The SERK1 protein complexes

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To Nikolay, Sophia and Johanna

“Tiger got to hunt, bird got to fly; Man got to sit and wonder, ‘Why, why, why?’
Tiger got to sleep, bird got to land; Man got to tell himself he understand.”
Cat’s Cradle, Kurt Vonnegut

Contents

Chapter 1

Somatic embryogenesis receptor like kinases, structure, function and signaling

Rumyana Karlova and Sacco de Vries
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Plants are constantly challenged by changes in temperature, light, nutrient conditions, and exposure to pathogens and by other fluctuations in their environment. Plants also lack extracellular antibodies and specific cells that defend against pathogen attack. For example specialized cell types (macrophages, neutrophils, and dendritic cells), which as part of a circulatory blood system are the key players of the animal immune system, are not found in plants. Nevertheless, plants have developed remarkable strategies to detect and adapt to environment cues by the use of a wide array of receptors at the surface level of each single cell (Nurnberger et al., 2004; Torii, 2004). Transmembrane receptor kinases (RKs) mediate signal transduction pathways leading to cell proliferation, growth and differentiation in animals as well as in plants. Unlike animal RKs almost all plant RKs phosphorylate serine/threonine residues, where as animal kinases are predominantly ligand activated tyrosin kinases (Shiu and Bleecker, 2001). The Plant RKs are classified into several groups based on the structure of the extracellular domains. The largest super family of plant RKs with more than 222 members in Arabidopsis contain extracellular leucine-rich repeat (LRR) motifs (Shiu and Bleecker, 2001). Initial steps in a general signal transduction cascade include perception of the external signal (ligand) or sets of signals that bind to the perception domain which than leads to protein conformational changes of the receptor (for example due to phosphorylation) and changing the interaction partner. This is followed by transmission of secondary signals through the plasma membrane and introduction of specific phosphorylation and dephosphorylation cascades (Citri and Yarden, 2006). Eventually the signal is transmitted to the nucleus, where the expression of specific genes is affected. The attenuation of the signaling cascade primarily is controlled by receptor dephosphorylation and eventually receptor degradation. Coupled to this degradation pathway is receptor internalization which is considered the most effective process that robustly attenuates signaling. The receptors are targeted to the proteasome for degradation via the endosomal trafficking pathway. The plant receptor class that is the focus of this chapter is the SOMATIC EMBRYOGENESIS RECEPTOR LIKE KINASES.

Structure and homology classification

The proteins of the SERK family consist of a signal peptide followed by a Leu- rich domain of 45 amino acids fitting the Leu-zipper (LZ) pattrn Lx6Lx6Lx6L (Hecht et al., 2001) and Figure 1. The LLR domain is composed of five units. LRRs have been found in a variety of proteins with diverse functions from yeast to flies, humans, and plants, and are implicated in protein-protein interactions. The plant extracytoplasmic LLR motif has the consensus sequence L--L--L--L-L--N-L-G-IP--, where the dashes represent nonconserved residues (Torii, 2004). Although the majority of plant LRRs does not posses any introns, single LRR units of some LRR-RLKs like SERK, ERECTA and SYMRK/NORK, are intercepted by introns at the identical positions, between nucle-otides 2 and 3 of the codon for the first leucine underlined in Figure 1 A. In most LRR receptor kinases, the transmembrane domain immediately follows the LLR domain. However, in AtSERK1 as in DcSERK, a Pro-rich region containing a repeated SPP motif separates these domains.

A

BRI1	a--a--L--L-LS-N-aSG-IP- -	25
FLS2	a--L--L--L-L--N-LTG- IP- -	28
SERK	L- -a--L--**L**-L-NN-LSTPIP- -	5
ERECTA	LG-L--L--**L**-L--N-L-G-IP- -	20

B

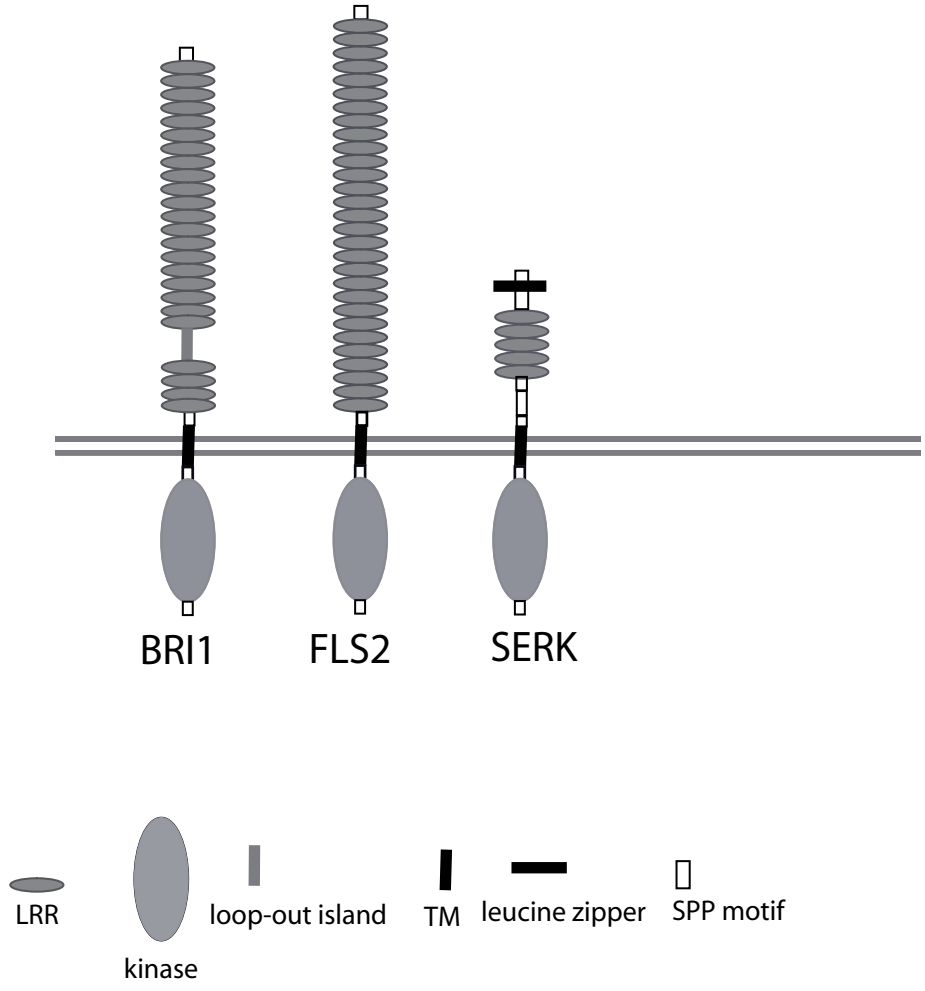

Figure 1. (A). Alignments of the LRR consensus sequence for a plant extracellular LRR- RLKs. Symbol -, Nonconserved amino acid; a, aliphatic amino acid. Numbers on the right indicate the total number of repeats, including both compleat and incomplete repeats. At the left are the protein names. The bolded leucine indicates the position of the introns, located between codons 2 and 3. (B). Structural composition of plant LRR-RLKs adopted from Torii (2004). N-terminal signal peptides are not included. Domains represented are listed below the diagram. LRR, Leucine-rich repeat; TM, transmembrane; SPP, serine-proline-prolene.

This motif has been suggested to act as a hinge providing flexibility to the extracellular part of the receptor or as a region for interaction with the cell wall (Schmidt et al., 1997; Hecht et al., 2001). As shown on Figure 1 B, SERKs contain only 5 LRRs in contrast to two of their main ligand binding receptors, BRI1 and FLS2 (Li et al., 2002; Nam and Li, 2002; Chinchilla et al., 2007). BRI1 has 24 LRRs, with LRR21 being an unusual methionine-rich repeat, interrupted with the ligand binding island domain and 28 LRRs for FLS2 (Vert et al., 2005; Dunning et al., 2007). Direct binding of BL (biosynthetic brassinosteroid) was demonstrated with native and recombinant BRI1 protein (Kinoshita et al., 2005). Moreover, recombinant protein consisting of the island domain and the neighboring C-terminal LRR repeat were sufficient to bind radioactive BL with an affinity comparable to that observed for full-length BRI1 from plants. In addition to BRI1, three highly similar homologs have been characterized (Cano-Delgado et al., 2004). Two of them, BRL1 and BRL3 display high BL-binding affinity. FLS2 was shown to bind bacterial flagellin or the flagellin-derived flg22 peptide through a presumed physical interaction with the FLS2 extracellular domain. Site-directed randomizing mutagenesis of solvent-exposed LRR residues across LRRs 9 to 15 identified mutations that disrupt flg22 binding and showed that flagellin perception is dependent on a limited number of tightly constrained residues of LRRs 9 to 15 (Dunning et al., 2007).

The intracellular region of SERKs and their main receptors BRI1 and FLS2, contains the 11 subdomains characteristic of the catalytic core of Ser/Thr protein kinases (Stone and Walker, 1995). The kinase domain ranges from 250-300 amino acid residues, corresponding to about 30 kD. All the sub-domains contain a characteristic pattern of conserved residues. The smaller N-terminal lobe containing sub-domain I-IV is primarily involved in anchoring and orienting the ATP molecules. The larger C-terminal lobe containing sub domains VI-IX is largely responsible for binding the substrates and initiating phosphotransfer (Hanks and Quinn, 1991; Hanks and Hunter, 1995). The juxtamembrane and C-terminal regions, that show less sequence conservation are believed to generate docking sites for specific kinase substrates rather than a more general response to kinase activation (Pawson, 2004). This was not the case for the C-terminal tail of BRI1, that was found to inhibit the kinase activity of the receptor and to be relieved upon ligand binding to the extracellular domain (Wang et al., 2005a). Depending on the amino acid residues that are phosphorylated, the receptor kinases have been classified into two families; those that phosphorylate on tyrosine residues

and those that phosphorylate on serine and/or threonine residues. The exception from this classification are proteins like SERK1 of which the kinase domain can trans- phosphorylate on Ser, Thr and Tyr residues and represents a dual specificity kinase (Shah et al., 2001a). As found for the SERK family, the kinase domain sequence of various plant receptor kinases is highly conserved, yet their phosphorylation peptides are highly variable as well as their modes of phosphorylation. For example the disease-resistant RLK Xa21 from rice contains a kinase domain that phosphorylates in an intramolecular manner (Liu et al., 2002). In contrast, the Arabidopsis SERK1 and Lotus japonicus SYMBIOSIS RECEPTOR KINASE (SYMRK) are reported to autophosphorylate intermolecularly (Shah et al., 2001a; Yoshida and Parniske, 2005). Although there is no crystal structure established for the kinase domain of plant RLKs, biochemical experiments have provided detailed information on the mechanism of phosphorylation catalyzed by these kinases. It was shown for the SERK1 kinase domain, that mainly autophosphorylates on threonine residues, that the reaction is Mg2+ dependent and inhibited by Mn2+, where for BRI1 Mg2+ was also important for the activity but Mn2+ did not have an inhibition effect (Oh et al., 2000). A lysine to glutamic acid substitution (K330E) in the ATP binding pocket of SERK1-KD abolishes all kinase activity and an active SERK1 kinase can phosphorylate inactive SERK1K330E proteins suggesting an intermolecular mechanism of autophosphorylation. Comparable substitutions in another member of this receptor family, SERK3, that is identical to BRI1-Associated Receptor Kinase 1 (BAK1), also abolishes the kinase activity (Li et al., 2002). Replacing Thr-468 in the activation loop with alanine completely obliterated the ability of SERK1 to autophosphorylate (Shah et al., 2001a). Kinase activity was also shown for the other members of the family, SERK4 and SERK5 and is discussed in Chapter 4. Autophosphorylation activity was shown also for FLS2, which was found to hetero-dimerize with SERK3 (Gomez-Gomez et al., 2001; Chinchilla et al., 2007). In the activation loop of the SERK proteins, there are 4 conserved threonines representing Thr-459, Thr-462, Thr-463 and Thr-468 in SERK1 protein. These residues were identified to be phosphorylated also in vivo in BRI1 and are functionally equivalent to Thr-1039, Ser-1042, Ser/Thr-1044/45 in BRI1 (Wang et al., 2005b).

Based on their sequence homology with SERK1, more LRR-RLKs were identified as shown in Figure 2 with 54% to 32% sequence identities. From these receptors, most of which also contain 5 LRRs in the extracellular domain, only five members had an SPP domain in front of the transmembrane domain, and thus define the SERK family (Figure 2).

Biological function of SERKs

Steroid hormone signal transduction

Steroid hormones play key roles in growth and development of multicellular eukaryotes. Plant steroid hormones are called brassinosteroids (BRs) and regulate cellular expansion, differentiation, and proliferation. BRs are involved in diverse processes, such as stem elongation,

vascular differentiation, male fertility, timing of senescence and flowering, leaf development, and resistance to biotic and abiotic stresses (Li and Chory, 1997; Clouse and Sasse, 1998;

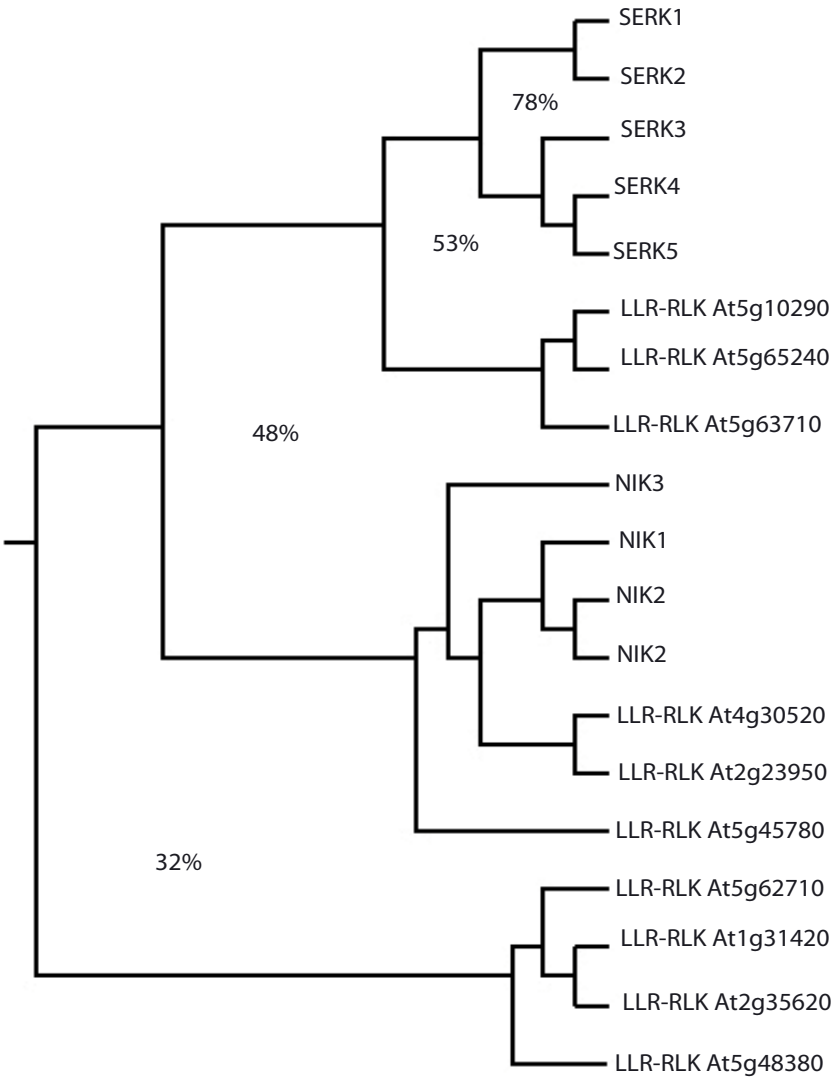

Figure 2. Arabidopsis LRR-RLKs and their phylogenetic relationships with SERK family. Amino acid sequence of the proteins were aligned using Clustal W (Thompson et al., 1994).

; Altmann, 1999). Unlike animal steroids that are perceived by nuclear receptors, plant steroids are perceived by transmembrane receptor kinases that initiate a phosphorylation-mediated signaling cascade to transduce the steroid signal. BRs bind to a subdomain within the extracellular leucine-rich repeats (LRRs) of the plasma membrane receptor kinase BRI1 (BRASSINOSTEROID-INSENSITIVE 1), as well as to two of its close relatives BRL1 (BRI1-Like 1) and BRL3 (Cano-Delgado et al., 2004; Zhou et al., 2004; Kinoshita et al., 2005). Both genetic and biochemical approaches have begun to provide further details of the various components of the BR signal transduction pathway.

On the basis of genetic screens and yeast-based protein-protein interaction screens, a second LRR receptor–like kinase (RLK) designated BAK1 (BRI1 ASSOCIATED KINASE 1) was identified (Li et al., 2002; Nam and Li, 2002) that most likely does not bind BR itself (Kinoshita et al., 2005). Two elegant studies of the in vivo phosphorylation properties of BRI1 revealed that after binding of BR to the BRI1 receptor, homodimerization was required (Wang et al., 2005a) to promote hetero-oligomerization with BAK1 (Wang et al., 2005b), which may result in a tetrameric receptor complex (Figure 3). Auto- or trans-phosphorylation of the C terminus of BRI1 is instrumental in enhancing the kinase activity of BRI1, as well as promoting its affinity for the co-receptor BAK1 (Wang et al., 2005b; Wang et al., 2005a; Wang et al., 2008). BAK1 is identical to the third member of the SERK family. After hetero-oligomerization, BAK1 (SERK3) accelerated internalization by endocytosis of BRI1 (Russinova et al., 2004). In planta as well as in protoplasts the localization and turnover of BRI1 appear independent from the presence of the ligand (Russinova et al., 2004; Geldner et al., 2007). However, increasing endosomal localization of BRI1 enhances activation of the signaling pathway and genomic responses, dtected by an increase in dephosphorylation of one of the targets of BR signaling, BES1 (BRI1 EMS SUPPRESSOR 1) (Geldner et al., 2007). These data suggest that BRI1 can use the endosomes as signaling compartments like has been shown for other receptors, for example TGF-β (Panopoulou et al., 2002). Another member of the SERK family, SERK1, is also able to interact with BRI1 and appears to modulate BRI1 signaling in a manner similar to that observed for BAK1 (chapter 2). The emerging picture suggests that SERK and BRI receptors exist as homodimers in the absence of ligands and form ligand-induced tetrameric complexes, analogous to the receptor complexes of mammalian transforming growth factor–β (TGF-β) receptors. Precise combinatorial interactions in the tetrameric TGF-β receptor complex allow differential ligand binding or differential signaling in response to the same ligand (Feng and Derynck, 1997). Whether this also applies to the BRI1-SERK complexes is not known. BRI1 also interacts with TRIP-1, a cytosolic protein that is homologous to a TGF-β receptor interacting partner (Ehsan et al., 2005).

On the basis of genetic screens, other downstream components of BR signaling have been identified. BIN2 (BR INSENSITIVE 2) is a glycogen synthase kinase 3 (GSK3) homolog. Genetic and biochemical evidence suggested that BIN2 is a negative regulator of BR signaling and functions by phosphorylating and thereby inactivating two nuclear proteins, BZR1 (BRASSINAZOLE RESISTANT 1) and BES1.

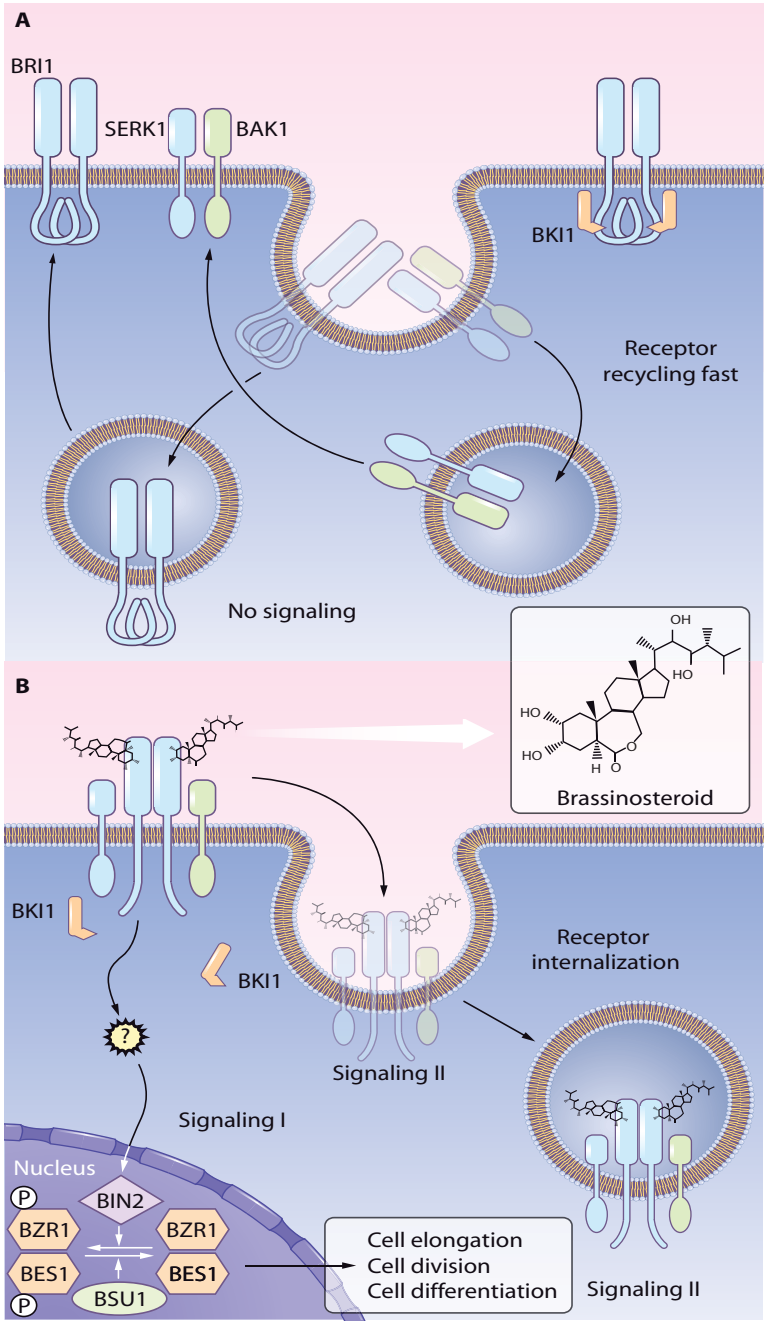

Figure 3. Model of the BR signal transduction pathway. The plasma membrane-localized receptor kinase BRI1 is the major BR receptor. (A) Without BR, BRI1 and its co-receptors BAK1 or SERK1, or both, recycle through endosomes. The kinase activity of BRI1 is kept in a basal state by both its own C- terminal domain and by an interaction with BKI1. (B) BR binding to the extracellular domain of

BRI1 induces a conformational change of the kinase domain, leading to the phosphorylation of BRI1 and dissociation of BKI1 from the plasma membrane. Following the activation of BRI1, together with BAK1 or SERK1, a multimeric complex is formed. This complex may consist of different members of both BRI and SERK families; only one possible configuration is shown. This activation leads to dephosphorylation of the nuclear proteins BES1 and BZR1 by inhibiting the negative regulator BIN2. BES1 and BZR1 then homodimerize and bind to DNA to regulate BR-responsive genes. In the absence of BR, the kinase BIN2 phosphorylates BES1 and BZR1. BSU1 is a nuclear phosphatase involved in the dephosphorylation of BES1. The entire hetero-oligomeric receptor complex can be internalized by endocytosis. It is not certain whether internalization of the receptor complex requires BR or whether the receptor complex is capable of propagating the BR signal following internalization.

Both are required for BR-regulated gene expression in a mechanism proposed to be analogous to that of Wnt signaling in fruit fly and vertebrates (Choe et al., 2002; Li and Nam, 2002; Yin et al., 2002; He et al., 2005). When over-expressed in Arabidopsis plants as fusion proteins with the green fluorescent protein (GFP), BES1 and BZR1 are rapidly dephosphorylated after treatment with BR (He et al., 2002; Yin et al., 2002), probably by the phosphatase BSU1 (BRI1 SUPPRESSOR PROTEIN 1), a nuclear protein (Mora-Garcia et al., 2004) (Figure 3). Vert and Chory (Vert and Chory, 2006) showed that BR signaling differs from the previously proposed canonical Wnt signaling mechanism in which the GSK3β protein is inactivated in the presence of the ligand, leading to accumulation and nuclear translocation of the now dephosphorylated β-catenin (Staal et al., 2002). Individual loss-of-function alleles of BIN2 and two of its closest homologs revealed no effect on BR signaling, but the triple knockout displayed constitutive BR responses and strong resistance to the BR biosynthetic inhibitor brassinazole, suggesting that GSK3 kinases act redundantly to negatively regulate BR signaling. Furthermore, BIN2-GFP was localized in the nucleus, in the cytosol, and at the plasma membrane. In contrast, a GFP fusion with *bin2* containing the E263K mutation that produced the original gain-of-function phenotype was found mostly localized to the nucleus. After expressing the mutant *bin2* protein fused to a nuclear localization signal, severe dwarf phenotypes resembling the genetic defects observed in the absence of synthesis or perception of BRs were observed. These results showed that BIN2 activity is required inside the nucleus and not in the cytoplasm. The conversion of BES1 proteins from the dephosphorylated to the phosphorylated state was consistent with the observed dwarf phenotypes. After expression in transgenic plants the BES1-GFP protein was constitutively nuclear, which further supports the model that BIN2-mediated phosphorylation of BES1 is exclusively nuclear. Neither protein stabilization nor nuclear translocation of BES1 is apparently required for BR signaling, emphasizing the importance of phosphorylation in regulating BES1 activity. Finally, BIN2-mediated phosphorylation of BES1 resulted in a complete loss of its DNA-binding activity to downstream target promoters, and its ability to multimerize was also clearly decreased. These results indicate that rather then regulating the nuclear translocation and accumulation

of BES1, BIN2 acts in the nucleus to regulate the genomic response to BRs through phosphorylation of BES1, which blocks the transcriptional activity of BES1. Recently a different model was proposed for BIN2 and BZR1 model of action by (Gampala et al., 2007; Ryu et al., 2007). These authors showed that BZR1 functions as a nucleocytoplasmic shuttling protein and BIN2 induces the nuclear export of BZR1 by modulating BZR1 interaction with the 14-3-3 proteins. BR-activated phosphatase mediated rapid nuclear localization of BZR1. It was proposed that the spatial redistribution of BZR1 is critical for proper BR signaling in plant growth and development. In these studies protein over-expression in protoplasts are used, which could only partially mimic the natural signaling process in planta.

Wang and Chory described another negative regulator of BR signaling, BKI1 (BRI1 KINASE INHIBITOR 1) (Wang and Chory, 2006). BKI1 interacts directly with the kinase domain of BRI1, as did transthyretin-like (TTL) protein (Nam and Li, 2004), both of which were identified by yeast two-hybrid screening. The C-terminal domain of BKI1 was both necessary and sufficient to bind the BRI1 kinase domain and the interaction was highly specific, because BKI1 did not interact with TTL, BIN2, or the kinase domain of other RLKs. Immunoprecipitation experiments confirmed that BRI1 interacted with a BKI1-FLAG fusion in vivo, and with the use of promoter–β-glucuronidase (GUS) fusions, BKI1 was shown to be coexpressed with BRI1 in a number of plant tissues (Friedrichsen et al., 2000; Nam and Li, 2004; Wang and Chory, 2006). RNA interference (RNAi) lines of BKI1 showed that BKI1 represses BR-related growth, whereas over-expression of BKI1 resulted in dwarf plants resembling plants harboring weak alleles of *bri1*. As could be predicted from the phenotype, dephosphorylated BES1 in the BKI1 over-expression lines was almost undetectable, suggesting that BRI1 signaling was suppressed by BKI1 over-expression. The BKI1-YFP (yellow fluorescent protein) fusion was localized both at the plasma membrane and the cytosol. Within 5 min of BR application, the plasma localization of BKI1 was shifted completely to the cytosol. No BR-induced dissociation of BKI1-YFP from the plasma membrane was observed in a bri1 kinase–inactive mutant background (Friedrichsen et al., 2000), suggesting that the kinase activity of BRI1 is required for BKI1-YFP relocalization. On the basis of these findings, a new model is proposed where in the absence of BRs, BRI1 homodimers are kept localized at the plasma membrane in association with BKI1 proteins. This may in fact keep BRI1 from association with its co-receptors, such as BAK1 or SERK1 (Figure 3). These new findings are summarized in a model based on various previous ones and shows that BR binding to the extracellular domain of BRI1 induces receptor phosphorylation and activation, as well as its dissociation from BKI1. In the absence of BRs, BIN2 negatively regulates BR signaling by phosphorylating the transcriptional regulators BES1 and BZR1. In the presence of BRs, BIN2 is inhibited by an unknown mechanism, leading to dephosphorylation of BES1 and BZR1, which then homodimerize or cooperate with other transcription factors, which allows DNA binding and regulation of BR-responsive genes (Yin et al., 2005; Vert and Chory, 2006).

This new signaling paradigm raises several interesting questions. The first is the stoichiometry and conformation of the BR receptor complex in the plasma membrane in the absence

and presence of BRs. To what extent are the proposed tetrameric complexes preformed, or are these tetrameric complexes only assembled upon ligand application? Both homodimeric and heterodimeric configurations appear to coexist in heterologous plant cells (Russinova et al., 2004). A second question is whether the complex is the same in all cells where the receptors are expressed or whether there are a multiple forms composed of different family members. This is important for determining whether the proteins so far identified are capable of integrating or separating the different cellular and developmental effects of BRs.

A third question concerns the role of the co-receptors in BR signaling. One hypothesis, which is modeled on TGF-β signaling, suggests that BR binding activates BRI1, which can then phosphorylate BAK1 to propagate BR signaling (Li et al., 2002; Karlova et al., 2006). This hypothesis is supported by Wang et al. (Wang et al., 2005b), who showed that BR treatment not only stimulates the phosphorylation of both BRI1 and BAK1, but also enhances their physical interaction in transgenic plants coexpressing tagged BRI1 and BAK1. Thus, downstream components intermediate between BRI1 and BIN2 may interact with BAK1 rather then BRI1 itself. However, using the SERK1 protein as bait to identify interacting proteins, we did not identify any known components of BR signaling other than the BRI1 and BAK1 receptors (Karlova et al., 2006; and chapter 2). Instead, we found components of the plant variant of the VCP/p97 complex of yeast and mammals (Aker et al., 2006; Karlova et al., 2006; Aker et al., 2007) and two putative targets in the form of the transcriptional regulator AGL15 and a Zn-finger protein. An alternative hypothesis, which is based on the epidermal growth factor (EGF) receptor model, suggests that BRI1-BAK1 dimerization is essential for the activation of both receptor kinases by trans-phosphorylation (Nam and Li, 2002) and may represent the first bifurcation in the pathway in which both BRI1 and BAK1 participate in downstream signaling. Recently Wang at al., (Wang et al., 2008) provided a proof for this model by showing a sequential trans-phosphorylation for BRI1 and its co-receptor BAK1. In this model BRI1 kinase function is initially activated to a basal level by BR binding to BRI1 in the absence of BAK1. The activated BRI1 then associates with BAK1 and trans-phosphorylates it on kinase domain residues. BRI1-activated BAK1 subsequently trans-phosphorylates specific juxtamembrane and C-terminal residues in BRI1, thereby increasing BRI1 kinase activity and substrate phosphorylation, leading to enhanced BR signaling and increased plant growth.

A final question concerns the role of endocytosis in BR-mediated signaling. Although animal EGF and TGF receptors remain capable of downstream signaling after internalization, so far the ligand triggered endocytosis has only been shown for the plant receptor FLS2 (Robatzek et al., 2006), which is involved in innate immunity and bacterial flagellin perception. BAK1 is reported to enhance endocytosis of BRI1 (Russinova et al., 2004). It is also unknown whether the BRI1-BAK1 complex may have functions that are independent of kinase activity. For example, the kinase activity of another plant receptor, CRINKLY4, is not instrumental for its biological activity (Gifford et al., 2005). If this is a general feature of plant receptors, properties other than the catalytic activity of BRI1 and BAK1 could also be essential for signaling.

In conclusion, it appears that although plant signaling pathways have many components in common with their animal counterparts, their individual properties may be quite different and may help to fully explain how the problem of perceiving and responding to steroid hormones as essential signal molecules has been tackled in different species.

Plant disease resistance pathway trough FLS2-SERK3 receptor

Plants, like animals, have the capacity to detect phathogens in a broad combinatorial way by detecting several general elicitors. Examples are flagellin (the main component of the bacterial flagellum, chitin (fungal carbohydrate polymer), pilin (proteins that are part of the pilus structures in bacteria), and ergosterol (the main sterol of the higher fungi) (Afzal et al., 2008). The plants response to general elicitors includes the production of ROS (reactive oxygen species) and ethylene and activation of the partial defense response genes. Genetic analysis in Arabidopsis has shown that FLS2, an LRR-RLK, is essential for flagellin perception (Gomez-Gomez and Boller, 2000). FLS2 was shown to be essential for the flg22-mediated activation of defense mechanisms through the mitogen-activated protein kinase (MAPK) cascade (Asai et al., 2002). Recently, using chemical crosslinking and immunoprecipitation, FLS2 was shown to bind flg22 and determine the specificity of flagellin perception (Chinchilla et al., 2006). Functionally, FLS2 is similar to animal Toll-like receptor 5 (TLR5), which also recognizes flagellin through its LRR domain (Andersen-Nissen et al., 2007) and belongs to a family of evolutionarily conserved membrane receptors. These receptors are demonstrated to recognize a diverse set of microbial molecules, collectively termed pathogen-associated molecular patterns 'PAMPs', which are absent from the host, making these receptors ideal for distinguishing self from non-self (Akira and Takeda, 2004). Unlike BRI1 (Russinova et al., 2004) and several toll-like receptors, Arabidopsis FLS2 does not undergo homodimerization either before or after flg22 treatment (Ali et al., 2007). Recently, it was shown that FLS2 localizes to the plasma membranes and also to intracellular vesicles (Robatzek et al., 2006). FLS2 was found to undergo rapid endocytosis in ligand-dependent manner leading to degradation. Two independent groups (Chinchilla et al., 2007; Heese et al., 2007) recently have shown that FLS2 rapidly becomes complexed with BAK1 (SERK3) in a ligand dependent manner, suggesting that BAK1 is a partner of FLS2 in PAMP signaling similar to being a partner of BRI1 in BR signaling. Plants carrying *bak1* mutations show a decrease in the response to PAMPs, which is not due to reduced sensitivity to brassinosteroids. However, *bak1*- mutant plants did not reduce flagellin binding, suggesting that BAK1 is not involved in flagellin perception (Chinchilla et al., 2007). Thus, BAK1 seems to regulate the function of both receptors, BRI1 and FLS2, at a step after ligand binding. Recent findings also show that *bak1* mutants have altered susceptibility to microbial pathogens such as oomycetes and true fungi (Kemmerling et al., 2007), indicating that BAK1 might regulate plant resistant responses other then FLS2 and EFR. Interestingly, compared to the wild type plants, *bak1-3* mutants showed markedly reduced endocytosis of FLS2 (Chinchilla et al., 2007). However, *bak1* mutants clearly retain

partial sensitivity to flagellin and elongation factor Tu (EF-Tu). One possible explanation for this is that a closely related protein might substitute for BAK1, like SERK1 in case of BRI1-mediated signaling. However, non of the other single mutants from the SERK family, *serk1*, *serk2*, *serk4* and *serk5* showed an effect in flg22 and elf18 responses (Chinchilla et al., 2007) or were susceptible to Alternaria brassicicola (Albrecht et al., 2008). The only exception being that a *serk4* mutant allele enhances the *bak1 (serk3)* susceptibility towards Alternaria brassicicola. Interestingly, in rice the OsSERK1 gene was found to act in two signaling pathways as positive regulator; somatic embryogenesis as well as the host defense response against fungal infection (Hu et al., 2005). Recently Shan et al., (Shan et al., 2008) showed that bacterial effectors like AvrPto and AvrPtoB target the common signaling partner, BAK1 to disrupt multiple PAMP receptor-signaling complexes and impede plant immunity. Remarkably, AvrPto and AvrPtoB are sufficient to interfere with the bacterial-induced formation of FLS2-BAK1 receptor-signaling complex in plant leaves. Moreover the interaction between AvrPto and BAK1 also reduced the association between BRI1 and BAK1 in the presence or absence of BL, consistent with the BR-insensitive phenotypes observed in transgenic plants over expressing AvrPto protein (Shan et al., 2008).

Cell death signaling pathway mediated trough SERK3 and SERK4

Developmentally controlled programmed cell death is critical for normal plant growth and development, as well as being part of a defense mechanism against numerous biotic and abiotic stresses. Recently two of the SERK proteins were shown to play a role in a cell death control. Although SERK3 and SERK4 share high sequence homology and a BAK1 null allele, *bak1-4* showed a subtle *bri1*-like phenotype, the SERK4 null allele *bkk1* did not exhibit any defective phenotypes (He et al., 2007; Albrecht et al., 2008). However the double-null mutant *bak1-4 bkk1-1*, illustrated extremely dwarfed phenotypes, yet completely distinct from typical *bri1* null mutants. *bak1-4 bkk1-1* double mutants exhibit a seedling lethal phenotype due to constitutive defense-gene expression, callose deposition, reactive oxygen species accumulation, and spontaneous cell death even under aseptic conditions. This indicates that SERK3 and SERK4 negatively regulate a BR-independent defense-related cell-death pathway. Albrecht et al., (Albrecht et al., 2008) extended these studies and showed that from the SERK family only SERK3 and SERK4 are partially redundant in cell death control. The authors demonstrated that a combination of weaker alleles of both SERK3 SERK4 genes no longer shows seedling lethality but exhibits reduced growth and more severe pathogen induced cell death than in the *serk3-1* single mutant lines. None of the double or triple mutant combination of the SERKs genes show any additional effect on growth or cell death responses compared to *serk3-1* single or *serk3-1 serk4-1* double mutants. It would be interesting to find the composition of the different receptor protein complexes in plants, that are exposed to pathogen attacks or BR or flg22 ligands to further understand how SERK3 protein play a role in separate signaling pathways.

Signaling pathways mediated trough SERK1 receptor

The SERK1 gene was first identified in carrot as a marker for single cells competent to form somatic embryos (SE) (Schmidt et al., 1997; Hecht et al., 2001). In Arabidopsis ectopic expression of SERK1 increases somatic embryogenesis in culture (Hecht et al., 2001), suggesting that SERK1 mediated signaling is required for acquisition of embryogenic competence in somatic cells. Cells acquiring embryogenic competence in Arabidopsis are derived from procambium cells that express the SERK1 gene in the vascular tissue of the hypocotyl (Kwaaitaal and de Vries, 2007). Orthologs of the SERK genes were discovered in several plant specie, where the gene was also found to be a marker for embrygenic potential, like sunflower (Thomas et al., 2004) and rice (Hu et al., 2005). Putative SERK genes were also identified in Medicago truncatula (Nolan et al., 2003) and maize (Baudino et al., 2001) and show a predominant expression during somatic embryogenesis and shoot organogenesis. These results suggest that SERK genes may constitute a small gene family and are functionally conserved in plants with a specific role in somatic embryogenesis. Another gene that was shown to increase SE formation, when over-expressed is the MADS box transcription factor AGL15 (Harding et al., 2003). Recently AGL15 was shown to act redundantly with its homolog, AGL18 in two processes: somatic embryogenesis (Thakare et al., 2008) and in the control of flowering time (Adamczyk et al., 2007). Both SERK1 and AGL15 (Hecht et al., 2001; Nolan et al., 2003; Zhu and Perry, 2005; Kwaaitaal and de Vries, 2007) and MtSERK1 (Nolan et al., 2003) genes expression is increase in response to auxin treatment (Hecht et al., 2001; Zhu and Perry, 2005; Kwaaitaal and de Vries, 2007). It was shown in protoplasts that about 15 % of the SERK1 receptor kinase is present in a predimerized state by Fluorescence Cross Correlation Spectroscopy (FCCS) (Hink et al., 2008). SERK1 dimerization and correct targeting depends on the extracellular LZ domain and N-linked glycosylation of the LLR domains (Shah et al., 2001b). Several downstream partners interacting with the SERK1 kinase domain have been isolated. The Kinase Associated Protein Phospatase (KAPP), the AAA-ATPase Cell Division Cycle (CDC) 48 and the 14-3-3 protein GF14λ (Shah et al., 2002; Rienties et al., 2005). SERK1 and KAPP bind in a phosphorylation-dependent manner and KAPP dephosphorylates SERK1 and possibly negatively regulates SERK1 signaling (Shah et al., 2002). Co-expression of fluorescently tagged KAPP and SERK1 in protoplasts resulted in internalization of the two proteins, where the interaction could be measured by FRET. This suggested that KAPP function is linked to inactivation of the SERK1 receptor by dephosphorylation and internalization and possible degradation in the vacuole (Shah et al., 2002). The closest homolog of SERK1 in the SERK family is SERK2 (Figure 2) and both genes show a complex expression pattern throughout development. Both are expressed in anther primordia up to the second parietal division. After this point, expression ceases in the sporocytes and is continued in the tapetum and middle layer precursors (Albrecht et al., 2005; Kwaaitaal et al., 2005). Single knockout mutants of SERK1 and SERK2 show no obvious phenotypes. Double mutants of SERK1 and SERK2 are completely male sterile due to a failure in tapetum specification. Fertility can be restored by a single copy of either gene. The SERK1 and SERK2

proteins can form homodimers or heterodimers *in vivo*, suggesting they are interchangeable in the SERK1/SERK2 signaling complex (Albrecht et al., 2005). *In vitro* studies showed that *serk1-1* truncated protein had lost its autophosphorylation activity. By contrast to the *serk1-1 serk2-1* double mutant, the strong *bri1-201* mutant, which is also male sterile, does produce pollen and the anther phenotype of the *serk1-1 serk2-1* could not be rescued by the *bes1-D* mutant construct (Albrecht et al., 2008). These data indicate that similar to *serk3 serk4* dwarf phenotype, *serk1 serk2* male sterility phenotypes are not related to BRI1-mediated BR signaling.

In summary members of the SERK family have in part redundant functions, can act as co-receptors with different main receptors and individual members can act in different signaling pathways. Although the different SERK members are expressed in all tissues during development and share a large overlapping pattern of expression (Albrecht et al., 2005; Kwaaitaal et al., 2005; Kwaaitaal and de Vries, 2007) these receptors have different functions. SERK1 and SERK2 are fully interchangeable in male sterility (Albrecht et al., 2005). SERK1 and SERK3 have additive effects in BR signaling (Albrecht et al., 2008) and SERK3 (BAK1) and SERK4 (BKK1) are redundant in mediating cell death (He et al., 2007). SERK3 alone also controls innate immunity (Kemmerling et al., 2007) and it is involved in flagellin perception by being a co-receptor of FLS2 (Chinchilla et al., 2007; Heese et al., 2007). SERK4 mutant allele enhances the *serk3* susceptibility towards Alternaria brassicicola (Albrecht et al., 2008). These data support the hypothesis for heterotetrameric receptor complex formation of two ligand-binding receptors and two co-receptors. Different specificities could then be attributed to a specific combination of main and co-receptors in analogy to the mammalian ERBB (erythroblastosis oncogene B) receptors (Citri and Yarden, 2006).

Scope of the thesis:

Plants have developed remarkable strategies to detect and adapt to environmental cues by the use of a wide array of receptors at the cell surface. Plant transmembrane receptors are prime components of many signaling cascades and thus are involved in cell fate, growth, differentiation and defense against pathogen attacks. One amazing properties of the plants is their ability to regenerate very fast. Single somatic plant cells in culture can be induced to shift their fate towards totipotency by application of exogenous auxins. The acquisition of the totipotent state coincides with the expression of the Somatic Embryogenesis Receptor Kinase (SERK). The main focus of this study is to unravel the signaling pathways in which the SERK proteins are involved.

In Chapter 2, the composition of the SERK1 signaling complex in vivo was investigated. Two other LRR-RLKs, the brassinosteroid insensitive 1 (BRI1) receptor and its co-receptor the SERK3 (BAK1) protein were identified as interacting partners. In addition the kinase associated protein phosphatase (KAPP), CDC48A and 14-3-3ν were found. Finally, the MADS-box transcription factor AGL15 and an uncharacterised zinc-finger protein, member of the CON-

STANS (CO) family were identified as part of the SERK1 complex. The role of the SERK1 receptor in BR signaling was also established. Collectively our results suggest that apart from SERK3 (BAK1) also SERK1 is involved in the brassinosteroid signaling pathway.

In Chapter 3, using a modified and improved method for isolation of transmembrane receptor complexes, the interacting partners of SERK3 and BRI1 were identified. It appears that SERK1 and SERK3 share a similar, but not identical protein complex composition, in line with their genetically determined functions.

In Chapter 4 the autophosphorylation activity of the kinase domains of the five SERK members was compared and the phosphorylated residues were identified by LC-MS/MS. Differences in the activity that range from a high activity of SERK1, intermediate activities for SERK2 and SERK3 to the low activity of SERK4 and SERK5 proteins were noted. In the SERK1 kinase, residue Ser-562 was found to control the full authophosphorylation activity. In vivo SERK1 phosphorylation was induced by brassinosteroids and seven in vivo phosphorylation sites were identified. The trans-phosphorylation sites in BRI1 and SERK1 were detected and the importance of the kinase activity for the interaction between SERK1 and BRI1 was investigated.

In Chapter 5 the role in the SERK1 protein complex of AGL15, 14-3-3ν and BRI1 proteins was investigated. The steroid hormone brassinolide, perceived by the BRI1 receptor and its co-receptor SERK1 was shown to be instrumental in changing the differentiated cell fate towards embryogenic competence in Arabidopsis explants. AGL15 was also involved in this process and was found to be a positive regulator of both somatic embryogenesis and brassinosteroid signaling. Upon BL-dependent nuclear translocation, mediated by the adaptor protein 14-3-3ν, the AGL15 protein was shown to be able to bind to the promoter elements of the BRI1 and SERK1 genes and most likely is a positive regulator of the expression of both genes.

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

Chapter 2

The Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1 protein complex includes BRASSINOSTEROID-INSENSITIVE1

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Abstract

The Arabidopsis thaliana Somatic Embryogenesis Receptor Kinase 1 (SERK1) is a leucine-rich repeat receptor-like kinase (LRR-RLK) involved in the acquisition of embryogenic competence and in male sporogenesis. To determine the composition of the SERK1 signaling complex in vivo we generated plants expressing the SERK1 protein fused to the cyan fluorescent protein under control of the SERK1 promoter. The membrane receptor complex was immunoprecipitated from seedlings and the co-immunoprecipitating proteins were identified using Liquid Chromatography / Matrix Assisted Laser Desorption Ionization-Time of Flight / Mass Spectrometry (LC/MALDI-TOF/MS) of the trypsin-released peptides. This approach identified two other LRR-RLKs, the brassinosteroid insensitive 1 (BRI1) receptor and its co-receptor the SERK3 or BRI1-associated kinase 1 (BAK1) protein. In addition the kinase associated protein phosphatase (KAPP), AtCDC48A and 14-3-3nu were found. Finally, the MADS-box transcription factor AGL15 and an uncharacterised zinc-finger protein, member of the CONSTANS (CO) family were identified as part of the SERK1 complex. Using Blue Native Gel Electrophoresis we show that SERK1 and SERK3 are part of BRI1 containing multiple protein complexes with a relative mass between 300 and 500 kDa. The SERK1 mutant allele *serk1-1* enhances the phenotype of the weak BRI1 allele *bri1-119*. Collectively, these results suggest that apart from SERK3 also SERK1 is involved in the brassinolide signaling pathway.

Introduction

Membrane-located leucine-rich repeat receptor like kinases (LRR-RLKs) play important roles in plant signaling pathways. For example, the Arabidopsis CLAVATA1 (CLV 1), BRI1, ERECTA, rice Xa21, HAESA-RLK5 and FLS2 genes were shown to function in shoot meristem maintenance, hormone perception, organ elongation, disease resistance, abscission and flagellin signaling respectively (reviewed in (Torii, 2004). Extensive genetic and biochemical studies have been undertaken to identify additional components of different signaling pathways. For example negative and positive regulators of brassinosteroid (BR) signaling were identified as the GSK-3/Shaggy-like kinase called Brassinosteroid Insensitive 2 (BIN2), (Li and Nam, 2002) and the nuclear-localized Ser/Thr phospatase bri1 Suppressor 1 family (Mora-Garcia et al., 2004), respectively. Their potential substrates are the nuclear proteins BRASSINAZOLE RESISTANT1 (BZR1) (Wang et al., 2002b) and bri1- EMS-SUPPRESSOR1 (BES1) (Yin et al., 2002).

We have previously identified the Arabidopsis Somatic Embryogenesis Receptor-like Kinase 1, an LRR-RLK that marks formation of embryogenic cells in culture and is expressed in ovule primordia and in both male and female gametophytes. In sporophytic tissues SERK1 has a complex expression pattern, with expression being highest in the vascular tissue of all organs (Hecht et al., 2001; Kwaaitaal, 2005; Albrecht, 2005). Ectopic expression of SERK1 does not result in an obvious phenotype in Arabidopsis plants but increases somatic embryo formation in culture (Hecht et al., 2001). SERK1 is a member of a small family of five related RLKs all of which have 5 LRRs and a typical serine-proline rich juxtamembrane region (Hecht et al., 2001). The SERK1 knock-out alleles *serk1-1* and *serk1-2* did not have a morphological phenotype but in combination with a *serk2* null mutant resulted in complete male sterile plants (Albrecht, 2005; Colcombet, 2005). SERK3 or BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) is part of the BR receptor complex and is proposed to function as a co-receptor of the BRASSINOSTEROID INSENSITIVE 1 (BRI1) protein (Li et al., 2002; Nam and Li, 2002). Recently we showed that in living plant cells BRI1 and SERK3 interaction occurred in restricted areas of the membrane and recycled upon internalisation by endocytosis (Russinova et al., 2004).

SERK1 was shown to interact with the Kinase Associated Protein Phosphatase (KAPP) that is postulated to play a role in receptor internalization (Shah et al., 2002). Employing a yeast two-hybrid screen, two additional SERK1 interacting proteins, AtCDC48A and 14-3-3λ were found (Rienties et al., 2005). These interactions suggested analogy with the mammalian CDC48 homologue p97/VCP complex, where p97/VCP can be phosphorylated by the JAK-2 kinase and dephosphorylated by the phosphatase PTPH1 that associates with a 14-3-3 protein (Zhang et al., 1997; Zhang et al., 1999). KAPP has also been reported to interact with other LRR-RLKs such as HAESA; WALL-ASSOCIATED KINASE1 (WAK1); FLS2 and CLAVATA1 (reviewed in (Becraft, 2002). Transgenic studies indicate that KAPP functions as a negative regulator of CLV1 and FLS2 signaling (Williams et al., 1997; Stone et al., 1998; Gomez-Gomez et al., 2001).

Identification of proteins can now be performed at high sensitivity by specific proteolytic digestion and determination of the peptide masses by Mass Spectrometry (MS). This technique has been used for a systematic study of multiprotein complexes. For the epidermal growth factor receptor (EGFR), a human receptor tyrosin kinase, immunoprecipitation combined with matrix-assisted laser desorption/ionisation MS (MALDI) was used to identify components of the EGFR signaling complex (Pandey et al., 2000). For only a few plant RLKs the signaling complex has been defined. One example is the 105 kDa CLV1 receptor that is found in two distinct protein complexes of 450 and 185 kDa. The larger 450 kDa complex requires functional CLV1 and CLV3 proteins for assembly and includes KAPP and a Rho GTPase-related protein (Trotochaud et al., 1999).
In this study we have determined the composition of the SERK1 complex/es in vivo. We combined immunoprecipitation of CFP-tagged SERK1 with a rapid Liquid Chromatography (LC)-MALDI-MS based protein identification. Using this method we confirmed the presence in the SERK1 signaling complex of AtCDC48A (Rienties et al., 2005) and KAPP (Shah et al., 2002). Additional proteins were found such as another member of the 14-3-3 family, 14-3-3nu, SERK3 (BAK1) and BRI1, the MADS-box transcription factor AGL15 and an uncharacterised zinc-finger protein. In particular the interaction between SERK1 and BRI1 was confirmed by a genetic experiment and Fluorescence Lifetime Imaging Microscopy (FLIM) to determine Förster Resonance Energy Transfer (FRET) between fluorescently tagged receptors. Our data show that the method used here can distinguish between individual isoforms of related members of the same protein family, confirms and extends previous screens carried out using the more commonly applied yeast two hybrid method to find interacting partners.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia was used as the wild type. Seeds were surface sterilized and sown on germination medium (½ Murashige and Skoog (MS) salt and vitamins medium (Duchefa, Haarlem, The Netherlands) supplemented with 0.8 % (w/v) agar (Daishin) and 2 % (w/v) sucrose). Seeds were kept for 2 days at 4oC and then transferred to a growth chamber. Seedlings were grown at 22oC under 16 h light/ 8 h dark periods for 7 days. Arabidopsis plants expressing the SERK1 receptor fused to cyan fluorescent protein (CFP) under its native promoter were similar to the SERK1-yellow fluorescent protein (YFP) expressing plants described previously by Kwaaitaal et al. (2005). The BRI1-GFP transgenic plants used for immunoprecipitation and BAK1-GFP plants used for BN-PAGE were described before (Russinova et al., 2004). *bri1-4* strong allele (nr 3953 obtained from Frans Tax – Arabidopsis stock center) were previously described by Noguchi et al., 1999.

Generation of *bri1-119 serk1-1*, *bri1-119 serk3* and *bri1-119 serk2-2* double mutants

The *bri1-119* mutant was obtained from the Arabidopsis Biological Resource Center (Ohio

State University, Columbus, stock CS399, ecotype Enkheim-2) and was previously described by (Noguchi et al., 1999) and (Friedrichsen et al., 2000). The SERK1 and SERK3 knockout lines, *serk1-1* and *serk3*, were obtained from the SIGnAL TDNA-Express collection (Alonso et al., 2003) as insertion lines SALK_544330 and SALK_034523.56.00 respectively and the serk2-2 insertion line (nr 119-G03) from the SAIL lines, previously described by Albrecht et al., 2005 in Columbia background. *bri1-119 serk1-1* and *bri1-119 serk2-2* were generated by crossing *bri1-119* with *serk1-1* or *serk2-2*, *bri1-119 serk3* was generated by crossing *bri1-119* with *serk3*. In each case, the F1 was allowed to self-fertilize, and candidate double F2 plants were genotyped phenotypically and by PCR.

Transient expression in protoplasts and Fluorescence Lifetime Imaging Microscopy (FLIM)

The full-length SERK1, SERK3 and BRI1-CFP and YFP fusion constructs were previously described (Shah et al., 2001b, Russinova et al., 2004). FLIM was performed using a Bio-rad Radiance 2100 MP system in combination with a Nikon TE 300 inverted microscope as described before (Russinova et al., 2004). From the intensity images obtained, complete fluorescence lifetime decays were calculated per pixel and fitted using a double exponential decay model. The fluorescence lifetime of one component was fixed to the value found for SERK1-CFP (2.5 ns).

Protein extraction, SDS-PAGE and Western Blot analysis

To prepare total protein extracts, plant material was ground in liquid nitrogen and thawed in extraction buffer containing 50 mM Tris pH 7.5, 150 mM NaCl and 0.5% Triton X-100, and a protease inhibitor cocktail according to the manufactures instructions (Roche, Germany). Samples were incubated on ice for 30 min. The suspension was centrifuged at 200g (Beckman-Microfuge 18 centrifuge) at 4 oC for 3 minutes. The concentration of extracted proteins was determined with the Biorad Protein assay (Biorad). After boiling 30 µg of total protein for 5 min in sample buffer containing 100mM Tris HCl at pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol and 100mM dithiothreitol (DTT) the proteins were separated on 8% or 12% SDS polyacrylamide gels (Laemmli, 1970) and transferred onto nitrocellulose (Schleicher & Schuell, Germany) by wet electroblotting (Mini-Protean II system; Biorad). Proteins were analysed by immunoblotting with anti-GFP (IgG fraction, 1500 x diluted, Molecular Probes, The Netherlands) and a polyclonal antibody generated against the GST-SERK1 kinase domain fusion protein (3000 x diluted, Rienties et al., 2005). Anti-BRI1 specific antibodies were kindly provided by Joanne Chory, (the Salk Institute for Biological Studies, La Jolla, California USA and used at 1000 x dilution), anti-CDC48A antibodies (Rancour et al., 2002) were kindly provided by Sebastian Bednarek, (University of Wisconsin, Department of Biochemistry, Madison, USA and used at 1000 x dilution), and-14-3-3 antibodies that have been previously described (Rienties et al., 2005). Bound primary antibodies were detected with horseradish peroxidase-conjugated IgG purified donkey anti-rabbit at 10000 x dilution

(Rockland Laboratories) or goat anti-chicken at 5000 x dilution (Rockland Laboratories) secondary antibodies and visualized using reagents for chemiluminescent detection (ECL Plus reagent, Amersham Biosciences, England).

Blue Native Gel Electrophoresis

Preparation of microsomes and Blue Native Gel electrophoresis were performed as described (Rivas et al., 2002). Microsomes from 7d old seedlings from wild type plants or transgenic plants expressing the SERK1 promoter-SERK1-CFP construct were solubilized in extraction buffer supplemented with 750mM aminocaproic acid and incubated for 30 min on ice. Aliquots of supernatants were incubated with 0.5% Coomassie Brilliant Blue G 250, 5% v/v glycerol and loaded on gel. Blue native PAGE then was performed following according to (Schagger and von Jagow, 1991) on a 5 to 15% gradient polyacrylamide gel. Albumin bovine monomer (66 kD), albumin bovine dimer (132 kD), urease trimer (272 kD) and urease hexamer (545 kD) from Sigma, were used as marker proteins.

Immunoprecipitations

Routinely we used 4 plates (9 cm) with about 400 seven days old seedlings (100 per plate) to prepare a protein extract suitable for immunoprecipitation. Ten millilitres of total plant protein extract (1mg.ml-1) in extraction buffer (isolated as described above) were incubated for 1 hour at 4°C with 100µl 50% v/v protein A-Sepharose CL-4B beads slurry (Amersham Biosciences), in 50mM Tris, 150mM NaCl, pH 7.5. After centrifugation at 200g (Beckman-Microfuge 18 Centrifuge) for 2 min at 4°C, the supernatant was incubated with 7µl of anti-GFP, anti-BRI1, anti-14-3-3, anti-CDC48A or rabbit preimmune serum. After incubation with gentle mixing for 1 h at 4°C, 200µl of fresh 50% slurry of Protein A beads was added and incubation continued for 4 h. Protein A beads were pelleted by centrifugation at 200g for 1 min and the supernatant was removed. The beads were washed three times with 1 ml wash buffer (50 mM Tris pH 7.5, 150 mM NaCl) and once with 10mM NH4HCO3, pH 8. Before the last wash step the beads together with the wash buffer were moved to a new tube. After centrifugation, the wash buffer was removed and the beads were used in this form directly for digestion with trypsin and subsequent MALDI-TOF/MS analysis.

For immunodetection, the proteins were eluted from the beads by adding SDS sample buffer and boiling for 5 min at 95 °C. In case of co-immunoprecipitation of SERK1-CFP with rabbit anti-GFP antibodies and detection with rabbit anti 14-3-3 antibodies no DTT was included in the SDS sample buffer. This prevents separation of the heavy and the light chain of the antibodies and improved the resolution of the blots since the detection system also recognized immunoglobulins of which the light chain overlaps with the 14-3-3 proteins.

Protein Digestion

Proteins bound to protein A beads in 50µl 10mM NH4HCO3 were incubated with 100ng trypsin (Sequencing Grade, Boehringer Mannheim, Germany). After overnight incubation

with gentle shaking at room temperature, the pH was decreased to 2 by adding trifluoro-acetic acid (TFA) in water up to 0.4% v/v.

Liquid Chromatography and MALDI-TOF Mass Spectrometry Analysis

Fifty microliters of peptide sample was injected into a 100x 0.25 mm Prontosil 3 µm C18H column (prepared in house) and eluted at a flow of 4 µl/min with 5% v/v acetonitril in water containing 0.05% TFA. A gradient from 5% to 20% v/v acetonitril in water containing 0.05% v/v TFA was applied in 90 s, followed by a slower linear increase to 40% v/v acetonitril in water containing 0.05% v/v TFA in another 4 min. (Alliance 2690 running at 0.25 ml/min equipped with a flow splitter, Waters, the Netherlands). Samples were directly collected onto a sample plate (MTP AnchorChip 200/384, Bruker Daltonics, Germany) in fractions of 15 s taking into account the system gradient delay time and column volume before starting collecting. After air-drying, 1µl of a 20x diluted α-cyano-4-hydroxy-cinnamic acid solution was added to each spot. This solution was prepared by diluting a saturated α-cyano-4-hydroxy-cinnamic acid solution (prepared in 50% v/v acetonitril, 49% v/v water, 1% v/v TFA) 20 times into the same solution. Sample spots were recrystallized by applying 1.5 µl of 60% v/v ethanol, 30% v/v acetone, 10% v/v water and 0.1% v/v TFA to all samples and allowing them to air-dry. Mass spectra were measured with Bruker Ultraflex MALDI-TOF/MS equipped with a reflectron (Bruker Daltonics, Germany) in positive mode with a high precision method. Spectra were measured after external quadratic calibration with a peptide standard mixture containing 9 peptides in the mass range between 757 and 3146 Da (Bruker Daltonics, Germany). In general, spectra were summed for about 500 – 1000 laser pulses (20 Hz, N2-laser at 337 nm) and internally recalibrated with Protein A derived peptides and/or trypsin autolysis peaks when present. All m/z values also present in the control peptide sample were subsequently removed from the m/z list obtained for the immunoprecipitated SERK1 protein complex. Remaining m/z values were checked for sodium and/or potassium adducts (difference of 22.990 and 38.946 Da respectively). When found, these m/z values were deleted too. For identification of SERK1 the program GPMAW (http://welcome.to/gpmaw/) was used to determine which m/z values correspond to tryptic peptides from the predicted protein sequence of SERK1. These selected m/z values were used as input for the MASCOT program to search either the Arabidopsis database or all entries, without selecting a taxonomy. Posttranslational modifications phosphorylation (of serine, threonine and tyrosin) and oxidation of methionines were allowed and a peptide m/z tolerance of 100 ppm was used. After identification, the m/z values that matched the SERK1 protein were removed from the list before searching for other proteins. Alternatively, the searches were performed with the complete m/z list directly using the MASCOT search engine (http://www.matrixscience.com) (Perkins et al., 1999).

Results

Immunoprecipitation and detection of SERK1-CFP protein

In order to purify and detect the SERK1 protein and its interacting partners directly from seedlings, C-terminal SERK1-CFP fusion proteins were stably expressed in Arabidopsis wild type plants under control of the SERK1 promoter as described by (Kwaaitaal et al., 2005). The expression pattern of SERK1-CFP was identical to the expression pattern of a previously characterized SERK1-YFP protein (Kwaaitaal et al., 2005). Similar to SERK1-YFP expressing plants no unusual phenotype was observed, while introduction of the same SERK1 promoter-SERK1-CFP construct as used here in the SERK1 knock-out mutant background restored the male sterility phenotype of the double serk1-1 serk2-2 mutant (Albrecht et al., 2005). This confirms that the fusion protein did not interfere with its endogenous counterpart and was fully functional. Total proteins extracted from 7 day old SERK1-CFP expressing seedlings were separated on SDS-PAGE and after blotting on a membrane, the fusion proteins were detected with anti-GFP antibody (Figure 1, lane 1). The same protein extract was detected with anti-GST-SERK1 kinase antibodies, that recognize endogenous SERK1 as well as the SERK1-CFP fusion proteins, confirming the specificity of the anti-GFP antibodies (Figure 1, lane 2). After immunoprecipitation an aliquot was analysed with anti-GFP antibody to confirm that SERK1-CFP was indeed recovered in the immunoprecipitation. A band of 100 kDa was recognized only in protein extracts of the transgenic line (Figure 1 lane 4) and not in extracts of wild type seedlings (Figure 1, lane 3).

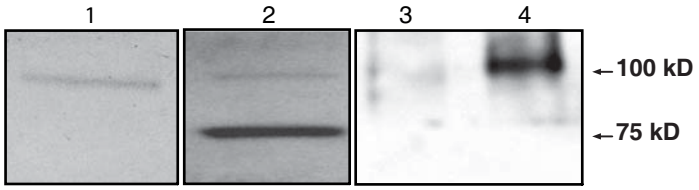


Figure 1. Detection of SERK1 and SERK1-CFP from wild type and transgenic plants. Total protein extracts from SERK1-CFP transformed seedlings and wild type seedlings were immunoprecipitated (IP) with anti-GFP antibodies. Thirty µg of total protein extracted from SERK1-CFP seedlings (lane 1 and 2) and the IP products from SERK1-CFP seedlings (lane 4) and wild type (lane 3) were separated on a 10% SDS-PAGE gel. The blots were incubated with anti GFP antibodies (lanes 1, 3 and 4) or with anti-GST-SERK1 kinase antibodies (lane 2). The size of the marker proteins is indicated by arrows.

LC/MALDI-TOF/MS identification of SERK1 and its interacting partners in vivo

To identify proteins that bind to SERK1 as extracted from SERK1-CFP expressing seedlings we subjected the immunoprecipitated proteins (bound to the protein A-Sepharose beads) to trypsin digestion. To reduce the influence of ion suppression effects in MALDI-TOF/MS measurements and obtain more peptide peaks, separation of the tryptic peptides by reverse phase column chromatography was performed before subjection to MALDI-TOF/MS measurements. As a negative control, samples obtained from an immunoprecipitated protein extract from wild type seedlings with anti GFP antibody were analysed in the same way by LC/MALDI-TOF/MS.

A representative LC/MALDI-TOF/MS spectrum, recorded for fraction 9 from the 24 LC fractions obtained after trypsin digestion of the immunoprecipitated SERK1 complex is shown in Figure 2A. In Figure 2B a spectrum recorded for fraction 9 of the trypsin-digested immunoprecipitated negative control extract is shown. All mass over charge (m/z) values also present in the negative control, that is values belonging to Protein A, GFP antibodies and proteins aspecifically immunoprecipitated by the GFP antibodies were removed from the m/z list. The remaining values were then compared with the predicted tryptic peptide masses for the Arabidopsis proteome as described in Materials and Methods. The upper panels of Figure 2C, 2D, 2E and 2F show four representative spectra, expanded from the spectrum shown in Figure 2A, that led to the identification of the SERK1 interacting partner 14-3-3nu. Both peaks with m/z of 1492.7 (Figure 2C) and 1611.8 (Figure 2E) are predicted with one phosphorylated residue present. The peak with m/z value of 1531.8 (Figure 2D) has the same predicted peptide sequence as the one predicted for m/z of 1611.8 (Figure 2E), but without phosphorylation of the serine residue. The same peptide sequence but now extended due to 2 missed tryptic cleavage sites is predicted for the peak with a m/z value of 1716.8 (Figure 2F). All lower panels of Figure 2C-F show the corresponding expanded spectra recorded for the negative control sample shown in Figure 2B. The combined results of comparable analysis carried out for the list of remaining m/z values were used to compile the list of identified proteins as presented in Table 1. As expected the SERK1 protein was identified to be part of the complex. This identification was based on 13 tryptic peptide matches and a Mascot score of 84. All of the peptides found covered the kinase domain of SERK1 and are located on the exterior of the protein including the phosphorylated peptides based on structural model for SERK1 protein (Shah et al., 2001a). None of the peptides were matching the sequence of the extracellular domain, which may be due to post-translational modification by glycosylation (Shah et al., 2001b) or the presence of two disulfide bounds between the four cysteines present, making trypsin digestion more difficult. One peptide could be identified in which oxidation of methionine had occurred, while three other peptides were identified that were modified by phosphorylation. The second receptor in the SERK1 complex was identified as the SERK3 or BAK1 protein. LC/MALDI-TOF/MS fingerprint analysis revealed tryptic peptide m/z values from both SERK1 and SERK3, but because of the high homology in their protein sequences (78%) 8 out of 13 of the predicted m/z values are matching with the sequence of both proteins.

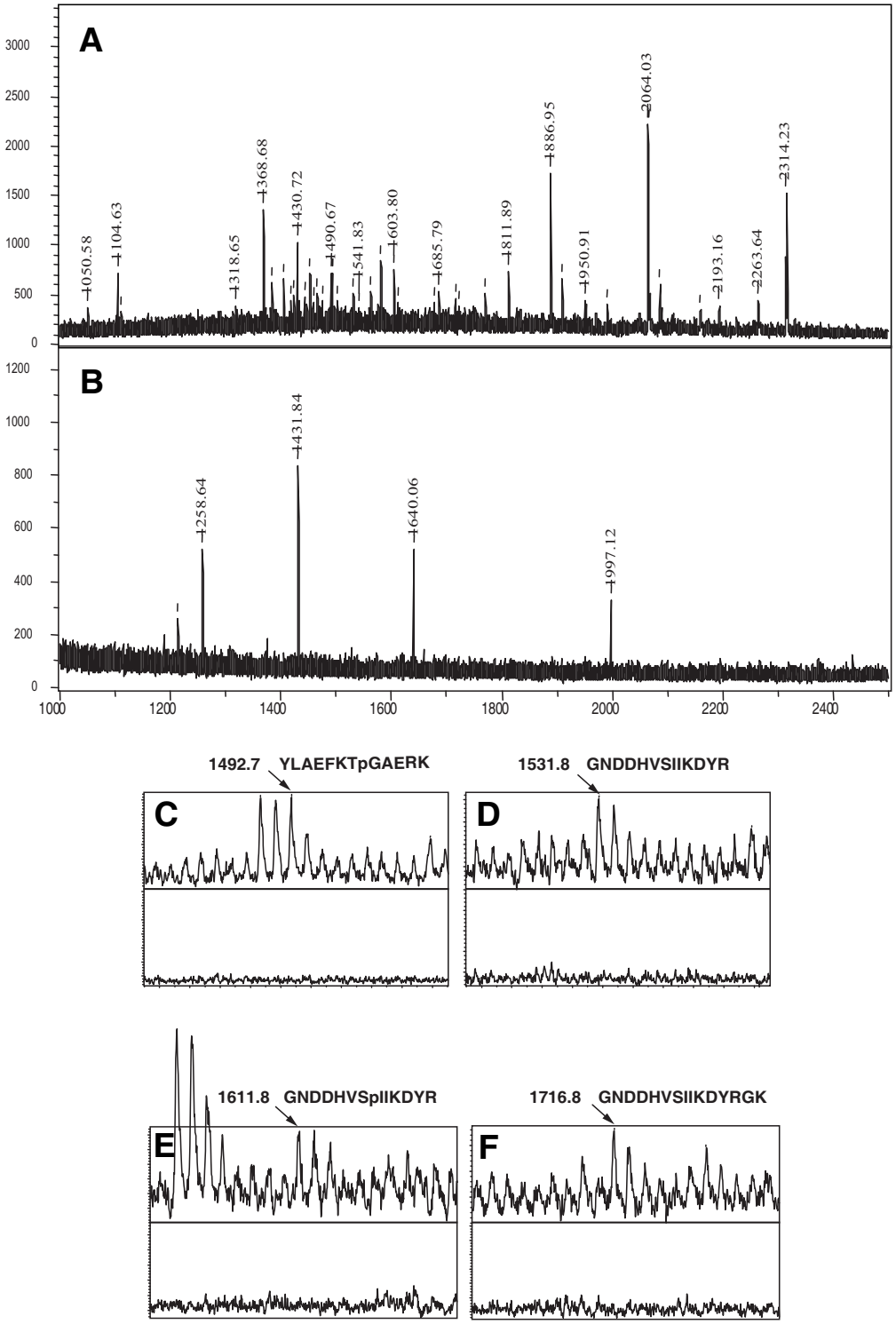
Five peptides unique for SERK3 were identified, 3 without and 2 with modification by phosphorylation. By inspecting the model of the SERK1 protein structure four of the five SERK3-specific peptides identified are from a region also yielding peptides used for identification of the SERK1 protein. This finding corroborates the identification of SERK1 and SERK3. For SERK2, which is 90% homologous to SERK1 on the protein level, five m/z values are identical for both proteins. No m/z values unique for SERK2 were found. The third receptor found in the SERK1 complex was the BRASSINOSTEROID INSENSITIVE 1 (BRI1) receptor (Li and Chory, 1997) identified on the basis of 17 peptides, most of them covering the kinase domain. Most of the peptides identified with phosphorylations were also found as non-phosphorylated or as oxidized peptides, which provide additional evidence for identification as the BRI1 protein. Since SERK3 (BAK1) is reported to be the co-receptor of BRI1 (Li et al., 2002; Nam and Li, 2002), the presence of both these receptors suggests that there is a direct link between SERK1 activity and brassinolide signaling. In addition to SERK1, SERK3 and BRI1, several proteins were found that were previously reported to be interacting with SERK1 and therefore were expected to be present in the SERK1 immunoprecipitated complex. One of them is the Arabidopsis kinase associated protein phosphatase (KAPP). KAPP was shown to interact only with a catalytically active kinase domain of SERK1, involving Thr-468 (Shah et al., 2001a). In a yeast two-hybrid screen two other proteins were found to interact with the kinase domain of SERK1, CDC48A and 14-3-3λ (Rienties et al., 2005). MALDI-TOF/MS fingerprint analysis indeed revealed tryptic peptides derived from CDC48A (Table 1). In our search 14-3-3nu, rather then 14-3-3λ was identified with a high score after searching even with the complete m/z list. Apparently only these two members of the large Arabidopsis family of 14-3-3 proteins can interact with the SERK1 receptor. Finally, two unexpected proteins were found. The first is the MADS box transcription factor AGL15 (Heck et al., 1995) and a putative CONSTANS-like B-box zinc finger protein (At2g33500). The zinc finger protein is annotated as one large 401 aa protein and also as a shorter protein of 186 aa. We only found peptides covering the shorter protein. To validate that the proteins as found above indeed interacted with the SERK1 protein, we treated control samples with SDS or with 0,5 M NaCl prior to co-immunoprecipitation in order to destroy protein-protein interactions. The resulting list of m/z values revealed only the presence of the SERK1 protein (results not shown). Finally, we employed our co-immunoprecipitation procedure to the GFP-tagged BRI1 receptor and to the GFP-tagged integral membrane protein PIN1. For BRI1, the resulting m/z lists did not reveal the presence of any of the SERK1 interacting proteins as listed in Table 1 (results not shown). We therefore conclude that the coimmunoprecipitation/LC/MALDI-TOF/MS procedure for identifying protein complexes in Arabidopsis as presented here is reliable and identifies specific interacting partners.

Table 1. SERK1-interacting proteins identified by MALDI-TOF-MS. The proteins identified in the SERK1complex all have a MASCOT score over 60 and were grouped according to postulated function. Interactions were confirmed using co-immunoprecipitation (CoIP), in case of SERK1 alone by immunoprecipitation (IP), yeast-twohybrid interaction (Y2H), (Rienties et al., 2004) and by detection of FRET, which is indicative of protein proximity in transient protoplast transfection assays (Sha et al., 2002; Rienties et al., 2004; Aker et al., 2006 and this study). ND is not determined.

Protein name	At number	Number of peptides identified	Sequence coverage (%)	Mascot Score obtained	Protein size (kD)	Conformation from additional experiments
SERK1	At1g71830	13	15	84	69	IP
SERK3	At4g33430	13	17	96	68	FRET
BRI1	At4g39400	17	9	76	130	CoIP, FRET
KAPP	At5g19280	18	25	102	65	FRET, Y2H
CDC48A	At3g09840	14	19	80	89	CoIP, Y2H, FRET
14-3-3nu	At3g02520	12	23	110	30	CoIP
AGL15	At5g13790	7	37	62	30	ND
Zinc finger protein	At2g33500	11	45	195	21	ND

BRI1, SERK3, CDC48A and 14-3-3nu interact in vivo with SERK1

To confirm our results obtained by LC/MALDI-TOF/MS we employed co-immunoprecipitation and FLIM based analysis to detect the interaction between SERK1 and its interacting partners in vivo. CFP-tagged SERK1 protein was immunoprecipitated with anti-GFP antibodies and the precipitated proteins were detected using anti BRI1 antibodies. A band with the expected mobility of BRI1 was visualized (Figure 3A, lane 1), that was absent in an immunoprecipitation of wild type plants with the GFP antibody (Figure 3A, lane 2). BRI1 antibodies recognize endogenous BRI1 as well as BRI1-GFP fusion proteins, and can precipitate BRI1 from a total plant protein extract (Figure 3B, lane 1-3). BRI1 antibodies are specific for BRI1, since they do not detect a band with the expected mobility for BRI1 in the protein extract from strong *bri1-4* allele homozygous plants (data not shown). Reciprocal co-immunoprecipitation with anti BRI1 antibodies revealed the presence of SERK1-CFP in the co-immunoprecipitated proteins (Figure 3C, lane 1). Previously we have shown that FRET can be detected between the cyan and yellow fluorescent GFP variants (CFP and YFP) of SERK3 and BRI1 when transiently expressed in cowpea protoplasts. Co-expression of BRI1 and SERK3 results in a change of the steady-state distribution of both receptors due to accelerated endocytosis (Russinova et al., 2004). To determine whether the SERK1 protein can form heterodimers with either BRI1 or SERK3 in the plasma membrane we employed a similar transient assay in protoplasts to detect FRET by FLIM. In Figure 3D, we show the fluorescence intensity images of protoplasts transfected with SERK1-CFP alone (Figure 3 D-I) , SERK1-CFP together with BRI1-YFP (Figure 3 D-II) and of SERK1-YFP together with


A
1050.58
1104.63
1318.65
1368.68
1430.72
1490.67
1541.83
1603.80
1685.79
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1886.95
1950.91
2064.03
2193.16
2263.64
2314.23
B
1258.64
1431.84
1640.06
1997.12
1492.7 YLAEFKTpGAERK
1531.8 GNDDHVSIIKDYR
C
D
1611.8 GNDDHVSpIIKDYR
1716.8 GNDDHVSIIKDYRGK
E
F

Figure 2. LC/MALDI-TOF/MS obtained after trypsin digestion of SERK1-GFP immunoprecipitated complex.

(A). MALDI-TOF/MS spectra from SERK1-CFP immunoprecipitated complex (upper panel, fraction number 9 out of 24) compared to corresponding spectra (fraction number 9) from the control IP performed with wild type plants (B). The spectra were obtained after LC separation from the trypsin-digested proteins after IP. The X-axis represents m/z (mass over charge) values. The Y-axis represents the intensity of the peaks in arbitrary units. (C), (D), (E), (F). Four representative spectra expanded from A (upper panels) and B (lower panels), which led to identification of one of the SERK1 interacting partners 14-3-3nu are shown. The corresponding predicted peptides are also shown. The peaks are present only in the spectra from SERK1-CFP immunoprecipitation (upper panel) and not in the control (lower panel).

SERK3-CFP (Figure 3 D-III). The corresponding fluorescence lifetime images are compared in Figure 3 D-IV to VI. The results presented in Figure 3 D-V show that SERK1 and BRI1 heterodimerize in small patches in the plasma membrane as indicated by the significant reduction in the average lifetime (τ) from 2.5 to 2.1ns of the donor (SERK1-CFP) molecules in those areas indicated by an arrow. A similar phenomenon was described for the SERK3/BRI1 heterodimers (Russinova et al., 2004). SERK1-YFP also formed heterodimers with SERK3-CFP protein in the plasma membrane as shown by the green coloured plasma membrane in Figure 3 D-VI that corresponds to an average lifetime of 1.9 ns for the donor (SERK3-CFP) molecules. Thus, the co-localization in the plasma membrane and the ability to form heterodimers in plant protoplasts strongly suggest that SERK1, SERK3 and BRI1 proteins can be part of the same complex in intact plant membranes. To confirm the in vivo interaction of CDC48A with SERK1, we co-immunoprecipitated SERK1 from Arabidopsis tissue-cultured cells with anti GST-SERK1 kinase antibodies (Rienties et al., 2005). The precipitated proteins were analysed by immunoblotting using anti CDC48-antibodies (Rancour et al., 2002). A band with the expected mobility for monomeric CDC48A was detected in the total protein extract (Figure 4A, lane 1), and in the immunoprecipitated sample (Figure 4A, lane 3), showing that AtCDC48A indeed co-immunoprecipitates with SERK1. No CDC48A band was detected in the negative control using rabbit preimmune serum for immunoprecipitation (Figure 4A, lane 2). Similarly the presence of a 14-3-3 protein in the complex with SERK1 in vivo was verified by co-immunoprecipitation with GFP antibodies of CFP-tagged SERK1 followed by immunodetection with anti 14-3-3 antibodies (Figure 4B, lane 1). No 14-3-3 band was detected in the negative control using wild type seedlings for immunoprecipitation (Figure 4B, lane2). The 14-3-3 antiserum can recognize in vitro produced 14-3-3nu protein (data not shown) as well as 14-3-3λ, confirming that SERK1 can interact with both 14-3-3λ (Rienties et al., 2005) and 14-3-3nu isoforms (this study). A summary of the experiments providing additional evidence for the presence of proteins in the SERK1 complex is shown in Table 1.

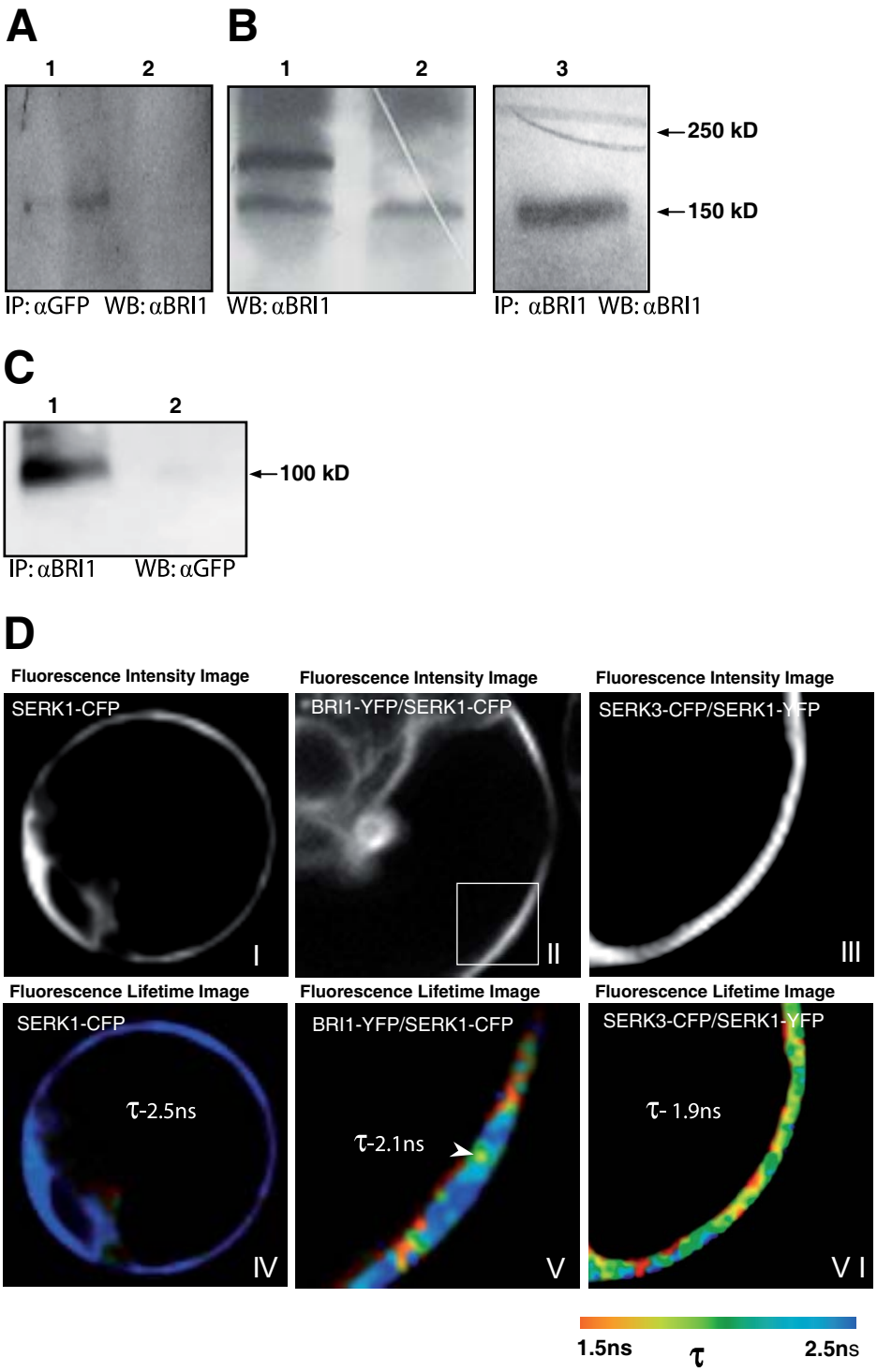

A
1
2
IP: αGFP WB: αBRI1
B
1
2
WB: αBRI1
3
250 kD
150 kD
IP: αBRI1 WB: αBRI1
C
1
2
100 kD
IP: αBRI1 WB: αGFP
D
Fluorescence Intensity Image
SERK1-CFP
I
Fluorescence Intensity Image
BRI1-YFP/SERK1-CFP
II
Fluorescence Intensity Image
SERK3-CFP/SERK1-YFP
III
Fluorescence Lifetime Image
SERK1-CFP
τ-2.5ns
IV
Fluorescence Lifetime Image
BRI1-YFP/SERK1-CFP
τ-2.1ns
V
Fluorescence Lifetime Image
SERK3-CFP/SERK1-YFP
τ- 1.9ns
V I
1.5ns
τ
2.5ns

Figure 3. Direct interaction of SERK1 with BRI1 and SERK3

(A). Total protein extract from SERK1-CFP transgenic seedlings was immunoprecipitated using anti-GFP antibodies (lane 1). As a negative control proteins extracted from wild type plants were precipitated using anti GFP antibodies (lane 2). (B) Total protein extracts from BRI1-GFP transgenic seedlings (lane 1) and wild type (lane2) and immunoprecipitated products from wild type seedlings (lane 3) were separated on 8% SDS-PAGE gel and were detected using anti BRI1-antibodies. (C) Protein extract from SERK1-CFP transgenic plants was precipitated using anti BRI1-antibodies (lane 1). As a negative control protein extract from wild type plants was precipitate with the same antibodies (lane2). The resulting immunoprecipitates were separated on 8% SDS-PAGE, transferred to a membrane, and probed with anti-GFP for detection of SERK1-CFP fusion proteins (C) or anti-BRI1 antibodies for detection of BRI1 protein (A and B). Arrows indicate the size of the marker proteins. (D) FRET between SERK1, SERK3 and BRI1 imaged by FLIM on cowpea protoplasts transiently expressing SERK1-CFP (I and IV), BRI1-YFP/SERK1-CFP (II and V) and SERK3-CFP/SERK1-YFP (III and VI) for 16 hours. The fluorescence intensity images representing a steady state of the donor SERK1-CFP or SERK3-CFP fluorescence are presented in (I), (II) and (III) respectively. The mean fluorescence lifetime values (τ) and the lifetime distribution for the images in (I), (II) and (III) are presented as pseudocolour images in (IV), (V) and (VI). The arrowhead points to an area with a short lifetime, indicative for FRET. Note the colour bar where dark blue colour is used to display a τ -of 2.5 ns (no interaction) and light green (τ)- of 1.9 ns (interaction). The red to dark orange colour corresponding to a τ-of 1.5 ns is due to autofluorescence effects.

SERK1 Genetically Interacts with BRI1

Analysis of the single T-DNA insertion mutant *serk1-1*, an allele that produces a kinase-dead receptor protein, did not reveal a morphological plant phenotype as well as *serk2-2* (Albrecht et al., 2005). We therefore crossed the *serk1-1* allele into the weak BRI1 mutant allele *bri1-119*. This allele contains the G644D missense mutation in the island domain of BRI1 (Fredrichsen et al. 2000). At the seedling level 7-day old *bri1-119* mutants exhibit mild morphological characteristics of the bri1 phenotype such as small seedlings, short petiols and round, dark green, inwardly curved true leaves (Figure 5A). *Bri1-119* plants developed smaller, compact rosettes (Figure 5D) as compared to the wild type. The weak *bri1-119* allele is a semi-fertile dwarf that has less reduced stature (Figure 5D) when compared to severe *bri1* alleles (Noguchi et al., 1999). The *serk1-1* mutation clearly enhanced all BRI1 associated phenotypes, especially the length of the petioles and excessive inward curling now seen in double mutant (*bri1-119 serk1-1*) of 7d old seedlings (Figure 5B), as well as a reduction in size of the rosette (Figure 5E). The stature of the double *bri1-119 serk1-1* mutant was also reduced (Figure 5H). For comparison, we also introduced the *serk3* mutation (Russinova et al., 2004), an allele of the BAK1 co-receptor, into the *bri1-119* mutant. The resulting *bri1-119 serk3* double mutant had even smaller rosette (Figure 5F) and grew with a severely reduced stature (Figure 5I) resembling strong *bri1* alleles, indicating that *serk3* mutation had even stronger effect on the *bri1-119* mutation

than *serk1-1*. In comparison with *serk1-1* and *serk-3 bri1-119* double mutants *serk2-2* mutant allele (Albrecht et al., 2005) had no effect on *bri1-119* mutation, showing that the effect of the double *serk1-1* and *serk3 bri1-119* mutants is not influenced from the two different ecotypes used (Columbia for *serk1-1*, *serk2-2* and *serk3* and Enkheim-2 for *bri1-119*). These results provide genetic evidence that SERK1 is involved in BRI1 signaling and confirm the role of the SERK3 (BAK1) gene as a BRI1 co-receptor.

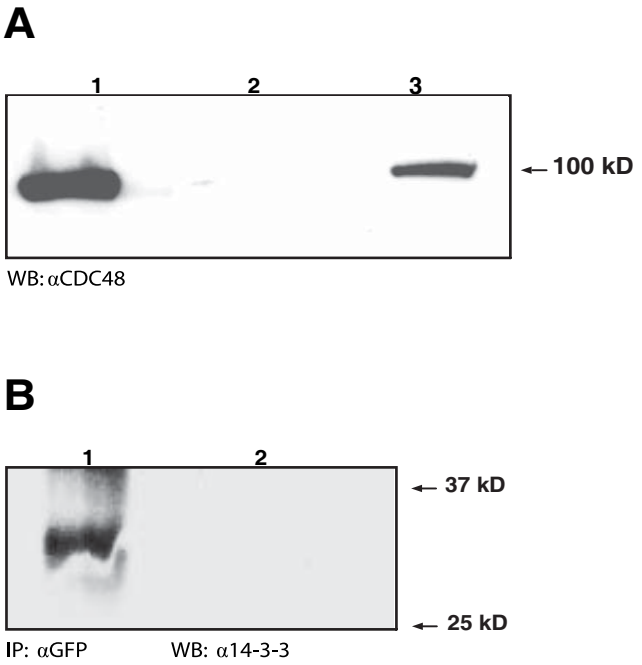


Figure 4. SERK1 interacts in vivo with AtCDC48A and 14-3-3(A). Co-immunoprecipitation of SERK1 and AtCDC48A. Total protein extract was isolated from Arabidopsis tissue cell culture and directly analysed by SDS-PAGE and immunodetected with anti-CDC48 antibody (lane 1) or immunoprecipitated with anti- SERK1-kinase antibody (lane 3) or preimmune sera (lane 2) and detected with anti AtCDC48 antibody. (B). Co-immunoprecipitation of SERK1 and 14-3-3. Total protein extract was isolated from SERK1-CFP transgenic plants (lane 1) or wild type plants (lane 2) and precipitated with GFP antibody. Precipitated proteins were immunodetected with anti 14-3-3 antibodies. The size of the marker proteins is indicated by arrows.

Identification of a Membrane-Associated SERK1-Containing Complex using Blue Native PAGE

In previous studies, we have shown that SERK1 is located at the membrane in protoplasts and in plants (Russinova et al., 2004; Kwaaitaal, 2005), and can directly interact in vitro and in vivo with KAPP and 14-3-3λ , and in vitro with CDC48A (Shah et al., 2002; Rienties et al., 2005). Our results presented here confirm the previously observed interactions and showed that SERK1 can be present in a complex with several other proteins. It is not clear if the SERK1 complex harbors all of the proteins listed in Table 1 at the same time or that we immunoprecipitated multiple forms of the SERK1 complex. Different forms could exist in different cell types or in different subcellular locations such as plasma membrane or after internalization (Russinova et al., 2004). To determine the size of the SERK1 complex in planta, microsomal preparations from SERK1-CFP and BAK1-GFP expressing seedlings and wild type seedlings were solubilized with Triton X-100 and subjected to blue native (BN) PAGE. In this technique, coomassie dyes and aminocaproic acid are employed to induce a charge shift and improve the solubilization of membrane-associated proteins (Rivas et al., 2002). To detect SERK1 within the potential protein complex, single lanes of the BN-PAGE were cut and mounted on a denaturing SDS gel. After electrophoresis the gel was subjected to immunoblot analysis using anti-GFP-antibody or anti-GST-SERK1 kinase antibody. Using the mass standards about 85 % of the protein complex containing SERK1 was determined to have a mean relative mass of 350 kDa. The remaining 15% appears to exist as complex with a mean mass of 450 kDa. The maximum size is approximately 500 kDa (Figure 6A). This variability in protein complex size is also seen when the tagged- SERK1-CFP complex is detected by anti-GFP antibodies (Figure 6B). About 90 % of the CFP-tagged complex has a mean mass of 400 kDa and 10 % has a mass of about 500 kDa. Although there is a clear difference in the mean size of the complex with and without the CFP tag we cannot precisely estimate if this difference is 25 kDa (one molecule of CFP protein) or 50 kDa (two molecules of CFP protein). The results show that there is one abundant “core” complex for SERK1 of about 350 kDa. We first examined whether BRI1 was part of the “core” SERK1 complex. A microsomal fraction prepared from SERK1-CFP transgenic plants was therefore examined for the presence of BRI1 proteins. BRI1 was found in complex with the same size range of 400 to 500 kDa (Figure 6C1) as the SERK1-CFP proteins. In contrast to SERK1, most of the BRI1 protein was found as probably a homodimeric form of approximately 250 kDa (Figure 6C2). The occurrence of potential BRI1 homodimers is consistent with our observation of BRI1 homodimerization observed in protoplast (Russinova et al., 2004) and was recently shown in plants (Wang et al., 2005a). The observed smaller and higher forms of BRI1 are probably due to recognition of differentially processed forms (Figure 6C). The distribution of BAK1 in the microsomes prepared from BAK1-GFP transgenic plants was then studied by BN-PAGE. BAK1-GFP was found in complex(es) with the same size range as the SERK1-CFP proteins (Figure 6D). These data suggest that BRI1, SERK1 and SERK3 are indeed present in the main 350 kDa “core” complex and are also present in complexes of higher molecular mass. No signal was

detected when solubilized microsomes from wild type seedlings were analyzed for the presence of GFP, confirming that the observed signal in Figure 6B and 6D are indeed derived from microsomal SERK1-CFP or BAK1-GFP proteins (Figure 6E). Collectively, the results obtained from the BN-PAGE suggest that the "core" SERK1 complex of approximately 350 kDa as well as the larger complexes consist of the membrane receptors SERK1, SERK3 and BRI1. Assuming that all proteins listed in Table 1 are present as monomers, a predicted size of about 500 kDa is obtained. Although this is about the maximum size of the SERK1complex as estimated by BN-PAGE, it exceeds the size of the predominant complex of 350 kDa.
In summary, we propose that in Arabidopsis seedlings a "core" SERK1 membrane complex consists of SERK1, BRI1 and SERK3. Collectively this would account to approximately 270 kDa when all proteins are present as monomer or between 350 and 400 kDa in a more conventional tetrameric arrangement. This is clearly in range of the observed sizes of BN-PAGE gels. KAPP is only interacting with the active SERK1 receptor (Shah et al., 2002) and could represent one of the lesser abundant higher Mr forms.

Discussion

In this work we have used a combination of co-immunoprecipitation based on the GFP variant CFP as a protein tag and LC/MALDI-TOF/MS to identify proteins associated with the membrane receptor SERK1. Based on our data with other membrane proteins from Arabidopsis this technique appears to be of general use. When compared with other techniques to identify interacting proteins such as in vitro pull-down assays and yeast two-hybrid screen the main advantage is that the immunoprecipitation combined with LC/MALDI-TOF/MS allows protein complex isolation under native conditions, therefore allows functional studies in which the activity or post-translational modification of one or several proteins in the complex can be examined (Drewes and Bouwmeester, 2003). Another advantage is that the CFP tag allows directly following all subcellular localisations of the fusion proteins. This allows predictions concerning the nature of the proteins recovered, especially in the case of membrane receptors undergoing recycling (Russinova et al., 2004). Other tags have been employed such as tandem affinity purification (TAP) tags for plant protein complex isolation in a transient expression system or in stably transformed transgenic lines, using the cauliflower mosaic virus 35S promoter (Rohila et al., 2004; Rubio et al., 2005). Our procedure differs in several aspects; we have used an endogenous promoter-based construct and only employed CFP as a protein tag, thereby minimising potential biological problems arising from the use of strong constitutive promoters. Also, we have used integral membrane protein as bait rather then soluble proteins so far employed.

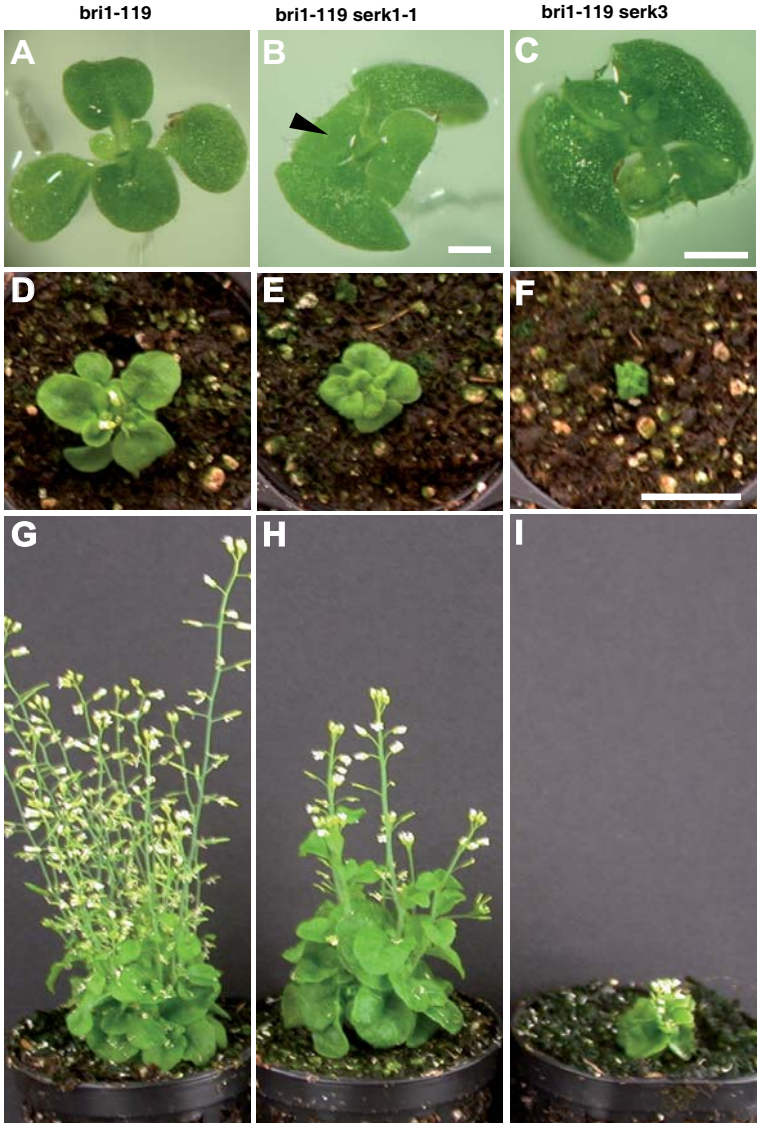
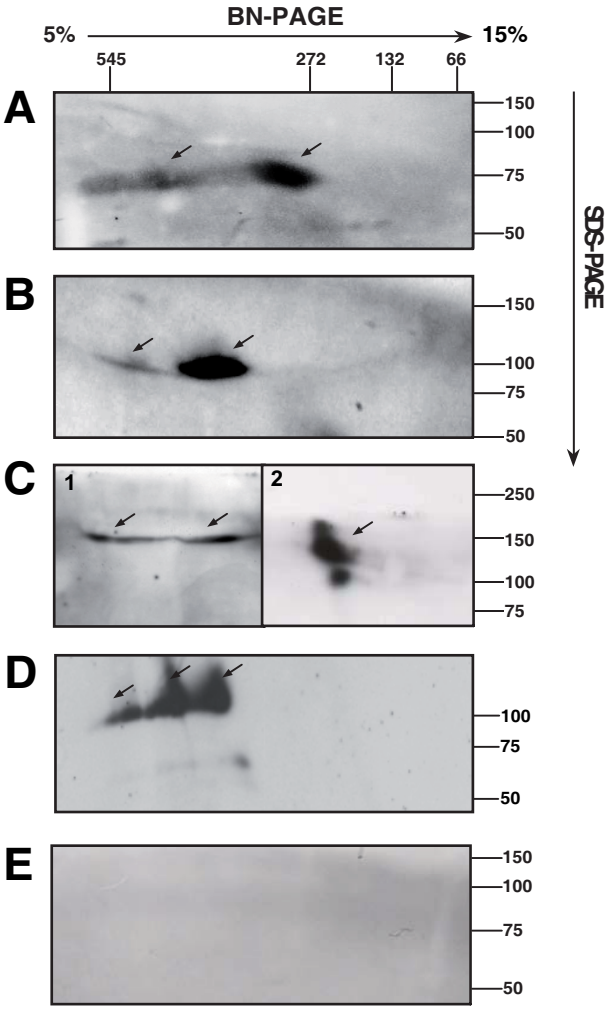


Figure 5. SERK1, SERK3 and BRI1 function together. serk1-1 and serk3 mutations enhance the weak bri1-119 mutation. 7-day old seedlings are shown in A, B and C. 3-weeks old plants are shown in D, E and F and 6-weeks old plants in G, H and I. (A, D and G) bri1-119, (B, E and H) bri1-119 serk1-1 and (C, F and I) bri1-119 serk3 mutations. Note the increase in the curliness of the first true leaves and reduction in the petiole length in the double mutant seedlings as well as the reduced rosette width of the double mutant plants.

Figure 6. BN-PAGE of solubilized WT and SERK1-CFP microsomes.
Microsomes were isolated from wild type plants (A, E) and SERK1-CFP (B and C) or BAK1-GFP (D) expressing transgenic plants and subjected to BN- PAGE in the first dimension and SDS-PAGE in the second dimension. Proteins were immunodetected using anti SERK1-kinase antibodies (A) or anti GFP antibodies (B, D and E) or anti BRI1 antibodies (C). C1 is part of the membrane exposed for 3h and C2 is part of the same membrane exposed for 3min. The molecular masses of standard proteins used are indicated at the top (BN-PAGE) and on the right (SDS-PAGE). Arrowheads in (A) indicate the positions of main SERK1 complex sizes of 350 and lower amount of 450 kDa and in (B) the positions of the main SERK1-CFP complex of 400 and the lower amount of 500 kDa. Arrowheads in (C) indicate the position of the main BRI1 complexes of 250, 350 and 450 kDa (C) and in (D) indicate the main BAK1-GFP complexes ranging from 350 kDa to 450 kDa.



SERK1 and brassinosteroid signaling

A highly interesting finding was to identify two members of the brassinosteroid (BR) signaling pathway, the main BR receptor BRI1 (Li and Chory, 1997) and its co-receptor BAK1 (SERK3) (Li et al., 2002; Nam and Li, 2002) as components of the SERK1 complex. This implies that SERK1 is a so far unrecognized element of BR signaling. Recently we showed that the SERK3 receptor may have a role in internalisation of the BRI1 protein after heterodimerisation and that BRI1 can also form homodimers in the plasma membrane (Russinova et al., 2004). Further biochemical evidence comes from the finding that BRI1 and SERK1 as well as SERK1 and SERK3 can interact in the plasma membrane of protoplasts (this study). FRET studies between SERK1-CFP and SERK1-YFP have shown that only 15% of SERK1 exists as homodimers on the plasma membrane of protoplast cells (Shah et al., 2001b). Our results showed that both SERK1 and SERK3 heterodimerize with the BRI1 receptor suggesting that both co-receptors can have a comparable activity in terms of BRI1 signaling and/or internalization. Another piece of evidence is that a *bak1* null allele shows less severe phenotypes than *bri1* loss-of-function alleles indicating that partially redundant activity could be attributed to different SERK family members (Li et al., 2002; Nam and Li, 2002). Genetic analysis of the *serk1-1 bri1-119* double mutants suggests that SERK1-BRI1 interactions indeed affect BRI1-mediated signaling in planta, but to a lesser extend than SERK3-BRI1 interactions. Notably, none of the proposed downstream components of BRI1, such as BES1, BZR1, or BIN2 (Yin et al., 2002; Wang et al., 2002b; Li and Nam, 2002) were detected in the SERK1 complex.

It is unlikely that all proteins (as identified by MALDI-TOF/MS) listed in Table 1 are present in a complex together with SERK1 at the same time. Especially since proteins such as receptors and CDC48A (Rancour et al., 2002; Rancour et al., 2004) are known to shuttle between mono and multimeric forms and can change their interaction properties upon activation and relocalization, multiple forms of the same complex are to be expected. An indication for the presence of different complexes is the localization of SERK1 in the plasma membrane as well as in internalised membrane compartments (Russinova et al., 2004; Kwaaitaal, 2005).

It was proposed that BRI1 forms heterodimers with SERK3 to initiate brassinosteroid signaling upon BR binding (Nam and Li, 2002; Li et al., 2002). This was recently confirmed in an elegant demonstration of the in vivo phosphorylation properties of the BRI1 receptor (Wang et al., 2005b). In that work it was clearly shown that the interaction between the BAK1 (SERK3) proteins and BRI1 is brassinolide dependent. Since we did not apply exogenous ligands before isolation of the SERK1 complex, it is likely that only a small fraction of the complexes we isolated are actively signalling via the SERK1 protein. This seems to be supported by the fact that most of the BRI1 receptors were found to be unassociated with SERK1 in seedlings. Although our results do not allows us to precisely predict the composition, it is likely to assume that SERK1, SERK3 and BRI1 receptors can form tetrameric complexes in analogy to the functional complex of TGF- β receptors. Each type of TGF- β receptor is also present as a ligand-independent dimer on the cell surface. TGFβ binding to the TβRII homodimers promotes the formation of (TβRI)2/(TβRII)2 heterodimers where TβRI is phosphorylated by

the constitutively active TβRII and becomes activated to propagate the TGFβ signal. Combinatorial interactions in the tetrameric receptor complex allow differential ligand binding or differential signaling in response to the same ligand. One receptor combination often binds different ligands, and patterns of ligand and receptor expression dictate which receptor-ligand combination are activated (Feng and Derynck, 1997). This so-called tetrameric model was also recently proposed for BRI1 and BAK1 (SERK3) (Wang et al., 2005a).

Other SERK1 complex components

The presence of the PP2C phosphatase KAPP, the 14-3-3nu protein and CDC48A in the SERK1 complex confirm previously obtained results using yeast two hybrid screening and in vitro interaction studies (Rienties et al., 2005). Collectively, these proteins appear to be involved in dephosphorylation, protein interaction and membrane interaction and protein degradation respectively. It is likely that these proteins represent receptor maintenance or trafficking functions. Previously it was shown that SERK1 could interact with 14-3-3λ (Rienties et al., 2005). Our results presented here suggest that in seedlings the receptor preferentially interacts with 14-3-3nu and perhaps in siliques with 14-3-3λ, given the origin of the Arabidopsis cDNA library from young silique tissue that was used for yeast two-hybrid screening. The kinase domain of SERK1 is able to trans-phosphorylate and bind in vitro to CDC48A, 14-3-3λ as well as KAPP (Rienties et al., 2005). In vitro, the interactions are all phosphorylation-dependent. In vivo, it was shown that SERK1 interacts with KAPP only in intracellular vesicles and was proposed to play a role in receptor internalisation as well as in dephosphorylation (Shah et al., 2002). For two other LRR-RLKs, CLV1 (Williams et al., 1997) and FLS2 (Gomez-Gomez et al., 2001) it has been show that KAPP functions as a negative regulator, and may have the same role in controlling signaling trough the SERK1 receptor.

CDC48 protein assembles mainly in hexameric forms (Rancour et al., 2002). In Arabidopsis suspension-cultured cells, soluble (cytosolic) CDC48A protein was found in high molecular order protein of 640 kDa. Recently it was proposed that CDC48A interacts with PUX1 as a monomer and may have a function in regulating plant growth (Rancour et al., 2004) and also in the plant endoplasmic reticulum-associated protein degradation (ERAD) system (Muller et al., 2005).

Plant 14-3-3 proteins have also been found to be associated with G-box transcription factors (Lu et al., 1992). Five Arabidopsis thaliana 14-3-3s, including 14-3-3λ have been shown to interact with other transcription factors (Pan et al., 1999) and current models propose that the interaction with members of the 14-3-3 family is “client-driven” (Paul et al., 2005).

Two transcription factors were found to be associated with the SERK1 complex, AGL15 and a putative CONSTANS-like B-box zinc finger protein. Most likely these two proteins do not interact directly with the receptor, but require 14-3-3 proteins as adaptor proteins. It has been shown that 14-3-3 proteins can also promote the cytoplasmic localization or, conversely, the nuclear localization of transcription factors (reviewed in (Muslin and Xing, 2000). One of the other MADS box transcription factors, AGL24, was shown to directly interact with the

kinase domain of the Arabidopsis Meristematic Receptor-Like Kinase (RLK-MRLK) and to be phosphorylated by the kinase domain of the receptor in vitro (Fujita et al., 2003). The AGL15 protein was shown to accumulate in nuclei but to be also present in the cytoplasm (Perry et al., 1996). SERK1 and AGL15 are highly expressed during embryogenic cell formation in culture and during early embryogenesis. As found for SERK1 (Hecht et al., 2001), AGL15 promotes somatic embryo production from SAM in liquid culture when ectopically over-expressed (Harding et al., 2003; Perry et al., 1996). Interestingly, AGL15 over-expressing tissues also had elevated expression of SERK1 (Harding et al., 2003). It was shown that AGL15 could bind directly to the promoter regions of different targets (Wang et al., 2002a). The authors described that after chromatin immunoprecipitation they obtained DNA fragments containing cis regulatory elements targeted by AGL15, which may contribute to the regulation of the SERK gene by directly binding to its promoter. These data and our findings suggest that SERK1 and AGL15 can be involved in the same signaling pathway.

The other putative transcription factor found in the SERK1 complex belongs to a large family of CONSTANS (CO)-like zinc finger transcription factors, where the zinc finger region regulates protein-protein interactions as found for several animal transcription factors (reviewed in (Griffiths et al., 2003). Recently it was shown that CO protein accumulation is regulated by photoreceptors in photoperiodic flowering (Valverde et al., 2004).

In conclusion, we propose that in Arabidopsis seedlings signaling mediated by the SERK1 receptor combines elements of the BL pathway with a short signal transduction chain where the plasma membrane receptor is in a complex with its cognate transcriptional regulators such as AGL15. Further studies are needed to evaluate this model and to map the phosphorylation sites that are responsible for the activation of receptor kinases and recruitment of downstream signaling components.

Accession Numbers

Sequence data and seed stocks from this article can be found in the GenBank/EMBL data libraries under the following accession numbers; SERK1 cDNA clone (A67827, At1g71830), SERK3/BAK1 cDNA clone (AF384970, At4g33430) and BRI1 cDNA clone (AF017056, At4g39400). The serk1-1 and serk3 insertion lines were obtained from the SIGnAL TDNA-Express collection with the accession numbers SALK_544330 and SALK_034523.56.00 respectively. The serk2-2 insertion lene was obtained from the SAIL lines, with the accession number 119-G03. The bri1-119 and bri1-4 mutants were obtained from the Arabidopsis Biological Resource Center stock number CS399 and 3953 respectively.

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Chapter 3

In vivo complex isolation of BRI1 and SERK3 receptors

Rumyana Karlova, Sjef Boeren, Jacques Vervoort and Sacco de Vries

Introduction

Membrane located plant receptor like kinases (RLKs) play important role in plant signaling pathways. There are more than 600 RLKs in *Arabidopsis* but for only a few the function is known. One of the best studied plant receptors is the LRR-RLK BRASSINOSTEROID-INSENSITIVE1 (BRI1), the main receptor in brassinosteroid (BR) signaling. Brassinosteroids are plant steroid hormones which are involved in diverse processes such as stem elongation, vascular differentiation, male fertility, timing of senescence and flowering, leaf development, and resistance to biotic and abiotic stresses (Li and Chory, 1997; Clouse and Sasse, 1998; Altmann, 1999).Biochemical studies have shown that BRI1 can bind BRs directly (Kinoshita et al., 2005) and can form heterodimers in vivo with SERK1 (Karlova et al., 2006), SERK3 (BAK1) (Li et al., 2002; Nam and Li, 2002) as well as with SERK4 (BKK1) (He et al., 2007). Recently it was shown that the phosphorylation status of the co-receptors of BRI1- SERK1 (Karlova et al., 2008 in press), BAK1 (Wang et al., 2005b) and BKK1 (He et al., 2007) is increased upon BR stimulation. Other negative and positive regulators of BR signaling were identified as the GSK-3/Shaggy-like kinase- BRASSINOSTEROID-INSENSITIVE2 (BIN2) (Li and Nam, 2002) and Ser/Thr phosphatase *bri1* Suppressor1-BSU1 (Mora-Garcia et al., 2004), respectively and based on genetic studies their potential substrates are the BRASSINOZOLE-RESISTANT1 (BZR1) (Wang et al., 2002) and *bri1*-EMS-SUPPRESSOR1 (BES1) (Yin et al., 2002). BRI1 has been shown to interact at the plasma membrane with BKI1, which is a negative regulator of BR signaling (Wang and Chory, 2006). The WD-domain protein TRIP-1 has been also shown to interact in vivo with BRI1 in analogy to the TGF-β RII receptor kinase in mammals, which could also interact with a WD-40 repeat protein (Ehsan et al., 2005). Recently scaffold/adaptor proteins like 14-3-3s were shown to be involved in BR signaling by playing a role in the cytoplasmic translocation of BZR1 upon BIN2-mediated phosphorylation (Ryu et al., 2007). Despite this progress in identifying proteins involved in BR signaling, there is still a big gap in our understanding of BR signal transduction between BR perception at the cell surface and subsequent inactivation of BIN2 in the cytoplasm. Therefore, identification of the in vivo status and composition of BRI1 and BAK1 protein complexes is an important step for the complete understanding of BR signaling.

Three members of the SERK family SERK1 (Karlova et al., 2006), SERK3 (Li et al., 2002; Nam and Li, 2002) and SERK4 (He et al., 2007) were shown to interact in vivo with BRI1. SERK1 and SERK3 can also trans-phosphorylate the kinase domain of BRI1 or to be trans-phosphorylated by BRI1 and participate in BR signaling. The redundancy between SERK3 and SERK4 in regulating BR signaling was proposed (He et al., 2007). Members of the SERK family can act as co-receptors with different main receptors and individual members can act in different signaling pathways. SERK1 was found to act redundantly with SERK2 in tapetum specification and male sterility (Albrecht et al., 2005). SERK1 was found to be in one complex together with SERK3 and BRI1. Using blue native gel electrophoresis we showed that SERK1 and SERK3 are part of BRI1-containing protein complexes with relative masses between 300 and 500 kD. SERK3 was recently implicated to be involved in plant innate immunity

responses (Heese et al., 2007; Kemmerling et al., 2007) and as a component of the flagellin-perceiving FLS2 receptor complex (Chinchilla et al., 2007). SERK3 also has a redundant function with SERK4 in negatively regulating a BR-independent cell death pathway (He et al., 2007). Unraveling the composition of the protein complexes in which SERK receptor are involved will shed light on how this receptor can function in different signaling pathways.

In order to identify the composition of the protein complexes of transmembrane plant receptors we have develop a fast single step purification method based on GFP tag as a bait protein (Karlova et al., 2006). Using this method we have now identified a number of interacting partners for BRI1 and SERK3 with or without exogenous application of BRs.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia was used as the wild type. Seeds were surface sterilized and sown on germination medium (½ Murashige and Skoog (MS) salt and vitamins medium (Duchefa, Haarlem, The Netherlands) supplemented with 0.8 % (w/v) agar (Daishin) and 2 % (w/v) sucrose). Seeds were kept for 2 days at 4°C and then transferred to a growth chamber. Seedlings were grown at 22°C under 16 h light/ 8 h dark periods for 7 days. Arabidopsis plants expressing the BRI1 or SERK3 (BAK1) receptor fused to green fluorescent protein (GFP) under the control of the native promoters were described before (Russinova et al., 2004). The seedlings were induced for 1h with 100 nM epibrassinolide in liquid MS media on a rotary shake platform.

Immunoprecipitations

To prepare total protein extracts, plant material was ground in liquid nitrogen and thawed in extraction buffer containing 50 mM Tris pH 7.5, 150 mM NaCl and 0.5% Triton X-100, supplemented with a protease inhibitor cocktail (Roche, Germany). Samples were incubated on ice for 30 min. The suspension was centrifuged at 200g (Beckman-Microfuge 18 centrifuge) at 4 °C for 3 minutes. The concentration of extracted proteins was determined with the Biorad Protein assay (Biorad). Routinely we used 4 plates (9 cm) with about 400 seven days old seedlings (100 per plate) to prepare a protein extract suitable for immunoprecipitation. Ten millilitres of total plant protein extract (1mg.ml^{-1}) in extraction buffer (isolated as described above) were incubated for 2-3 hour at 4°C with 30 µl 50% v/v CNBr-activated Sepharose 4B beads (GE Healthcare) cross linked to anti-GFP antibodies (Produsedby Eurogentech). CNBr-activated beads were prepared according to the manufacturers instructions. The beads were pelleted by centrifugation at 200g for 1 min and the supernatant was removed. After that the beads were washed three times with 1 ml wash buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.01% triton X-100) and twice with 10mM NH_4HCO_3, pH 8. Before the last wash step the beads together with the wash buffer were moved to a new tube. After centrifugation, the wash buffer was removed and the beads were used in this form directly for digestion with trypsin and subsequent MS/MS analysis.

In-liquid protein treatment

The protein reduction was performed by adding 2 µL 50 mM DTT in 50 mM NH_4HCO_3 and incubated for 1h. at 56 °C. Alkylation was performed with 2 µL 100mM iodacetamide in 50 mM NH_4HCO_3 solution. To remove the excess of iodoacetamide 1 µL of 125 mM cystein dissolved in 50 mM NH_4HCO_3 was added followed by 1 µL of trypsin.

In-gel protein treatment

All chemical products used were of analytical or HPLC grade quality. For MS analysis the protein gels were stained with Colloidal Coomassie staining kit (Invitrogen), according to the manufacturers' instructions. The protein bands were excised and the gel parts cut into 1 mm pieces. In-gel digestion was performed as described by Shevchenko et al. (Shevchenko et al., 1996) with the following modifications. Destaining was obtained by two washes with water and two times with 50 mM NH_4HCO_3/ CH_3CN (1/1 mixture) for 15 min. at 37 °C. Reduction was performed with 10 µL of 50 mM DTT (dithiotreitol) in 50 mM NH_4HCO_3 for 1h at 56°C. Alkylation was performed with 10 µL of 100 mM iodacetamide in 50 mM NH_4HCO_3 for 1h. at room temperature in the dark. The gel pieces were washed three times with 100 µL 50 mM NH_4HCO_3 and dried in a vacuum centrifuge. For proteolytic digestion, the gel was treated over night with 1 µL (in 10 µL 50 mM NH_4HCO_3) trypsin (sequencing grade, Boehringer Mannheim, Germany) or endoproteinase Lys C (sequencing grade, Roche Mannheim, Germany) at room temperature. The gel fragments were removed by centrifugation and the proteolytic peptides were recovered in the supernatant fraction. A second extraction step of the peptides was performed using 15-20 µL 5% TFA (trifluoro-acetic acid) in water followed by sonication for 1 min. A third gel extraction was done using 10-15 µL CH_3CN/TFA/H_2O (25/1/84 v/v/v) mixture and sonicated for 1 min. Finally all extracts were pooled together in µL and used as such for LC-MS/MS. (25/1/84 v/v/v) mixture and sonicated for 1 min. Finally all extracts were pooled together in µL and used as such for LC-MS/MS.

Tandem mass spectrometric analysis and data analysis

Peptides extracted from tryptic digests were subjected to LC-MS/MS analysis. The samples were analyzed by injecting 20 µL over a 0.10 mm x 32 mm Prontosil 300-3-C18H pre-concentration column (Bischoff, Germany, prepared in house) at a flow of 4 µL/min for 10 minutes or 6 µL/min for 5 minutes. Peptides were eluted from the pre-concentration column onto a Prontosil 300-3-C18H analytical column (0.10 mm x 200 mm) with an acetonitril gradient at a flow of 0.5 µL/min. The gradient consisted of an increase from 10 to 35% acetonitril in water with 1ml/L formic acid in 50 minutes followed by a fast increase in the percentage acetonitril to 80% (with 20% water and 1 ml/l formic acid in both the acetonitril and the water) in 3 minutes as a column cleaning step. Downstream of the column, an electrospray potential of 1.8 kV was applied via a platinum electrode that made direct contact with the eluent. Full scan positive mode MS spectra with 3 or 1 microscans were measured between m/z 350 and 1400 on a LCQ classic or LTQ-Orbitrap (Thermo Electron, San Jose,

CA, USA). MS/MS scans of the 3 (for LCQ) or 4 (for LTQ-Orbitrap) most abundant peaks in the MS scan were recorded in data dependent mode.
Each run with all MS/MS spectra obtained was analyzed with Bioworks 3.3 (Thermo Electron). For the identification of the proteins the searches were done with an *A. thaliana* database and allowing methionine oxidation and phosphorylation of serine, threonine and tyrosine as variable modifications and carboxamidomethylation of cysteines as fixed modification. The peptide identifications obtained were analyzed with Bioworks 3.3 with the following filter criteria: $\Delta Cn > 0.08$, Xcorr > 2 for charge state 1+ , Xcorr > 1.5 for charge state 2+ and Xcorr > 3.3 for charge state 3+ as described previously (Peng et al., 2003) and Xcorr > 4 for charge state 4+.

***In vitro* auto- and trans-phosphorylation assays**

14-3-3 λ, 14-3-3ν and BRI1-KD were cloned and expressed in pGEX-4T1 vector. Protein expression, GST purification and the *in vitro* phosphorylation reaction with (γ^{-32}P) ATP was done as described previously (Shah et al., 2001). After incubation for 30 min. at 30 °C, the reaction was quenched by adding Laemmli SDS-PAGE sample buffer, boiled at 95 °C for 5 min. and separated on 10% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue (CBB) to verify equal loading and then dried. The radioactivity was quantified with PhosphoImager using the ImageQuant program (Molecular Dynamics).

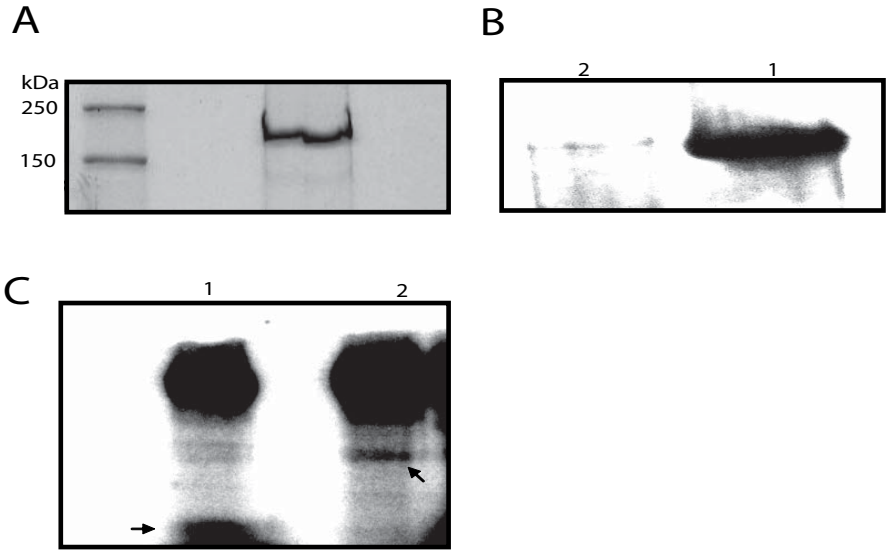
Results and Discussion

Immunoprecipitation and Detection of BRI1-GFP interacting partners in vivo

By using immunoprecipitation in combination with MS we previously identified proteins interacting with SERK1-CFP in vivo. In this way SERK3 and BRI1 as well as 14-3-3ν, CDC48A and AGL15 were found to be present in the SERK1 protein complex (Karlova et al., 2006). For the immunoprecipitation, protein A beads were used, which resulted in the presence of Protein A peptides after trypsin digestion in our precipitates. In order to avoid the presence of external peptides in our samples we optimized the procedure by crosslinking anti-GFP antibodies to CNBr-activated agarose beads (GE Healthcare). This resulted in better protein coverage of the precipitated receptor. For example for the BRI1 protein only 4 peptides were identified by using Protein A beads, in comparison with 83 BRI1 peptides found, when using crosslinked agarose beads and the precipitated complexes were digested in liquid with trypsin and directly (without extra separation) subjected to LC-MS/MS. By using this procedure we could isolate about 2 µg of BRI1-GFP protein from the plant extract (Figure 1 A). The identity of this comassie stained band of around 160 kDa was confirmed by western blotting (Figure 1B) using anti GFP antibodies and by LC-MS/MS. From the BRI1 protein sequence 43% was recovered and the relative abundance representing the percentage of the peak areas was calculated to be 35 % (Table 1). In the SEQUEST softer there is a possibility to obtain semi-quantitative information on peptides and proteins by calculating the peak areas of peptides identified, representing the relative abundance of a protein, using the formula; (the sum of

peak areas for protein X, divided by the sum of peak areas of all protein identified, times 100%). All of the proteins, that were identified in the negative control, sample consisting of proteins extracted from WT seedlings and precipitated with anti GFP antibodies, binding either aspecifically to the beads or to the anti GFP antibodies are listed in Table 3 and are subtracted from the list of the interacting protein (Table 1, 2 and 4). Not only BRI1 was identified but also the fusion protein tag GFP with 13 peptides (39.9% sequence recovered). Interestingly, although the protein concentration of BRI1 should be equal to its fusion protein, for the GFP protein the relative abundance was only 2%. There are two possible explanations for this difference. One is that the GFP protein is more difficult to be digested by trypsin than BRI1 (although similar sequence recovery was observed for both proteins, Table 1).

Figure 1. A. Detection of BRI1-GFP protein on coomassie stained SDS gel from BRI1-GFP transgenic plants after immunoprecipitation (IP) with anti GFP antibodies. The size of detected proteins is indicated on the right. B. Detection of BRI1-GFP protein from BRI1-GFP transgenic plants, lane 1 and wild type plants, lane 2 after immunoprecipitation with anti GFP antibodies. C. In vitro trans-phosphorylation of 14-3-3λ, and 14-3-3υ by BRI1 kinase domain protein. The GST tag only of 14-3-3λ was cleaved by trombin, which resulted in difference in the protein size between the two 14-3-3 isoformes. Phosphoimage of 14-3-3λ (lane 1) and 14-3-3υ (lane 2) trans- phosphorylated by BRI1. Arrows indicate the position of 14-3-3 proteins.



Another possibility is that BRI1 is homodimerizing with the endogenous receptor protein in vivo in agreement with data of (Russinova et al., 2004; Wang et al., 2005a) and thereby the amount of precipitated BRI1 protein is higher than the amount of the GFP fusion protein alone. Similar difference was observed for BRI1 and GFP proteins precipitated from the

pools of seedlings stimulated with BL (biosynthetic brassinosteroid), Table 2. Compared to the relative protein amount of the precipitated BRI1-35%, the protein amounts of the identified interacting partners is lower and ranges from 0.84 to 0.01%. This relative abundance of the identified proteins after immunoprecipitation shows how much of the bait protein was isolated compared to the background proteins and it is useful to estimate for the following experiments how much from the bait proteins is needed in order to detect a specific interacting partner with very low abundance. Amongst these proteins 14-3-3s were identified as interacting partners for BRI1 similar to what was found previously for SERK1 (Karlova et al., 2006). Recently 14-3-3 proteins were shown to play a role in the brassinosteroid signaling by interacting with phosphorylated BZR1 transcription factor and leading to cytoplasmic retention of BZR1 (Ryu et al., 2007). Our data suggests direct interaction of 14-3-3s with BRI1. We next investigated if 14-3-3s are substrates of BRI1. As shown on figure 1C BRI1 can trans-phosphorylate two 14-3-3 isoformes, 14-3-3υ and 14-3-3λ in vitro. Other interacting partners identified for BRI1 are the nodulin related protein (At1g13930) without a known function, a glycoprotein located to the plasma membrane with peptidase activity (At1g03220) and also an unknown cell wall protein (At5g25460) (Bayer et al., 2006). Another protein located in the cell wall was identified in the BRI1 complex, gibberellin (GA)-responsive GASA1 protein (At1g75750). These cell wall proteins probably interact with the extracellular (LLR) domain of BRI1 receptor. GASA1 encodes a small peptide of 98 residues of unknown function and accumulates markedly in siliques, about five days after pollination and correlates with the peak of GA biosynthesis at this stage (Aubert et al., 1998). GASA1 was found to be up-regulated by GA and down regulated by BR (Bouquin et al., 2001). In our complex isolation experiments we could identify the GASA1 protein only in non BR treated pools of seedlings, which is consistent with the down regulation of this protein by BRs. GASA1 gene expression co-localized with BRI1 expression in 1-week- old seedlings in roots and vasculature (Raventos et al., 2000). We could also identify (only in the BRI1 receptor complex not induced with BRs) a WD-40 repeat protein, RACK1A (Receptor for Activated C Kinase 1). Previously TRIP-1 WD-40 repeat protein was shown to interact with BRI1 and to be also involved in brassinosteroid signaling based on the phenotype of antisense suppression of TRIP-1 in Arabidopsis plants (Jiang and Clouse, 2001) and was also shown to be transphosphorylated by BRI1 (Ehsan et al., 2005). The mammalian counterparts of serine-threonine receptor like kinases are the transforming growth factor-beta (TGF-β) receptors. Based on heterodimerization of BRI1 with its co-receptors SERK1, SERK3 a tetrameric complex formation model was proposed for BRI1 in analogy with TGF-β receptors (Karlova et al., 2006). WD-40 domain proteins similar to TRIP-1 were shown to be interacting partners of TGF-β receptors (Choy and Derynck, 1998). TRIP-1 was found to inhibit the signal transduction of the activated TGF-β receptor complex, where the *Arabidopsis* TRIP-1 was proposed to positively regulate BRI1 signaling (Jiang and Clouse, 2001). WD-40 repeats, were first recognized in the β-subunit of hetero-trimeric G proteins (Fong et al., 1986) and have been involved in diverse functions, such as transcriptional regulation, RNA processing, signal transduction, cell cycle progression,

and vesicular trafficking (Neer et al., 1994). These proteins are often found in multi-subunit protein complexes, and it has been proposed that WD-40 repeats are important for protein-protein interactions. RACK1A was found to be ubiquitously expressed in *Arabidopsis* and loss-of-function mutations in RACK1A result in defects in seed germination, leaf production and flowering. *rack1a* mutant displayed reduced sensitivity to gibberellin and brassinosteroids in seed germination, hypersensitivity to abscisic acid in seed germination and early seedling development, and reduced sensitivity to auxin in adventitious and lateral root formation. Another link to brassinosteroid signalling is that in etiolated seedlings, the hypocotyls of *rack1a* mutants were shorter than those of the wild type (Chen et al., 2006). These results indicate that RACK1A is involved in multiple hormone responses and signal transduction pathways. Other proteins involved in protein degradation like ubiquitin or polyubiquitin and 26S proteosome were identified only in the BRI protein complex isolated from seedlings not induced with BRs. In both pool downs, treated and non treated with BRs we could identified as specific interacting partners of BRI1, the small Ras GTP binding proteins, three ADP-ribosylation factors (ARFs, At1g23490 or At1g10630 or At3g62290). Because of the high homology we could not distinguish between the three isoforms. ARFs are in general require for efficient membrane trafficking. Association of the GTPase with guanine nucleotide exchange factors (GEFs), catalize the conversion of the small GTP-binding protein to their GTP-bound "active" conformation (Matheson et al., 2007). In plants it was suggested that ARF1 is involved in retrograde protein transport from Golgi to ER by interaction with COPI vesicle coat protein component. Plant ARF1 is localized not only on Golgi apparatus with coatomers but also on additional structures that lack coatomers, which might be involved in protein transport to the vacuole (Matheson et al., 2007). BRI1 was shown to internalize and colocalize in vesicles in the protoplast system as well as in BFA bodies in plant cells (Russinova et al., 2004; Geldner et al., 2007). Most likely BRI1 interacts with these ARFs in these internal membrane vesicles. Although SERK1 (Karlova et al., 2006) as well as SERK3 (Li et al., 2002; Nam and Li, 2002) were shown to interact with BRI1 we did not find peptides for these receptors with the filter settings we used. One possible explanation could be that only very low amount of BRI1 protein is in a complex with these receptors and they probably are below the detection limit. This is in line with our previous observations that BRI1 receptor is mainly present as a homodimer on the membrane and only around 5% is in a higher order complex coinciding with SERK1 and SERK3. The co-receptors were only observed in higher molecular size complexes and not as a monomers or dimers (Karlova et al., 2006).
Finally it appears remarkable that none of the other genetically identified BRI1 interacting proteins as BKI1 (Wang and Chory, 2006), BIN2 (Li and Nam, 2002), BZR1 (He et al., 2005) etc were found. Apparently non of these proteins is interacting stably enough or long enough to appear in our list of BRI1 complex partners.

Immunoprecipitation and Detection of SERK3-GFP interacting partners in vivo

Previously it was shown that SERK3 is part of brassinosteroid signalling pathway and is a co-

receptor of BRI1 (Li et al., 2002; Nam and Li, 2002). BAK1 was reported to be also involved in resistance against bacterial pathogens by interacting with flagellin-sensitive 2 (FLS2) and elongation factor Tu (EFR) receptors (Chinchilla et al., 2007). BAK1 and FLS2 form a complex *in vivo*, in a ligand-dependent fashion, within the first minutes of stimulation with the ligand fagellin (Chinchilla et al., 2007). In order to identify interacting partners of SERK3 we use the same immunoprecipitation technique in combination with mass spectrometry as described above for BRI1. The proteins identified to be present in SERK3-GFP complex are listed in Table 4. SERK3 was identified with 38 peptides recovering 33 % of the protein sequence. No unique peptides were found for SERK1 or SERK4/5. Two other LRR-RLKs were identified, At1g27190 and At3g28450 with 44.5% and 20% sequence recovery respectively (Table 4). These two receptors share 54% sequence identity with each other and 27% with SERK3 (Figure 2) and consist of 5 LRR, a single transmembrane domain and a serine/threonine kinase domain but are lacking the Ser-Pro rich domain before the transmembrane domain, which is characteristic for the SERK family members (Hecht et al., 2001).

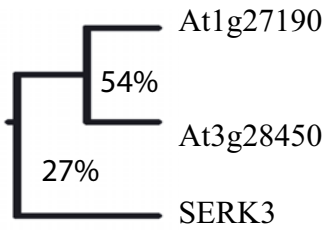


Figure 2. Phylogenic relationship of RLKs identified in SERK3 receptor complex. Amino acid sequence of the proteins were aligned using Clustal W (Thompson et al., 1994).

Interestingly we could observe the same receptors to be present also in the SERK1 protein complex, using this time the same improved technique as used for BRI1 (data not shown), which could point to the possibility that SERK1 and SERK3 are part of several multireceptor complexes or that exchange occurs between these related receptors and SERK3/SERK1, in similarity with TGF-β receptors in mammals. Combinatorial interactions within a family building up a tetrameric receptor complex would allow differential ligand binding or differential signaling in response to the same ligand. Patterns of ligand and receptor expression can then help to determine which receptor-ligand combination is activated (Feng and Derynck, 1997). SERK3 apparently has multiple functions and was found also to play a role in negative regulation of a cell death pathway independent of its role in brassinosteroid signaling (He et al., 2007). The double mutant *bak1 bkk1* (serk4) exhibits a seedling-lethality phenotype due to constitutive defense-gene expression, reactive oxygen species (ROS) accumulation, and

spontaneous cell death even under sterile growing conditions. Interestingly we identified the BAG1 (Bcl-2-associated athanogene) protein in SERK3 receptor complex. BAG proteins are evolutionarily conserved, and consist of a family of multifunctional proteins that promote cell survival. They are distinguished by a common conserved region located near the C terminus termed the BAG domain (BD) involved in interaction with Hsp70 proteins. Some of them, like AtBAG1, contain a ubiquitin-like domain at the N terminus (Doukhanina et al., 2006). BAG proteins may operate as bridging molecules that recruit molecular chaperones to target proteins or via the ubiquitin-like domain control protein turnover rates. Alternatively BAG1 protein can mediate binding to other proteins, including the 26S proteosome (Takayama and Reed, 2001). Mammalian BAG proteins have been reported to associate with and regulate the activity of steroid hormone receptors and to associate with the tumour necrosis factor (TNF) receptor that regulates signal transduction events preventing cell death (Takayama and Reed, 2001). The plant BAG proteins were also shown to have a function in cell protection under stress and inhibition of plant programmed cell death (PCD) that shares features associated with apoptosis (Doukhanina et al., 2006). This supports the idea that some mechanisms for PCD regulation are conserved between plants and animals. Similar to the SERK1 receptor complex, the CDC48A protein was also identified in the SERK3 protein complex. CDC48A in Arabidopsis was shown to play role in protein degradation of incorrectly folded proteins (Muller et al., 2005). As found for BRI1 we identified in SERK3 complex element of the 26S proteosome, the 14-3-3 chi isoform as well as ARF1 (Table 1, 2 and 4). The identification of small Ras related GTPase in both complexes could indicate that there are similar mechanisms in vesicle trafficking of both BRI1 and SERK3 receptors. This is in line with the fact that the two receptors can co-localize together in vesicles and also can physically interact with each other in endosomes (Russinova et al., 2004; Geldner et al., 2007).

In summary, by isolating the protein complexes of SERK3 and BRI1 and identifying their protein composition, several novel interacting partners were observed, that were shown to be involved in different functions, that range from vesicle trafficking (ARFs) to protein degradation (CDC48 and 26S proteosome), regulation of apoptosis (BAG1) and protein-protein interactions like 14-3-3s. Finding all these proteins to be associated with single receptors indicates that most probably we are isolating multiple receptor complexes, either at different stages of formation, or playing different roles in different signalling pathways. The composition of SERK1 and SERK3 protein complexes was found to be quite similar, both receptors interact with 14-3-3s, CDC48A and two other RLKs. This could be explained by the fact that they can share common functions like in brassinosteroid signalling (Karlova et al., 2006).

Table 1. Identification of the proteins interacting specifically with BRI1 using Bioworks 3.3 software. All the proteins present in the negative control were removed from the list of the BRI1 interacting partners.

Protein Name	TAIR number	N of peptides observed	% protein coverage	% Peak area
BRI1	At4g39400	83	42.9	34.75
GFP	P42212	13	39.9	2.13
Vacuol ATP ase	At1g20260	2	6.1	0.02
Nodulin related	At1g13930	3	25.2	0.01
Small GTP-ase	At1g23490 or At1g10630 or At3g62290	2 2	14.15 13.8	0.01
Ubiquitin or PoliU	At4g05320	4	17.7	0.84
GASA1	At1g75750	2	11.2	0.01
Glycoprotein	At1g03220	2	5.7	0.01
26S proteosome	At1g53750	2	6.8	0.0
14-3-3 psi	At5g38480	3	11.3	0.0
cell wall protein	At5g25460	3	11.65	0.02
RACK1A	At1g18080	2	11.3	0.0

Table 2. Identification of the proteins interacting specifically with BRI1 immunoprecipitated from seedlings induced with 100nM BL for 1h, using Bioworks 3.3 software. All the proteins present in the negative control were removed from the list of the BRI1 interacting partners.

Protein name	TAIR number	N of peptides observed	% protein coverage	% Peak area
BRI1	At4g39400	66	38.4	26.5
GFP	P42212	17	39	5.5
Vacuol ATP-ase	At1g20260	2	6.1	0.02
Small GTP -ase	At1g23490	2	14.5	0.01
	or At1g10630	2	19.9	0.01
	or At5g14670	2	19.5	0.01
Glycoprotein	At1g03220	2	5.7	0.02
14-3-3 psi	At5g38480	3	11.3	0.03

Table 3. Identification of proteins present in the negative control using Bioworks 3.3 software.

Protein Name	TAIR number	N of peptides observed	% protein coverage
RUBISCO	At2g39730	23	53%
12S seed storage protein	At5g44120	19	34%
Malate Dehydrogenase genase	At2g22780	13	55.6
Cupin	At4g28520	17	48.2
Homocystein methyl Transferase	At5g17920	10	19.6
Glyceraldehyde-3 Phosphate Dehydroge-nase	At3g04120	10	33.4
ATP syntase	Atcg00480	7	25.3
calcium -binding protein	At1g12900	7	25.3
Chlorophyl		7	38.5
membrane 30kd protein	At1g65260	6	25.4
beta-Glucosidase	At3g09260	6	26.1
Keratine		7	19
Photosystem 2	At2g34420	6	27
Fructose- Biphosphate aldolase	At3g52930	5	29.6
Prohibitin	At5g40770	5	34.6

Protein name	TAIR number	N of peptides	% protein coverage
Elongation factor 1-alpha	At5g60390	8	14.4
EPSIN-like	At1g03050	4	13.8
Jasmonate inducible protein	At3g16460	3	6.9
MATH domain protein	At5g26280	3	17.7
Glutamine synthetase	At5g35630	3	14.1
Glutamine rich protein	At1g10270	3	4.7
60S ribosomal protein	At2g01250	3	19.8
WHIRLY2	At1g71260	3	15.9
Catalase	At4g35090	2	7.5
Carbonic anhydrase 2	At5g14740	2	12.7
Luminal-binding protein 2	At5g42020	2	4.6
ribosome	At4g31700	2	17.5
Ribonucleoprotein 1	At4g14300	2	7.7
Glycolate oxidase	At3g14130	2	13.9
Probable peroxisomal (S)-2-hydroxy-acid oxidase 2	At3g14420	2	9.8
GloEL protein; chaperonin	At3g13470	2	4.1
Putative DNA binding protein	AT4g27000	2	11.4
MATH domain	At5g26280	2	13.4
Cytosolic invertase 1	At1g35580	2	7.6
Oxygen-evolving enhancer protein	At5g66570	2	12.6
DNA binding	At1g76010	2	11.7
Heat shock protein 70	At5g49910	2	3.9

Table 4. Identification of proteins interacting with BAK1 using Bioworks 3.3 software. All the proteins present in the negative control were removed from the list of the BAK1 interacting partners.

Protein name	TAIR number	Number of peptides	%protein coverage	% Peak area
BAK1	At3g28450	38	33.3	6.15
RLK	At1g27190	25	44.5	2.46
RLK	At3g28450	9	20	0.18
14-3-3 chi	At4g09000	4	15.3	0.00
Putative 26S proteosome	At5g20000	2	5.7	0.01
Ras related or ARA3 or	At3g46060 At5g59840	2 2	12.96 10.5	0.01
ARF1	At1g10630	2	10.5	
Pentatrico-peptide	At5g11310	3	4.4	0.01
CDC48C	At3g09840	2	3.7	0.01
BAG regulator of apoptosis	At5g52060	2	10.5	0.01

Chapter 4

Identification of in vitro phosphorylation sites in the Arabidopsis thaliana Somatic Embryogenesis Receptor-like Kinases

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Proteomics in press

Abstract

The Arabidopsis thaliana Somatic Embryogenesis Receptor-like Kinase (SERK) family consists of five leucine-rich repeat receptor-like kinases (LRR-RLKs) with diverse functions such as Brassinosteroid Insensitive 1 (BRI1) -mediated brassinosteroid perception, development and innate immunity. The autophosphorylation activity of the kinase domains of the five SERK proteins was compared and the phosphorylated residues were identified by LC-MS/MS. Differences in autophosphorylation that ranged from high activity of SERK1, intermediate activities for SERK2 and SERK3 to low activity for SERK5 were noted. In the SERK1 kinase the C-terminally located residue Ser-562 controls full autophosphorylation activity. Activation loop phosphorylation, including that of residue Thr-462 previously shown to be required for SERK1 kinase activity, was not affected. In vivo SERK1 phosphorylation was induced by brassinosteroids. Immunoprecipitation of CFP-tagged SERK1 from plant extracts followed by tandem mass spectrometry identified Ser-303, Thr-337, Thr-459, Thr-462, Thr-463, Thr-468 and Ser-612 or Thr-613 or Tyr-614 as in vivo phosphorylation sites of SERK1. Trans-phosphorylation of SERK1 by the kinase domain of the main brassinosteroid receptor BRI1 occurred only on Ser-299 and Thr-462. This suggests both intra- and intermolecular control of SERK1 kinase activity. Conversely, BRI1 was trans-phosphorylated by the kinase domain of SERK1 on Ser-887. BRI1 kinase activity was not required for interaction with the SERK1 receptor in a pull down assay.

Introduction

Plant genomes contain a large number of genes encoding predicted transmembrane receptor like kinases (RLKs) with a structure similar to animal receptor kinases (Shiu and Bleecker, 2003, Shiu et al., 2004). Membrane-located leucine-rich repeat receptor like kinases (LRR-RLKs) play important roles in plant signaling pathways (reviewed in (Torii, 2004). Plant RLKs consist of an extracellular domain, a single transmembrane-spanning domain and a cytosolic kinase domain that resembles those of serine/threonine receptor kinases of the transforming growth factor β (TGF β) family in metazoa.

We have previously identified the *Arabidopsis* Somatic Embryogenesis Receptor-like Kinase 1, an LRR-RLK that marks formation of embryogenic cells in tissue culture. Ectopic expression of SERK1 does not result in an obvious phenotype in *Arabidopsis* plants, but increases somatic embryo formation in culture (Hecht et al., 2001). SERK1 is a member of a small family of five related RLKs all of which have 5 LRRs and a typical serine-proline rich juxtamembrane region (Hecht et al., 2001). The C-terminal kinase catalytic domain is also conserved and has all of the 11 subdomains as described for serine/threonine protein kinases (Shah et al., 2001b). The GST-SERK1-KD (kinase domain) fusion protein mainly autophosphorylates on threonine residues and the reaction is Mg^{2+} dependent and inhibited by Mn^{2+}. A lysine to glutamic acid substitution (K330E) in the ATP binding pocket of SERK1-KD abolishes all kinase activity and an active SERK1 kinase can phosphorylate inactive $SERK1^{K330E}$ proteins, suggesting an intermolecular mechanism of autophosphorylation. A comparable substitution in another member of this receptor family, SERK3 that is identical to BRI1-Associated Receptor Kinase 1 (BAK1), also abolishes the kinase activity (Li et al., 2002). Replacing Thr-468 in the activation loop with alanine completely obliterated the ability of SERK1 to autophosphorylate. Trans-phosphorylation by SERK1 on myelin basic protein and casein showed tyrosine, serine and threonine as targets, demonstrating that SERK1 is a dual specificity kinase (Shah et al., 2001a). The SERK1 knock-out alleles *serk1-1* and *serk1-2* do not result in a morphological phenotype but in combination with the SERK2 mutant alleles *serk2-1* or *serk2-2* result in complete male sterility (Albrecht et al., 2005; Colcombet, 2005). Recently we have shown that hetero-oligomerization can occur between SERK family members (Russinova et al., 2004, Albrecht et al., 2005) and with the major brassinosteroid receptor BRI1 in living plant cells (Karlova et al., 2006). It was also shown that SERK3 (BAK1) as well as SERK1 are part of the BR receptor complex and are proposed to function as co-receptors of the BRI1 ligand-binding receptor (Li et al., 2002; Nam and Li, 2002; Karlova et al., 2006). SERK3 was recently implicated to be involved in plant innate immunity responses (Kemmerling et al., 2007; Heese et al., 2007) and as a component of the flagellin-perceiving FLS2 receptor complex (Chinchilla et al., 2007). BAK1 was also shown to have a redundant function with SERK4 (also called BAK1-Like Kinase 1 or BKK1) in positively regulating a BR-mediated plant growth pathway, and negatively regulating a BR-independent cell death pathway (He et al., 2007). It has also been shown that the interaction and phosphorylation of the BRI1 and BAK1 receptors is dependent on the presence of BRs

(Wang et al., 2005b; Wang et al., 2005a). Site-directed mutagenesis studies suggested that autophosphorylation of BRI1 in the juxtamembrane (JM) or C-terminal regions plays a role in recognition and/or phosphorylation of downstream targets (Albrecht et al.). The C-terminal tail of the BRI1 intracellular domain also plays a critical role in negatively regulating the kinase activity (Wang et al., 2005a). BAK1 and BRI1 can trans-phosphorylate each other, and the autophosphorylation activity of BAK1 is stimulated by BRI1 (Li et al., 2002). The proposed current model is that BR binding to the extracellular domain of BRI1 induces receptor phosphorylation and activation (Wang et al., 2005a; Karlova and de Vries, 2006). Which sites are trans-phosphorylated within the receptor pair and involved in their activation remains to be determined.

Mass spectrometry (MS) represents an efficient tool to perform such an analysis and has been used to identify the phosphorylation sites of several RLKs using liquid chromatography-tandem mass spectrometry (LC-MS/MS) of tryptic peptides from plasma membrane proteins (Nuhse et al., 2004). The kinase domains of numerous plant RLKs have been expressed as recombinant proteins and they do indeed behave as functional kinases and are capable of autophosphorylation on serine and threonine residues in vitro (Stone et al., 1998; van der Knaap et al., 1999; Fujita et al., 2003; Wang et al., 2005b). Only for a few plant receptors, including BRI1 and BAK1, the autophosphorylation sites have been identified (Oh et al., 2000; Wang et al., 2005b). After BR treatment the BRI1 receptor autophosphorylates at 6 residues that were identified *in vivo* and corresponded to the same residues as previously identified from the *in vitro* produced BRI1 protein.

Members of the SERK family have in part redundant functions, can act as co-receptors with different main receptors and individual members can act in different signalling pathways. Here we investigated the *in vitro* autophosphorylation properties of all SERK proteins in order to determine whether any link exists between particular phosphorylated residues and the observed biological activity. SERK1 is the most active kinase, resulting in 24 autophosphorylated residues. SERK1, SERK2 and SERK3 (BAK1) proteins were all found to be phosphorylated at the same threonines in the activation loop of the kinase domain, while fast degradation was responsible for SERK4 (BKK1) and SERK5 showing lower autophosphorylation activity *in vitro*. However, SERK1 and SERK2 are fully interchangeable in male sterility and require kinase activity (Albrecht et al., in preparation), SERK1 and SERK3 have additive effects in BR signaling (Karlova et al., 2006) and SERK3 (BAK1) and SERK4 (BKK1) are redundant in mediating cell death (Heese et al., 2007). The functional significance for the *in vitro* kinase activity of specific Ser and Thr residues of SERK1 was examined. We identified phosphorylated sites of SERK1 by using LC/MS/MS analysis of immunoprecipitated SERK1-CFP protein to show that the receptor is also phosphorylated on specific Ser and Thr residues in vivo. We also assessed the trans-phosphorylation between BRI1 and SERK1 and addressed the dependence of BRI1 and SERK1 association on the kinase activity of BRI1.

Materials and Methods

Bacterial strains and growth conditions

For cloning and expression of recombinant proteins *E. coli* DH5α (Clontech Laboratories, Palo Alto, USA) and *E. coli* BL21 (DE3) pLysS (Novagen, R&D Systems Europe, Albingdon, Oxford, United Kingdom) were used. All *E. coli* strains were grown and maintained in LB medium supplemented with 100 µg/mL ampicillin at 37°C.

Cloning, over-expression, and purification of cytosolic kinase domains (KD) of SERK proteins.

The SERK1-KD and SERK1-KD^{K330E} constructs used were described before (Shah et al., 2001a), BRI1-KD and BRI1-KD^{K911E} were kindly provided by John Walker (Division of Biological Sciences, University of Missouri-Columbia, Columbia, MO 65211 USA) and described before (Li et al., 2002). PCR fragments encoding the intracellular region corresponding to the kinase catalytic domain and the juxtamembrane linker of SERK2 (residue 282-629) and SERK3 (residue 248-616) were amplified by using synthesised cDNA from 10d old seedlings as a template with the following primers: 5'-CCGGAATTCGACCCTGAGGTTCACTTGG-3' and 5'-CCGCTCGAGTTATCTTGGACCAGACAACTC-3' for SERK2 and 5'-AAAAAGAATTCGACCACTTCTTTGATGTACC-3' and 5'-TTTTTCTCGAGTTATCTTGGACCCGAGG-3' for SERK3. SERK4 (residue 261-621) was amplified from cDNA (accession number AY056243) with primers 5'-AAAAAGAATTCGACCACTTCTTCGATGTACC-3' and 5'-TTTTTGCGGCCGCTTATCTTGGACCCGAGG-3'. SERK5 (residues 242-601) was amplified from cDNA (accession number U21915) with primers 5'-AAAAAGAATTCGGTCACTTTCTTGATGTAC-3' and 5'-TTTTTGCGGCCGCTTATCTTGGCCCCGAGG-3'. The SERK2 and SERK3 PCR fragments (amplified with proofreading-grade polymerase Pwo, Roche) were digested with EcoRI and XhoI and ligated into vector pGEX4T1. SERK4 and SERK5 PCR fragments were digested with EcoRI and NotI and ligated into pGEX4T1 vector as well. Site-directed mutagenesis was carried out using the QuickChange Site-Directed Mutagenesis Kit (Strategene) and was used to create the SERK1-KD^{T541A}, SERK1-KD^{S562A}, SERK1-KD^{S570A}, SERK1-KD$^{T541A/S570A}$, SERK1-KD$^{T559A/S562A}$, SERK1-KD$^{T541A/S570A/T559A/S562A}$ (SERK1-KD^{TSTS}) constructs. All DNA constructs were verified by sequencing before being transformed into *E. coli* strain BL21 for protein expression. SERK2-KD and all of the SERK1-KD kinase mutants, SERK3-KD, SERK4-KD, BRI1-KD and BRI1-KD^{K911E} were induced and purified as described previously (Shah et al., 2001a; Li et al., 2002).

In vitro auto- and trans-phosphorylation assays

In vitro phosphorylation of each kinase (1 µg) with [γ^{-32}P]ATP was done as described previously (Shah et al., 2001a). After incubation for 30 min at 30 °C, the reaction was quenched by adding

Laemmli SDS-PAGE sample buffer, boiled at 95 °C for 5 min. and separated on 10% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue (CBB) to verify equal loading and then dried. The radioactivity was quantified with a PhosphoImager using the ImageQuant program (Molecular Dynamics). For trans-phosphorylation assays the reaction conditions were the same as described for the autophosphorylation experiments.
In vitro phosphorylation with ATP for MS analysis was performed as described above except that 3 µg of each kinase protein was used and [γ^{-32}P]ATP was replaced by 3 mM of cold ATP.

Immunoprecipitation and Immunoblot analysis
SERK1-CFP was immunoprecipitated from extracts of 7 d old seedlings with cross- linked to agarose beads anti-GFP antibodies (produced by Eurogentec). Immunoprecipitation was essentially as described before (Karlova et al., 2006), except that kinase inhibitors (50 mM NaF, 1 mM Na3VO4 , 1 mM EDTA) and Phosphatase Inhibitor Cocktail (Sigma) were added during preparation of the plant extracts. Immunoprecipitated proteins were detected by immunoblot analysis on PVDF membrane with anti-GFP antibody (1: 1000) or anti-phosphothreonine antibody (Zymed laboratories, Invitrogen immunodetection) at 1:250. Blots were developed with horseradish peroxidase-linked secondary antibodies and the ECL chemiluminesence detection system (GE Healthcare).

***In vivo* pull down assay**
Plants were grown as described before (Karlova et al., 2006). To prepare total protein extracts, *Arabidopsis* 10 d old seedlings were ground in liquid nitrogen and thawed in extraction buffer containing 50 mM Tris pH 7.5, 150 mM NaCl and 0.5% Triton X-100, and a protease inhibitor cocktail according to the manufacturers instructions' (Roche, Germany). Samples were incubated on ice for 30 min. The suspension was centrifuged at maximum speed (Beckman-Microfuge 18 centrifuge) at 4 °C for 10 minutes. The extract was incubated with 30 µL of amylose resin (New England BioLabs) for 30 min at 4 °C and the resins were removed by centrifugation at 200g for 2 min. The supernatant was transferred to a new tube. For the identification of BRI1-KD^{K911E} interacting proteins, immobilized MBP-BRI1-KD^{K911E} on amylose resin was incubated with the protein extract at 4 °C for 2 h at 0.3 mg BRI1-KD^{K911E} per 10 mg of plant protein extract. Bound protein complexes were collected by centrifugation of the resins followed by washing two times with 1 ml of extraction buffer supplemented with 0.05% Triton X100 and two times with 50 mM NH_4HCO_3. The proteins bound to the resin were then subjected to trypsin digestion and LC-MS/MS.

In-gel protein treatment
All chemical products used were of analytical or HPLC grade quality. For MS analysis the protein gels were stained with Colloidal Coomassie staining kit (Invitrogen), according to the manufacturers' instructions. The protein bands were excised and the gel slices cut into 1 mm

pieces. In-gel digestion was performed as described by Shevchenko et al. (Shevchenko et al., 1996) with the following modifications. Destaining was obtained by two washes with water and two times with 50 mM NH_4HCO_3/ CH_3CN (1/1 mixture) for 15 min. at 37 °C. Reduction was performed with 10 µL of 50 mM DTT (dithiotreitol) in 50 mM NH_4HCO_3 for 1h at 56°C. Alkylation was performed with 10 µL of 100 mM iodacetamide in 50 mM NH_4HCO_3 for 1h at room temperature in the dark. The gel pieces were washed three times with 100 µL 50 mM NH_4HCO_3 and dried in a vacuum centrifuge. For proteolytic digestion, the gel was treated over night with 1 µL (in 10 µL 50 mM NH_4HCO_3) trypsin (sequencing grade, Boehringer Mannheim, Germany) or endoproteinase Lys C (sequencing grade, Roche Mannheim, Germany) at room temperature. The gel fragments were removed by centrifugation and the proteolytic peptides were recovered in the supernatant fraction. A second extraction step of the peptides was performed using 15-20 µL 5% TFA (trifluoro-acetic acid) in water followed by sonication for 1 min. A third gel extraction was done using 10-15 µL CH_3CN/TFA/H_2O (25/1/84 v/v/v) mixture and sonicated for 1 min. Finally all extracts were pulled together 30 µL and used as such for LC-MS/MS.

In-liquid protein treatment

The protein reduction was performed by adding 2 µL 50 mM DTT in 50 mM NH_4HCO_3 and incubating for 1h. at 56 °C. Alkylation was performed with 2 µL 100 mM iodacetamide in 50 mM NH_4HCO_3. To remove the excess of iodoacetamide 1 µL of 125 mM cysteine dissolved in 50 mM NH_4HCO_3 was added, followed by 1 µL of trypsin or endoproteinase Lys C.

Tandem mass spectrometric analysis and data analysis

Peptides extracted from tryptic or LysC digests of 3 µg of each kinase were subjected to LC-MS/MS analysis. The samples were analyzed by injecting 20 µL over a 0.10 mm x 32 mm Prontosil 300-3-C18H pre-concentration column (Bischoff, Germany, prepared in house) at a flow of 4 µL/min for 10 minutes or 6 µL/min for 5 minutes. Peptides were eluted from the pre-concentration column onto a Prontosil 300-3-C18H analytical column (0.10 mm x 200 mm) with an acetonitril gradient at a flow of 0.5 µL/min. The gradient consisted of an increase from 10 to 35% acetonitril in water with 1ml/L formic acid in 50 minutes followed by a fast increase in the percentage acetonitril to 80% (with 20% water and 1 ml/l formic acid in both the acetonitril and the water) in 3 minutes as a column cleaning step. Downstream of the column, an electrospray potential of 1.8 kV was applied via a platinum electrode that made direct contact with the eluent. Full scan positive mode MS spectra with 3 or 1 microscans were measured between m/z 350 and 1400 on a LCQ classic or a LTQ-Orbitrap (Thermo Electron, San Jose, CA, USA). MS/MS scans of the 3 (for LCQ) or 4 (for LTQ-Orbitrap) most abundant peaks in the MS scan were recorded in data-dependent mode.

Each run with all MS/MS spectra obtained was analyzed with Bioworks 3.3 (Thermo Electron). For the identification of the proteins the searches were done with an *A. thaliana* database or in case of the identification of the mutant proteins a homemade local database

was used, containing the protein sequence of the mutated and wild type protein kinases. In all cases, the following proteins were added to the databases; maltose binding protein (P02928), GST, trypsin (bovine, P00760), trypsin (porcine, P00761), keratin K2C1 (human, P04264) and keratin K1CI (human, P35527). The searches were done allowing methionine oxidation and phosphorylation of serine, threonine and tyrosine as variable modifications and carboxamidomethylation of cysteines as fixed modification. The peptide identifications obtained were analyzed with Bioworks 3.3 with the following filter criteria: $\Delta Cn > 0.08$, Xcorr > 2 for charge state 1+ , Xcorr > 1.5 for charge state 2+ and Xcorr > 3.3 for charge state 3+ as described previously (Peng et al., 2003) and Xcorr > 4 for charge state 4+.

Results

Expression, purification and autophosphorylation of the kinase domains of SERK1, SERK2, SERK3, SERK4 and SERK5

The kinase domains of SERK1, SERK2, SERK3, SERK4 and SERK5 were cloned into plasmid pGEX4T1 and transformed into *E.coli* BL21. Analysis of the GST fusion proteins by SDS-PAGE showed that the kinase domains were expressed in soluble form and migrated as a single band with the predicted molecular mass (Figure 1A). Exception was SERK4, where we observed fast degradation and the protein was running slightly higher than expected. Although we tried different conditions for expression only little amount of the SERK4 protein could be purified. However this problem was solved when the SERK4 protein was expressed as a MBP fusion, but then no autophosphorylation activity could be detected any more (data not shown). The SERK receptors have a kinase domain homology between 87% and 95% at protein level (Hecht et al., 2001). To examine the autophosphorylation activity of the recombinant kinases, an *in vitro* phosphorylation assay was performed by incubating the five different proteins in the presence of [γ^{-32}P] ATP. The reaction products were then separated by SDS-PAGE and the labeled proteins were identified by autoradiography using the ImageQuant program. The BRI1 protein was expressed both as a MBP or GST fusion protein and the tag used did not interfere with the kinase activity, where for SERK4 and SERK5 the MBP fusion proteins did not show autophosphorylation activity (data not shown). SERK1-KD, SERK2-KD, SERK3-KD, SERK4-KD and SERK5-KD exhibit variable autophosphorylation activity (Figure 1B).

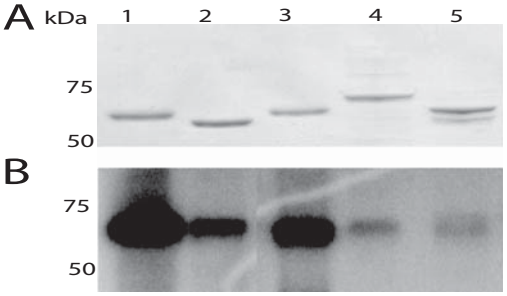


Figure 1. Autophosphorylation activity of the 5 SERK kinases.
A. Lane 1, cytoplasmic kinase domains of SERK1 (residue 269-626). Lane 2, SERK2 (residue 282-629). Lane 3, SERK3 (residue 248-616). Lane 4, SERK4 (residue 261-621). Lane 5, SERK5 (residue 242-601). The proteins were expressed, purified on a glutathione-Sepharose 4B matrix, submitted to gel electrophoresis and stained with Colloidal Coomassie Blue. B. *In vitro* phosphorylation assays were performed with [γ^{32}P]ATP for 30 min. Proteins were analysed by SDS-PAGE and radioactive bands were revealed by autoradiography. The size of the marker proteins is shown on the right.

Identification of the in vitro phosphorylated residues in SERK 1, 2, 3, 4 and 5 kinase domains

The five kinase domains were autophosphorylated with non radioactive ATP, digested with trypsin and subjected to tandem mass spectrometric analysis. To obtain better sequence coverage of the proteins both trypsin and endoproteinase Lys C were used in combination with in liquid and in gel digestion. An example of the results obtained is shown in Figure 2 and the combined results of all 5 SERK kinase domains are shown in Supplemental Figure 1. Tryptic digests yielded between 60 and 80% coverage. Non-tryptic proteases such as LysC typically yielded only 10-25% coverage, resulting in identification of an additional site in SERK1-KD, as well as confirmation for several other sites already identified from tryptic digests and also providing additional peptide coverage. Although a high sequence recovery was achieved for the five SERK proteins, it is possible that some of the non-recovered regions containing serine, threonine or tyrosine residues could also be potential phosphorylation sites. As shown in Table 1, SERKs are phosphorylated on multiple serine and threonine residues and in case of SERK1-KD also three phosphorylated tyrosine residues were found. In our MS/MS conditions, phosphoserine and phosphothreonine containing peptides displayed significant ion signals originating from neutral loss of phosphoric acid (-H_3PO_4, equal to –98 Da), as shown in Figure 2. Since this loss is not being taken into account in the calculation of the cross correlation Xscores (XC) given by the software, XC scores of phosphopeptides are generally relatively low compared to the non-phosphorylated peptides. Therefore, the assignment of phosphorylated sites was verified by manual inspection of the MS/MS spectra. Successive losses of two molecules of phosphoric acid were observed for *y* or *b* fragment ions in the case of doubly phosphorylated peptides. Most of the phosphorylated peptides assigned were also found as non phosphorylated residues. A peptide from SERK1-KD (Figure 2 A) was identified with an Xscore of 4.7, while a peptide with the same sequence was identified with one phosphorylated serine residue and an Xscore of 2.8 (Figure 2 B), and as a peptide with two phosphorylated residues with Xscore of 2.2 (Figure 2 C). In agreement with the autophosphorylation radioactive assay (Figure 1 B), SERK1-KD appears to be the most active amongst the family with at least 24 phosphorylated residues identified (Table 1). To distinguish between the phosphorylated sites that are resulting from autophosphorylation activity and those due to *E. coli* kinases, we investigated the autophosphorylation profile of the SERK1-

KD^{K330E} kinase-dead mutant (Shah et al., 2001a) and (Figure 4A, lane 6). As shown in Table 1, three sites (Ser-303, Ser-386 and Thr-559) were identified as being phosphorylated in the SERK1-KD^{K330E} mutant kinase.

domain	position in SERK1	SERK1	$SERK1^{K330E}$	$SERK1^{S562A}$	SERK2	SERK3	SERK4	SERK5
Juxtamem-brane	Ser-291							
	Ser-299				Thr-302			
I	Ser-303	*				Ser-290	Ser-295	
II	Thr-325							
II	Thr-337	*						
III	Thr-346							
	Ser-352							
V	Ser-386							
	Ser-394							
	Ser-415							
VII	Thr-402							
	Tyr-456							
	Thr-459	*			Thr-462	Thr-446		
VIII	Thr-462	*			Thr-465	Thr-449		
	Thr-463	*			Thr-466	Thr-450		
	Thr-468	*				Thr-455		Thr-441
	Tyr-476							
IX	Ser-478							
	Thr-479							
	Ser-482	or Ser-483						
	Ser-483	or Ser-482						
X	Thr-541							Ser-506
	Tyr-543							
XI	Thr-559							
	Ser-562							
	Ser-570	Possible						
C-terminal Tail	Ser-601				Ser-604	or Thr-589		
	Ser-606					or Ser-595		
	Ser-612	*						
	Thr-613	or*Tyr-614			Thr-616	Ser-604		
	Tyr-614	or*Thr-613						
	Ser-622				Ser-625	Ser-612		
Total number of sites		26	3	7	7	8	1	2

Table 1. Comparison of phosphorylated sites in SERK1-KD, SERK1-KD^{K330E}, SERK1-KD^{S562A} SERK2-KD, SERK3-KD, SERK4-KD and SERK5-KD. In the first column the kinase domains in which the phosphorylation occurs are listed. In the second column the position of the phosphorylated residues in the SERK1 protein are shown. Asterisks represent phosphorylated sites for SERK1 identified *in vivo*. In the third to nineth columns the grey boxes represent the phosphorylated residues, normalized to the corresponding positions in SERK1. Where text occurs in the boxes, this indicates whether the position differed from the one in SERK1 or that the precise phosphorylation site could be on either one of the residues given.

For the wild type SERK1-KD protein the same residues were found to be phosphorylated (Table 1), suggesting that out of the 24 phosphorylation sites identified, only 3 are due to bacterial trans-phosphorylation activity. However, comparable residues were not always phosphorylated in the proteins of the other SERK members (Table 1).

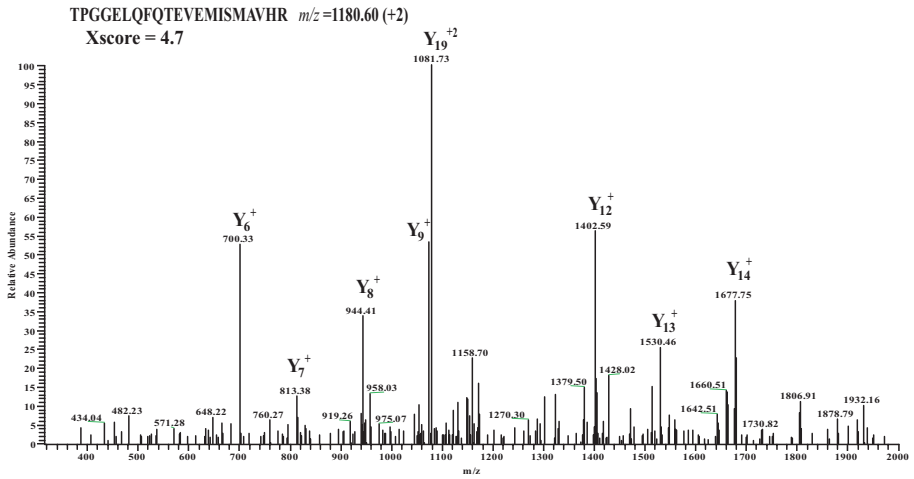
In contrast to SERK1-KD, in the most related kinase SERK2-KD only 7 phosphorylated sites were determined, 3 of which are in the activation loop one in the juxtamembrane region and 3 sites in the C-terminal tail and represent the same sites identified in SERK1. For SERK3 four phosphorylated sites Ser-290 in kinase sub domain I and Thr-446, Thr-449 and Thr-455 in the activation loop were previously identified by Wang et al., 2005 (Wang et al., 2005b). We could confirm these 3 autophosphorylation sites in the activation loop and identified 4 additional phosphorylated residues, one in the activation loop (Thr-450) and 3 in the C-terminal tail (Thr-589 or Ser-595, S-602 and Ser-612) of the SERK3-KD. For SERK4 and SERK5 a lower autophosphorylation activity was observed (Figure 1 B). SERK4 fused to GST was degrading very rapidly and we could obtain only 47 % of the protein sequence after trypsin digestion. This was not the case when SERK4 was expressed as an MBP fusion, of which 63% of the protein sequence could be determined, but this fusion protein did not show any autophosphorylation activity so we identified only one phosphorylated serine (Ser-295) (Figure 1 B and data not shown). This residue corresponds to Ser-303 in SERK1-KD^{K330E} and most probably is a site trans-phosphorylated by *E. coli* kinases. For SERK5-KD, cDNA from the *Arabidopsis* ecotype Columbia was used and we could identify 2 phosphorylated sites, one of which is in the activation loop and corresponding to Thr-468 in SERK1, which was shown to play an important role in regulating SERK1 activity (Shah et al., 2001a). Despite the high sequence homology amongst the SERK family a surprisingly large variation in phosphorylation status of the kinase domains *in vitro* was found.

Site directed mutagenesis of SERK1 phosphorylation sites identified in the C-terminal kinase subdomains X and XI affects kinase activity

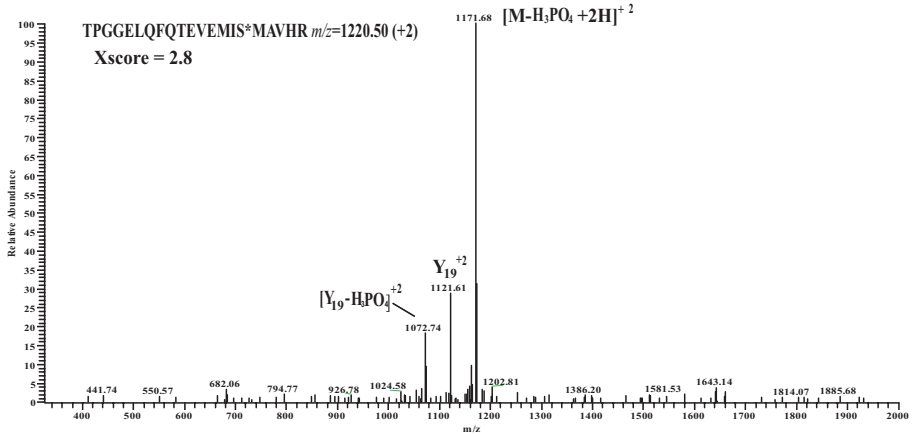
We chose to investigate the last subdomains of the kinase of SERK1 because of the *serk1-1* allele, in which the last 90 amino acids of the protein are missing, has no kinase activity left in vitro and confers male sterility in Arabidopsis plants together with *serk2* mutant alleles

(Albrecht et al., 2005). The potential role for SERK1 kinase activity of identified phosphorylation sites Ser-562, Thr-541, Ser-570 in the kinase subdomains X and XI was

A



B



C

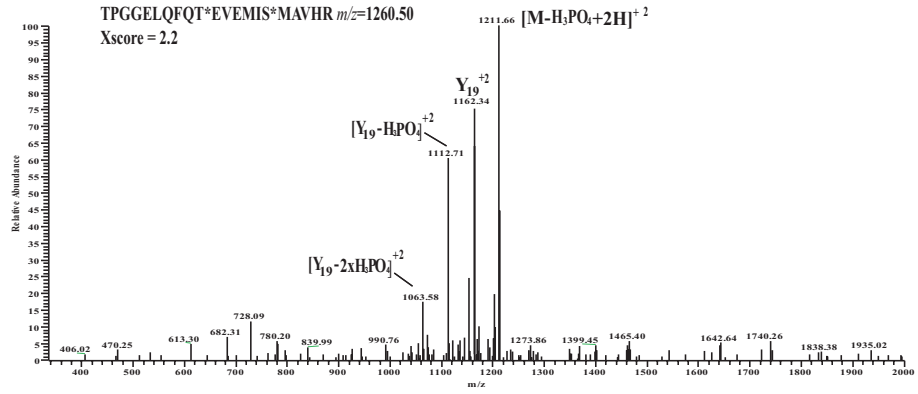


Figure 2. LCQ MS/MS spectra identifying SERK1 in vitro phosphorylation sites.
MS/MS mass spectrum of the non-phosphorylated (A), mono-phosphorylated (B) and doubly-phosphorylated (C) peptides. Fragment ions with relative abundance above 15%, which were measured, are shown. Neutral losses of phosphoric acid from the entire peptide or fragments are indicated.

ssessed by site-directed mutagenesis. All three residues were mutated into alanine alone or in combination and the resulting proteins were expressed and assayed for autophosphorylation activity. The role for authophosphorylation activity of the phoshorylated sites in the activation loop Thr-459, Thr-462, Thr-463 and Thr-468 was described previously by Shah et al., 2001 (Shah et al., 2001a). The mutation Ser-562-Ala nearly abolished the autophosphorylation activity of SERK1-KD. Substitutions at residues Thr-541 and Ser-570 alone did not, but in combination did result in a reduction in autophosphorylation activity (Figure 3 B). We next investigated the phosphorylation profile of the SERK1-KD^{S562A} mutant protein by LC-MS/MS. As shown on Table 1 only 7 phosphorylated residues remained in the mutant protein. In comparison with phosphorylated residues in the wild type SERK1-KD protein these 7 residues were identical. Four of these 5 phosphorylated sites in the SERK1-KD^{S562A} mutant are in the activation loop of the kinase. Apparently activation loop phosphorylation is not sufficient to regain the authophosphorylation activity of this mutant protein and suggest an important role of the SERK1-Ser-562 residue in regulating the overall kinase activity.

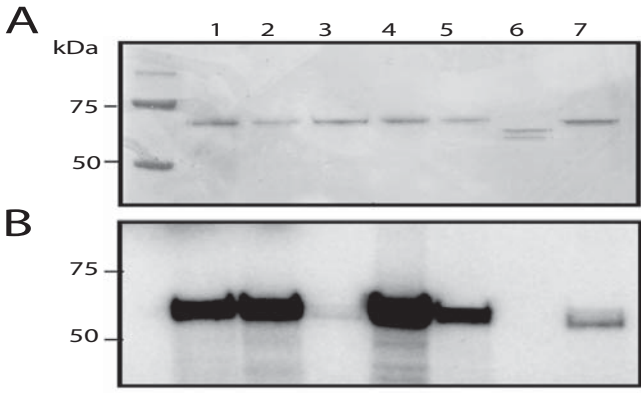


Figure 3. Effect of mutating specific Ser and Thr residues of the SERK1 cyoplasmic domain on autophosphorylation in vitro.
A. Lane 1, SERK1-KD. Lane 2, SERK1-KDT541A. Lane 3, SERK1-KDS562A. Lane 4, SERK1-KDS570A. Lane 5, SERK1-KDT541A/S570A. Lane 6, SERK1-KDT559A/S562A. Lane 7, SERK1-KDT541A/S570A/T559A/S562A. The proteins were expressed, purified and loaded on 10% SDS-PAGE in equal amounts and stained with Colloidal Coomassie. B. In vitro phosphorylation assays on the proteins in the same order as shown on A were performed with [γ-32P]ATP. The size of the marker proteins (in kDa) is shown on the right.

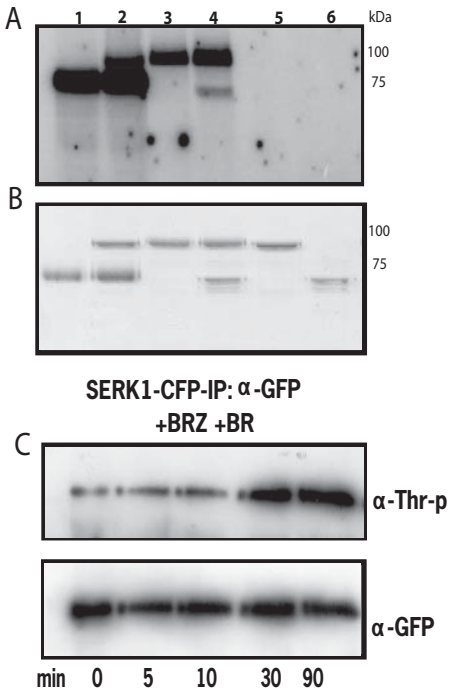


Figure 4. SERK1 and BRI1 both trans-phosphorylate kinase domains. A. Lane 1, SERK1-KD. Lane 2, SERK1-KD together with BRI1-KDK911E. Lane 3, BRI1-KD alone. Lane 4, BRI1-KD together with SERK1-KDK330E. Lane 5, BRI1-KDK911E alone and lane 6, SEK1-KDK330E alone. The proteins were loaded at equal concentrations and stained with Coomassie. B. The protein were incubated with [γ-32P]ATP. The size of the marker proteins (in kDa) is shown on the left. C. Exogenous application of BR increases the phosphorylation level of SERK1 in time. Liquid grown seedlings expressing SERK1-CFP were either treated or not with 100 nM BR for different time periods, after treatment with 1 μM BRZ. The levels of threonine phosphorylation were detected with anti-phospho threonine antibody (upper panel). Lower panel shows that equal amounts of SERK1-CFP proteins were used as depicted with anti-GFP antibody.

Identification of residues in SERK1-KD and BRI1-KD that can be trans-phosphorylated

We previously showed that not only SERK3 (BAK1) but also SERK1 interacts *in vivo* with BRI1 and is involved in the brassinosteroid pathway (Karlova et al., 2006). To determine whether SERK1 can be trans-phosphorylated by BRI1 or can trans-phosphorylate BRI1, we made use of enzymatically inactive forms of BRI1, BRI1-KD^{K911E} (Shah et al., 2001a; Li et al., 2002) and SERK1-KD^{K330E} proteins (Shah et al., 2001a; Li et al., 2002). Mixtures of mutant and wild-type proteins were tested for protein kinase activity (Figure 4 A). The results show that BRI1 and SERK1 can trans-phosphorylate each other *in vitro*, similar to results obtained for BRI1 and BAK1 (Li et al., 2002). We next identified the SERK1-KD trans-phosphorylation sites by using LC-MS/MS. More than 80% sequence recovery was achieved after proteolytic in gel digestion. We could not detect any phosphorylated residues in the BRI1-KD^{K911E} protein alone but in combination with active SERK1-KD proteins, only Ser-887 was found to be phosphorylated in the BRI1-KD^{K911E} protein (Figure 5). It was shown by Wang et al., (Wang et al., 2005b) that Ser-887 is phosphorylated *in vitro* in the active BRI1 protein and substitution with alanine did not affect the kinase activity of BRI1, but slightly increased trans-phosphorylation on a peptide substrate. For the SERK1-KD^{K330E} mutant trans-phosphorylated by BRI1-KD, the same three sites that were trans-phosphorylated by *E. coli* kinases were identified plus two additional ones, Ser-299 and Thr-462 were found to be phosphorylated (Figure 5). We conclude that these two sites are targets for BRI1-KD trans-phosphorylation in SERK1-KD. When Thr-462 was mutated to alanine the mutant protein SERK1-KD^{T462A} had decreased activity compared with the wild type SERK1-KD, and both casein and MBP cannot be trans-phosphorylated by SERK1-KD^{T462A} protein (Shah et al., 2001a). This suggests that the phosphorylation status of Thr-462 is essential for SERK1 activity and suggest a direct link to trans-phosphorylation by BRI1.

SERK1 is phosphorylated at Ser and Thr sites in vivo and the phosphorylation status is enhanced by BR

To test if the *in vivo* phosphorylation level of SERK1 was also regulated by exogenously applied brassinosteroids (BR), we grew SERK1-CFP expressing seedlings in liquid culture in the presence of brassinazole (BRZ; an inhibitor of brassinosteroid synthesis) for 3 days and then induced with 100 nM epibrassinolide for 0 to 90 min after which the SERK1-CFP protein was immunoprecipitated with anti-GFP antibodies. The depletion of endogenous BRs resulted in basal levels of threonine phosphorylation on SERK1 (Figure 4 C). After a 30 min treatment of these BRZ-pretreated seedlings with BR the phosphorylation status of SERK1 *in vivo* was enhanced. These data show that SERK1 is involved in brassinosteroid signaling in a similar fashion as BAK1 and SERK4 (BKK1) (Albrecht et al., 2005). To identify in vivo phosphorylation sites, transgenic plants expressing SERK1-CFP (Karlova et., 2006), were grown in liquid culture for 7 d, treated for 3 d with 2 μM BRZ and then induced with BR for 60 min. After immunoprecipitation (IP) with anti-GFP the IP was loaded on SDS-PAGE and

the SERK1-CFP band (around 0.2 µg) was excised, digested with trypsin, and subjected to MS/MS analysis. A high sequence recovery was achieved, 93% of the SERK1 cytoplasmic domain sequences and 7 phosphorylated sites were identified; Ser-303 in kinase subdomain I, Thr-337 in kinase subdomain II, Thr-459, Thr-462, Thr-463 and Thr-468 in the activation loop, kinase subdomain VIII and Ser-612 or Thr-613 or Tyr-614 in the C-terminal tail (Table 1, the *in vivo* sites identified are marked with an asterisk). Interestingly the phosphorylated sites in the C-terminal tail were only observed in the pull of SERK1-CFP seedlings treated with BRZ and BR and not in the ones treated only with BZR. All of these sites, except for Thr-337, were also identified as *in vitro* autophosphorylation sites of the GST-SERK1 cytoplasmic domain (Table 1).

SERK1 can interact with the inactive kinase domain of BRI1

We have demonstrated previously that SERK1 interacts *in vivo* with BRI1 (Karlova et al., 2006). To test whether this interaction depends on the phosphorylation status of BRI1 we expressed and purified the BRI1-KD^{K911E} mutant protein. *Arabidopsis* total protein extract was then incubated with immobilized MBP alone or MBP-BRI1-KD^{K911E} fusion protein. Proteins interacting specifically with BRI1-KD^{K911E} were identified by in liquid trypsin digestion and LC-MS/MS. As shown in Table 2, SERK1 was identified with 4 specific peptides, which were not found in the control experiment with MBP alone. Similar results were previously obtained for SERK3 and BRI1 in vitro (Li et al., 2002) and suggested that protein kinase activity is not required for the interaction between the SERK and BRI1 receptors.

Table 2. Identification of Arabidopsis SERK1 receptor interacting with MBP-BRI1^{K911E} fusion protein. The peptides were identified by ion trap LC-MS/MS.

Peptide- SERK1	Charge State	XC-score[a]	Ions[b]
R.LADGTLVAVKR.L	2	1.9	12/20
R.ELQVASDGFSNK.N	2	2.6	16/22
R.GTIGHIAPEYLSTGK.S	2	3.38	17/28
R.ERPPSQPPLDWPTR.K	2	2.48	11/26

[a]Bioworks 3.2 cross-correlation score (Xcorr) of the peptide is based on the fit of the ion trap MS/MS data to the theoretical distribution of ions produced for the peptide using a simple fragmentation model.
[b]The total number of b and y ions (identified/theoretical).

SERK1-KD^{K330E} : total sequence recovery= 80%

DIFFDVPAEEDPEVHLGQLKRFSLRELQVASDGFS*NKNILGRGGFGKVYKGRLADGTLVAV**E**
RLKEERTPGGELQFQTEVEMISMAVHRNLLRLRGFCMTPTERLLVYPYMANGSVAS*CLRERP
PSQPPLDWPTRKRIALGSARGLSYLHDHCDPKIIHRDVKAANILLDEEFEAVVGDFGLAKLMD
YKDTHVTTAVRGTIGHIAPEYLSTGKSSEKTDVFGYGIMLLELITGQRAFDLARLANDDDVML
LDWVKGLLKEKKLEMLVDPDLQTNYEERELEQVIQVALLCT*QGSPMERPKMSEVVRMLEG
DGLAEKWDEWQKVEILREEIDLSPNPNSDWILDSTYNLHAVELSGPRF

BRI1-KD^{K911E} ; total sequence recovery=86%

REMRKRRRKKEAELEMYAEGHGNSGDRTANNTNWKLTGVKEALSINLAAFEKPLRKLTFAD
LLQATNGFHNDSLIGSGGFGDVYKAILKDGSAVAI**E**KLIHVSGQGDREFMAEMETIGKIKHRN
LVPLLGYCKVGDERLLVYEFMKYGSLEDVLHDPKKAGVKLNWSTRRKIAIGSARGLAFLHHN
CSPHIIHRDMKSSNVLLDENLEARVSDFGMARLMSAMDTHLSVSTLAGTPGYVPPEYYQSFRC
STKGDVYSYGVVLLELLTGKRPTDSPDFGDNNLVGWVKQHAKLRISDVFDPELMKEDPALEIE
LLQHLKVAVACLDDRAWRRPTMVQVMAMFKEIQAGSGIDSQSTIRSIEDGGFSTIEMVDMSIK
EVPEGKL

BRI1-KD^{K911E} trans-phosphorylated by SERK1-KD; total sequence recovery=86%

REMRKRRRKKEAELEMYAEGHGNSGDRTANNTNWKLTGVKEALSINLAAFEKPLRKLTFAD
LLQATNGFHNDS*LIGSGGFGDVYKAILKDGSAVAIEKLIHVSGQGDREFMAEMETIGKIKHRN
LVPLLGYCKVGDERLLVYEFMKYGSLEDVLHDPKKAGVKLNWSTRRKIAIGSARGLAFLHHN
CSPHIIHRDMKSSNVLLDENLEARVSDFGMARLMSAMDTHLSVSTLAGTPGYVPPEYYQSFRC
STKGDVYSYGVVLLELLTGKRPTDSPDFGDNNLVGWVKQHAKLRISDVFDPELMKEDPALEIE
LLQHLKVAVACLDDRAWRRPTMVQVMAMFKEIQAGSGIDSQSTIRSIEDGGFSTIEMVDMSIK
EVPEGKL.

SERK1-KD^{K330E} trans-phosphorylated by BRI1: total sequence recovery= 82%

DIFFDVPAEEDPEVHLGQLKRFSLRELQVAS*DGFS*NKNILGRGGFGKVYKGRLADGTLVAV
ERLKEERTPGGELQFQTEVEMISMAVHRNLLRLRGFCMTPTERLLVYPYMANGSVAS*CLRE
RPPSQPPLDWPTRKRIALGSARGLSYLHDHCDPKIIHRDVKAANILLDEEFEAVVGDFGLAKLM
DYKDTHVT*TAVRGTIGHIAPEYLSTGKSSEKTDVFGYGIMLLELITGQRAFDLARLANDDDV
MLLDWVKGLLKEKKLEMLVDPDLQTNYEERELEQVIQVALLCT*QGSPMERPKMSEVVRML
EGDGLAEKWDEWQKVEILREEIDLSPNPNSDWILDSTYNLHAVELSGPRF

Figure 5. Sequence recovery after trypsin digestion and identification of the phosphorylated residues by LC/MS/MS. The phosphorylated residues are shown in bold and with a star.

Discussion

SERK1 is a member of a small family of five homologous LRR-RLKs (Hecht et al., 2001). Members of this family, such as SERK1 and SERK3, were shown to exhibit ATP-dependent autophosphorylation activity (Shah et al., 2001a; Li et al., 2002; Nam and Li, 2002). In this study we examined and compared the kinase activity of all five members of the SERK family, and determined the position of numerous autophosphorylated residues by LC-MS/MS. Differences in activity and in the number of autophosphorylated residues were found *in vitro*.

As found for the SERK family, kinase domain sequences of various plant receptor kinases are highly conserved, yet their phosphorylation properties are variable. A suggestion was made to distinguish between active and non-active kinases based on the mutations in the RD kinase motif (He et al., 2007). However, in the case of the SERK family all kinases have this motif, yet diverge widely in autophosphorylation activity. SERK5-KD, (since we used cDNA obtained from Col) did have a mutation in this RD domain, Leu to Arg at position 401, nevertheless the kinase remains active (Figure 1). Also the modes of phosphorylation in plant receptor kinases can be different. For example, the disease-resistant RLK Xa21 from rice contains a kinase domain that phosphorylates in an intramolecular manner (Liu et al., 2002). In contrast, the *Arabidopsis* SERK1 and *Lotus japonicus* SYMBIOSIS RECEPTOR KINASE (SYMRK) are reported to autophosphorylate intermolecularly (Shah et al., 2001a; Yoshida and Parniske, 2005). Recently it was shown that two of the SERK members, SERK1 and SERK2, have a redundant function in tapetum specification in anthers, while both the SERK1 and the SERK2 proteins are fully interchangeable genetically (Albrecht et al., 2005) and also need to be catalytically active (Albrecht et al., in preparation). Yet both proteins have a different auto-phosphorylation activity in vitro. SERK3 was also shown to be involved in different signaling pathways. Both SERK3 and SERK4 were recently shown to be required in preventing cell death in *Arabidopsis* (He et al., 2007), while SERK3 alone has multiple functions in BR mediated signalling (Li et al., 2002; Nam and Li, 2002) and in innate immunity (Kemmerling et al., 2007). Taken together, apparently the kinase activity or pattern of autophosphorylated residues identified *in vitro* does not give a direct clue for the biological significance of the kinase domains. A similar case was reported for the kinase activity of another plant receptor, CRINKLY4, that was shown to be not required for its biological activity (Gifford et al., 2005).

In animal systems, upon ligand binding receptor oligomerization occurs followed by activation of the cytoplasmic domain(s) through phosphorylation of one or more of the receptor components. We have shown that the first three members of the SERK family can heterodimerize with each other (Russinova et al., 2004; Albrecht et al., 2005). In agreement with our kinase activity data, SERK1-KD appears to be the most phosphorylated one with at least 24 phosphorylated residues identified. It was not surprising to find multiple phosphorylation sites in the catalytic core of the kinase sub domains I to XI. Only two phosphorylated residues were identified in the juxtamembrane region and 4 in the C-terminal tail, which is in contrast with BRI1 kinase, where 5 sites were identified in the juxtamembrane region (Wang et al., 2005b).

In the activation loop of the SERK proteins, there are 4 conserved threonines representing Thr-459, Thr-462, Thr-463 and Thr-468 in SERK1 protein. These residues were identified to be phosphorylated also *in vivo* and are functionally equivalent to Thr-1039, Ser-1042, Ser/Thr-1044/45 in BRI1 (Wang et al., 2005b). The conserved residues within the activation loop of SERK 1, 2, 3, 5 and perhaps SERK4 (where, due to fast degradation we could not detect phosphorylation sites, nevertheless low autophosphorylation activity could be detected) were found to be autophosphorylated, similar to what was shown previously for BRI1 (Wang et al., 2005b). Phosphorylation of one to three residues in the kinase activation loop was shown to be a common mechanism of general kinase activation and in some cases also affects binding of kinase substrates (Shah et al., 2001a; Adams, 2003). For example residue Thr-462 in SERK1, corresponding to BRI1 Ser-1042, was found to affect substrate phosphorylation more than autophosphorylation *in vitro* (Shah et al., 2001a), which was also observed for the $BRI1^{T1039A}$ mutant (Wang et al., 2005b). These data are also in agreement with our finding that Thr-462 in SERK1-KD is trans-phosphorylated by BRI1-KD *in vitro*. Mutation of Thr-462 completely abolishes the ability of the mutant protein to trans-phosphorylate artificial substrates (Shah et al., 2001a). Substitution in BRI1 of Ser-1044 and/or Thr-1045 and Thr-1049 by alanine strongly affected autophosphorylation, peptide substrate phosphorylation, and in planta BRI1 signaling and suggests an important role for these activation loop residues in BRI1 kinase function (Wang et al., 2005b). The equivalent of the $BRI1^{T1049A}$ mutation in $SERK1\text{-}KD^{T468E}$ has a similar effect on SERK1 kinase activity *in vitro* (Shah et al., 2001a). The SYMRK receptor was shown by LC-MS/MS to be autophosphorylated *in vitro* on the corresponding residue (Thr-760), and the $SYMRK^{T760A}$ substitution significantly reduced kinase activity (Yoshida and Parniske, 2005). Another activation loop residue, Ser-754, was also phosphorylated in vitro in SYMRK, and this residue aligns with Ser-1044 in BRI1 and with Thr-463 in SERK1. Autophosphorylation of juxtamembrane and C-terminal regions, that both show less sequence conservation is believed to generate docking sites for specific kinase substrates rather than a more general response to kinase activation (Pawson, 2004; Wang et al., 2005b). From the SERK family only for SERK1-KD and SERK2-KD the residues Ser-291 and Ser-299 (corresponding to Thr-302 in SERK2) were found to be phosphorylated in the juxtamembrane region and the Thr-606, Ser-612, Thr-613 and Ser-622 residue in SERK1-KD corresponding to Ser-604, Thr-616 and Ser-625 in SERK2-KD and Thr-589 or Ser-595, Ser-602 and Ser-612 for SERK3 in the C-terminal tail (Table 1). Autophosphorylation may be important not only as a modulator of trans-phosphorylation kinase activity but also as a means of altering protein binding that can affect macromolecular complex formation (Ikeda et al., 2000; Merkle et al., 2002). In SERK1-KD protein three phosphorylated tyrosines were found, that are unlikely to be the product of trans-phosphorylation by kinases in *E. coli* , because they are not found in the $SERK1\text{-}KD^{K330E}$ mutant protein. This is in line with the fact that SERK1-KD was found to trans-phosphorylate artificial substrates such as casein and MBP on both serine/threonine as well as tyrosine (Shah et al., 2001a). These findings confirm that SERK1 is a dual specificity-type receptor kinase.

Different biological functions were found for the members of the SERK family. SERK1

was found to act redundantly with SERK2 in tapetum specification and male sterility. The *serk1-1* allele misses the last 90 amino acids of the protein. To test the function of the identified phosphorylated residues in this region that include kinase subdomains X and XI we created mutant proteins. The substitution Ser-562-Ala nearly abolished the kinase activity of SERK1-KD *in vitro*, where Thr-541-Ala/Ser-570-Ala only in double combination reduced the autophosphorylation activity. All the residues in kinase subdomain I, II, III and IX, X, XI, found to be phosphorylated in wild type SERK1-KD were not found to be phosphorylated in the SERK1-KD^{S562A} mutant protein. Interestingly, 3 threonines in the activation loop remained phosphorylated, including Thr-462, a residue essential for the full autophosphorylation activity (Shah et al., 2001a). The Thr-468 residue shown to be the most important residue for autophosphorylation in the activation loop was no longer phosphorylated in the SERK1-KD^{S562A} mutant protein. These data, in combination with the phosphorylation profile of the SERK1-KD^{S562A} protein shows the importance of this region and the Ser-562 residue for a positive regulation of the kinase function of SERK1. The identification of *in vivo* phosphorylation sites of SERK1 receptor confirms our *in vitro* identified phosphorylated sites and the important role of the activation loop phosphorylation for kinase activity.

Other members of the SERK family, SERK3 and SERK4 (BKK1) were also shown to have multiple functions. Our *in vitro* autophosphorylation data showed that with the conditions used, SERK4 and SERK5 are active kinases, but with lower activity compared to the other members of the family. These differences in autophosphorylation activity could be also due to expression and purification of the kinase domains in this *in vitro* system. Like in case of SERK4 protein the lower autophosphorylation activity could be a result of the rapid degradation of the SERK4 protein observed. He et al, (He et al., 2007) showed that SERK4 functions redundantly with SERK3 in negatively regulating a BR-independent cell death pathway. Also BR treatment increased phosphorylation levels on threonine residues in SERK3 and SERK4 (He et al., 2007). Similar induction of phosphorylation status was observed for SERK1 after stimulation with BR *in vivo.* This observation is most probably due to trans-phosphorylation upon BR treatment from the main co-receptor BRI1, which is in line with the proposed model for signaling of BRI1 upon ligand stimulation (Karlova and de Vries, 2006). These data also fit with our observation that upon BR stimulation phosphorylation in the C-terminal tail is detected *in vivo.* Receptor homo and heterodimerization occurs in the SERK family. SERK1 was found to form dimers at the plasma membrane of *Arabidopsis* protoplasts with SERK3 as well as with BRI1 (Russinova et al., 2004). SERK1 was also found in a complex with SERK3 and BRI1 *in vivo*, suggesting the formation of tetrameric complexes in analogy with animal TGF-β receptors that consist of heterotetramers of the receptor pairs Tβ-RI and Tβ-RII. Tβ-RII homodimerizes in the absence of ligand and exhibit constitutive autophosphorylation. TGF-β binding by Tβ-RII induces the formation of heterotetramer and results in phosphorylation of Tβ-RI by Tβ-RII on residues in the juxtamembrane domain (Wagner et al., 1998; Huse et al., 2001) while activated Tβ-RI is responsible for downstream signaling. It was shown that BRI1 and BAK1 can trans-phosphorylate each other (Li et al., 2002; Nam and Li, 2002). Our data showed that the same is true for BRI1 and SERK1. The current model for BRI1 activation is

that binding of BR to the extracellular domain of BRI1 (Kinoshita et al., 2005) activates BRI1 by phosphorylation of Thr-1049 and Ser-1044 and induces a conformational change in the BRI1 homodimer. After dissociation from the BKI1 protein (Wang et., 2006) a negative regulator of BR signalling, the activated BRI1 then interacts with BAK1 via the kinase domains (Wang et al., 2005b; Wang et al., 2005a). Our data showed that SERK1 can also interact with the *in vitro* produced kinase domain of catalytically inactive BRI1 (BRI1-KD^{K911E}) protein, suggesting a possibility for heterodimerization in the absence of BR, similar to what was shown *in vitro* for BAK1 (Li et al., 2002). Since *in vitro* produced BRI1-KD^{K911E} protein was used in the pull down the observed interactions could differ from the *in vivo* situation where the BKI1 (Wang et al., 2006) protein bound to BRI1 could prevent the heterodimerization of kinase-inactive BRI1 with SERK1. SERK1 and BAK1 co-immunoprecipitate in vivo with BRI1 and BR treatment can increase the amount of BAK1-GFP associated with BRI1-Flag (Wang et al., 2005b; Karlova et al., 2006). Our data support this model and indicate that SERK1 and BRI1 can both trans-phosphorylate each other. The SERK1 Thr-462 residue, trans-phosphorylated by BRI1-KD, was shown to play an important role in auto- and trans-phosphorylation activity (Shah et al., 2001a). The BRI1 activation model also involves inhibition of kinase activity by its C-terminal tail, which is relieved upon ligand binding to the extracellular domain. After activation BRI1 can trans-phosphorylate the SERK1 kinase domain on Thr-462, that subsequently leads to activation and increased kinase activity, including phosphorylation of the C-terminally located Ser-562. Our data therefore suggest a complex pattern of both intra- and intermolecular control of SERK1 kinase activity.

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Chapter 5

Brassinosteroid signaling is essential for plant cell totipotency

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In preparation

Abstract

Cell fate in plant cells is highly flexible and even differentiated cells can assume a different fate. One dramatic example of such a fate change is the ability of fully differentiated cells in tissue cultures to revert back to the totipotent state upon appropriate stimulation with plant growth regulators such as auxin and cytokinin. The molecular mechanism underlying this fate change is poorly understood. Here we show that the plant steroid hormone brassinolide (BL) is instrumental to change the differentiated cell fate towards totipotency in Arabidopsis explants. The BL-dependent cell fate change is mediated by the BR-perceiving receptor BRI1 and one of its co-receptors SERK1. We found that BRI1/SERK1 phosphorylate directly the transcriptional regulator AGL15. Upon BL induction, AGL15 protein translocates to the nucleus, where directly controls BRI1/SERK1 gene expression, thereby completing a novel positive feedback control loop in BR signaling pathway.

Introduction

Single somatic plant cells in culture can be induced to shift their fate towards totipotency or embryogenic competence by application of exogenous auxins (Mordhorst et al., 1998). The SOMATIC EMBRYOGENESIS RECEPTOR LIKE KINASE (SERK) gene was first identified in carrot as a marker for single cells competent to form somatic embryos (SE) (Schmidt et al., 1997). In *Arabidopsis* ectopic expression of SERK1 increases somatic embryogenesis in culture (Hecht et al., 2001), suggesting that SERK1 mediated signaling is required for acquisition of embryogenic competence in somatic cells. Cells acquiring embryogenic competence in Arabidopsis are derived from procambium cells in the vascular tissue that express the SERK1 gene (Kwaaitaal and de Vries, 2007). The AGAMOUS-like 15 (AGL15) (Harding et al., 2003) also results in enhanced somatic embryogenesis upon ectopic over-expression, while loss-of-function mutants of AGL15 exhibit decreased ability to form somatic embryos (Thakare et al., 2008). Previously we identified AGL15 as a component of the SERK1 protein complex (Karlova et al., 2006), suggesting a direct link in the form of a very short signal transduction chain between both. SERK1 is a member of a small family of five related RLKs, all of which have five LRRs and a typical Ser-Pro-rich extracellular region missing in related LRR-RLKs (Hecht et al., 2001). The SERK1 protein complex also contains the main brassinosteroid receptor BRI1 (Li and Chory, 1997) as well as its co-receptor SERK3, also known as BAK1 (Li et al., 2002; Nam and Li, 2002) suggesting that SERK1 also mediates BR signaling (Karlova et al., 2006). BR signaling commences with the ligand binding to the main receptor BRI1 (Kinoshita et al., 2005), followed by a conformational change and phosphorylation of BRI1 (Wang et al., 2005a) resulting in dissociation of the inhibitor BKI1 (Wang and Chory, 2006) and the heterodimerization with BAK1 (Wang et al., 2005b) Via a so far unknown mechanism, BRI1 activation then leads to inactivation of the kinase BIN2 (Li et al., 2001) resulting in dephosphorylation of the nuclear proteins BES1 and BZR1 (Mora-Garcia et al., 2004; Vert and Chory, 2006). Dephosphorylated BES1 and BZR1 than homodimerized and bind to DNA to regulate BR-responsive genes. Recently it was shown that BR signaling also involves a member of the family of 14-3-3 proteins serving as a cytoplasmic retention signal of phosphorylated BZR1 (Gampala et al., 2007; Ryu et al., 2007).

BRs regulate cellular expansion, differentiation and proliferation and are involved in diverse processes, such as stem elongation, vascular differentiation, male fertility, timing of senescence and flowering and leaf development (Li and Chory, 1997; Clouse and Sasse, 1998; Altmann, 1999), but to date have not been implicated in the acquisition of embryogenic competence. In this study we provide evidence that the main BRI1-mediated BR signaling pathway is required for the acquisition of embryogenic competence. Both exogenous application of BRs and overexpression of BRI1 enhances somatic embryogenesis while *bri1* strong mutant alleles and the BR biosynthesis mutant *det2* show strongly reduced embryogenic competence. In *det2* but not in the perception mutant *bri1-201* somatic embryogenesis can be rescued by exogenous BRs application. The loss-of-function alleles s*erk1* alone or in combination

with *agl15-4* significantly impair somatic embryogenesis. *agl15* also aggravates general phenotypes of a weak *bri1-301* mutant allele suggesting that it acts as a positive regulator in BR signaling. By using Fluorescence Lifetime Imaging Microscopy (FLIM) to determine Förster Resonance Energy Transfer (FRET) between fluorescently tagged proteins we found that AGL15 interacts via 14-3-3ν at the plasma membrane with SERK1. Translocation of AGL15 to the nucleus appears to be dependent on BRs. This suggests that the SERK1 co-receptor and its direct target AGL15 represent a novel short positive feedback loop in BR signaling reminiscent of the SMAD pathway in TGFß signaling in animals (Feng and Derynck, 1997).

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia was used as the wild type plant, loss-of-function mutants (*serk1-1*, *agl15-4*, *serk1-1 agl15-4, det2*, *bri1-201*, *bri1-301*, *bri1-116* all in Col) and transgenic seeds were surface sterilized and sown on MS media (½ Murashige and Skoog (MS) salt and vitamins (Duchefa, Haarlem, The Netherlands) supplemented with 0.8 % (w/v) agar (Daishin) and 2 % (w/v) sucrose. Seeds were kept for 2 days at 4oC and then transferred to a growth chamber where the seedlings were grown at 22oC under 16 h light/ 8 h dark periods. Arabidopsis plants expressing the SERK1 receptor fused to cyan fluorescent protein (CFP) under its native promoter was described previously (Kwaaitaal et al., 2005). The BRI1-GFP transgenic plants used for SE cultures was described before (Russinova et al., 2004).

The *Arabidopsis* Somatic Embryo System

The embryo culture system were initiated and maintained using the seedling assay as described previously (Mordhorst et al., 1998). Briefly seeds were surface sterilized and about 30 seeds were incubated in 20ml of liquid MS medium, containing 2 % (w/v) sucrose, 4.5µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 10mM 2-(*N* –morpholino)-ethanesulfonic acid (MES), pH 5.8. After two days at 4 °C the cultures were transferred to 22 °C in the light (16h light/8h dark photoperiod). After 2 weeks of culture, and subsequently every week after, the medium was replaced with fresh one. For *bri1-116* and *bri1-201* after 2 weeks, homozygous seedlings were discriminated based on their smaller size and anthocyanin accumulation and were subcultured separately. After 5 weeks of culturing, the percentage of calli containing green embryonic clusters was determined and this percentage was used as a measure for embryogenic competence of the culture. The significance of the difference in the mean embryogenic competence values compared to wild type or mutants was assessed using student-T test; p values equal or lower than 0.05 were regarded as significant. The *det2* mutant was previously described by Chory et al (2001) as well as the *bri1-201* and *bri1-116* (Li and Chory, 1997; Bouquin et al., 2001). For the AGL15 overexpression lines, the coding region (cDNA) of AGL15 under 35S promoter was used (the construct was kindly provided by Stefan de Folter and Gerco Angenent, Plant Research International, Bornsesteeg 65, 6708PD, Wageningen).

Generation of agl15-4 *bri1-301* and agl15-4 *serk1-1* double mutants

The *bri1-301*mutant, ecotype Columbia was kindly provided by Jianming Li (Department of Molecular, Cellular, and Developmental Biology, university of Michigan) and was previously described by Li and Nam, 2002 (Li and Nam, 2002). AGL15 and SERK1 knockout lines, *agl15-4* and *serk1-1* were obtained from the SALK insertional mutant population (Alonso et al., 2003) as insertion lines SALK_076234 and SALK_544330, respectively, described before by Lenti-Shiu et al., (2005) (Lehti-Shiu et al., 2005) , Albrecht et al., (2005) (Albrecht et al., 2005) in Columbia background. *bri1-301 agl15-4* mutant line was generated by crossing *bri1-301* with agl15-4, *agl15-4 serk1-1* was generated by crossing *agl15-4* with *serk1-1.* In each case, the F1 was allowed to self-fertilize, and candidate double F2 plants were genotyped phenotypically and by PCR.

Construction of the CFP/YFP and GST tagged proteins

PCR fragments containing the coding sequence (cDNA) of AGL15 (At5g13790) and 14-3-3υ (At3g02520) were digested with NcoI for AGL15 or BglII/NcoI for 14-3-3υ and were C-terminally linked to CFP or YFP in pMON999 plasmid (van Bokhoven et al., 1993). For the expression of the protein with GST tag, pGEX4T1 vector was used and the PCR fragments of AGL15 and 14-3-3υ were digested with EcoRI/XhoI in both cases and inserted into pGEX4T1 for N-terminal fusion with GST. All constructs were checked by restriction analysis and were verified by sequencing. Protoplast isolation and transfection were performed as described before (Aker et al., 2006). FRET-FLIM was measured 16h after transfections as described previously (Karlova et al., 2006; Aker et al., 2007).

Confocal laser scaning microscopy.

The CFP and YFP fluorescence in protoplast was imaged using a confocal laser scanning microscope 510 (Carl Zeiss, Jena, Germany) excited using the 458 nm and 514 nm laser lines. The fluorescence was detected using a band-pass filter (CFP: 470-500 nm, YFP: 535-590 nm). Chlorophyll fluorescence was detected using a 650 nm long pass filter.

Fluorescence Lifetime Imaging Microscopy

FLIM was performed using a Biorad Radiance 2100 MP system in combination with a Nikon TE 300 inverted microscope as described before (Russinova et al., 2004). From the intensity images obtained, complete fluorescence lifetime decays were calculated per pixel and fitted using a double exponential decay model.

***In vitro* trans-phosphorylation and LC-MS/MS analysis**

Protein expression and GST purification were performed as described before (Shah et al., 2001). For the *in vitro* trans-phosphorylation reaction 1μg SERK1-KD was added to 1μg of AGL15 or 14-3-3υ with (γ^{32}P) ATP as described previously by Shah et al (Shah et al., 2001). After incubation for 30 minutes at 30 °C, the reaction was stopped by adding Laemmli SDS-

PAGE sample buffer, boiled at 95°C for 5 minutes and separated on 10% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue (CBB) to verify equal loading and then dried. The radioactive signal was quantified using ImageQuant program of the PhosphoImager scanner (Molecular Dynamics). *In vitro* phosphorylation for MS analysis was performed as described above except that 3 µg of each kinase protein was used and (γ^{-32}P) ATP was replaced by 3 mM of cold ATP. In-gel digestion was performed as described by Shevchenko et al. (ref) and Aker et al. Ref and the tandem mass spectrometric analysis and data analysis were done as described before (Aker et al., 2007).

Chromatin immunoprecipitation of AGL15-DNA complex

AGL-15 specific antibodies and pre-imune sera were used as a negative control and were kindly provided by Sharyn Perry (University of Kentucky, Department of Plant and Soil Sciences, Lexington, USA). The chromatin immuno precipitation (ChIP) was performed with wild type plant extract from 5 days old seedlings as described before by Wang et al (Wang et al., 2002). Co-precipitated DNA was amplified by PCR using the following primers: BRI1PF1: CAATCGCACTCACCTCCATGA and BRI1PR1:TGTAACTATCTATCG-CAAGTG and BRI1P2F: CATCTCGTACAGTGTACTACA and BRI1P2R: AGAATA-CTATATTGGTACAAG, and SERK1P1F:TATGAGAACACACTGGTAGAT and SERK1P1R: GATAATATCCTAGTGCAAGTG and SERK1P2F:GTGCATAGCCTTAAGAGCTT, and SERK1P2R:CTACGTGAAGTAATAATAAC. Typically 35 to 40 cycles of PCR were performed.

Gel retardation assays

The binding reactions for the gel retardation assays were performed as described by (Winter et al., 2002). 120 ng of protein were used per binding reaction. A CArG box sequence from the second intron of AGAMOUS was used as DNA probe (5'-GAAATTTAATTATATTCCAAATAAGGAAAGTATGGAACGTT-3') (Gomez-Mena et al., 2005). The 5' ends of the double-stranded DNA probes were biotinylated, and 13 fmol of probe was used per lane. For competition experiments, unlabelled CArG-box-containing DNA, or unlabelled DNA with randomized sequence was added to the binding reaction in 100-fold molar excess. After native polyacrylamide gel electrophoresis, the DNA was blotted to positively charged nylon membranes (Hybond N+, Amersham) and signal detection was done using the chemiluminescent nucleic acid detection module (Pierce, Rockford, IL, USA).

Immunoprecipitation and protein detection.

Immunoprecipitation was done as described before (Karlova et al., 2006). For immno detection, the proteins were eluted from the beads by adding SDS sample buffer and boiling for 5 min at 95°C. The proteins were separated on 10% SDS-polyacrylamide gel (Laemmli, 1970) and transferred onto nitrocellulose (Schleicher & Schuell) by wet electroblotting (Bio-Rad). Bound primary anti-GFP antibodies (produced by Invitrogen) were detected with horseradish

peroxidase-conjugated IgG purified donkey anti-rabbit secondary antibodies at 10,000 x dilution (Rockland Laboratories) and visualized using reagents for chemiluminiscence detection (ECL Plus reagent; Amersham Biosciences).

Results

BRI1 dependent BR signaling is required for embryogenic competence

To investigate the role of BRs and BRI1 in embryogenic competence, somatic cultures were initiated by exposure to 2,4-dichlorophenoxy acetic acid (2,4-D) from wild type, *bri1-116* (Li and Chory, 1997), *bri1-201* (Bouquin et al., 2001) and BRI1-GFP over-expressors (Russinova et al., 2004; Geldner et al., 2007) according to the seedling assay (Mordhorst et al., 1998).

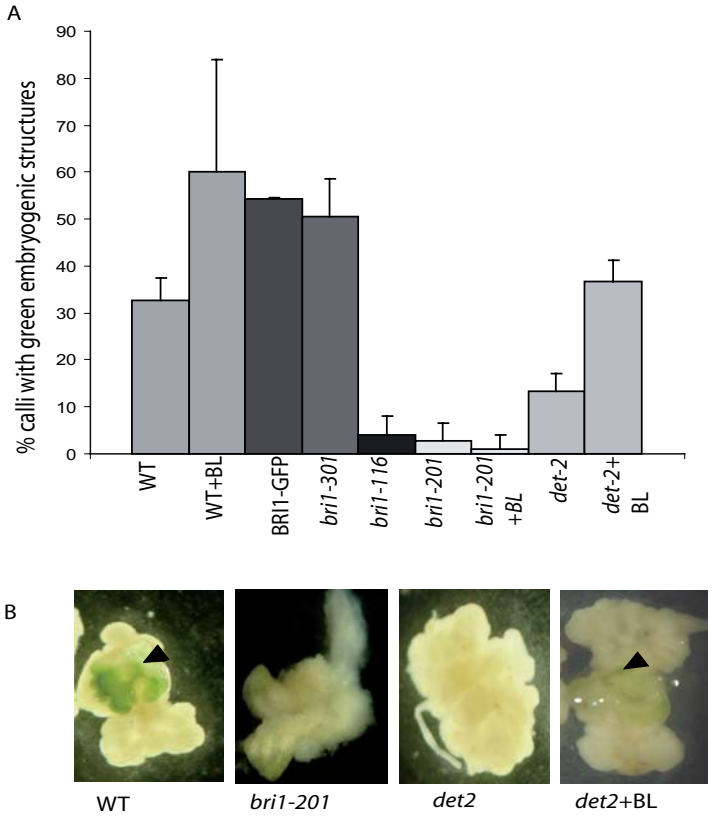


Figure 1. BRI1 and BRs are involved in somatic embryogenesis signaling. A. The percentages of calli with green embryonic structures after 5 weeks of culture were accounted. Mean and standard error of mean are shown. B. Images are representative of the development of callus and green embryonic structures (arrowhead) in wild type Col, *bri1-201* and *det2* mutants.

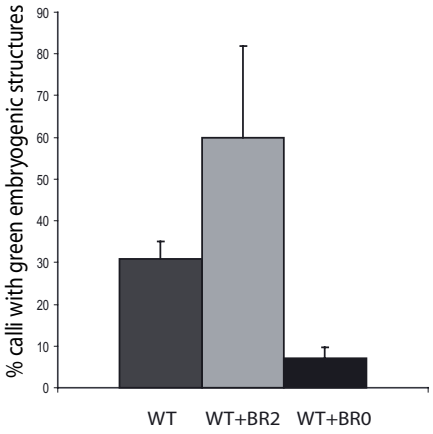


Figure 2. BRs are involved in somatic embryogenesis signaling. A. The percentages of calli with green embryonic structures after 5 weeks of culture were accounted. Mean and standard error of mean are shown. BR0, Brassinosteroids were added from day 0 of the culturing. BR2, Brassinosteroids were added after 2 weeks of culturing.

After five weeks, the seedling-derived calli that showed green embryonic masses (Figure 1B) were counted. As shown in Figure 2, exogenous application of only 2.5 nM of BL (biosynthetic BR) at the beginning of the cultures effectively inhibited somatic embryogenesis while application of only 2.5 nM of BL after 2 weeks increased somatic embryogenesis about two-fold (Figure 1 and Table 1). Therefore in all further analyses BL was added after 2 weeks. Over-expression of BRI1 also resulted in a clear increase in somatic embryogenesis, while the BRI1 alleles *bri1-201* and *bri1-116* had a very strong reduction in embryogenic competence compared to wild type (Figure 1 and Table 1). Both mutants not only suffered from impaired embryogenic competence, but also failed to develop a normal proliferating callus as the wild type (Figure 1B). Surprisingly, the weak *bri1-301* mutant did not show any reduction but rather an increase in embryogenic competence comparable to that of an over-expressor line. The *bri1-301* allele contains a 989G to I mutation in the kinase domain of BRI1. *bri1-301* plants developed smaller, compact rosettes and have less reduced stature compared to severe *bri1* alleles (Figure 4A) while maintaining full insensitivity to BR of the roots (Li et al., 2002). Embryogenic callus typically originates from the vascular procambial tissue close to the shoot apical meristem in seedlings (Kwaaitaal and de Vries, 2007) and *bri1-301* mutants typically have increased procambial cell layers, which might explain the response in embryogenic culture. However this increase was not observed in the *bri1-116* mutant which is in agreement with the strong reduction in the embryogenic competence (data not shown). For comparison with the BR perception mutants, homozygous seed stocks of the brassinosteroid biosynthetic

mutant (*de-etiolated*) *det2-1* (Chory et al., 1991) were included. The DET2 protein is a 5α-reductase in the second step of brassinolide biosynthesis and *det2-1* mutant plants accumulate less then 10% of wild type level brassinosteroids (Fujioka et al., 1997). The biosynthetic mutant *det2-1* showed a strong reduction in embryogenic competence (Figure 1 and Table 1) but did produce normal proliferating callus (Figure 1B). As expected, *det2* cultures could fully be restored by exogenous application of BL, while the perception mutants failed to respond, completely in line with what is observed in plant-based assays (Fujioka et al., 1997; Li and Chory, 1997) for *det2* and *bri1* mutants respectively (Figure 1). These results suggest that optimized BR signaling mediated by the BRI1 receptor is required for the acquisition of 2,4-D induced embryogenic competence in Arabidopsis explants.

Table 1 Percentage of seedlings developing embryonic clusters out of SAM upon germination in liquid 2,4-D containing media of wild type (WT), WT induced with BL, *bri1-116*, *bri1-201*, BRI1-GFP, *det-2*, *det-2* induced with BL, *bri1-301.*

Genotype	Total N[a] of seedlings tested	Percentage of EC[b]	TTEST-p
WT	1080	33%	
WT+BL	270	60%	p≥0.01
bri1-201	450	2.7%	p≥0.0000001
bri1-116	270	4%	p≥0.000002
BRI1-GFP	180	54%	p≥0.0001
det2	450	13%	p≥0.00008
det2+BL	270	36%	p≥0.0009
bri1-301	270	50.6	p≥0.003

[a]N, number
[b]EC, embryogenic clusters.

SERK1 and AGL15 are involved in BR-induced acquisition of embryogenic competence

Having established a clear link between the acquisition of embryogenic competence and BRI1-mediated BR signaling, our next question was whether SERK1 and AGL15 played a similar role in this signaling process. SERK1 and AGL15 showed an overlapping expression pattern starting from the mature unfertilized ovule, during embryo development as well as throughout the vascular system and shoot apical meristem in seedlings (Perry et al., 1996; Hecht et al., 2001; Adamczyk et al., 2007; Kwaaitaal and de Vries, 2007). Previously we reported that over-expression of SERK1 increased embryogenic competence (Hecht et al., 2001), and a similar phenotype was shown for AGL15 (Harding et al., 2003), while an *agl15* mutant exhibited mildly reduced embryogenic competence (Thakare et al., 2008). As

shown in Figure 3, over-expression of either SERK1 or AGL15 resulted in enhanced somatic embryogenesis in the Columbia ecotype, confirming previous observations made in other ecotypes (Figure 3 and Table 2). The SERK1-YFP line used in these experiments showed only approximately two-fold over-expression of the SERK1 gene (data not shown) compared to WT. Only the *serk1-1* (Albrecht et al., 2005) allele exhibits reduced embryogenic competence (Figure 3). None of the other *serk* mutants tested gave a significant reduction in somatic embryogenesis while none of the double mutant combinations with a *serk1* allele present showed more reduction (data not shown). This suggests a specific role of the SERK1 receptor in the acquisition of embryogenic competence. In contrast to what was observed for the *bri1-201* mutant (Figure 1), application of exogenous BL to the *serk1-1* somatic cultures restored embryogenic competence to slightly more then untreated WT level, possibly the result of enhanced signaling via the main BRI1 receptor as was seen in the WT and independent from the presence of one of its co-receptors. This appears to fit into the more general scheme for BRI1 mediated BR signaling, where it is observed that the BR-related phenotypes observed in *serk* mutants are always less severe when compared to those of BRI1 itself (Li et al., 2002; Nam and Li, 2002; Karlova et al., 2006; Albrecht et al., 2008); confirming that BRI1 can also perceive and transduce the BR signal in the absence of the co-receptors (Wang et al., 2005a).

Table 2 Percentage of seedlings developing embryonic clusters out of SAM upon germination in liquid 2,4-D containing media of SERK1-YFP, 35S-AGL15, *serk1-1*, *serk1-1*+35SAGL15, *serk1-1* induced with BL, *agl15-4*, *agl15-4 serk1-1*, *agl15-4 bri1-301*, *bri1-301.*

Genotype	Total N[a] of seedlings tested	Percentage of EC[b]	TTEST-p
SERK1-YFP	450	60	p≥0.00001
35S-AGL15	270	70	p≥0.00002
s*erk1-1*	900	23	p≥0.01
serk1-1+ 35SAGL15	270	34	
s*erk1-1*+BL	180	39.7	p≥0.03
agl15-4	450	30.5	p≥0.45
agl15-4 serk1-1	540	17.7	p≥0.006
agl15-4 bri1-301	180	399	p≥0.17

[a]N, number
[b]EC, embryogenic clusters.

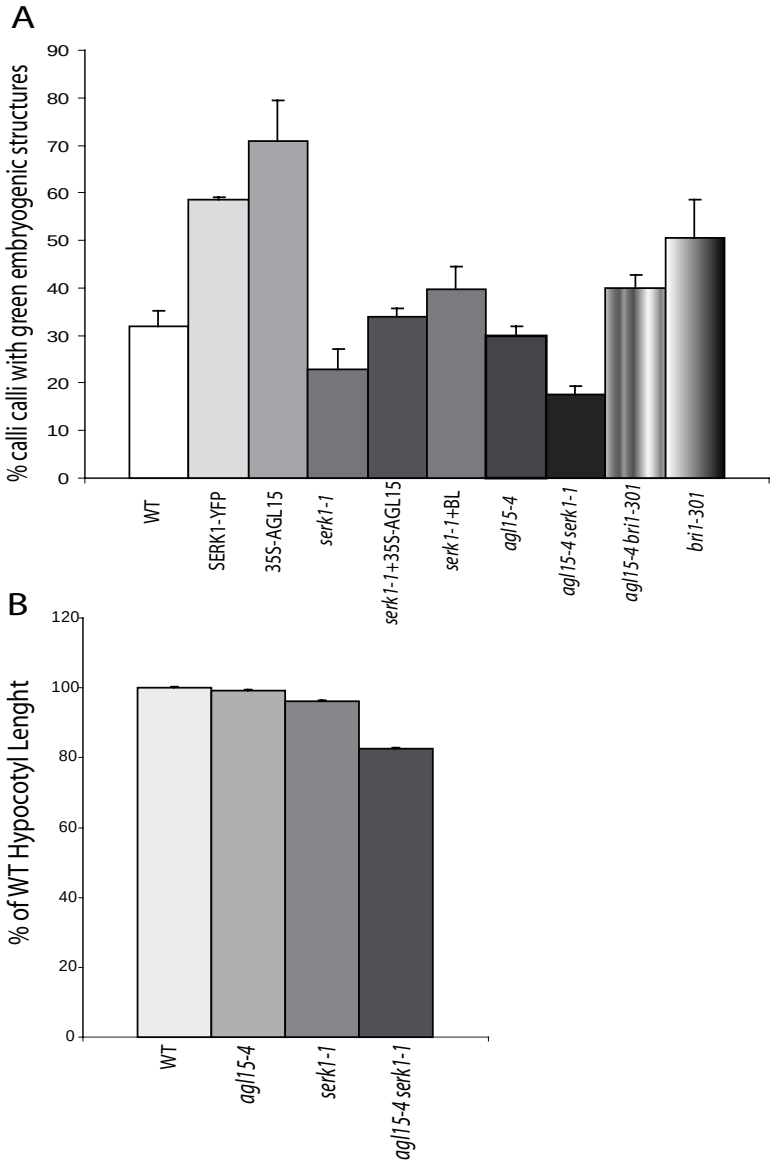


Figure 3. The effect of SERK1, and AGL15 on embryogenic potential of seedlings. A. Percentage of seedlings that showed somatic embryo development from the SAM, when seeds of the indicated genotypes were allowed to germinate for 5 weeks in liquid media containing 2,4-D. Mean and standard deviations are shown. B. Double mutant agl15-4 serk1-1 has shorter hypocotyls when seedlings were grown on dark for 7 days. At least 20 seedlings were measured.

We next tested the *agl15-4* mutant (Lehti-Shiu et al., 2005), but could not confirm the previously reported mild reduction (Thakare et al., 2008) in somatic embryogenesis for this allele (Figure 3 A) probably due to the different light conditions used. However, the *agl15-4 serk1-1* double mutant did show a significant reduction compared to single *serk1-1* or *agl15-4* (Figure 3 and Table 2) mutants, suggesting a synergistic interaction between both genes. Next question was if the double *agl15-4 serk1-1* mutant has impaired BR signaling. First the root insensitivity to BRs was examined. The double mutant was as sensitive as the *serk1-1* alone (data not shown), which is consistent with the fact that AGL15 is not expressed in roots (Adamczyk et al., 2007). Second the double mutant had no stature phenotype different from the wild type, but, analyzing the hypocotyls length as an assay for impaired BR signaling, *agl15-4 serk1-1* double mutant showed a significant reduction in hypocotyls length, when grown in the dark (Figure 3 B, $p \leq 0.01$). Next the *agl15-4 bri1-301* double mutant was compared with its respective parents. The *agl15-4* mutation clearly enhanced the BRI1-associated phenotypes such as a reduction in the size of the rosette and the inflorescence length (Figure 4A and B). No effect was observed on the root insensitivity or the hypocotyls length probably due to the fact, that *bri1-301* mutant itself is already insensitive to the high concentrations of BL. Embryogenic competence was also determined in the *agl15-4 bri1-301* double mutant (Figure 3 A). The results showed a reduction of bri1-301 embryogenesis to a level intermediate of both single mutants, confirming that similar to the double mutant *agl15-4 serk1-1*, also *agl15* mutation can influence the embryogenic competence of the weak *bri1-301* allele. This is in agreement with the observed enhancement of the stature phenotype. These findings indicate that the MADS box transcription factor AGL15 is involved as a novel positive regulator in general aspects of BR signaling besides embryogenic competence. Over-expression of AGL15 restored the observed limited reduction in embryogenic competence in *serk1-1* mutant calli to wild type level. Surprisingly the strong over-expression phenotype of AGL15 in WT is lost in the *serk1-1* mutant background, indicating that AGL15 is not only a direct target of BRI1 and SERK1 signaling, but also acts as a regulator of SERK1 expression. This is in line with the fact that AGL15 over-expression can enhance SERK1 expression in SE cultures (Harding et al., 2003). To verify whether AGL15 can directly bind to the promoter of SERK1, chromatin immuno precipitation using AGL15 specific antibodies (kindly provided by Sharyn Perry, University of Kentucky, Department of Plant and Soil Sciences, Lexington, USA) was performed. Five days old seedlings were used and immuno precipitation was performed as described by Wang et al., (Wang et al., 2002). The SERK1 and BRI1 genomic sequences were analyzed for precise canonical CArG motifs as putative MADS box transcriptional regulator targets (Tang and Perry, 2003). Four primer pairs were designed near the identified motifs for measurement of DNA enrichment by PCR. As shown in Figure 5 A, for both SERK1 and BRI1 promoter elements enrichment was observed, suggesting that AGL15 indeed is able to bind directly to these sites *in vivo* and could indeed be a regulator of SERK1 as well as BRI1 expression. The specific binding of *in vitro* produced GST-AGL15 protein to the CArG box sequence is shown on Figure 5 B. All three bands visible are specific since there are no longer

present after incubation with the unlabeled CArG box containing DNA (Figure 5 B and C).

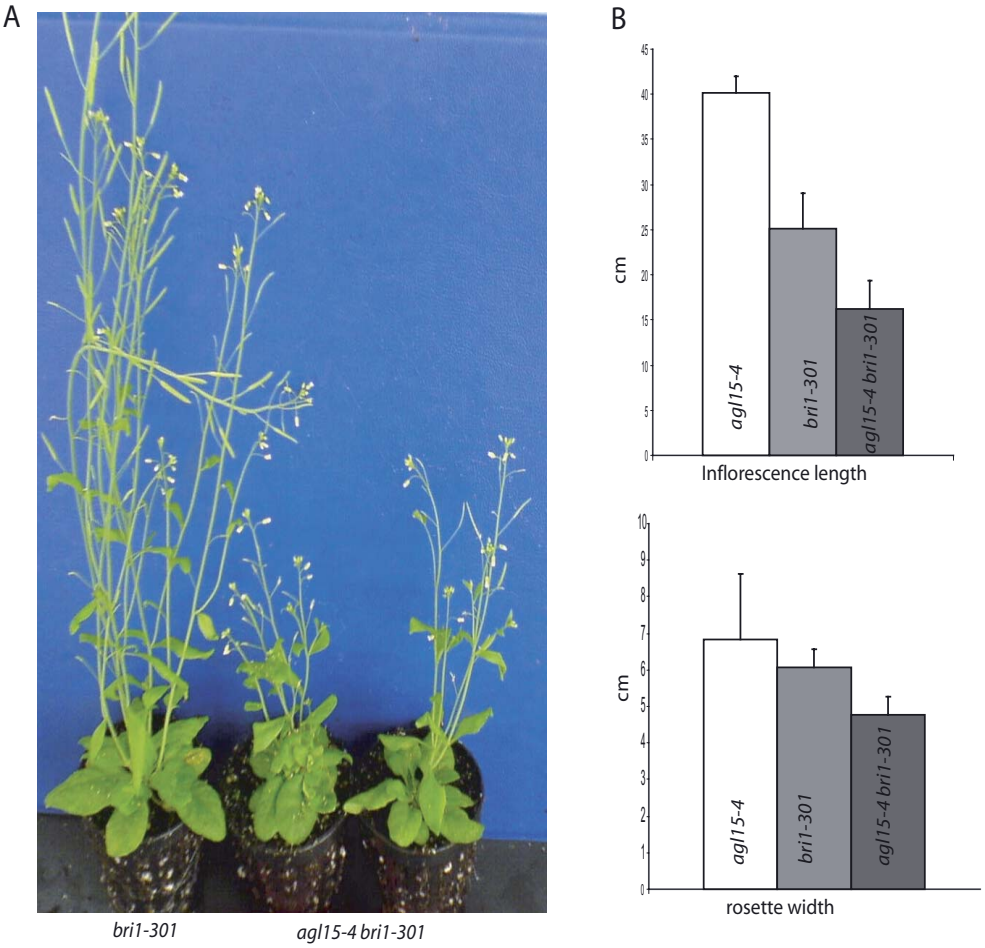
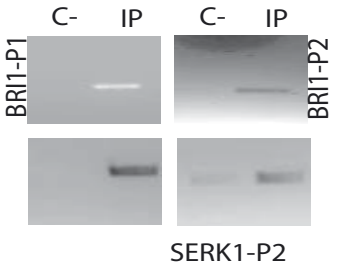


Figure 4. AGL15 function in brassinosteroid signaling. a*gl15-4* mutation enhances the weak *bri1-301* mutation. A, 5 weeks old plants are shown. B, Reduction of the inflorescence length and the rosette width of the double *agl15-4 bri1-301* mutant. At least 10 plants were measured.

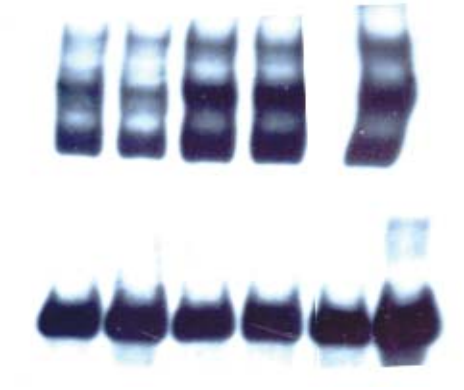
A



B

SERK1-P1

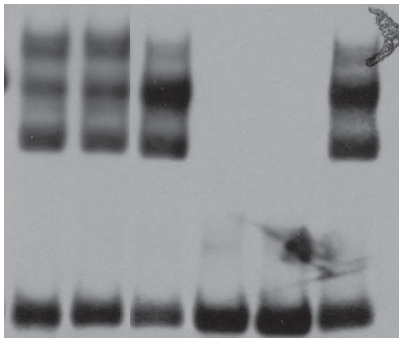
KB+ATP	+	+	-	-	-	-
AGL15	+	+	+	+	+	+
SERK1-KD	-	+	-	-	-	-
14-3-3nu	-	-	-	+	-	-
random DNA	-	-	-	-	+	-
unlabled CArGbox	-	-	-	-	-	+
labled CArGbox	+	+	+	+	+	+



Free Probe

C

14-3-3nu	-	-	+	+	-	-
KB+ATP	+	+	-	-	-	-
AGL15	+	+	+	-	-	+
SERK1-KD	+	-	-	-	+	-
SERK1^{K330E}-KD	-	+	-	-	-	-



Free Probe

Figure 5. A. Chromatin immunoprecipitation. Enrichment of the SERK1 and BRI1 promoter fragments. No enrichment was observed using preimmune serum as a negative control- C-. B and C, Gel retardation experiments with biotinylated DNA probes and with GST-AGL15 and/or GST-14-3-3ν, GST-SERK1 and GST-SERK1^{L330E} proteins *in vitro* purified.

BL induces nuclear localization of AGL15

Recently, it was shown that BR induces rapid nuclear trans-location of BZR1, a transcription factor involved in brassinosteroid signaling (Gampala et al., 2007; Ryu et al., 2007). To clarify whether BR regulates nuclear localization of AGL15, the transcription factor was coupled to either a cyan or yellow fluorescent protein (AGL15- YFP or AGL15-CFP) and analyzed in absence and presence of BR. When AGL15-CFP/YFP was transiently expressed in Col-0 Arabidopsis leaf protoplasts the labeled protein was localized both in the nucleus and in the cytoplasm (Figure 6A). In the *det2* (BR biosynthesis mutant) protoplasts, AGL15-CFP was completely absent from the nucleus in 70% of the transfected cells (Figure 6B). A similar result was observed when Col-0 protoplasts expressing AGL15-CFP were treated with brassinazole (BRZ), an inhibitor of BR biosynthesis (data not shown). To confirm wheteher the nuclear localization of AGL15-CFP is BL dependent, *det2* protoplasts expressing AGL15-CFP, were treated with BL. As shown in Figure 6C, AGL15-CFP clearly appeared in the nucleus after 3h of BL application. In contrast, in untreated cells AGL15-CFP remained in the cytoplasm (Figure 6D).

Interaction between SERK1 and AGL15 occurs at the plasma membrane and requires 14-3-3ν

Molecular interactions between SERK1 and AGL15 was demonstrated by applying Förster Resonance Energy Transfer (FRET) detected by Fluorescence Lifetime Imaging Microscopy (FLIM) in a transient protoplast assay (Russinova et al., 2004). In these experiments SERK1-CFP was used as a donor and the AGL15-YFP as acceptor fluorescent proteins. By comparing the donor fluorescence lifetimes in absence and presence of acceptor will be a read-out for monitoring molecular interactions spatially resolved. In figure 7A I confocal images show that SERK1-YFP and AGL15-CFP are localized at the PM however FRET-FLIM measurements showed no reduction of the donor lifetimes and no FRET was observed (Figure 7B IV). Therefore we asked whether the 14-3-3ν protein (Karlova et al., 2006) could be a mediator between both, perhaps in analogy to what was shown for 14-3-3λ in case of BZR1/BES1 (Gampala et al., 2007). As shown in Figure 7A II , SERK1 co-localizes with 14-3-3ν at the plasma membrane while AGL15-CFP and 14-3-3ν-YFP co-localize at the plasma membrane, in the cytoplasm as well as in the nucleus (Figure 7A III and IV). Subsequently FRET between the three proteins was determined in all 6 possible combinations (only 3 donor-acceptor combinations are shown in Figure 7 B). The mean fluorescence lifetime of the donor molecules in cells expressing AGL15-CFP alone was determined at the PM (Figure 7B I) and in the nucleus (Figure 7B II). The average lifetime of 14-3-3ν-CFP alone was also recorded at

the PM and found to be identical with a value of 2.5 ns (Figure 7B III and Table3). The results presented in Figure 7B IV show that AGL15-CFP and SERK1-YFP do not interact directly at the plasma membrane, since no reduction of the fluorescence lifetime compared to the τ of the donor molecules was observed in any of the 29 cells tested.

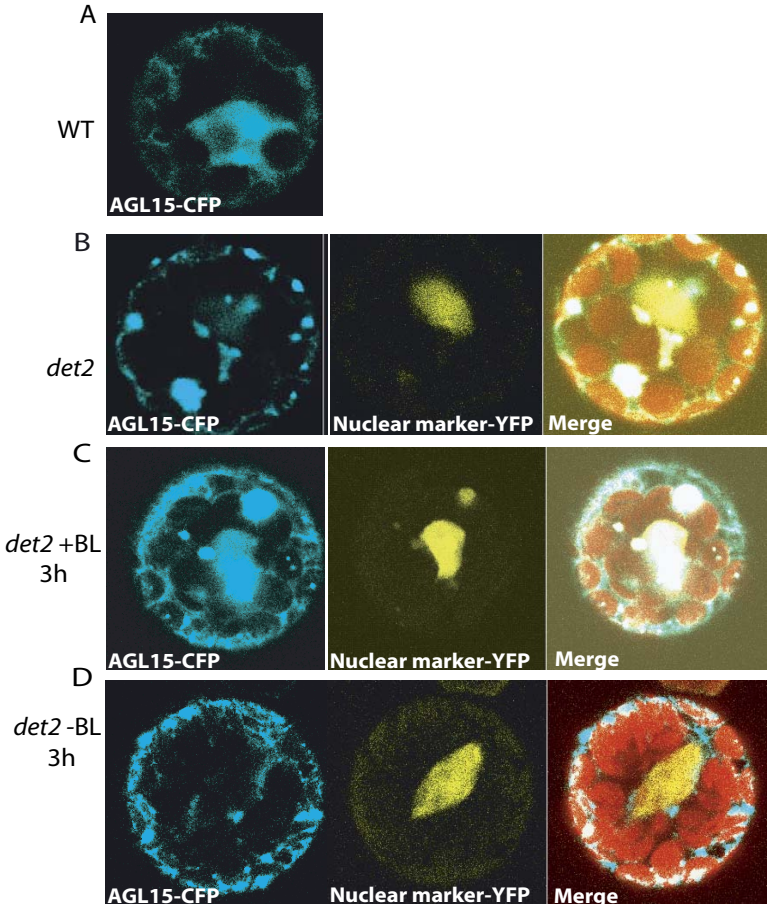


Figure 6. Brassinosteroids are essential for the nuclear localization of AGL15. *Arabidopsis* leaf protoplasts of wild type Col (A) or *det2* mutant plants (B, C and D) were transfected with AGL15-CFP alone (A) or co-transfected with AGL15-CFP and CDC48C-YFP (nuclear marker), (B, C and D). After incubation for 16h the transformed protoplasts were pictured on CLSM (B) or after 18h (D), or treated with 100μ epi-brassinolide for 3h (C).

A similar result was obtained when the CFP and the YFP tags were changed between both

proteins (data not shown). In contrast, AGL15-CFP and 14-3-3υ-YFP did show an interaction at the plasma membrane and exhibited a significant reduction in the average lifetime (τ) from 2.5 to 2.03 ns of the donor AGL15-CFP molecule (Figure 7B VII). This reduction was not equally distributed over the membrane and exhibited a typical punctuate pattern that was also observed previously when comparing the direct interaction between receptor molecules (Russinova et al., 2004) and between the receptors and CDC48 proteins (Aker et al., 2006). A similar phenomenon was observed in the nucleus, where AGL15-CFP and 14-3-3υ-YFP co-localize and interact in a spotted pattern (Figure 7B V and Table 3). Such spotted patterns are routinely observed when determining interaction properties between other members of the MADS box transcription factors family (Tonaco et al., 2006) and are not the result of over-expression of the proteins in the protoplasts. These results show that in the physiological conditions of the protoplasts, a subset of the AGL15-CFP protein is in a complex with 14-3-3υ-YFP. SERK1-YFP interacts with 14-3-3υ-CFP at the plasma membrane (Figure 7B VI), as evidenced by an average lifetime reduction of 2.00 ns for the donor (14-3-3υ-CFP). However, there appear to be less areas of strong interaction when compared to AGL15-CFP/14-3-3 υ-YFP. These data suggests that a subset of the SERK1 receptors reside in a protein complex at the PM with a subset of AGL15 molecules most probably by employing the adaptor or scaffold protein 14-3-3. To confirm that the observed subset of AGL15-CFP proteins interacting with 14-3-3υ-YFP is the same subset of SERK1-YFP interacting with 14-3-3υ-CFP, a co-immunoprecipitation experiment was performed using AGL15 specific antibodies (Wang et al., 2002) to detect AGL15 in transgenic seedlings expressing SERK1-CFP. The precipitated proteins were analyzed by immunobloting procedure using anti-GFP antibodies. A band with the expected mobility of SERK1-CFP was detected (Figure 7C a) which was not present in the negative control, precipitated with rabbit preimmune serum (Figure 7C b). These data confirm the presence of AGL15 in the SERK1 receptor complex *in planta* while previously the presence of 14-3-3 proteins in the SERK1 complex was established (Rienties et al., 2005; Karlova et al., 2006).

Protein	τ PM ns	FRET Eff. %	τ N ns	FRET Eff. %	Number of cells
A-C	2.44 ± 0.03	-	2.5	-	5
A-C/S-Y	2.44 ± 0.04	-	-	-	29
A-C/14-Y	2.03 ± 0.06	17	2.23 ± 0.10	11	30
14-C	2.45 ± 0.02	-	-	-	5
14-C/S-Y	2.00 ± 0.09	18	-	-	11

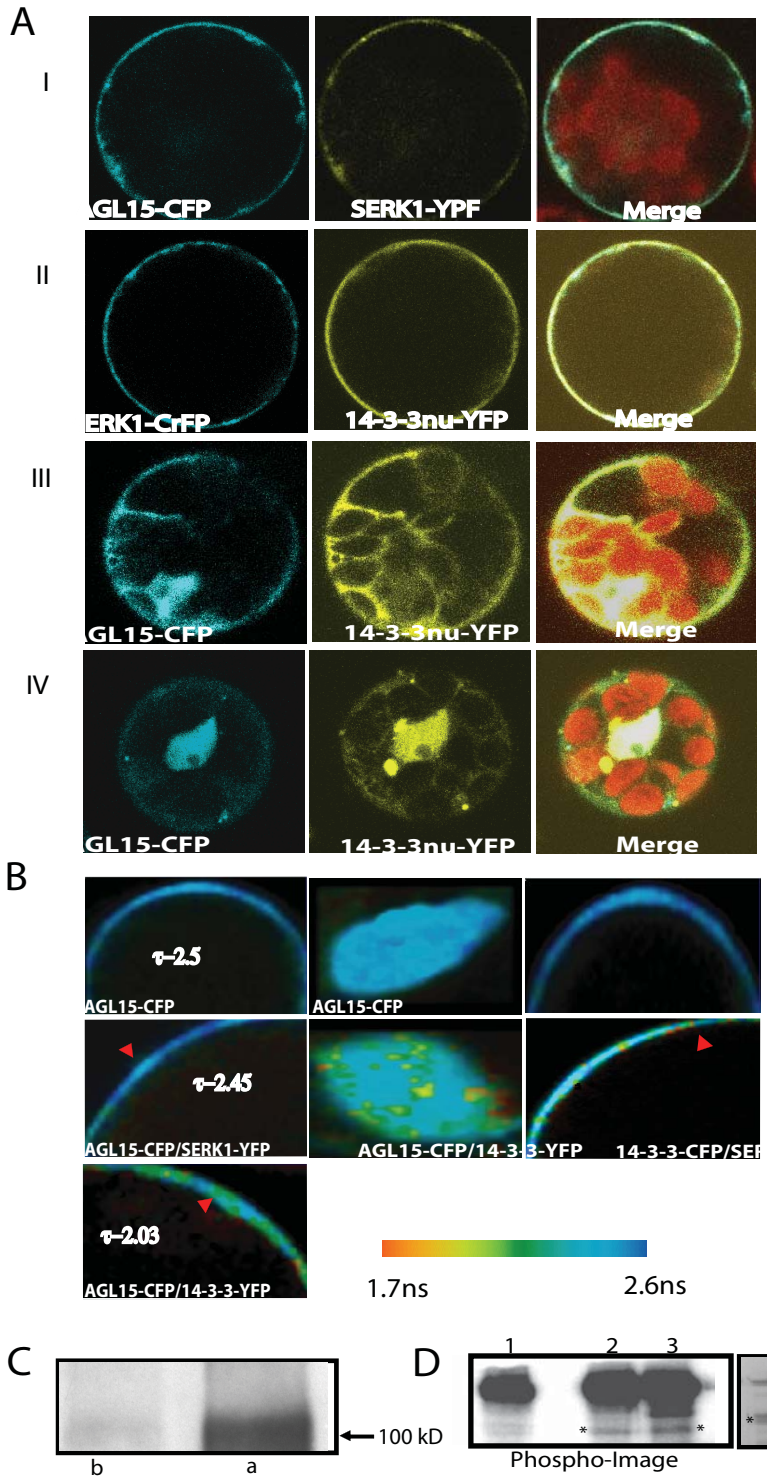

A
I
GL15-CFP
SERK1-YPF
Merge
II
ERK1-CFP
14-3-3nu-YFP
Merge
III
GL15-CFP
14-3-3nu-YFP
Merge
IV
GL15-CFP
14-3-3nu-YFP
Merge
B
τ-2.5
AGL15-CFP
AGL15-CFP
τ-2.45
AGL15-CFP/SERK1-YFP
AGL15-CFP/14-3-3-YFP
14-3-3-CFP/SE
τ-2.03
AGL15-CFP/14-3-3-YFP
1.7ns
2.6ns
C
100 kD
b
a
D
1 2 3
3 2 1
Phospho-Image
Coomassie

Table 3. FRET-FLIM analysis. Lifetime τ is determined as described in "Materials and Methods". PM is plasma membrane, N is nucleus. FRET Eff. Is FRET efficiency. A-C is AGL15-CFP, S-Y is SERK1-YFP, 14-Y is 14-3-3υ-YFP, 14-C is 14-3-3υ-CFP, A-C/S-Y is AGL15-CFP/SERK1-YFP, A-C/14-Y is AGL15-CFP/14-3-3υ-YFP, 14-C/S-Y is 14-3-3υ-CFP/SERK1-YFP.

Figure 7. A, I. Co-localization of SERK1-YFP with AGL15-CFP in transfected protoplast on the plasma membrane. II. Co-localization of SERK1-CFP with 14-3-3υ-YFP on the plasma membrane. III. Co-localization of AGL15-CFP and 14-3-3υ-YFP on the plasma membrane. IV. Co-localization of AGL15-CFP and 14-3-3υ-YFP in the nucleus. B. FRET between SERK1, AGL15 and 14-3-3υ imaged by FLIM on Arabidopsis protoplast transiently expressing AGL15-CFP (I and II) or 14-3-3υ-CFP (III) alone, AGL15-CFP/SERK1-YFP (IV), AGL15-CFP/14-3-3υ-YFP (V), 14-3-3-CFP/SERK1-YFP (VI) or AGL15-CFP/14-3-3υ-YFP (VII) for 16h. Mean fluorescence lifetime values (τ) and lifetime distribution for the images I to VII are presented as pseudo color images. The arrow heads point to an area with a short lifetime, indicative of FRET. Note the color bar, where dark blue is used to show a τ of 2.5 ns (no interactions) and light green or yellow is used to show a τ of 2 ns (interactions). C, protein extract from SERK1-CFP transgenic seedlings was precipitated with anti-AGL15 antibodies (a). As a negative control a protein extract was precipitated with preimune serum (b). D. In vitro trans-phosphorylation of AGL15 and 14-3-3υ by SERK1 kinase domain protein. Left, Phospho image of AGL15 (lane 2) and 14-3-3υ (lane 3) trans-phosphorylated by SERK1. Lane 1 shows autophosphorylation activity of SERK1 alone. Right panel, Equal loading and size control of GST-SERK1-KD (lane 1), GST-SERK1-KD together with GST-AGL15 (lane 2), GST-SERK1-KD and GST-14-3-3υ (lane 3).

AGL15 and 14-3-3υ are substrates of the SERK1 kinase domain

Having established that AGL15 and 14-3-3υ are part of the PM-located SERK1 receptor complex we next aimed to determine whether the SERK1 kinase domain (KD) (Shah et al., 2001) can trans-phosphorylate AGL15. The SERK1-KD, AGL15 and, the 14-3-3υ proteins were expressed in *E. coli* as N-terminal GST fusion proteins and purified on glutathione resins. Phosphorylation assays were performed by incubating the AGL15 or 14-3-3υ with SERK1-KD in presence of radioactive γP^{32} –ATP. As shown on Figure 7D, lane 1, SERK1 is heavily autophosphorylated and can trans-phosphorylate both AGL15 and 14-3-3υ proteins (Figure 7D, lane 2 and 3). In case of the 14-3-3υ protein these results confirmed previous work using the 14-3-3λ protein as a target of SERK1 (Rienties et al., 2005). To identify the phosphorylated sites, liquid chromatography (LC)-tandem mass spectrometry (MS/MS) was employed. The 50 kD GST-AGL15 and GST-14-3-3υ proteins were in gel digested with trypsin, and the peptides were measured using an LCQ. More than 75% sequence recovery of the proteins was achieved and phosphorylated residues in AGL15 or 14-3-3υ proteins were only identified after incubation with active SERK1 protein. For 14-3-3υ two threonines (T-34 and T-211) were found to be phosphorylated and for AGL15, three sites were found to be phosphorylated by SERK1 in a part of the protein that is rich on serines and threonines. As a result, we could not

precisely determine the trans-phosphorylated (S-58, S-59 or T-60, S-173 or T175, and T-209 or T-210) residues. 14-3-3 proteins are known to bind to specific phosphorylated sequences. A potential although not perfect 14-3-3 binding site sequence was identified in AGL15 at amino acids 168-175 (RSFLPSF), where S-173 was found to be phosphorylated by SERK1, corresponding to S-173 in the 14-3-3 binding site of BZR1 transphosphorylated by BIN2 (Ryu et al., 2007). Having determined that AGL15 can be trans-phosphorylated by SERK1, we examined now if the phosphorylation is affecting the binding of the transcription factor to the CArG box DNA sequence. As shown on Figure 5 B and C performing the binding reaction in presence of kinase buffer (KB) and ATP already inhibit the reaction. Addition of 14-3-3 does not change the binding capacity of AGL15 as well as SERK1-mediated phosphorylation, since the binding of AGL15 is the same in presence of an active SERK1 kinase or of the mutated $SERK1^{K330E}$ kinase, which has no auto and transphosphorylation activities (Shah et al., 2001) (Figure 5 C). Apparently AGL15 phosphorylation does not affect its transcriptional activity, in contrast to BIN2-mediated phosphorylation of BES1, which resulted in a complete loss of its DNA-binding activity (Vert and Chory, 2006).

Discussion

In this study we aimed to investigate the role of SERK1, AGL15 and BRI1 in the somatic embryo formation from SAM. SERK1 and AGL15 previously were shown to play a role in this signaling pathway (Hecht et al., 2001; Harding et al., 2003; Thakare et al., 2008). Our data shows that loss of function of SERK1 alone or in combination with loss-of function of AGL15 significantly decreases somatic embryogenic competence in culture. Furthermore to extend our previous observation that SERK1, AGL15 and BRI1 are found to be present in one protein complex (Karlova et al., 2006) we examined the role of BRI1 in the acquisition of embryonic competence. Both BRs and BRI1 were found to be essential for this process. The loss of function allele of AGL15 was found to enhance aspects of defective brassinosteroid signaling in combination with a weak *serk1-1* or *bri1-301* alleles, suggesting that it is a positive regulator in both somatic embryogenesis and BR signaling. The nuclear localization of AGL15 was BR dependent and using FRET by FLIM we proposed that AGL15 interacts via 14-3-3ν with SERK1 at the plasma membrane.

Somatic embryogenesis and BR signaling mediated trough BRI1, SERK1 and AGL15

Plant stem cells, contained in specialized structures called meristems, have amazing regenerative powers. They enable plants to grow and produce new organs throughout lifetimes, that can span hundreds of years (Weigel and Jurgens, 2002). Plant cells can undergo an alternative form of embryo formation called somatic embryogenesis, a process that does not require formation of a zygote by fertilization (Mordhorst et al., 1998). Thus in contrast with the mammals a single differentiated plant cell in culture can be induced to shift their fate towards stem cell fate by application of exogenous auxins. Understanding how the process of differentiation can be reversed in plant cells could contribute to stem-cell biology in other

systems as well. In *Arabidopsis* SERK1 ectopic expression increases somatic embryo formation in culture (Hecht et al., 2001) and in several plant species, homologs of SERK gene were discovered, where the gene was also found to be a marker for embryogenic potential like sunflower (Thomas et al., 2004) and rice (Hu et al., 2005). Another gene that was shown to increase SE formation, when over-expressed is the MADS box transcription factor, AGL15 (Harding et al., 2003). Recently AGL15 was shown to act redundantly with its homolog, AGL18 in two processes: somatic embryogenesis (Thakare et al., 2008) and in the control of flowering time (Adamczyk et al., 2007). Interestingly BRI1 and BRs were found to predominantly functioned as a flowering-time enhancer (Domagalska et al., 2007). AGL15 together with BRI1 and SERK3 have been identified as a components of SERK1 protein complex (Karlova et al., 2006). Relevant to this finding in this study we could confirm a synergistic interaction between both AGL15 and SERK1 genes. Although SERK1 (Karlova et al., 2006; Albrecht et al., 2008) was shown to be in a complex with SERK3 and both proteins can participate in brassinosteroid signaling by being co-receptors of BRI1 (Li et al., 2002; Nam and Li, 2002; Karlova et al., 2006), only SERK1 and none of the other members of the SERK family was found to have a role in somatic embryogenesis. Somatic embryogenesis can be induced in addition of over-expression of specific genes by use of specific hormone treatments. Both SERK1 and AGL15 are expressed in response to auxin treatment (Hecht et al., 2001; Zhu and Perry, 2005; Kwaaitaal and de Vries, 2007). In *Arabidopsis*, AGL15 was shown to act via control of gibberillin (GA) metabolism. Chromatin immunoprecipitation (ChIP) analysis showed that a GA2-oxidase (AtGA2ox6) is a downstream target of AGL15. GA2-oxidase converts biologically active GA to inactive forms. A decrease in biologically active GA caused an increased in SE, whereas addition of active GA led to a decrease in SE production (Wang et al., 2004). Using the same ChIP method we found that AGL15 can bind specifically to the promoter elements of SERK1 and BRI1, and probably as shown before for the embryogenic callus (Harding et al., 2003) to positively regulate their expression. Since BRI1 was found in the SERK1 protein complex and BRI1 can bind directly BRs (Kinoshita et al., 2005) we first investigated the role of brassinosteroids in the process of somatic embryogenesis. BRs were shown to regulate cellular expansion, differentiation and proliferation and are involved in diverse processes (Karlova and de Vries, 2006), but were never shown to play a role in somatic embryogenesis. Our data showed that BRs can enhance the somatic embryo competence in liquid culture in presence of auxin, which points to a synergetic effect of the two hormones in agreement to the cross talks observed before (Nakamura et al., 2003). Both BRs and auxin signaling pathways were shown to activate the transcription of IAA biosynthesis and auxin-responsive genes (Nakamura et al., 2003). Moreover, BRs were shown to stimulate polar auxin transport and to modify the distribution of endogenous auxin (Li et al., 2005). Interestingly our data suggests that BRs can enhance as well as inhibit the somatic embryogenesis and more specifically the proliferation, depending on the time of the application. When BRs are applied from the begging of the liquid culture no proliferation and formation of somatic embryos was observed (Figure 2) and even more the

wild type seedlings in these cultures were mimicking the phenotype of the strong homozygous *bri1-201* seedlings, when grown in liquid. Moreover the BR biosynthetic mutant *det2* (Chory et al., 1991) showed strong reduction in the embryogenic competence, which could be rescued by exogenous application of BL. BRI1 is the receptor perceiving and transducing the BR signaling (Li and Chory, 1997; Kinoshita et al., 2005). In this study we determined a new role of BRI1 receptor in acquisition of embryogenic competence in somatic cells. BRI1 over-expression in analogy to SERK1 and AGL15 and addition of BRs can enhance, while *bri1* strong mutant alleles showed very limited embryogenic competence. An exception of these findings was the weak *bri1-301* mutant, which did not show any reduction, but instead an increased embryogenic competence. In Arabidipsis the property to form embryogenic cells in tissue culture is restricted to the vascular (pro-cambium) stem cell or transit amplifying (TA) cell and is not shared with the stem cells in the apical meristem, because mutants lacking a functional SAM like *shoot meristemless*, *wuschel* and *zwille/pinhead* did not show a reduction in embryogenic competence (Mordhorst et al., 2002). Therefore we analyzed further the inflorescence stem vascular organization of *bri1-301* mutant and found out that *bri1-301* mutant had increased number of procambial cell layers, which might explain the increased embryogenic competence. Unlike *det2*, which embryogenic competence was fully rescued by exogenous application of BL, strong *bri1* mutants failed to respond, in line with their insensitivity to the BRs on hypocotyls, root and plant level (Clouse et al., 1996; Fujioka et al., 1997; Li and Chory, 1997). In this study we could establish also a direct link between the three proteins identified in the SERK1 receptor complex, which participate in the process of somatic embryogenesis. Previously SERK1 was identified to participate in BR signaling as a co-receptor of the main receptor, BRI1 (Karlova et al., 2006). The SERK1 mutant allele *serk1-1* was found to enhance the phenotype of the weak BRI1 allele *bri1-119*. Moreover recent data of Albrecht et al., (Albrecht et al., 2008) showed that from the SERK family only *serk1* but not *serk2*, *serk4* or *serk5* mutant alleles enhance the BR insensitivity of *serk3-1* mutant roots and hypocotyls. It was shown that the interaction between SERK3 and BRI is enhanced upon BR stimulation (Wang et al., 2005b). Although our data suggest that the interaction of BRI1 and SERK1 is not dependent on the kinase activity of BRI1, the phosphorylation status of SERK1 is also brassinosteroid-dependent (Karlova et al., 2008 in press). Auto- or trans phosphorylation of the C- terminus of BRI1 was shown to be instrumental in enhancing the kinase activity of BRI1, as well as promoting its affinity for the co-receptor BAK1 (Wang et al., 2005b; Wang et al., 2005a). After hetero-oligomerization, BAK1 accelerated internalization by endocytosis of BRI1 (Russinova et al., 2004). SERK1, is also able to interact with BRI1 and appears to modulate BRI1 signaling in a manner similar to that observed for BAK1 (Karlova et al., 2006; Albrecht et al., 2008). Although the precise stoichiometry is not yet known, the emerging picture suggests that SERK and BRI receptors exist as homodimers in the absence of ligands and form ligand-induced tetrameric complexes, analogous to the receptor complexes of mammalian transforming growth factor–β (TGF-β) receptors. At the cell surface, the ligand binds a complex of transmembrane receptor serine/threonine kinases (types I and II) and

induces trans phosphorylation in the type I receptor by the type II receptor kinases. The consequently activated type I receptor phosphorylate selected Smads at C-terminal serines. Activated Smad (R-Smad) complexes translocate into the nucleus, where they regulate transcription of target genes. R-Smads shuttle between nucleus and cytoplasm (Derynck and Zhang, 2003). In analogy to this model BRI1 in *Arabidopsis* could be the mechanistic counterpart to the type II receptor, and SERK3 or SERK1 have a role similar to the type I receptor. Then upon activation by BRI1 (type II), SERK1 is activated and phosphorylate AGL15 (R-Smad) at the C-terminus as shown by our trans-phosphorylation data. This activation of AGL15 leads to its translocation from the cytosol to the nucleus, which is dependent in analogy to the Smad protein on the presence of the ligand, in our case BRs. In agreement with our data, AGL15 is a positive regulator of somatic embryogenesis as well as BRs signaling. The AGL15 mutant allele *agl15-4* was found to enhance the phenotype of the weak BRI1 allele *bri1-301* as well as *serk1-1.* Moreover we show that the nuclear translocation of AGL15 is dependent on the presence of BRs and that AGL15 interacts with SERK1 at the plasma membrane via the 14-3-3ν protein.

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Chapter 6

Summarizing discussion

Rumyana Karlova and Sacco de Vries

Most biological processes are governed by multiprotein complexes rather than individual proteins. Identification of protein complexes therefore is becoming increasingly important to gain a molecular understanding of cells and organisms. Receptors for example represent an abundant class of integral membrane proteins that transmit information on various types of signals within the cell. Receptor signaling and regulation can be characterized as a dynamic process that is dependent on direct as well as indirect coupling between receptors and their interacting partners (Kabbani, 2008). Besides that the receptors are distributed throughout the cell at different subcellular localization at different time points. This translocation also requires different interacting partners and assembly of different complexes. Therefore the capture of receptor complexes in their full integrity and in sufficient purity and quantity remains a primary challenge. Several methods have been developed to identify receptor complexes in cells. The most common method used is immunoprecipitation, which makes use of specific antibodies to isolate target proteins out of complex sample mixtures. The immunoprecipitation is often followed by 1- or 2-D electrophoresis for separating the isolated protein complexes followed again by, nano HPLC mass spectrometric analysis to identify proteins. In this thesis an approach was establish for isolating complexes of transmembrane plant receptors (chapter 2 and 3) based on single step purification using GFP as a bait protein fused to the protein of interest in our case SERK1, SERK3 and BRI1 receptors. Quite often tandem affinity purification (TAP) is used for protein complex isolation. The first TAP tag used consisted of three components: a calmodulin-binding peptide, a TEV protease cleavage site, and Protein A as a Ig-binding domain. TAP of protein complexes was first demonstrated in yeast (Rigaut et al., 1999). Because proteins are present in a high dynamic range, varying from only 10-100 copies to more then 10^7 copies per cell (Van Leene et al., 2008), and because they cannot be amplified like polinucleotides by PCR, the success rate of TAP and single tags purification depends on the amount of protein complexes purified and the sensitivity of the MS as well as the amount of the background proteins binding. In general, using tandem affinity purification will reduce the amount of the background proteins but also the less abundant and/or stable interacting partners will be lost. From the other hand the single tag purification (for example GFP used in this thesis) is a quicker method (which will benefit the purification of less stable interactors) but also more “dirty” then the TAP method and will result in more background proteins which subsequently will reduce the number of the identified proteins. Actually for unraveling the entire composition of receptor protein complexes the combination of methods is required as well as combination of different mass spectrometers which use different ionization methods. In any case the identified interacting partners then should be validated by other methods like western blotting, co-localization of the receptor and the putative binding protein within the cells, FLIM etc. One of the best studied plant receptors is BRI1. BRI1 can bind directly to its ligand, brassinosteroids and regulate cellular expansion, differentiation and proliferation (Li and Chory, 1997; Clouse and Sasse, 1998; Altmann, 1999). On the basis of genetic screens, a second LRR-RLK, a member of the SERK family, BAK1 (SERK3) was identified as interacting partner of BRI1. Another receptor from

the SERK family, SERK1 was found to function redundantly with SERK2 in male sterility (Albrecht et al., 2005). Because of protein redundancy and high sequence homology in the SERK family we developed an alternative to the genetic screens approach to determine the functions of a protein by identifying its interacting partners as described in chapter 2 and 3. The Immunoprecipitation of the membrane receptors with specific antibodies was followed by mass spectrometry and identification of the protein present in the receptor complex. This method identified not only specific partners for SERK1, which help to unravel the function of this receptor for example in the brassinosteroid signaling (chapter 2), but also for SERK3 and BRI1 (chapter 3). As described in chapter 2, two other receptors were identified to be present in SERK1 protein complex, BRI1 and SERK3 and further genetic analysis showed that indeed the SERK1 mutant allele *serk1-1* could enhance the phenotype of the weak BRI1 allele *bri1-119*. Furthermore, using Blue Native Gel Electrophoresis we showed that SERK1 and SERK3 are part of BRI1 containing multiple protein complexes with a relative mass between 300 and 500 kDa and in contrast to the co-receptors, only a small percentage of the BRI1 protein is present in high order complex(es) and most of it is present as a homo or heterodimer (chapter 2). In agreement with these data recent studies of Albrecht et al., (Albrecht et al., 2008), showed that from the five SERK family members indeed only SERK1 and SERK3 are involved in the BRs signaling pathway. SERK3 was also shown to be differentially phosphorylated upon treatment with BRs, which also enhanced its affinity to interact with BRI1 (Wang et al., 2005b). Similar to these data in chapter 4 we showed that BRs could enhance the phosphorylation status also of SERK1 and that the kinase activity of BRI1 is not necessary for their dimerization. Other interacting partners were identified to be present in SERK1 as well as in SERK3 receptor complexes like CDC48A and 14-3-3s proteins. Interestingly we identified the BAG1 (Bcl-2-associated athanogene) protein in SERK3 receptor complex. BAG proteins are distinguished by a common conserved region located near the C terminus termed the BAG domain (BD) involved in interaction with Hsp70 proteins. Some of them, like AtBAG1, contain a ubiquitin-like domain at the N terminus (Doukhanina et al., 2006). The plant BAG proteins were also shown to have a function in cell protection under stress and inhibition of plant programmed cell death that shares features associated with apoptosis. This observation is in line with the postulated function of SERK3 to play a role in negative regulation of a cell death pathway independent of its role in brassinosteroid signaling (He et al., 2007). Further analyses are needed in order to understand how this signaling pathway is regulated. It was shown that after hetero-oligomerization, SERK3 accelerated internalization by endocytosis of BRI1 (Russinova et al., 2004), although the localization and turnover of BRI1 are independent of ligand (Russinova et al., 2004; Geldner et al., 2007). In line with the observed presence of the two receptors in endosomal compartments we identified members of the small Ras related GTPases in both SERK3 and BRI1 complexes, which could indicate that there are similar mechanisms in the vesicle trafficking for both BRI1 and SERK3 receptors (chapter 3).

Activation of many kinases is a key regulator in their signaling. For example auto-or transphosphorylation of the C-terminus of BRI1 was shown to be important for enhancing

the kinase activity of BRI1, as well as promoting its affinity for the co-receptor BAK1 (Wang et al., 2005b; Wang et al., 2005a). Only for a few plant receptors, including BRI1 and BAK1, the autophosphorylation sites have been identified (Oh et al., 2000; Wang et al., 2005b). After BR treatment the BRI1 receptor autorphosphorylates at least 6 residues that were identified *in vivo* and corresponded to the same residues as previously identified from the *in vitro* produced BRI1 protein. To test if the in vivo phosphorylation level of SERK1 was also regulated by exogenously applied brassinosteroids (BR) we identified the *in vivo* phosphorylation sites in SERK1 receptor. Interestingly and in contrast with BRI1 most of the sites were already phosphorylated in SERK1 *in vivo* and only one site in the C-terminal tail was observed to be differentially phosphorylated after BRs stimulation (chapter 4). Since members of the SERK family were found to participate in different signaling pathways we compared in chapter 4 the kinase activity and identified the auto-phosphorylation sites in all 5 members in order to investigate if there is a link between the different kinase activity and functions. Surprisingly although SERK1 and SERK2 were found to have redundant function, SERK1 was found to be more active than SERK2 with 22 compared to 7 sites phosphorylated *in vitro* respectively. For SERK3, 8 phosphorylated sites were identified, which were similar to the one identified in SERK1 and SERK2, especially the sites in the activation loop, which point to the similar mechanism of activation of all the members of the family. Low autophosphorylation activity was observed for SERK4 and SERK5, where protein stability problems were observed for SERK4. Using mutagenesis in the SERK1 kinase domain, residue Ser-562 was found to control the full autophosphorylation activity. As shown before, SERK1 (chapter 2) and BAK1 (Wang et al., 2005b) co-immunoprecipitate in vivo with BRI1 and BL treatment can increase the amount of BAK1-GFP associated with BRI1-Flag. Our data from chapter 4 support this model and indicate that SERK1 and BRI1 can both trans-phosphorylate each other. Also the SERK1 Thr-462 residue, that was found to be trans-phosphorylated by BRI1-KD in chapter 4, was shown to play an important role in auto- and trans-phosphorylation activity before (Shah et al., 2001). The BRI1 activation model involves inhibition of the kinase activity by its C-terminal tail, which is relieved upon ligand binding to the extracellular domain. After activation, BRI1 can trans-phosphorylate the SERK1 kinase domain on Thr-462, that subsequently leads to activation and increased kinase activity, including phosphorylation of the C-terminally located Ser-562 as found in chapter4. Our data therefore suggest a complex pattern of both intra- and intermolecular control of SERK1 kinase activity and point to a tetrameric active complex formation of SERKs with BRI1 in analogy to the TGF-β family in animals. SERK1 was also shown to increases somatic embryogenesis in culture, when overexpressed (Hecht et al., 2001), suggesting that SERK1 mediated signaling is required for acquisition of embryogenic competence in somatic cells. Plant cells can undergo an alternative form of embryo formation called somatic embryogenesis upon appropriate stimulation with plant growth regulators such as auxin and cytokinin. This reveals the underlying totipotent character of a plant cell, but the question of how fully differentiated plant cells become stem cells again remains. In chapter 5 we aimed to answer this question by investigating the

signaling pathway required for acquisition of the embryogenic competence in somatic cells. These cells in Arabidopsis are derived from procambium cells in the vascular tissue and were shown to express the SERK1 gene (Kwaaitaal and de Vries, 2007)**.** Another protein, which overexpression result in enhanced somatic embryogenesis is AGAMOUS-like 15 (AGL15) (Harding et al., 2003). A loss-of-function mutants of AGL15 exhibits decreased ability to form somatic embryos (Thakare et al., 2008). We identified AGL15 as a component of the SERK1 protein complex (chapter 2), suggesting a direct link in the form of a very short signal transduction chain between both. In chapter 5 the functional involvements in the somatic embryogenesis and BR signaling of the proteins found to be present in the SERK1 receptor complex was investigated. The loss-of-function alleles s*erk1* alone or in combination with *agl15-4* significantly impair somatic embryogenesis. Furthermore AGL15 mutant allele *agl15-4* also aggravated general phenotypes of a weak *bri1-301* and *serk1-1* mutant alleles, suggesting that it acts as a positive regulator in BR signaling. Moreover in this study (chapter 5) for the first time we provided evidence that the main BRI1-mediated BR signaling pathway is required for the acquisition of embryogenic competence. Both exogenous application of BRs and overexpression of BRI1 enhances somatic embryogenesis while *bri1* strong mutant alleles and the BR biosynthesis mutant *det2* show strongly reduced embryogenic competence. In *det2*, but not in the perception mutant *bri1-201*, somatic embryogenesis can be rescued by exogenous BRs application. The physical interaction of all the proteins found in the SERK1 receptor complex was investigated by using fluorescent lifetime imaging microscopy to determine FRET between fluorescently tagged proteins. Using this technique we fond that AGL15 interacts via 14-3-3nu at the plasma membrane with SERK1 (chapter 5). 14-3-3 proteins are scaffold proteins that are highly conserved in all eukaryotes. They participate in various signal transduction and regulatory processes by interacting with diverse target proteins in a sequence-specific and phosphorylation-dependent manner (Ferl, 1996; Fulgosi et al., 2002). Recently it was show that 14-3-3s participate also in BR signaling by interacting with the BZR1 protein that has been phosphorylated by BIN2 (Gampala et al., 2007; Ryu et al., 2007). Our data from the complex isolation of the BRI1 receptor showed that 14-3-3 is a part of this complex *in vivo* (chapter 3). Brassinosteroids were also shown to regulate the nuclear localization of BZR1 and BZR2 proteins. In chapter 5 we observed similar effect of BRs on AGL15 nuclear translocation. Moreover using chromatin immunoprecipitation AGL15 was found to specifically bind to the promoter elements of SERK1 and BRI1, thus forming a novel positive feedback control loop in their signaling.

The research described in this thesis provides a new powerful technique for transmembrane protein complex isolation as well as advance in our understanding of brassinosteroid signaling and stem cell fate in *Arabidopsis.*

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Summary

Receptors represent an abundant class of integral membrane proteins that transmit information on various types of signals within the cell. Since receptor signaling and regulation can be characterized as a dynamic process that is dependent on direct as well as indirect coupling between receptors and their interacting partners immunoprecipitation based on single GFP tag purification technique was employed in chapter 2 to identified interacting partners of plant trans-membrane receptor SERK1. SERK1 is a member of small family of 5 receptors. Two other receptors were identified to be present in SERK1 protein complex, BRI1 and SERK3 and further genetic analysis showed that indeed the SERK1 mutant allele *serk1-1* could enhance the phenotype of the weak BRI1 allele *bri1-119*. Other interacting partners were identified to be present in SERK1 as well as in SERK3 receptor complexes (chapter 3) like CDC48A and 14-3-3s proteins and a MADS-box transcription factor AGL15 in case only of SERK1 receptor. Furthermore, using Blue Native Gel Electrophoresis we showed that SERK1 and SERK3 are part of BRI1 containing multiple protein complexes with a relative mass between 300 and 500 kDa and in contrast to the co-receptors, only a small percentage of the BRI1 protein is present in high order complex(es) and most of it is present as a homo or heterodimer (chapter 2). These data help to unravel the function of SERK1 receptor in the brassinosteroid signaling. Brassinosteroids are plant steroid hormones, which regulates cellular expansion, differentiation, and proliferation. BRI1 is the plant receptor that can bind directly to brassinosteroids. In chapter 3 using the same immunoprecipitation technique the protein complexes of BRI1 and its co-receptor SERK3 were investigated. Interestingly we identified the BAG1 (Bcl-2-associated athanogene) protein in SERK3 receptor complex. The plant BAG proteins were also shown to have a function in cell protection under stress and inhibition of plant programmed cell death that shares features associated with apoptosis. This observation is in line with the postulated function of SERK3 to play a role in negative regulation of a cell death pathway independent of its role in brassinosteroid signaling (He et al., 2007). BRI1 as well as its co-receptor SERK1 and SERK3 are found to be present in endosomal compartments and we identified members of the small Ras related GTPases in both SERK3 and BRI1 complexes, which could indicate that there are similar mechanisms in the vesicle trafficking for both BRI1 and SERK3 receptors (chapter 3). The SERK receptors as well as BRI1 have functional kinase domains which activities were compared in chapter 4. Furthermore the phosphorylated residues were identified by LC-MS/MS. The in vivo phosphorylation level of SERK1 was regulated by exogenously applied brassinosteroids (BR), like it was shown before for SERK3 and the *in vivo* phosphorylation sites in SERK1 receptor were identified. Different members of the SERK family were found to have different autophosphorylation activity and SERK1 and BRI1 were found to transphosphorylate each other as well as to interact independent of the kinase activity of BRI1. In chapter 5 the role of the SERK1 interacting partners AGL15, 14-3-3ν and BRI1 in the somatic embryogenesis

was investigated. In this chapter we provided evidence that the main BRI1-mediated BR signaling pathway is required for the acquisition of embryogenic competence. Both exogenous application of BRs and overexpression of BRI1 enhance somatic embryogenesis while *bri1* strong mutant alleles and the BR biosynthesis mutant *det2* show strongly reduced embryogenic competence. AGL15 is also involved in this process and was found to be a positive regulator of both somatic embryogenesis and brassinosteroid signaling. Upon BL-dependent nuclear translocation, mediated by the adaptor protein 14-3-3ν, the AGL15 protein is able to bind to the promoter elements of the BRI1 and SERK1 genes and probably is a positive regulator.

Samenvatting

Een belangrijke klasse integrale membraaneiwitten zijn de receptoren. Deze receptoren verzorgen signaal overdracht van verschillende signaal typen in de cel. Zowel de regulatie van deze receptor aktiviteit als de afgegeven signaal intensiteit kan worden gezien als een dynamisch proces dat afhankelijk is van zowel direkte als indirekte koppeling tussen receptoren en hun interactoren. Immunoprecipitatie met behulp van een enkelvoudige GFP additie was gebruikt in hoofstuk 2, om interacterende partners van de planten trans membraan receptor SERK1 te identificeren. SERK1 behoort tot een kleine klasse met 5 receptoren. Twee andere receptoren (BRI1 en SERK3) zijn geidentificeerd in een SERK1 eiwitcomplex. Verdere genetische analyse toonde aan dat de SERK1 mutant versie serk1-1 het fenotype van het zwakke BRI1 versie bri1-119 kon versterken. Andere interakterende partners zoals CDC48A en 14-3-3-s eiwitten zijn aangetoond in zowel SERK1 als SERK3 receptor complexen (hoofdstuk 3). In het geval van de SERK1 receptor is ook nog een MADS box transcritie faktor aangetoond. Met behulp van natieve gel electroforese (Blue Native Gel Electroforese) toonden wij aan dat SERK1 en SERK3 onderdeel zijn van een meervoudig BRI1 eiwitcomplex met een massa tussen 300 en 500 kDalton. In tegenstelling tot de co-receptoren, is alleen een klein percentage BRI1 aanwezig in hogere orde complexen, terwijl het grootste deel voorkomt als homo- of heterodimeer (hoofdstuk 2). Deze data helpen om de functie van de SERK1 receptor in de Brassinosteroide signalering te ontrafelen. Brassinosteroiden zijn planten steroid hormonen die de celgroei, diferentiatie en ontwikkeling reguleren. BRI1 is de planten receptor die direct brassinosteroiden kan binden. In hoofdstuk 3 werd dezelfde immunoprecipitatie techniek gebruikt om de eiwit komplexen van BRI1 en zijn co-receptor SERK3 te onderzoeken. We identificeerden BAG1 (Bcl-2-associated athanogene) eiwit in SERK3 receptor complexen. Voor de planten BAG eiwitten is aangetoond dat ze een beschermende funktie hebben onder stres condities en dat ze de geprogrammeerde planten cel dood (welke veel overeenkomsten vertoond met apopotose) remmen. Deze observering past in de voorgestelde functie van SERK3, namelijk dat SERK3 een rol speelt bij de (negatieve) regulering van een cel dood proces onafhankelijk van zijn rol in brassinosteroid signalering (He en anderen, 2007). Zowel BRI1 als zijn co-receptoren SERK1 en SERK3 zijn aanwezig in endosomale compartimenten en voor zowel SERK3 als voor BRI1 komplexen hebben we onderdelen van kleine Ras gerelateerde GTPases geïdentificeert. Dit zou er op kunnen duiden dat transport van zowel BRI1 als SERK receptoren via vergelijkbare mechanismen plaats vinden (hoofdstuk 3). SERK receptoren en BRI1 hebben functionele kinase domeinen en de kinase aktiviteiten hiervan zijn vergeleken in hoofdstuk 4. De gefosforyleerde aminozuren zijn geïdentificeert met behulp van LC-MSMS. Het nivo van de in vivo fosforylering van SERK1 was gereguleerd door exogeen aangeboden brassinosteroiden (BR), zoals eerder gevonden voor SERK3. De in vivo gefosforyleerde aminozuren van SERK1 receptor werden geïdentificeert

door middel van LC-MSMS. Voor verschillende kinases behorende tot de SERK familie is aangetoond dat ze een verschillende autofosforylerings aktiviteit bezitten en SERK1 en BRI1 transfosforyleren en interakteren met elkaar onafhankelijk van de kinase aktiviteit van BRI1. In hoofdstuk 5 wordt de rol van SERK1 z'n interakterende partners AGL15, 14-3-3v en BRI1 in de somatische endogenese onderzocht. In dit hoofdstuk verstrekken wij bewijs dat de door BRI1 beinvloedde BR signaleringsroute nodig is voor de competentie om embryo's te kunnen vormen. Zowel exogene toevoeging van brassinosteroiden als overexpressie van BRI1 versterken de somatische embryogenese. Sterke bri1 mutanten als ook de BR biosyntese mutant det2 daarentegen, laten een sterk gereduceerde embryogene competentie zien. Voor AGL15, welke ook betrokken is bij dit proces, is aangetoond dat deze een positieve regulator is van zowel somatische embryogenese als brassinosteroid signalering. Bij BL afhankelijke kern translocatie of AGL15, zoals verzorgt door het transport eiwit 14-3-3v, kan het AGL15 eiwit binden aan promotor delen van BRI1 en SERK1 genen en is hierdoor waarschijnlijk een positieve regulator.

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Rumyana

List of publications

Karlova, R., Weemen-Hendriks, M., Naimov, S., Ceron, J., Dukiandjiev, S., and de Maagd, R.A. (2005). Bacillus thuringiensis delta-endotoxin Cry1Ac domain III enhances activity against Heliothis virescens in some, but not all Cry1-Cry1Ac hybrids. *Journal of invertebrate pathology* 88, 169-172.

Karlova, R., Boeren, S., Russinova, E., Aker, J., Vervoort, J., and de Vries, S. (2006). The Arabidopsis SOMATIC EMBRYOGESIS RECEPTOR-LIKE KINASE1 protein complex includes BRASSINOSTEROID-INSENSITIVE1. *Plant Cell* 18, 625-638.

Karlova, R., and de Vries, S.C. (2006). Advances in understanding brassinosteroid signaling. *Science STKE*, pe36

Aker, J., Borst, JW., **Karlova, R**., and de Vries, S.C. (2006). The Arabidopsis thaliana AAA protein CDC48A interacts in vivo with the somatic embryogenesis receptor-like kinase 1 receptor at the plasma membrane. *J Struct Biol* 156, 62-71.

Aker J, Hesselink R, Engel R, **Karlova R**, Borst JW, Visser AJ, de Vries SC (2007). In vivo hexamerizatio and characterization of the Arabidopsis AAA ATPase CDC48A complex using foster resonance energy transfer-fluorescence lifetime imaging microscopy and fluorescence correlation spectroscopy. *Plant Physiol.* Vol 145, pp. 339-350.

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Naimov S, Boncheva R, **Karlova R**, Dukiandjiev S, Minkov I, de Maagd RA (2008). Bacillus thuringiensis serovar thompsoni HD542 Crystal Proteins: Solubilization, Activation, and Insecticidal Activity. *Appl Environ Microbio.* In Press

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de Vries. Brassinosteroid signaling is essential for plant cell totipotency. *In Preparation*

CURRICULUM VITAE

Rumyana Borisova Karlova was born on 13 of April 1978, in the town of Pazardjik, Bulgaria. From 1995-1999, she stadied Biology at University of Plovdiv "Paisii Hilendarski", Bulgaria. After the completion of the general courses, she started 2 years specialization in "Plant Biotechnology" at the department of Plant Physiology and Molecular Biology. In 2000, she received a TEMPUS grand for six months internship at the Business Unit "Cell cybernetics" at Plant Research International in Wageningen, The Netherlands. On completion of her MSc in 2001, she continued working on a fellowship project in the same group. In 2003, Rumyana started a PhD program at the Department of Biochemistry, Wageningen University. The research was funded by the Center for BioSystems Genomics (CBSG). In her PhD research she worked on "Isolation and Identification of the SERK1 interacting proteins". The results from this study are summarized in this thesis. In august 2008, Rumyana was appointed as a post-doctoral researcher at the Laboratory of Biochemistry at Wageningen University.

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: Rumyana Karlova
Date: 17 December 2008
Group.

1) Start-up phase	*date*
▶ **First presentation of your project**	
Proteins interacting with SERK1 and SERK3	Sep 16, 2003
▶ **Writing or rewriting a project proposal**	
▶ **Writing a review or book chapter**	
Advances in Understanding Brassinisteroid signaling, Sci. STKE 2006 (354), pe36	Sep 2007
▶ **MSc courses**	
▶ **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	*date*
▶ **EPS PhD student days**	
PhD student day 2004, Vrije Universiteit Amsterdam	Jun 03, 2004
PhD student day 2005, Radbout Universiteit, Nijmegen	Jun 02, 2005
PhD student day 2006, Wageningen University	Sep 19, 2006
PhD student day 2007, Wageningen University	Sep 13, 2007
▶ **EPS theme symposia**	
EPS Theme 1 'Developmental Biology', Wageningen	Apr 26, 2005
EPS Theme 4 'Genome Plasticity', Wageningen	Dec 09, 2005
▶ **NWO Lunteren days and other National Platforms**	
NWO-ALW meeting Lipids and Biomembranes, Lunteren	Mar 08-09, 2004
NWO-ALW meeting Experimental Plant Sciences, Lunteren	Apr 06-07, 2004
NWO-ALW meeting Lipids and Biomembranes, Lunteren	Feb 14-15, 2005
NWO-ALW meeting Experimental Plant Sciences, Lunteren	Apr 04-05, 2005
NVMBC Fall Meeting 2005 Regulatory Protein Networks	Nov 04, 2005
NWO-ALW meeting Proteins, Lunteren	Dec 12-13, 2005
Lipids and Biomembrane, Lunteren	Mar 06-07, 2006
NWO-ALW meeting Experimental Plant Sciences, Lunteren	Apr 03-04, 2006
NWO-ALW meeting Proteins, Lunteren	Dec 11-12, 2006
NWO-ALW meeting Experimental Plant Sciences, Lunteren	Apr 02-03, 2007
▶ **Seminars (series), workshops and symposia**	
Flying Seminar prof. Dr. A.G.M. Gerarts 'Gene duplication&functional diversification in the Petunia MADS box gene family	Dec 16, 2004
Flying Seminar Prof. Dr. Joseph R. Ecker, 'Discovery of Functional Elements in the Arabidopsis Genome', Wageningen	Sep 26, 2005
Flying Seminar Prof. Dr. Philip Benfey, 'A systems biology approach to understanding root development'	Oct 25, 2005
▶ **Seminar plus**	
▶ **International symposia and congresses**	
15e International Conference on Arabidopsis Research , Berlin	Jul 11-14, 2004
16e International Conference on Arabidopsis Research , Madison	Jun 15-19, 2005
▶ **Presentations**	
16e International Conference on Arabidopsis Research (poster)	Jun 15-19, 2005
EPS theme 1 synposium, 2004 Wageningen (oral)	Feb 17, 2004
NWO-ALW meeting Experimental Plant Sciences, Lunteren (poster)	Apr 05-06, 2005
NWO-ALW meeting Proteins, Lunteren (oral)	Dec 12-13, 2005
Lipids and Biomembrane, Lunteren (poster)	Mar 06-07, 2006
NWO-ALW meeting Experimental Plant Sciences, Lunteren (poster)	Apr 03-04, 2006
NWO-ALW meeting Experimental Plant Sciences, Lunteren (oral)	Apr 02-03, 2007
▶ **IAB interview**	Sep 18, 2006
▶ **Excursions**	
Subtotal Scientific Exposure	*15.4 credits**

3) In-Depth Studies	*date*
▶ **EPS courses or other PhD courses**	
2nd Bioinformatics course Bioinformation Technology-1	Dec 08-16, 2003
EPS Summer School (Wageningen): Signaling in Plant Development and Defence:towards Systems Biology	Jun 19-21, 2006
▶ **Journal club**	
Biweekly Biochemistry literature discussions	2003-2008
▶ **Individual research training**	
Subtotal In-Depth Studies	*6.0 credits**

4) Personal development	*date*
▶ **Skill training courses**	
Interpersonal Communication for PhD students	Nov 01, 2007
Dutch Pre-Intermediate	Jan-Apr 2007
▶ **Organisation of PhD students day, course or conference**	
Organisation PhD proteomics course	Feb 27-Mar 03, 2006
▶ **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*4.2 credits**

TOTAL NUMBER OF CREDIT POINTS*	**33.1**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*

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