.3 ± 0.1
	cluster size (μm^2)	0.29 ± 0.04	0.31 ± 0.04	0.29 ± 0.02
BRI1-GFP line 2	clusters/ μm^2	nd	Nd	nd
	cluster size (μm^2)	0.45 ± 0.06	0.49 ± 0.06	0.41 ± 0.05
SERK3-mCherry	clusters/ μm^2	5.1 ± 0.5	5.8 ± 0.3	8.0 ± 0.5
	cluster size (μm^2)	0.12 ± 0.02	0.08 ± 0.03	0.13 ± 0.02

BRI1 and SERK3/BAK1 distribution is influenced by tyrphostin A23

All three integral membrane receptors investigated in this study, BRI1, SERK3 and BIR3, showed the same nanocluster distribution, which was not influenced by ligand induced activation. This suggests that the observed distribution is not coupled to the biological function of these receptors, but might have a more structural origin. To further investigate the underlying mechanisms causal to the observed nanoclusters, the receptor distribution of SERK3 and BRI1 was visualised after treatment with various inhibitors of the endocytic, cytoskeleton or biosynthetic pathways. Treatment of BRI1-GFP and SERK3-mCherry seedlings with the protein synthesis inhibitor cycloheximide, latrunculin B or brefeldin A either alone or in combination with BRZ or with BRZ and BL did not result in any consistent change in distribution of either receptor (data not shown). In particular, the absence of clear effects on nanocluster distribution of actin-depolylarizing agent such as latrunculin B is puzzling. The only compound that gave a more consistent result was tyrphostin A23, a tyrosine kinase inhibitor. Tyrphostin A23 is thought to both interfere with endocytosis (Ortiz-Zapater et al. 2006; Dhonukshe et al. 2007) and with Golgi-based secretion of proteins (Li et al. 2012). Tyrphostin A23 inhibits endocytosis of BRI1-GFP bound to fluorescent ligand, and in this way increases BR signalling (Irani et al. 2012). However, tyrphostin A23 also seemed to increase the intracellular amount of BRI, perhaps due to inhibition of Golgi-based secretion. To see whether interference with the normal endocytic route would alter the receptor distribution on the membrane, BRI1-GFP and SERK3-mCherry were visualised on the PM in seedlings treated with tyrphostin A23 or the inactive variant tyrphostin A51. As can be seen in Figure 4, the overall PM fluorescence diminishes for both receptors, causing the clustering to be less pronounced. However, both receptors are still present in their nanocluster arrangement. Unfortunately, reliable quantification of the SERK3-mCherry clusters was not possible due to the decreased

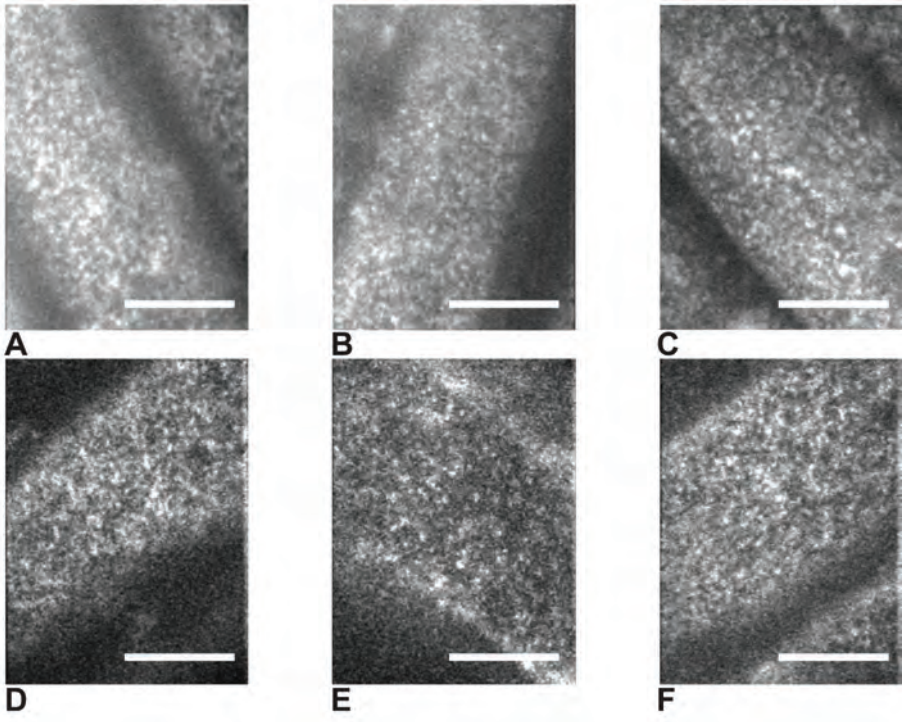


Figure 4 Receptor distribution upon tyrphostin application
(A) BRI1-GFP 1 PM distribution in untreated seedlings (left), seedlings treated with 50 µM tyrphostin A23 for 1 h **(B)** and seedlings treated with 50 µM tyrphostin A51 **(C)**. **(D)** SERK3-mCherry PM distribution in untreated seedlings, seedlings treated with 50 µM tyrphostin A23 for 1 h **(E)** and seedlings treated with 50 µM tyrphostin A51 **(F)**. Receptor clustering is less pronounced after tyrphostin A23 treatment because overall fluorescence intensity appears diminished. Scale bars represent 10 µm.

Table 4 Quantification of clusters per μm^2 of BRI1 upon tyrphostinA23 application
Given are average numbers of clusters per μm^2 observed in the respective plant lines. Numbers are given with their respective standard error of the mean (SEM). The number of clusters does not significantly differ between the treatments, as assessed via a Student's t-test.

	tyrphostin A23		tyrphostin A51	
	+BL	-BL	+BL	-BL
Bri1-GFP line1	2.2 ±0.1 (n=14)	2.5 ± 0.2 (n=17)	2.7 ±0.2 (n=8)	3.0 ± 0.2 (n=12)

n= number of individual measurements

fluorescence intensity. Quantification of the clusters in the tyrphostin A23 treated BRI1-GFP roots indicated that the number of clusters per μm^2 was not significantly affected by the treatment, neither in the presence, nor in the absence of the brassinosteroid ligand BL (see Table 4). Thus, although the amount of PM located BRI1 receptors did change upon tyrphostin treatment, it appeared that the nanocluster density was not affected.

Discussion

Our results using Variable Angle Epifluorescence Microscopy (VAEM) to visualise SERK3 and BRI1 plasma membrane (PM) distribution clearly demonstrate that these receptors are arranged in nanoclusters in the PM of *Arabidopsis* root cells.

Cluster formation of receptors has been observed more often; for instance the Epidermal Growth Factor Receptor (EGFR) is present in oligmeric clusters in the membrane (Clayton 2005). These clusters consist of approximately 2 receptors in an unstimulated situation, which is in the same range as our results for the brassinosteroid receptor BRI1 and its coreceptor SERK3. For EGFR, the nanocluster distribution is thought to be coupled to the biological activity of the receptor (Ariotti 2010), and a marked increase in the number of receptors per cluster is observed upon ligand binding (from 2.2 to 3.7 receptors per nanocluster; Clayton, 2005). However, no change in distribution was observed for either SERK3 or BRI1 upon ligand application, or depletion of endogenous ligand. In addition, the fact that a third PM receptor, BIR3, is also observed in a nanocluster distribution, suggests that the formation of nanoclusters is a general feature of plant receptors.

Receptors that are arranged in nanoclusters are considered to be part of larger arrangements of signalling proteins (Clayton et al. 2005). In addition, the stoichiometry between the different components can be altered without affecting the arrangement (Plowman et al. 2008). Similar observations were made here; when comparing the PM of two BRI1-GFP lines, that differed about 3-fold in receptor density while retaining the same nanocluster density. Also, a decrease in PM localised BRI1-GFP by tyrphostin A23, did not change the nanocluster density. Changing the receptor stoichiometry within the confinements of the nanoclusters could be a mechanism of the plant cell to regulate signalling output, especially in the situation of SERK3, which is part of different signalling complexes in the same cell (Robatzek et al. 2006; Savaldi-Goldstein et al. 2007).

Using FRAP, the BRI1-GFP nanoclusters appeared largely immobile. As in the case of EGFR, animal receptor nanoclusters are thought to be confined by the cortical actin filament network and cholesterol rich domains (Orr et al. 2005). However, for BRI1 and SERK3, treatment with Latrunculin B (an actin depolymerising agent) did not result in consistent changes in nanocluster distribution. Lateral diffusion of plant receptors is thought to be restricted directly or indirectly by the presence

of the plant cell wall (Martinière et al., 2012). Restricted diffusion of receptor proteins due to physical barriers from, for instance, the underlying cytoskeleton, or, in the case of plant cells, the cell wall, could induce clustering (Jaqaman and Grinstein 2012). The classical model for PM receptor activation assumes ligand-induced endocytosis. This could involve removal of entire nanoclusters from the PM or a change in their stoichiometry. In the BRI1-GFP and SERK3-mCherry VAEM recordings both events were observed. Due to the absence of a clear link with ligand availability this was not investigated further at this time.

Given the observed restrictions in lateral movement, it is unlikely that BRI1 nanoclusters are formed after arrival of the proteins at the PM. At present, it is unknown where plant PM receptor nanocluster assembly takes place. One way in which this could be accomplished is via the preformation of the higher order signalling complexes, inserted in their respective position in the membrane as a fully assembled unit. Preformation of complexes has been observed for BRI1 and SERK3 (Bücherl, 2013), corroborating this idea. It would be of great interest to investigate whether these proteins are indeed inserted in the membrane together, or whether minor mobility within the confinements of the clusters is sufficient to form receptor complexes after insertion in the membrane. A combination of VAEM and FRET might indicate the location of preformed and ligand induced complexes between main receptor and co-receptors in respect to the nanoclusters, and could provide interesting answers into the dynamics of BR signal activation within the complex confinements of the plant PM and cell wall.

The receptor clusters observed with VAEM in this study were rather big; 300-500 nm across, which does not seem to correspond to the 2-6 receptor molecules which were calculated to be part of these clusters. Most likely this is due to the diffraction limit of the technique used. The VAEM technique can greatly increase resolution due to selective illumination of a thin surface layer; however, on its own it does not allow going below the diffraction limit caused by the Gaussian distribution of fluorescence. Since the expected cluster size of the receptors is probably below the diffraction limit, the use of VAEM is not sufficient to investigate the clusters size in detail. It can thus be expected that the 300- 500 nm clusters observed in this study is an over-estimate of the actual receptor cluster size. To further investigate the clustering of BRI1 and SERK3, optical super-resolution techniques, namely photo-activated localisation microscopy (PALM), should be employed in combination with VAEM. However, this report clearly shows that VAEM can be used in combination with live *A. thaliana* root cells to visualise both the PM and the underlying cellular compartments and clearly demonstrates the nanocluster formation of several plant membrane proteins.

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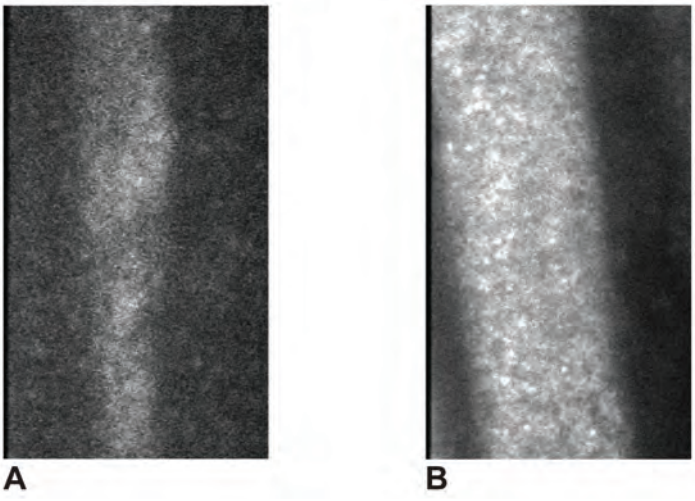
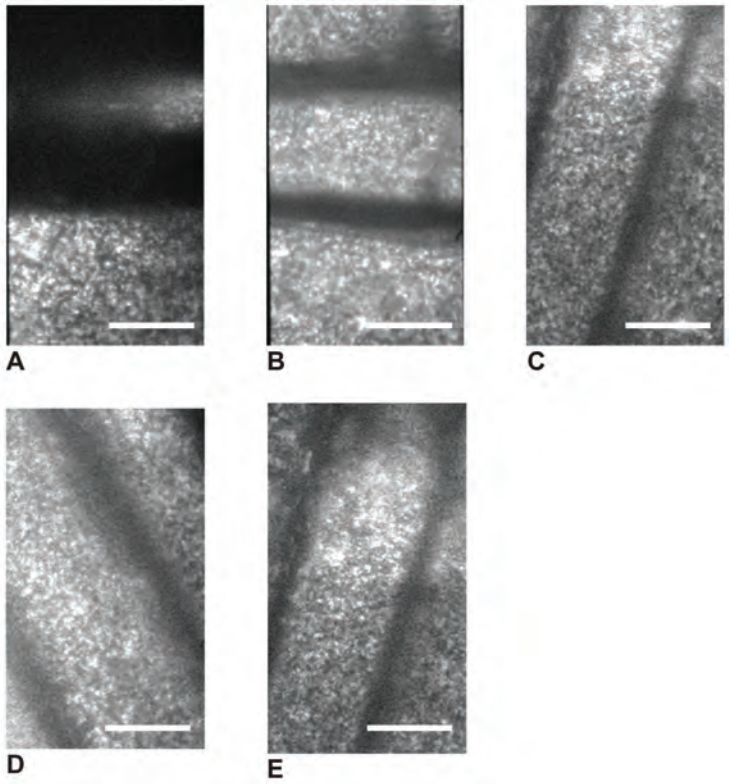
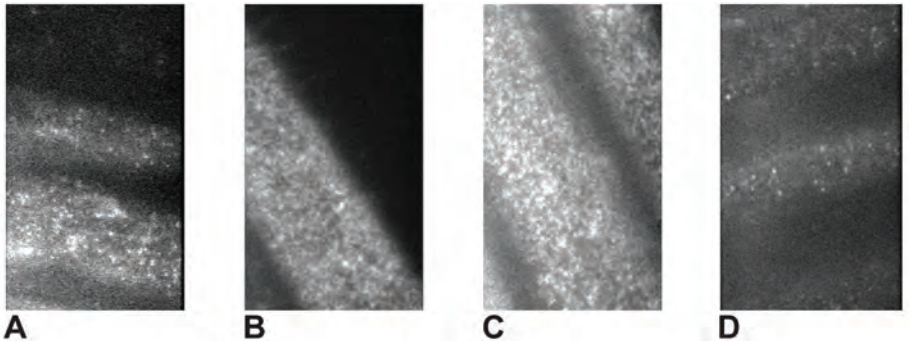
Supplemental Figures and Files



Supplemental Figure S1 PM distribution of BRI1-GFP using full TIRF and VAEM.
(A) Full TIRF image of BRI1- GFP line 1; **(B)** VAEM image of the same root at the same place as in A.



Supplemental Figure S2 BRI1 distribution upon signal activation and ligand depletion.
Live root cells of BRI1-GFP untreated seedlings **(A)**, treated for 3 days with 5 μM brassinazole,**(B)** and BRZ treated seedling roots incubated with 1 μM 24-epi-brassinolide for 1 h . Live root cells of BRI1-GFP untreated seedlings **(D)**. BRI1-GFP in live root cells of the det2 mutant **(E)**.



Supplemental Figure S3 PM distribution of PIN2-GFP, BIR3-GFP compared with BRI1-GFP and wild-type controls using VAEM.
Live root cells of PIN2-GFP seedlings **(A)**, BIR-GFP seedlings **(B)**, BRI1-GFP seedlings **(C)** and Col[0] seedlings **(D)**.

Supplemental File S1 FRAP data analysis

Analysis of the FRAP data was done essentially as described previously (Kwaaitaal et al., 2011). All fluorescence intensities were recorded in arbitrary units (a.u.) and corrected for autofluorescence in the wild type control according to:

$$I_{norm,t} = \frac{I_t - I_{background}}{I_{t,scanbleach} - I_{background} / I_{0,scanbleach} - I_{0,background}}$$

where $I_{norm,t}$ is the normalised fluorescence intensity at time t, I_t is total intensity at time t, $I_{background}$ is the intensity in the wild type control, $I_{t,scanbleach}$ is the signal level of a spot of the same size as the bleached area for measuring the scan-bleaching, $I_{0,scanbleach}$ is the average signal of the scan-bleaching before the bleaching procedure, and $I_{0,background}$ is the average signal of the background before bleaching. The normalised fluorescence intensities were calculated according to:

$$I_{data,norm} = \frac{I_{norm,t} - I_{norm,bleach}}{I_{norm,0} - I_{norm,bleach}}$$

where $I_{data,norm}$ is the calculated normalised intensity, $I_{norm,bleach}$ is the normalised intensity just after the bleach, and $I_{norm,0}$ is the average normalised intensity before bleaching.

The mobile fraction is defined as:

$$F_m = \frac{I_{norm,end} - I_{norm,bleach}}{I_{norm,0} - I_{norm,bleach}}$$

Where $I_{norm,end}$ is the normalised intensity after full recovery. The recovery curve can be fitted for a molecule undergoing two-dimensional diffusion to the next formula:

$$I(t) = F_m \times \left(1 - e^{-kt}\right)$$

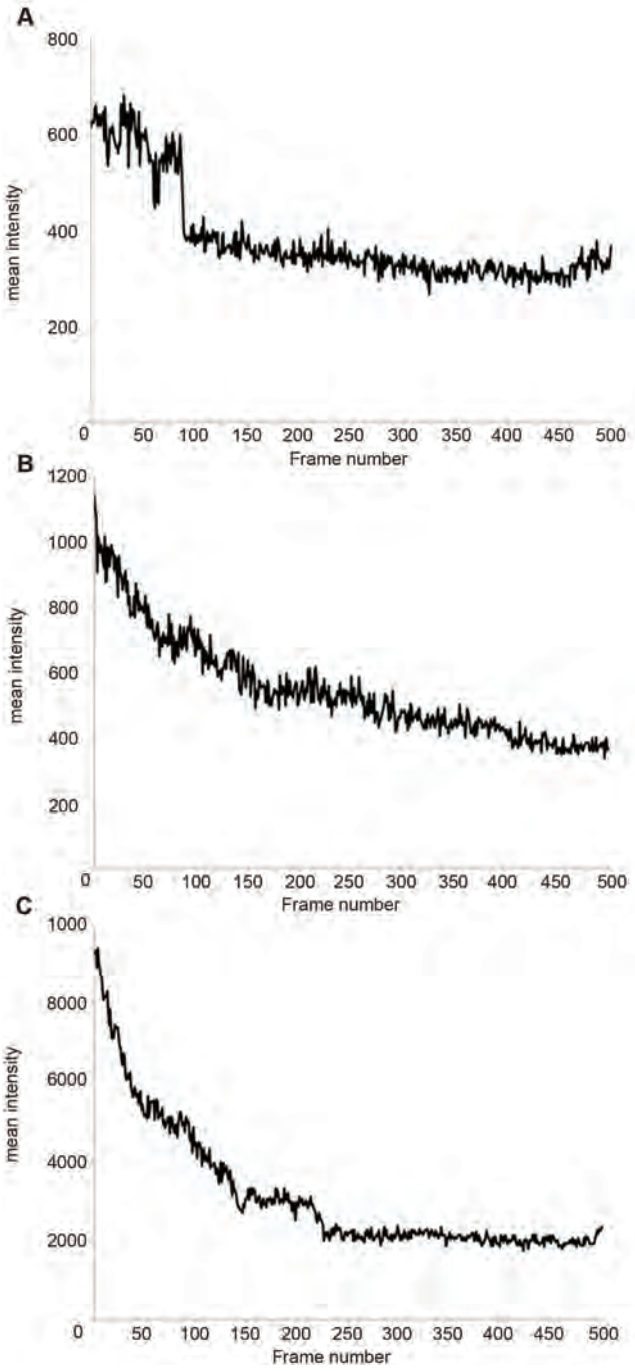
where k is the recovery constant, this is related to the half-time of diffusion as follows:

$$t_{1/2} = \ln(2) / k$$

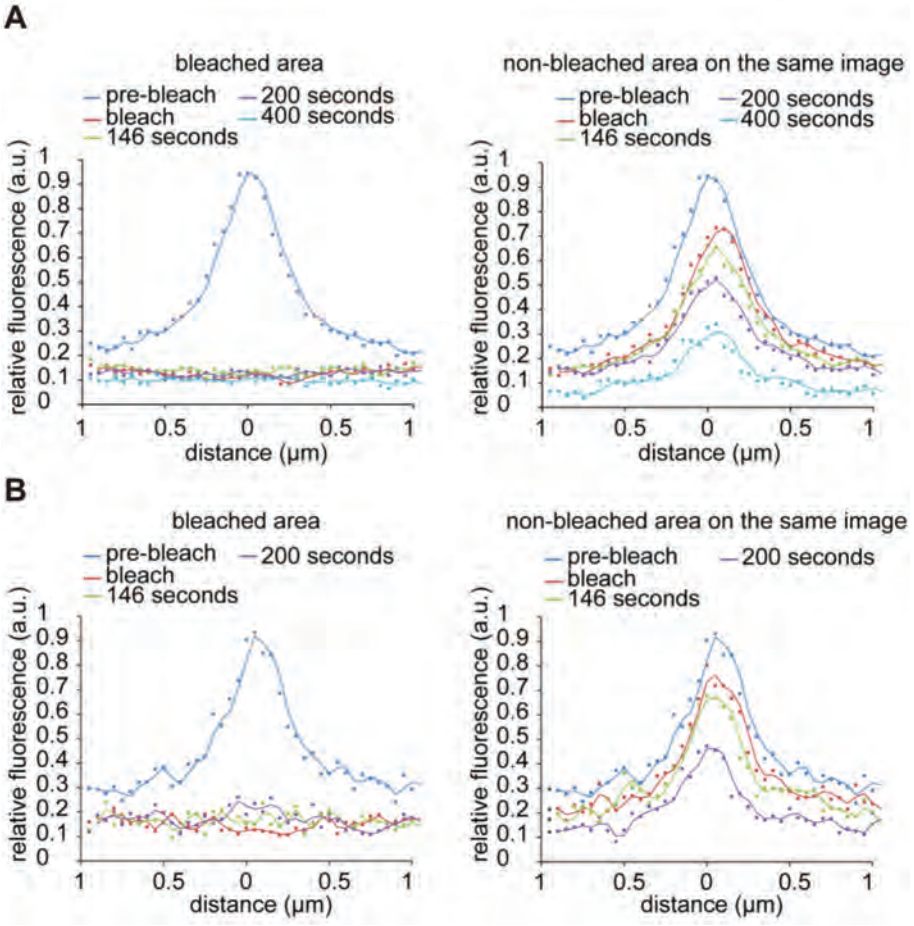
Supplemental File S1 Continued

Assuming that there is an unrestricted two-dimensional diffusion the diffusion coefficient, D_c, is related to the half-time of diffusion, as:

$$D_c = \frac{A}{4 \times t_{1/2}} [\mu m/s^2]$$



Supplemental File S2
VAEM fluorescence decay curves.
(A) Single molecule decay curve of SERK3-mCherry. **(B)** Representative decay curve of SERK3-mCherry. **(C)** Representative decay curve of BRI1-GFP.
For both receptors, discreet steps in fluorescence decay are observed, indicating that the number of receptors in the cluster is limited.



Supplemental File 3 Gaussian fits of receptor molecules in PM surrounding the anticlinal cell wall before and after photobleaching.
The line represents the fit of a Gaussian distribution on the fluorescence intensity data across the anticlinal cell wall. Distance 0 is the midpoint of two adjacent plasma membranes in a confocal image. The cytoplasm is situated between 1-05 μm on either side of the midpoint. **(A)** Fluorescence intensity of BRI1-GFP at the bleached area (left) compared to the intensity at a non-bleached area of the PM (right). After 400 seconds, the fluorescence intensity at the non-bleached area (right panel) was reduced significantly due to scan bleaching. **(B)** Same as A, except now for SERK3-GFP. N=5 different roots; 20 fits per image (n ≥100 data points).

Chapter 6

Summarising Discussion

M. aan den Toorn

The transduction of extracellular signals to intracellular responses is of vital importance for all living organisms. The perception of signalling molecules outside the cell is often accomplished via ligand binding proteins spanning the plasma membrane, i.e. receptor proteins. In plants, the majority of plasma membrane receptors are part of the family of leucine-rich-repeat receptor-like-kinases (LRR-RLKs) (Shiu and Bleecker 2001a). These receptors contain an extracellular LRR domain, coupled to an intracellular kinase domain via a single pass transmembrane helix. The plant RLKs and their animal counterparts, the receptor-tyrosine-kinases (RTKs), are thought to form dimers or oligomers to make activation of their cytoplasmic kinase domains possible. Sometimes, additional membrane spanning proteins are part of these higher order complexes. If so, we can distinguish between the main ligand binding receptor, which contains both the domains to perceive signal and to confer this across the membrane to intracellular reactions and co-receptors (or auxiliary receptors), which on their own cannot perceive the signal or transduce the signal into the cell. The somatic embryogenesis receptor kinases (SERKs) are a family of co-receptors found in plants and show multitasking functions in several, seemingly unrelated, pathways (Albrecht et al. 2005, Albrecht et al. 2008, Chinchilla et al. 2009, Roux et al. 2011). Except for SERK5, that has no reported function at this time, the SERK genes in *Arabidopsis thaliana* show partial redundancy, with each SERK functioning in its own specific subset of signalling pathways.

In this thesis I have taken a closer look at the SERK family of receptor kinases to understand their role as co-receptors in plant signalling cascades and the regulation of their activity. Studies on animal signalling indicate that co-receptors can have many different functions in signalling complexes and form an additional layer of regulation in signalling cascades, which I have discussed in more detail in the introductory **Chapter 1**. Also in this chapter, I have reviewed the current crystal structures available on plant leucine-rich-repeat receptor-like-kinases (Santiago et al. 2013, Sun et al. 2013). These structures provide us with the first clues into the molecular basis of plant RLK function. Interestingly, the current crystal structures of SERK extracellular domains indicate that these co-receptors have interactions not only with the main ligand binding receptor, but also with the different ligands. The SERK residues that are implicated in ligand interaction are conserved in the SERK protein family, so this new evidence does not seem to be able to explain the functional divergence observed between the SERK family members. Current structural, biochemical and genetic evidence define the *A. thaliana* SERK proteins as true co-receptors, part of active signalling complexes via interaction with different main ligand binding receptors (Albrecht et al. 2012, Gou et al. 2012, Santiago et al. 2013, Sun et al. 2013).

The wide functional diversity seen between different members of the SERK protein family must be inherent to differences in protein sequence. In some ways, this is rather counter-intuitive, because the SERK proteins are very homologous

and show only minor changes. In **Chapter 2,** I pinpoint which residues/motifs or domains are important for the different functions of the SERKs. A sequence comparison with SERK sequences from different plant species showed that SERK genes are ancient, present already with multiple copies in genomes of non-vascular plants (e.g. the moss *Physcomitrella patens*). The SERKs present in these evolutionary older genomes closely resemble the *A. thaliana* SERK1 and 2 sequences; apparently the SERKs have not changed much throughout 2 M years of evolution. This is a strong indication that SERKs are indeed essential for important plant signalling pathways. The *A. thaliana* SERK3 and 4 genes, however, have diverged more in protein sequence and belong to a SERK-clade only present in dicotyledons. This divergence is probably linked to different functionality of these proteins. Chimeric SERK proteins, in which the extracellular domain of one SERK is coupled to the cytoplasmic domain of another, show that SERK specificity is rather complex and cannot be attributed to any one residue, motif or even domain.

To further investigate the functional plasticity of SERKs, we aimed to obtain additional high-resolution structural data on these co-receptors. In recent years, the first crystal structures of plant LRR-RLK domains have been reported. These include the kinase domain of SERK3/ BAK1, the extracellular domain of SERK1 in complex with that of the main receptor BRI1 (Brassinosteroid Insensitive 1) and of SERK3 with the main receptor FLS2 (Flagellin Sensing 2). These structures have given important clues into the function of SERK co-receptors. High-resolution structural data on other SERKs could provide insights into the functional plasticity and specificity observed in the SERK protein family. In **Chapter 3** I describe our attempts to solve the crystal structures of the kinase domains of different *A. thaliana* SERK proteins. This was hampered by low protein production and difficulties in purification. We managed to obtain two crystals of the SERK1 kinase domain, which diffracted to around 2.5 Å resolution. However, structure elucidation was not possible.

Besides structural information, biochemical investigations into receptor activation mechanisms are essential and in **Chapter 4** I used a combination of spectroscopic data and activity assays to understand more of the regulation of activity of the SERK kinase domain. Co-receptors can modulate signalling activity, but only if their activity is also tightly regulated to avoid aberrant activation of the entire receptor complex. In this thesis, and in other reports, it has been shown that kinase domains of receptors and co-receptors are intrinsically active when produced *in vitro*. This means that mechanisms need to be in place *in vivo* to keep kinase domains in their inactive state. In **Chapter 4** we investigated one such way of keeping proteins inactive, namely intrinsic disorder. In this manner, proteins are kept disordered, or unfolded/unstructured, until their activity is needed. Folding, or structuring, can subsequently be induced when the protein is at its proper location or in the proper complex conformation. Our results show that the SERK1 kinase domain has the ability to refold itself to an active kinase domain *in vitro,*

thus in the absence of any cellular components. About 80% of the kinase activity is regained after only 30 minutes of refolding. We conclude that the SERK1 kinase is not only intrinsically active, but folds efficiently to its active state. Therefore it seems unlikely that intrinsic disorder is a manner in which the phosphorylation activity of the kinase domain is regulated.

Protein function is closely coupled to its location in the cell. SERKs, as RLKs, are located at the plasma membrane (PM), which seems to be the major site for signalling. The PM is not homogenous and many proteins do not display a free diffusion through this lipid bilayer. In **Chapter 5** the distribution of the SERK3/ BAK1 co-receptor and BRI1 main receptor on the plasma membrane is shown, using Variable Angle Epifluorecence Microscopy (VAEM), a technique that makes use of the difference in refractive index between the sample and cover slide in order to create an evanescence wave of excitation light. This evanescence wave only penetrates the sample to a certain depth (depending on the angle of the laser) and thus excites only those fluorophores in close proximity to the cover slide. The technique makes it possible to visualise fluorescent proteins with high resolution and low background signal in the plane of the plasma membrane or just below. In this chapter we show that this technique can be used to visualise the PM of *A. thaliana* live epidermal root cells, and several intracellular membrane compartments in the cytoplasm just below the PM. In addition, VAEM was used to show that SERK3/ BAK1 and BRI1 are not homogenously distributed across the PM, but in fact are present in nanoclusters. These nanoclusters are not influenced by addition or depletion of ligand, and BRI1 is still present in clusters in the absence of SERK1 and SERK3/ BAK1. Overexpression of BRI1, or diminishing the number of PM localised BRI1 receptors by tyrphostin A23 treatment, influenced the number of receptors per cluster, but not the cluster density. We propose that the nanocluster distribution in the PM is a general feature of plant PM receptors.

In summary, this thesis provides new insights in the role of SERKs as co-receptors, their origin, regulation and localisation. A combination of different techniques, biochemical, molecular, bioinformatics and structural, was used to understand how SERKs function as co-receptors in plant signalling complexes.

General discussion

Constant interaction to environmental changes by cellular responses is of utmost importance for the survival of any organism. This is illustrated by the fact that as many as 10% of the proteins in mammalian cells are involved in signal transduction (Good et al. 2011). As the cell is bombarded at any given time by numerous signals to which response is needed, efficient signalling is only possible if the proteins and substrates of the signalling cascade or tightly regulated in their spatiotemporal

organisation (Scott and Pawson 2009). The current view is that cells achieve their specific output to extracellular signalling, by organising discreet subsets of signalling components together in space and time (Good et al. 2011) providing the cell with the specificity and sensitivity to correctly respond to changes in its environment. In multicellular organisms, this response must take into account not only the signal, but also the response of the neighbouring cells in the tissue. Thus, complex mechanisms must be in place to tailor the cellular responses to the same ligand for different tissues or developmental stages.

Response to an extracellular signal starts with the perception of this signal by a receptor molecule, which relays this signal via a cascade of second messengers to a final cellular response (such as increased transcription, or altered enzyme activity). To tailor the response to the signal, a ligand binding receptor can form higher order complexes, or signalosomes, of distinct composition (Rub et al. 2009). In this manner, the association of ligand binding receptors with different co-receptor molecules (receptors which by themselves cannot perceive ligand or transduce the signal) can provide specificity in the signalling output (Kirkbride et al. 2005). Co-receptors have been reported to influence affinity to ligands, modulate signalling output, change subcellular localisations and create cross talk between different signalling pathways (see Chapter 1).

The SERK co-receptors in *A. thaliana* have been shown to be indispensable players in several plant signalling pathways (Gou et al. 2012; Albrecht et al. 2005). SERK3/ BAK1 transphosphorylation on the Brassinosteroid receptor 1 (BRI1) was found to be essential for full downstream signalling activity (Wang et al. 2008), both SERK3 and SERK1 extracellular domains have been reported to interact with ligand (*in vitro)* (Santiago et al. 2013, Sun et al. 2013), and SERK3/ BAK1 increases the endocytosis of BRI1, while impairing the endocytosis of Flagellin Sensing 2 (FLS2) (Chinchilla et al. 2007; Russinova et al. 2004). Although these studies provide much insight in the roles the SERK co-receptors play within plant signalling complexes, not much is known on the mechanisms underlying SERK specific functions. The SERK protein family in *Arabidopsis* consists of five highly homologous members, that each function in a specific subset of signalling pathways (Schmidt et al. 1997, Albrecht et al. 2005, Lewis et al. 2010, Li et al. 2002, Nam and Li 2002, Karlova et al. 2006, Chinchilla et al. 2007, He et al. 2007, Heese et al. 2007, Kemmerling et al. 2007, Albrecht et al. 2008, Postel et al. 2010). How these similar proteins function in such different pathways is still puzzling. Also the question why these proteins have been incorporated in so many, seemingly unrelated, pathways has not been addressed. In this thesis, I employed several biochemical and microscopic techniques to investigate the molecular basis of SERK specificity, regulation and function in plant signalling pathways.

Essential roles of SERKs in signalling complexes

The classic concept of signal transduction mediated by single pass transmembrane receptors, such as receptor tyrosine kinases (RTKs), is through ligand induced dimerization, which allows for transphosphorylation between kinase domains (Hubbard and Miller 2007). These transphosphorylation events cause structural changes in the kinase domain which stabilises the active state (Hubbard 2004). The formation of receptor-dimers, or oligomers, is essential for activation of these RTKs; to be able to translate ligand binding at the extracellular domain to activation of cytoplasmic kinase domains, conformational changes in different parts of the receptor is required. An example is the activation of the epidermal growth factor receptor (EGFR). In the absence of ligand, preformed dimers of EGFR exist, but are in the inactivate state (Moriki et al. 2001); ligand binding induces conformational changes leading to activation via asymmetric kinase dimers (Zhang et al. 2006).

The plant counterparts of the animal RTKs are the receptor-like-kinases (RLKs), belonging to the same superfamily of receptor kinases (Shiu and Bleecker 2001b). Examples of RLKs are the brassinosteroid insensitive 1 (BRI1) and flagellin-sensing 2 (FLS2) receptors and the somatic embryogenesis receptor-like-kinases (SERKs). Although knowledge on the dimerization or oligomerization of these receptors is not as extensive as for their animal counterparts, the emerging theme is that complexes exist already prior to ligand binding. Both FLS2 and BRI1 were found to form homodimers in absence of ligand (Wang et al. 2005, Gendron and Wang 2007, Hink et al. 2008, Sun et al. 2012) and preformed complexes have also been observed between SERK3 and BRI1 (Bücherl et al. 2013). However, for both FLS2 and BRI1, ligand binding to their extracellular domains did not induce homodimerization, nor did it induce large conformational changes (Hothorn et al. 2011, Sun et al. 2013). Thus, these *in vitro* experiments provide indications that the 'simple' mode of activation, where ligand binding induces a particular dimer conformation that leads to kinase activation, does not hold true for these receptors. As a consequence, additional players, i.e. the SERK co-receptors, are needed within the active signalling complex. This idea is corroborated by the fact that signalling is completely lost in a plant lacking the necessary members of the SERK family (Albrecht et al. 2005, Gou et al. 2012). However, a major question that still remains is how the SERKs accomplish the activation of the signalling complex. If mere proximity of the SERK kinase domain is needed for activation via transphosphorylation events, preformed complexes between BRI1 and SERK3 would not be expected (as are observed in Bücherl et al. 2013). In addition, this would suggest that the different functions of the different SERK members can be attributed to differences in the cytoplasmic domain. As was shown in Chapter 2, the mechanisms underlying SERK specificity are much more complex, and more than only the cytoplasmic domain is essential for proper SERK function. For this reason, it would be of great interest to elucidate how the (inactive) preformed complexes of SERK3 and BRI1 are acti-

vated and whether this involves asymmetric kinase dimers as in EGFR (Zhang et al. 2006). For this, more structural information (especially of the kinase domain dimers) of these receptor complexes is needed.

Even though the crystal structures of extracellular domain complexes have indicated that the SERKs associate with both the ligand and the extracellular domain of the main ligand binding receptor (Santiago et al. 2013, Sun et al. 2013) the data presented in Chapter 2 demonstrates that also the extracellular domain cannot be the sole determinant for SERK specificity. Moreover, although the crystal structures indicate a role for the SERKs in ligand binding, functional data is still missing to evaluate the importance of these interactions. The presence of SERKs in receptors is essential for signalling (Albrecht et al. 2005, Gou et al. 2012), but whether the SERK-ligand interactions contribute to this, or whether the SERK-main receptor interactions are sufficient, remains to be investigated. As Kinoshita et al. (2005)has calculated the binding affinity of BRI1 for BL in absence and presence of the co-receptor SERK3/ BAK1 and found no difference, the affinity of the SERK co-receptors for the respective ligands might be too low to be of any significance *in planta*. Determining the binding affinity of SERKs for different ligand *in vitro* via, for instance, fluorescent binding assays with fluorescent casasterone (Irani et al. 2012), could be of great interest to evaluate the possible contribution of this interaction to the ligand affinity of the receptor complexes. In addition, it might provide answers to the question why SERK2 can form complexes with BRI1 (although only in protoplasts), but cannot transmit BR signalling (Albrecht et al. 2008, and unpublished data); as perhaps the affinity of the BRI1-SERK2 complex for the BR-ligand is too low for signalling function *in planta*.

Incorporation of SERKs in different signalling complexes

Phylogenetic analysis shows that the SERK co-receptors predate the BRI1 and FLS2 main receptor molecules. This is remarkable, as co-receptors traditionally have not been thought to signal independently of their main signalling receptors. Either another ancestral main receptor (such as an ortholog of EMS1/EXS, the proposed ligand-binding receptor of the SERK1-SERK2 pair that is involved in male sporogenesis; Albrecht et al. 2005) was present in these earlier genomes, or the SERKs have an autonomous function not yet uncovered. An example of a co-receptor that also has autonomous signalling activity is the $p75^{ntr}$ receptor, initially identified as a co-receptor to the Trk tyrosine receptor kinase. $p75^{ntr}$ can directly, and without activity of a main ligand-perceiving receptor, signal in apoptosis, Swann-cell migration and neuronal myelination (Nykjaer et al. 2005). It is not unimaginable that SERKs can transmit signals without the presence of main receptors or can be activated independently of the main signalling pathway using ligand-binding proteins such as MSBP1 (Shi et al. 2011). To elucidate the ancient role of the SERKs, it will be of interest to further investigate the genes that originate from evolutionary

older genomes, such as *Physcomitrella patens,* to determine how they function in modern plants genomes. This may provide insight in how SERKs function, and especially how they have been recruited in the many, seemingly unrelated, signalling pathways and complexes.

Signalling pathways in which SERKs function take place in the same cell often at the same time (Robatzek et al. 2006, Savaldi-Goldstein et al. 2007). This calls for tight regulation on the formation of signalling complexes, as the SERKs need to be divided amongst the different complexes. One way in which this could be accomplished is by the preformation of signalling complexes in the ER or Golgi network, after which they are transported to the plasma membrane (PM) as a full signalling unit. Preformed complexes shortly after biosynthesis have been observed for SERK3 and BRI1 (Bücherl et al. 2013), although only a minor amount. In addition, clear ligand-induced hetero-oligomer formation has been observed between SERK3 and FLS2 (Roux et al. 2011) and SERK3 and BRI (Chapter 2). Whether preformation of complexes alone is sufficient to regulate the division of the co-receptors amongst the different signalling pathways remains to be determined.

In order to have further insight into the organisation of receptors in the PM-located complexes, it was of interest to see how BRI1 and SERK3 are present in the PM. Previous work using confocal microscopy suggested that most of the receptors co-localised in the PM but few physically interacted. Because the BRI1-SERK3 heterodimers were not uniformly present, spatial segregation between active and non-active complexes in the PM appears to be the case (Bücherl et al. 2103). As is seen in Chapter 5, BRI1 and SERK3 are present in nanoclusters in the PM that are almost completely immobile. Therefore it seems unlikely that a large exchange of receptors can occur between clusters before or after ligand binding. Most likely, the clusters already contain all the components needed for the formation of an active signalling complex, which is in line with the almost instantaneous association and transphosphorylation observed between SERK3 and FLS2 upon ligand application (Schulze et al. 2010). However, it is puzzling how this relates to the slow kinetics of heterodimer formation between SERK3 and BRI1 observed after only 30 min with FRET-FLIM (Bücherl et al. 2013) and co-immunoprecipitation (Albrecht et al. 2012). This could suggest that different stoichiometry within the nanoclusters greatly influence the kinetics of complex formation and activation and thereby the timing of signalling output. Nanoclusters can be formed by physical features, such as lipid microdomains or the cytoskeleton (He and Marguet 2011). In plant cells, the ordering in the PM is reported to be caused by the presence of the cell wall (Martinière and Runions 2013), and some indications exists that cluster formation might be coupled to signalling activity (Demir et al. 2013, Wang et al. 2013). However, for BRI1 and SERK3, this remains to be investigated.

Regulation of SERK activity

Being essential components of active signalling complexes, SERK kinase phosphorylation activity needs to be tightly regulated. Protein phosphorylation can be controlled in various ways, such as transcriptional regulation, availability of substrates via recruitment or control of subcellular localisation (Pawson and Scott 1997, Scott and Pawson 2009) or regulation at the protein level, via structural or chemical modifications to the kinase domain (Hubbard and Till 2000). BRI1 activity is regulated via several mechanisms; auto-inhibition mediated by the C-terminal domain (Wang et al. 2005); binding of the inhibitor protein BKI (Wang and Chory 2006), and degradation mediated via dephosphorylation by PP2A (Di Rubbo et al. 2011). For the SERK proteins no such regulatory elements are presently known. As was shown, the SERK1 kinase domain is intrinsically active *in vitro* (Chapter 4 and Karlova et al. 2009) suggesting that inhibition mechanisms must be in place *in vivo*. Regulation of kinase activity employing intramolecular regulatory domains that cause auto-inhibition, as is reported for BRI1 (Wang et al. 2005) can take place in various ways. Intramolecular regulatory domains may keep kinase domains inactive, either by allosteric regulation (binding to sites distinct from the active site, usually stabilising an inactive conformation) or intrasteric regulation (binding in the catalytic site, hindering kinase activity using the specificity and structure of the active site) (Krueger et al. 1995, Kobe and Kemp 1999). These regulatory domains are often found in the regions flanking the kinase domain; the juxtamembrane domain and the C-terminal tail segment. Examples of such regulation mechanisms can be seen for the TIE2 kinase domain, where the C-terminal tail blocks access to the substrate binding site (Shewchuk et al. 2000), or the EphB2 tyrosine kinase receptor, in which the juxtamembrane domain adopts a structure which prevents the formation of the active conformation of the kinase activation loop (Wybenga-Groot et al. 2001). Phosphorylation of specific sites in these auto-regulatory domains is often required for activation of the kinase (Huse and Kuriyan 2002, de Castro et al. 2010). However, several other methods are also reported to release inhibition of intramolecular regulatory domains, such as proteolysis, reduction of disulfide bonds or protein activators or ligands (Kobe and Kemp 1999). Phosphorylation events play a major role in the activation of the signalling pathways involving SERKs, often in a sequential fashion. Initial phosphorylation events of BRI1 on SERK3/BAK1 activate the co-receptor kinase domain, which subsequently transphosphorylates the main receptor on specific sites (Wang et al. 2008). The following phosphorylation of the inhibitor proteins BRI1 Kinase Inhibitor 1 (BKI1) and the BR-signalling kinase 1 (BSK1) by the fully activated BRI1 are thought to be the first steps downstream of the receptor complex (Tang et al. 2008 , Jaillais et al. 2011,). Mathematical modeling indicates that the SERK co-receptors mainly function by increasing the maximal output of the BRI1 main receptor (van Esse et al. 2013). The phosphorylation events in flagellin signalling also occur in strict sequential

fashion; the receptor-like-cytoplasmic kinase BIK1 is likely first phosphorylated by SERK3/BAK1 and subsequently transphosphorylates the FLS2-BAK1/SERK3 complex to propagate flagellin signalling (Lu et al. 2010). These reports clearly indicate that plant signalling pathways employ phosphorylation (both regulated in sequence of phosphorylation and specific residues) to regulate activation. As the sequence analysis in Chapter 2 uncovered that especially the C terminal domain of the SERKs is divergent and less constraint, and because several phosphorylation sites are present in this domain, this could be a very interesting domain for further investigations into regulation of SERK kinase activity.

SERK specificity and redundancy

The *Arabidopsis* SERK proteins have very similar protein sequences, function in specific subsets of signalling pathways and are partially redundant (Albrecht et al. 2005, Albrecht et al. 2008, Roux et al. 2011). The mechanisms underlying SERK functionality and redundancy are therefore rather complex; while most, but not all SERKs are capable of interacting with a main ligand binding receptor, signalling capacity is coupled to the presence of domains specific for a particular SERK family member. For example, as discussed in Chapter 2, the SERK1 and SERK2 cytoplasmic domains are interchangeable in male sporogenesis, while the SERK3 cytoplasmic domain is essential for SERK3 specific functionality in both the BR and PTI pathways. To complete this puzzle, SERK1 and SERK3 are partially redundant in BR signalling. It is presently not clear to which properties of the different proteins these properties can be traced back to. The C-terminal tail is the most divergent region of the cytoplasmic domain and could have an important role. In addition, differences between SERK3 and SERK2 tyrosine phosphorylation sites points could also be of importance. In contrast to the animal receptor tyrosine kinases, plant RLKs were traditionally classified as serine/threonine kinases (Shiu and Bleecker 2001b). However, tyrosine phosphorylation has now been reported for BRI1, SERK1, SERK3 and several other plant RLKs (Shah et al. 2001, Oh et al. 2009; Oh et al. 2011) and is suggested to be as important in plants as in animals (Ghelis 2011). Tyrosine phosphorylation of BIN2 and BKI are essential steps in the BR signalling pathway (Kim et al. 2009, Jaillais et al. 2011). Because of this, it is tempting to suggest that differences in tyrosine phosphorylation sites between the various SERK proteins could be linked to distinct functionalities in the BR, and perhaps also in other, SERK-mediated pathways.

However, initial results using the SERK chimeric proteins indicate that tyrosine phosphorylation cannot be the sole determinant, as the SERK3 kinase domain was not sufficient to confer BR signalling capacity to SERK2. This is markedly different from most main ligand binding receptors, where mostly the cytoplasmic domain is solely responsible for the specificity of downstream signalling (Riedel et al. 1986, Riedel et al. 1989). For instance, a chimer between Nod-factor receptors from *Lotus*

japonicas (involved in rhizobium symbiosis) and chitin receptor from *A. thaliana* (involved in defense signalling) created a receptor that activates the downstream signalling components corresponding to the cytoplasmic receptor domain, when induced with the ligand corresponding to the extracellular receptor domain (Wang et al. 2014). For SERKs, it has been established that their extracellular domain can bind to multiple ligands (Santiago et al. 2013, Sun et al. 2013) and their intracellular domain can phosphorylate multiple associated receptors of different signalling pathways (Schwessinger et al. 2011). This might lead to the assumption that SERKs specificity is solely created by affinity for complex formation and phosphorylation of the main receptor.

In conclusion, of the various functions that coreceptors may have in transducing the signal originating from an activated main ligand-binding receptor, to add specificity, modulate or determine the output, mediating cross-talk, stabilising the multiprotein membrane complex and translocating the main receptor to an internal compartment it appears that in the case of the SERK proteins, only a function in cross-talk (Albrecht et al. 2012) and in receptor translocation (Bücherl et al. 2013) have been ruled out so far. For all other aspects, evidence has been found and in part presented in this thesis of this small class of intriguing Arabidopsis co-receptors.

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Addendum

Dankwoord

List of Publications

Nederlandse samenvatting

Overview of the completed training activities

Dankwoord

Dit is de laatste lege pagina waar ik naar staar. Het voelt nog wat onwerkelijk, maar het staat toch allemaal op papier, de afgelopen vier en een half jaar en in een beetje minder dan 200 pagina's. Het was niet allemaal even gemakkelijk en ik heb dan ook veel mensen te danken voor hun hulp, steun, vriendschap en liefde.

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List of Publications

Aan den Toorn, M., M. M. Huijbers, S. C. de Vries and C. P. van Mierlo (2012). "The Arabidopsis thaliana SERK1 kinase domain spontaneously refolds to an active state in vitro." PloS one **7**(12): e50907.

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Aan den Toorn, M., C. Albrecht, S.C. de Vries. “On the Origin of SERKs- Bioinformatics analysis of the Somatic Embryogenesis Receptor Kinases” Manuscript in preparation

Aan den Toorn, M., S.J. Hutten, G. W. van Esse, A. Nolles, J. Hohlbein, S.C. de Vries. “Visualization of SERK3 and BRI1 Plasma Membrane Distribution using VAEM” Manuscript in preparation

Nederlandse samenvatting

Het kunnen vertalen van een extracellulair signaal naar een corresponderende intracellulaire reactie is essentieel voor alle levende organismen. Het waarnemen van deze extracellulaire signalen, of extracellulaire signaal moleculen, gebeurt over het algemeen via ligand bindende eiwitten die over het plasma membraan heen steken, de zogenaamde receptor eiwitten. In planten vallen de meeste plasma membraan receptor eiwitten onder de familie van 'leucine-rich-repeat receptor-like-kinases' of LRR-RLKs. Deze eiwitten bevatten een extracellulair domein met een leucine rijk motief, gekoppeld aan een intracellulair kinase domein via een enkel trans-membraan helix. Zowel de plant LRR-RLKs als hun dierlijke tegenhangers, de receptor tyrosine kinases (RTKs)worden gedacht dimeren of oligomeren te vormen om op die wijze hun intracellulaire kinase domeinen te activeren. Soms maken ook andere transmembraan eiwitten onderdeel uit van deze receptor complexen. Wanneer dit het geval is, kunnen we onderscheid maken tussen de 'hoofd' receptor, die zowel het domein bevat dat het extracellulaire signaal molecuul kan herkennen, als de mogelijkheid bezit om dit signaal over het membraan heen te kunnen vertalen naar een cellulaire reactie. Daarnaast kunnen er ook nog co-receptoren onderdeel uitmaken van het receptor complex. Deze receptoren kunnen op zichzelf staand óf het signaal niet herkennen, óf dit signaal niet omzetten in een cellulaire reactie. De somatische embryogenese receptor kinases (SERKs) zijn een familie van co-receptoren in planten, die meerdere functies in zeer verschillende, en ogenschijnlijk niet gekoppelde, signaal transductie routes bewerkstelligen. Zo werken de SERKs in het vormen van sporen, de groei en ontwikkeling van de plant, en het afweermechanisme. In *Arabidopsis thaliana* bestaat de SERK familie uit vijf leden, waarvan alleen van SERK5 nog geen functie bekend is. De andere vier SERK leden in *Arabidopsis thaliana* hebben deels overlappende functies, maar elk functioneert wel in zijn eigen specifieke subset aan signaal transductie routes.

In deze thesis heb ik gekeken naar de SERK familie van receptor kinases, om hun rol als co-receptoren in plant signalering en de regulatie van deze eiwitten beter te begrijpen. Door studies naar receptor functie en signalering in dierlijke systemen, weten we dat co-receptoren veel verschillende functies kunnen vervullen binnen signaal complexen, waardoor zij een additionele vorm van regulatie bewerkstelligen voor de signaal transductie. De kennis die hiervan al aanwezig is, heb ik bediscussieerd in **Hoofdstuk 1.** In dit hoofdstuk heb ik ook een overzicht gegeven van de huidige kristal structuren die al gepubliceerd zijn over plant LRR-RLKs. Deze structuren geven de eerste aanwijzingen over de onderliggende moleculaire mechanismen van de functie van plant receptor kinases. Interessant genoeg laten deze structuren zien dat de extracellulaire domeinen van de SERK eiwitten interacteren met niet alleen de hoofd-receptor, maar ook met de verschillende liganden. De aminozuren van SERK die deze interactie met de hoofd-receptor en het ligand aangaan zijn aanwezig in de hele familie en kunnen dus de verschillende functies van de

verschillende SERK leden niet verklaren. Op dit interessante punt ga ik verder in **Hoofdstuk 2.** Door genetische studies is het duidelijk geworden dat de functionele verschillen tussen de SERK leden niet verklaard kan worden door verschillen in expressie, maar door verschillen op eiwit niveau. Echter, doordat de SERK-leden enorm op elkaar lijken op aminozuur niveau, is het ietwat onverwacht dat zij zulke verschillende functies vervullen in plant signalering. In **Hoofdstuk 2** zoom ik verder in om te kijken welke residuen/motieven of domeinen belangrijk zijn voor de verschillende functionaliteiten binnen de SERK familie. Een vergelijking van de sequentie van SERK eiwitten van verschillende plant soorten (van mos, tot rijst en tomaat) laat zien dat de SERK genen oude genen zijn, en al met meerdere kopieën te vinden zijn in de genomen van niet-vasculaire planten (zoals *Physcomitrella patens)*. De SERK genen uit deze evolutionair oudere genomen lijken erg op de SERK1 en 2 van *Arabidopsis thaliana*, wat aangeeft dat de eiwitten niet veel veranderd zijn in 2 miljoen jaar evolutie. Dit is een sterke aanwijzing dat de SERK genen essentieel zijn in belangrijke plant signaal routes. De *A. thaliana* SERK3 en 4 genen zijn echter wel meer veranderd in hun sequentie, en vormen een aparte groep SERK genen die alleen in tweelobbige planten aanwezig is. Hoogstwaarschijnlijk is deze verandering van de SERK3/4 genen gekoppeld aan een andere functionaliteit van deze eiwitten. Het koppelen van het extracellulair domein van een SERK aan het intracellulair domein van een andere SERK (waarmee chimere eiwitten ontstaan) laat zien dat de specificiteit tussen de verschillende SERK leden een complex geheel is, welke niet kan worden toegeschreven aan één specifiek aminozuur/motief of zelfs domein.

Het vergelijken van de driedimensionale eiwit structuur van de SERK leden kan helpen bij het begrijpen van functionele specificiteit en overlap binnen deze familie. In de afgelopen jaren zijn de eerste kristal structuren van plant LRR-RLKs gepubliceerd. Dit waren het kinase domein van SERK3/BAK1, het extracellulair domein van SERK1 in complex met het extracellulair domein van BRI1 (Brassinosteroid Insensitive 1, de receptor voor het plant steroïde hormoon brassinosteroid) en het complex van het extracellulair domein van SERK3 met FLS2 (Flagellin Sensing 2, de receptor die bacterieel flagelline herkent). Deze structuren geven belangrijke informatie over de structuur-functie relatie van plant LRR-RLKs. Extra structuren, van de andere SERKs of van andere complexen, kunnen veel informatie opleveren over hoe de verschillende functionaliteiten van de SERKs gekoppeld zijn aan hun structuur. In **Hoofdstuk 3** beschrijf ik onze werkwijze om de kristalstructuren van de kinase domeinen van de verschillende SERK eiwitten op te helderen. Onze aanpak werd gehinderd door lage eiwitopbrengst en moeilijkheden tijdens de zuivering. Het is gelukt om twee kristallen te groeien van het SERK1 kinase domein, welke een diffractie vertoonde van ongeveer 2.5 Å resolutie. Echter, hiermee was het niet mogelijk om de structuur te bepalen.

Naast structurele informatie, kan biochemische informatie over de eiwitten ook veel duidelijkheid geven. In **Hoofdstuk 4** gebruik ik een combinatie van

spectroscopische technieken en activiteit assays om te onderzoeken hoe de activiteit van het kinase domein gereguleerd is. Co-receptoren hebben vaak een functie als extra regulatie van een signaal transductie route, maar kunnen dit natuurlijk alleen wanneer hun eigen activiteit ook strikt gereguleerd is. Zowel in deze thesis, als in andere studies, is het aangetoond dat veel kinase domeinen van receptoren en co-receptoren intrinsiek aan staan wanneer zij *in vitro* geproduceerd worden. Dit betekend dat er *in vivo* mechanismen moeten bestaan die het eiwit inactief houden wanneer hun activiteit niet wenselijk is. In **Hoofdstuk 4** heb ik een mechanisme onderzocht wat eiwitten inactief kan houden, namelijk het houden van het eiwit in een intrinsiek wanordelijke staat. Dit betekend dat het eiwit niet de correcte structuur heeft, en ongestructureerd of ontvouwen is tot dat de activiteit nodig is. Het vouwen of structureren van het eiwit kan dan geïnduceerd worden wanneer het eiwit op de juiste locatie is, of onderdeel is van het juiste eiwitcomplex. Onze resultaten laten zien dat het SERK1 kinase domein kan hervouwen tot een actief kinase *in vitro,* dus zonder enige vorm van cellulaire context of interactie partners. Na 30 minuten hervouwen, is ongeveer 80% van de kinase activiteit herkregen (in vergelijking met de kinase activiteit voor het ontvouwen). Hieruit concluderen wij dat het SERK1 kinase domein niet alleen intrinsiek actief is, maar zich ook efficiënt vouwt tot een actief eiwit. Hierdoor is het onwaarschijnlijk dat intrinsiek ontvouwing een manier is waarop de fosforylatie activiteit van SERK1 geregeld is.

De functie van een eiwit is nauw gekoppeld aan zijn locatie in een cel. De SERK eiwitten, aangezien zij RLKs zijn, zijn gelokaliseerd in het plasma membraan. Dit lijkt ook de belangrijkste plaats te zijn waarvandaan veel van de signaal transductie routes beginnen waarin SERKs een rol spelen. Het plasma membraan is niet homogeen en veel eiwitten ondervinden beperkingen in hun diffusie door het plasma membraan. In **Hoofdstuk 5** van deze thesis is de distributie van de SERK3 co-receptor en de BRI1 hoofdreceptor op het plasma membraan te zien. Om deze distributie te visualiseren is gebruik gemaakt van de techniek VEAM, welke gebruik maakt van het verschil in de brekingsindex tussen het monster en het dekglaasje om een zogenaamde ‘evanescence wave’ te creëren. Deze evanescence wave kan enkel tot een bepaalde diepte het monster ingaan (dit ligt aan de hoek waaronder het laserlicht op het monster schijnt), en kan dus die fluoroforen aanslaan die zich dicht bij het dekglaasje bevinden. Deze techniek maakt het mogelijk om fluorescente eiwitten in het plasma membraan of net daaronder met hoge resolutie en lage achtergrond fluorescentie te laten zien. In **Hoofdstuk 5** laten we zien dat met VEAM fluorescente eiwit op het plasma membraan van levende *A. thaliana* epidermis wortelcellen gevisualiseerd kunnen worden, maar ook sommige intracellulaire compartimenten die dicht bij het plasma membraan liggen. Daarnaast is VEAM gebruikt om te laten zien dat SERK3/ BAK1 en BRI1 niet homogeen over het membraan zijn verspreid, maar nanoclusters vormen. Deze nanoclusters kunnen niet beïnvloed worden door toevoeging of verwijdering van ligand. Daarbij zit BRI1 ook nog steeds in nanoclusters wanneer SERK3 of SERK1 niet aanwezig zijn. Overex-

pressie van BRI1, of verlaging van het aantal membraan gelokaliseerde receptoren door middel van het middel tyrphostin A23, zorgde ervoor dat de hoeveelheid receptoren per cluster veranderde, maar niet de verspreiding van de clusters. We stellen dat de nanocluster distributie een algemeen kenmerk is van plant receptor kinases.

Deze thesis geeft nieuwe inzichten in de rol van de SERK eiwitten als co-receptoren, hun evolutionaire oorsprong, hun regulatie en lokalisatie. Met een combinatie van verschillende technieken uit de biochemie, bio-informatica en structurele biologie, hebben wij de functie van SERKs onderzocht in plant signaleringscomplexen.

Overview of the completed training activities

Discipline specific activities

Annual Dutch meeting on Molecular and Cellular Biophysics	2009 -2011
EMBO Con. Catalytic Mechanisms by Biological Systems, Hamburg	2010
ALW/EPW Annual Meeting Lunteren + presentation 2012	2011 -2013
1st International Brassinosteroid Conference, Barcelona	2012
Protein Crystallography training at IRB Barcelona, 8 weeks total	2009 -2013
CBSG conferences + presentation 2009 and Poster Slam 2012	2009 -2013

General courses

Advanced course guide to scientific artwork, WUR	2013
Philosophy and Ethics of Food Science & Technology, VLAG	2013
Career perspectives, VLAG	2013

Optional activities

Biochemistry Seminars	2009 -2013
Bi-weekly Journal club Signal Transduction Group, Biochemistry	2009 -2013
PhD week Biochemistry	2013
Preparing PhD research proposal	2009

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