The Arabidopsis kinase-associated protein phosphatase controls internalization of the somatic embryogenesis receptor kinase 1

Khalid Shah, Eugenia Russinova, Theodorus W.J. Gadella, Jr., Joost Willemse, and Sacco C. de Vries

Laboratory of Molecular Biology, Department of Plant Sciences, Wageningen University, 6703 HA Wageningen, The Netherlands

The AtSERK1 protein is a plasma membrane-located LRR receptor-like serine threonine kinase that is transiently expressed during plant embryogenesis. Our results show that AtSERK1 interacts with the kinase-associated protein phosphatase (KAPP) in vitro. The kinase interaction (KI) domain of KAPP does not interact with a catalytically inactive kinase mutant. Using mutant AtSERK1 proteins in which Thr 462, Thr 463, and Thr 468 in the A-loop of the AtSERK1 kinase domain were replaced by alanines, we show that phosphorylation status of the receptor is involved in interaction with KAPP. KAPP and AtSERK1 cDNAs were fused to two different variants of green fluorescent protein (GFP), the yellow fluorescent protein (YFP) or the cyan fluorescent protein (CFP). Both KAPP and AtSERK1 proteins are found at the plasma membrane. Our results show that AtSERK1-CFP becomes sequestered into intracellular vesicles when transiently coexpressed with KAPP-YFP proteins. AtSERK1T463A-CFP and AtSERK13T→A-CFP proteins were partially sequestered intracellularly in the absence of KAPP-YFP protein, suggesting an active role for KAPP dephosphorylation of threonine residues in the AtSERK1 A-loop in receptor internalization. The interaction between the KAPP-CFP/YFP and AtSERK1-CFP/YFP fusion proteins was investigated with fluorescence spectral imaging microscopy (FSPIM). Our results show that AtSERK1-CFP and KAPP-YFP proteins are colocalized at the plasma membrane but only show fluorescence energy transfer (FRET) indicative of physical interaction in intracellular vesicles. These results suggest that KAPP is an integral part of the AtSERK1 endocytosis mechanism.

[Key Words: Interaction; phosphorylation; localization; fluorescence; phosphatase]

Received December 5, 2001; revised version accepted May 8, 2002.

Dephosphorylation of transmembrane receptor kinases catalyzed by phosphatases is an essential control element in receptor-mediated signaling. Two major groups, tyrosine kinases and phosphatases, and serine/threonine kinases and phosphatases are distinguished [Cohen 1989]. Phosphatases that dephosphorylate serine and threonine residues are encoded by the PPP and the PPM gene families that are defined by distinct amino acid sequences and crystal structures. The PPP family includes the signature phosphatases, PP1, PP2A, and PP2B whereas the PPM family comprises the Mg2+-dependent protein phosphatases that include PP2C and pyruvate dehydrogenase phosphatase [Cohen 1997].

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

KAPP is comprised of an amino terminal type 1 signal anchor followed by a kinase interaction [KI] domain and a C-terminal type 2C-protein phosphatase catalytic do-

Present addresses: 1Department of Neurology, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA 02129, USA; 2Section of Molecular Cytology, Swammerdam Institute for Life Sciences, 1098 SM Amsterdam, The Netherlands.
3Corresponding author.
E-MAIL sacco.devries@mac.mb.wau.nl; FAX 31-317-483-584.
Article and publication are at http://wwwgenesdev.org/cgi/doi/10.1101/gad.220402.
Somatic embryoogenesis is the process whereby somatic cells can develop into plants via characteristic and does not bind kinase-inactivemutantsofRLKs. The physical interaction between KAPP and the RLKs in vitro in a phosphorylation-dependent manner (Braun et al. 1997). The KIdomain of KAPP bindstothe CLV1kinasedomain (Williamset al. 1997), the HAESA KAPP hasalsobeenshowntointeractinvitrowiththe derivedfrommeristematictissue (Trotochaud et al. 1999). The Ki-domain of KAPP binds RLKs in vitro in a phosphorylation-dependent manner and does not bind kinase-inactive mutants of RLKs.

Somatic embryogenesis is the process whereby somatic cells can develop into plants via characteristic morphological stages. In cultured *Daucus carota* suspension cells, the SERK [somatic embryogenesis receptor kinase] gene encoding a leucine-rich-repeat [LRR]-RLK was found to be a marker for single somatic cells capable of forming embryos [Schmidt et al. 1997]. During somatic and zygotic embryogenesis, SERK expression continues up to the early globular stage and is absent in later stages of embryo development. The most closely related *Arabidopsis* AtSERK1 gene encodes a receptor kinase that is expressed during ovule development and early embryogenesis [Hecht et al. 2001] and has serine/threonine kinase activity [Shah et al. 2001a]. The predicted AtSERK1 protein contains an N-terminal leucine zipper (LZ), five leucine-rich repeats [LRRs] and a proline-rich region in its predicted extracellular domain. This is followed by a single transmembrane domain that is attached to an intracellular kinase domain with all 11 conserved subdomains found in serine/threonine kinases [Shah et al. 2001a]. We showed previously that Thr 468 in the AtSERK1 A-loop is essential for transphosphorylating common substrates like casein and MBP (Shah et al. 2001a).

Different spectral variants of GFP exhibiting yellow or cyan fluorescence [YFP and CFP, respectively] provide an opportunity to label proteins with two different fluorophores within the same living cell [Rizzuto et al. 1996]. YFP and CFP retain their characteristic fluorescence when fused to other protein sequences, allowing fluorescence microscopy to visualize the GFP-fusion protein localization [Carey et al. 1996, Gerdes et al. 1996, Ludin et al. 1996] and the interaction between two proteins by fluorescence resonance energy transfer [FRET] [Sorkin et al. 2000] in intact cells. FRET is a quantum mechanical process by which the excitation energy is transferred from a donor fluorophore to an appropriate acceptor fluorophore. FRET is manifested in different ways: a decrease in donor fluorescence quantum yield, a decreased donor fluorescence lifetime, and an increased [sensitized] emission acceptor fluorescence emission [if the acceptor is a fluorophore] [Jovin and Arndt-Jovin 1989, Gadella et al. 1999]. In our previous studies with AtSERK1-YFP/CFP proteins using FSPIM, we showed that AtSERK1 is expressed mainly as a monomer at the plasma membrane (Shah et al. 2001b).

In the present study we investigated whether the KAPP protein is involved in AtSERK1 signaling. Our results suggest that KAPP is required for AtSERK1 endocytosis.

**Results**

**Interaction between AtSERK1 and KAPP proteins**

The AtSERK1-GST, KAPP-GST, AtSERK1-YFP/CFP, and KAPP-YFP/CFP constructs used are shown in Figure 1. To determine whether the KAPP Ki domain biochemically and physically interacted with AtSERK1 in vitro, both the kinase domain of AtSERK1 and the Ki domain of KAPP were expressed as GST fusion proteins in *E. coli*. The affinity-purified AtSERK1kin and KAPPki fusion proteins were then used in phosphorylation assays [Fig. 2A]. Increasing phosphorylation of AtSERK1kin was seen on increasing its concentration [Fig. 2A, lanes 1–3]. KAPPki was phosphorylated in the presence of AtSERK1kin protein [Fig. 2A, lanes 4–8]. Since both proteins are substrates for phosphorylation, the level of phosphorylation was determined as a function of protein concentration [Fig. 2A]. Above an approximately equimolar ratio, increasing amounts of KAPPki led to an increase in the phosphorylation of KAPPki and a decreased phosphorylation of AtSERK1kin [Fig. 2A, cf. lanes 6 and 3]. The KAPPki phosphorylation was less affected when the AtSERK1kin protein concentration was decreased in the incubation mix [Fig. 2A, cf. lanes 7 and 8 with lanes 4 and 5]. KAPPki protein was unable to phosphorylate alone [Fig. 2A, lane 9]. This demonstrates that KAPP is a substrate for AtSERK1kin and that the two proteins biochemically interact in vitro.

AtSERK1 A-loop that is formed from subdomains VII and VIII has three threonine residues that are potential phosphorylation sites [Shah et al. 2001a]. To determine whether the interaction between AtSERK1kin and KAPPki is phosphorylation-dependent, we tested the AtSERK1kin, the catalytically inactive AtSERK1K330E, and the AtSERK1ST→F mutant proteins in which three threonine residues in the AtSERK1 A-loop have been mutated, for their association with KAPPki by in vitro binding assays. The AtSERK1kin proteins were incubated in the presence of unlabeled ATP and transferred after electrophoresis onto a membrane and incubated with [γ-32P]ATP-labeled KAPPki probe. AtSERK1kin [Fig. 2B, lane 1] interacted with KAPPki probe, and no interaction was observed between the KAPPki and the AtSERK1K330E and AtSERK1ST→F mutant [Fig. 2B, lanes 2 and 3]. To further test whether the interaction between AtSERK1kin and KAPPki was dependent on the phosphorylation state of the AtSERK1kin protein, the AtSERK1kin was treated
Interaction between AtSERK1 and KAPP proteins

Figure 1. Plasmid constructs used. Different domains of AtSERK1 (A) and KAPP (Q) (Stone et al. 1994). AtSERK1-GST fusion (B–H) and KAPP-GST fusion (R, S) constructs used for expression in E. coli. AtSERK1-CFP/YFP fusion (I–P) and KAPP-CFP/YFP fusion (T–Y) constructs used for transient expression in plant protoplasts. The AtSERK1-GST transgenes were constructed in the PGEX-4T1 vector under the control of the inducible tac promoter. AtSERK1kn [B] contains cDNA encoding for the intracellular kinase domain of AtSERK1 as an N-terminal fusion with the GST. Site-directed mutagenesis in the AtSERK1kn construct generated mutant constructs (C–H): AtSERK1K330E (C), AtSERK1T462E (D), AtSERK1T463A (E), AtSERK1T468A (F), AtSERK1V466A (G), AtSERK1T468A (H). Each mutation is noted at the top of the construct. The KAPP-GST transgenes were constructed similarly. The KAPPkn (R) encodes only the kinase interaction domain of KAPP fused to GST. The KAPPkn constructs were constructed in the PMON999 vector under the control of the 35S promoter. AtSERK1-YFP/CFP (K, L) contains cDNA encoding for the full-length AtSERK1 as a C-terminal fusion with the YFP/CFP genes. AtSERK1kn-YFP/CFP (K) encodes only the intracellular kinase domain of AtSERK1 fused to YFP/CFP (L). The AtSERK1T462E-YFP/CFP and AtSERK1T463A-YFP/CFP constructs contain the full-length AtSERK1 in which Thr 462, Thr 463, Thr 468 in AtSERK1T462E or Thr 463 in AtSERK1T463A were mutated into alanine residues and fused to YFP/CFP genes (M–P). The KAPP-YFP/CFP transgenes were constructed similarly. KAPP-YFP/CFP (Q, R) encodes cDNA encoding for the full-length KAPP as a C-terminal fusion with the YFP/CFP genes. KAPPkn-YFP/CFP (V, W), the same as T and U but missing the N-terminal signal anchor. KAPPkn-YFP/CFP (X, Y) encodes only the kinase interaction domain of KAPP fused to YFP/CFP.
Different concentrations of AtSERK1<sup>1Kin</sup> proteins were purified, and aliquots ranging from 0 to 800 ng were used. Different concentrations of AtSERK1<sup>1Kin</sup> (top) and KAPP<sup>KI</sup> (bottom) proteins were incubated with [γ-<sup>32</sup>P]ATP as described inMaterials and Methods. After separation on 10% SDS-PAGE, the gels were autoradiographed using a PhosphorImager. [β] Immobilized KAPP<sup>KI</sup> protein was phosphorylated with 10 units of bovine heart muscle kinase (Sigma) in the presence of [γ-<sup>32</sup>P]ATP. The resulting eluent was used to probe the nitrocellulose filter containing AtSERK1<sup>1Kin</sup> (lane 1), AtSERK1<sup>K330E</sup> (lane 2), AtSERK1<sup>T459A</sup> (lane 3), AtSERK1<sup>1Kin</sup> treated with alkaline phosphatase inhibitor (API) (lane 4), AtSERK1<sup>1Kin</sup> treated with alkaline phosphatase (AP) (lane 5), and AtSERK1<sup>1Kin</sup> treated with AP and API (lane 6), and then allowed to phosphorlyate in the presence of ATP and MgCl<sub>2</sub>. The filters were autoradiographed using a PhosphorImager. [C] Phosphoaminoacid analysis of the HCl hydrolyzed KAPP<sup>KI</sup> protein phosphorylated by AtSERK1<sup>1Kin</sup>. The hydrolyzed protein was spotted onto a TLC plate and resolved by ascending chromatography in two dimensions. The autoradiograph of the phosphorylated amino acids and the position of the standard amino acids visualized by 0.25% ninhydrin spray are phosphoserine (pS), phosphothreonine (pT), and phosphotyrosine (pY), respectively.

Furthermore, the threonine residues in the AtSERK1 A-loop seem to play an essential role in the transphosphorylation of the KAPP<sup>KI</sup> protein.

In a previous work, we showed that the effect on the phosphorylation activity was similar when AtSERK1 A-loop threonines were mutated to either glutamic acid residues or to alanine residues (Shah et al. 2001a). To investigate the role of individual threonine residues in the AtSERK1 A-loop in AtSERK1<sup>1Kin</sup>-KAPP<sup>KI</sup> transphosphorylation, all four threonine residues in the AtSERK1 A-loop, that is, Thr 459, Thr 462, Thr 463, and Thr 468 were mutagenized individually to alanine residues. The transphosphorylation assays with affinity-purified KAPP<sup>KI</sup> and AtSERK1<sup>1Kin</sup> threonine mutant proteins showed that there was a reduced transphosphorylation of KAPP<sup>KI</sup> when incubated with AtSERK1<sup>T459A</sup> (Fig. 3A, lane 2) compared to KAPP<sup>PS</sup> phosphorylation by AtSERK1<sup>1Kin</sup> (Fig. 3A, lane 1). KAPP<sup>PS</sup> was not phosphorylated when incubated with AtSERK1<sup>T466A</sup> [Fig. 3A, lane 3] or AtSERK1<sup>T463A</sup> [Fig. 3A, lane 4] or AtSERK1<sup>T466A</sup> with AtSERK1<sup>1Kin</sup> or AtSERK1<sup>T463A</sup> proteins (lane 1) or with KAPP<sup>KI</sup> [lane 2] or with KAPP<sup>SA</sup> [lane 3], or with both KAPP<sup>KI</sup> and KAPP<sup>SA</sup> proteins [lane 4]. The autoradiographs are shown.

**Figure 2.** In vitro KAPP interactions with AtSERK1<sup>1Kin</sup>. [A] Bacterially produced, affinity-purified AtSERK1<sup>1Kin</sup> and KAPP<sup>KI</sup> proteins were purified, and aliquots ranging from 0 to 800 ng were used. Different concentrations of AtSERK1<sup>1Kin</sup> (top) and KAPP<sup>KI</sup> (bottom) proteins were incubated with [γ-<sup>32</sup>P]ATP as described inMaterials and Methods. After separation on 10% SDS-PAGE, the gels were autoradiographed using a PhosphorImager. [B] Immobilized KAPP<sup>KI</sup> protein was phosphorylated with 10 units of bovine heart muscle kinase (Sigma) in the presence of [γ-<sup>32</sup>P]ATP. The resulting eluent was used to probe the nitrocellulose filter containing AtSERK1<sup>1Kin</sup> (lane 1), AtSERK1<sup>K330E</sup> (lane 2), AtSERK1<sup>T459A</sup> (lane 3), AtSERK1<sup>1Kin</sup> treated with alkaline phosphatase inhibitor (API) (lane 4), AtSERK1<sup>1Kin</sup> treated with alkaline phosphatase (AP) (lane 5), and AtSERK1<sup>1Kin</sup> treated with AP and API (lane 6), and then allowed to phosphorlyate in the presence of ATP and MgCl<sub>2</sub>. The filters were autoradiographed using a PhosphorImager. [C] Phosphoaminoacid analysis of the HCl hydrolyzed KAPP<sup>KI</sup> protein phosphorylated by AtSERK1<sup>1Kin</sup>. The hydrolyzed protein was spotted onto a TLC plate and resolved by ascending chromatography in two dimensions. The autoradiograph of the phosphorylated amino acids and the position of the standard amino acids visualized by 0.25% ninhydrin spray are phosphoserine (pS), phosphothreonine (pT), and phosphotyrosine (pY), respectively.

**Figure 3.** (A) KAPP interactions with AtSERK1<sup>1Kin</sup> mutant proteins. Five hundred nanograms of affinity-purified AtSERK1<sup>1Kin</sup> or AtSERK1<sup>1Kin</sup> mutant proteins were incubated with KAPP<sup>KI</sup> protein in the presence of [γ-<sup>32</sup>P]ATP in a phosphorylation assay as described in Materials and Methods. After separation on 10% SDS-PAGE, the gels were autoradiographed using a PhosphorImager. AtSERK1<sup>1Kin</sup> and AtSERK1<sup>1Kin</sup> mutants [top] and KAPP<sup>KI</sup> [bottom] proteins are shown. AtSERK1<sup>1Kin</sup> (lane 1), AtSERK1<sup>T459A</sup> (lane 2), AtSERK1<sup>T466A</sup> (lane 3), AtSERK1<sup>T463A</sup> (lane 4), AtSERK1<sup>T466A</sup> (lane 5), AtSERK1<sup>T463A</sup> (lane 6), and AtSERK1<sup>K330E</sup> (lane 7). (B) Dephosphorylation of AtSERK1 by KAPP<sup>KI</sup> protein. Matrix-bound AtSERK1<sup>1Kin</sup> was phosphorylated by [γ-<sup>32</sup>P]ATP as described in Materials and Methods. The phosphorylated AtSERK1<sup>1Kin</sup> (lane 1) was incubated with KAPP<sup>SA</sup> protein and the aliquots were taken after 30, 60, 90, 120, and 240 min (lanes 2–6). After separation on 10% SDS-PAGE, the gels were autoradiographed using a PhosphorImager. (C) Phosphorylation of a mix of 500 ng of each AtSERK1<sup>1Kin</sup> and KAPP<sup>KI</sup> proteins in the presence of [γ-<sup>32</sup>P]ATP was performed as described in Materials and Methods. AtSERK1<sup>1Kin</sup> was incubated alone (lane 1) or with KAPP<sup>KI</sup> [lane 2] or with KAPP<sup>S</sup> [lane 3], or with both KAPP<sup>KI</sup> and KAPP<sup>S</sup> proteins [lane 4]. The autoradiographs are shown.

1710 GENES & DEVELOPMENT
The transphosphorylation assays with AtSERK1T468A (Fig. 3A, lane 5). The transphosphorylation assays with AtSERK1A-loop have a major role in both the autophosphorylation activity of AtSERK1 and transphosphorylation activity on the KI domain of KAPP. This confirms previous work using casein and myelin basic protein (MBP) as artificial transphosphorylation targets for AtSERK1 (Shah et al. 2001a). In addition, Thr 463 also influences the transphosphorylation of the KI domain of KAPP. This is a different observation than that obtained with AtSERK1 transphosphorylation on casein and MBP, where only Thr 462 and Thr 468 have any influence on the transphosphorylation (Shah et al. 2001b). This finding implies a role of other threonine residues in the AtSERK1 A-loop in substrate phosphorylation.

To determine the protein phosphatase catalytic activity of KAPP, the full-length KAPP cDNA missing only the N-terminal signal anchor was fused to GST and expressed in E. coli. The affinity-purified KAPP protein [KAPP−SA] was incubated with matrix-bound [γ-32P]ATP phosphorylated AtSERK1kum, and aliquots were taken out at different time intervals [Fig. 3B, lanes 2–6]. The phosphorylated ASERK1kin was dephosphorylated by KAPP−SA with increased incubation time [Fig. 3B, lanes 2–5]. Phosphorylated AtSERK1kin protein was almost completely dephosphorylated by KAPP−SA after 4 h [Fig. 3B, lane 6]. We were also interested in knowing whether the AtSERK1kin1 protein would phosphorylate in the presence of KAPP−SA protein. This led us to perform experiments in which we incubated affinity-purified AtSERK1kin1 alone [Fig. 3C, lane 1] with KAPPK1YFP [Fig. 3C, lane 2] or KAPP−SA [Fig. 3C, lane 3] or with both KAPPK1YFP and KAPP−SA proteins in the presence of [γ-32P]ATP. In the presence of the KAPP−SA protein, there was a reduced phosphorylation of AtSERK1kin [Fig. 3C, lane 3] or both AtSERK1kin1 and KAPPK1YFP [Fig. 3C, lane 4]. These results demonstrate that KAPP protein not only dephosphorylates AtSERK1 protein but also actively prevents auto-phosphorylation of the receptor, suggesting that the KAPP protein can maintain the dephosphorylated state of AtSERK1.

Colocalization of AtSERK1 and KAPP proteins in living plant cells

Because AtSERK1 and KAPP interact in vitro, it was of interest to determine whether the two proteins are colocalized and can interact in plant cells. For this we transiently expressed in plant protoplasts two different variants of the GFP, the cyan fluorescent protein (CFP), and the yellow fluorescent protein (YFP) that are fused to the C-terminal end of AtSERK1 and KAPP protein [Fig. 1, I–L, P–U]. The KAPP type 1 signal anchor (SA) acts as an uncleaved signal peptide, resulting in cytoplasmic orientation of the membrane-anchored protein (Stone et al. 1994). To study the localization of the KAPP protein in plant protoplasts, we performed transient transfections with KAPPK1YFP, KAPP−SA−YFP, and KAPP−YFP constructs. After transfection and incubation for 12 h, total protein was isolated, fractionated by denaturing SDS-PAGE, and immunoblotted using an antiserum against GFP. The results show that all KAPP−YFP/CFP fusion proteins of the correct size are expressed in plant protoplasts [Fig. 4A]. KAPPK1−YFP protein showed localization in the cytoplasm [Fig. 4B], whereas KAPP−YFP protein was expressed in the plasma membrane and in the cytosol [Fig. 4C]. When the protoplasts were transfected with KAPP−SA−YFP, a construct in which the KAPP protein lacks the SA signal peptide, the KAPP−SA−YFP protein was observed in the cytoplasmic compartments (Fig. 4D). This shows the importance of the KAPP−SA peptide in membrane anchoring of the KAPP protein.

In our previous studies with AtSERK1-YFP fusion protein in plant protoplasts, we showed that most of the AtSERK1−YFP protein is found in the plasma membrane and in a very small amount in endomembranes (Shah et al. 2001b). In Figure 5A, a protoplast expressing the AtSERK1−CFP is shown. CFP is represented by the cyan color and confirms the previously noted location of the AtSERK1 protein. The same protoplast was cotransfected with an ER marker (ER-YFP) that contains a C-terminal HDEL-ER retention signal fused to YFP (Fig. 5B). For comparison, the chlorophyll autofluorescence is shown in red [Fig. 5C]. From the combined image [Fig. 5D], it is clear that ER-YFP and AtSERK1-CFP have different localizations in the protoplast. AtSERK1-CFP is seen mainly in the plasma membrane, whereas the ER at

![Figure 4](https://example.com/figure4.png)
Figure 5. Colocalization of AtSERK1, AtSERK1T463, AtSERK13T→A, and KAPP proteins in cowpea mesophyll protoplasts. (A–D) Confocal images of protoplasts cotransfected with AtSERK1-CFP and ER-YFP. The two fluorophores CFP and YFP were simultaneously visualized. AtSERK1-CFP localization is shown in cyan (A), ER-YFP localization is shown in yellow (B). Chlorophyll autofluorescence is shown in red (C). The superimposed images of A and B result in D. (E–H) Confocal images of protoplasts cotransfected with KAPP-YFP and AtSERK1-CFP. The two fluorophores CFP and YFP were simultaneously visualized. Chlorophyll autofluorescence is shown in red (G). Colocalization is shown in bluish green fluorescence (H) resulting from the overlap of cyan (AtSERK1-CFP) (E) and green (KAPP-YFP) (F). (I–L) Confocal images of the protoplasts transfected with AtSERK1T463-YFP and visualized as yellow fluorescence (I), and a superimposed image showing the autofluorescence of the chlorophyll (J). (K, L) Confocal images of protoplasts transfected with AtSERK13T→A-YFP visualized as yellow fluorescence (K), and a superimposed image showing the autofluorescence of the chlorophyll (L). (M–P) Confocal images of protoplasts cotransfected with KAPP-YFP and AtSERK1T463-CFP. KAPP-YFP fluorescence is shown in yellow (N), and AtSERK1T463-CFP fluorescence is shown in blue (M). Chlorophyll autofluorescence is shown in red (O). The superimposed images of M and N result in P. (Q–T) Confocal images of the protoplasts cotransfected with KAPP-YFP and AtSERK13T→A-CFP. KAPP-YFP fluorescence is shown in yellow (R), and AtSERK13T→A-CFP fluorescence is shown in blue (Q). Chlorophyll autofluorescence is shown in red (S). The superimposed images of Q and R result in T. Bars, 10 µm.
this stage (12 h after transfection) is almost depleted of the AtSERK1 protein. The ER-YFP localization is visible as a complex pattern [Fig. 5B] that covers most of the cytoplasm-containing area and extends right up to the plasma membrane. It is notably excluded from the chloroplasts and the vacuole. To examine the subcellular localization of KAPP and AtSERK1 proteins together, we transiently expressed both AtSERK1-CFP and KAPP-YFP fusion proteins. When coexpressed, striking differences were noted in the localization pattern of both proteins. Substantial amounts of AtSERK1 and KAPP proteins are now seen internally [Fig. 5E–H]. At the periphery of the protoplast, AtSERK1 is found in the areas that coincide with the presence of ER membranes. Substantial CFP fluorescence is also seen in a rather diffuse dotted pattern almost in the center of the cell [Fig. 5E]. The KAPP protein is found mainly in the center of the cell and less in the peripheral areas [Fig. 5F]. The superimposed image [Fig. 5H] shows that KAPP and AtSERK1 fluorescence only substantially overlap in the central area and in the membrane. The comparison of the localization of AtSERK1 and KAPP proteins [Fig. 5E, F] with the ER marker [Fig. 5B] suggests that the diffuse punctate pattern observed in the colocalization experiment might represent intracellular vesicles such as endosomes, containing internalized AtSERK1-KAPP proteins.

We next investigated whether AtSERK1 threonine residues 462, 463, and 468, involved in the interaction with KAPP [Fig. 2B] and in the transphosphorylation reaction in vitro [Fig. 3A] were involved in the observed internalization of the AtSERK1 protein. A construct containing the AtSERK1T463A-CFP fusion was localized in the plasma membrane, with a rather higher signal seen in a diffuse area in the cytoplasm compared to the AtSERK1-CFP protein [Fig. 5J]. When AtSERK1T463A-CFP was transiently coexpressed with KAPP-YFP, substantial amounts of both proteins were found to be sequestered intracellularly [Fig. 5M, N]. The AtSERK1ST→A-CFP protein alone localizes at the plasma membrane but is also seen internally [Fig. 5K, L], similar to what was seen for the AtSERK1T463A-CFP protein. Some AtSERK1ST→A-CFP protein was also seen to be present in the ER, suggesting an additional effect of removing the three threonines on the correct secretion of the receptor via the Golgi. Cotransfection of AtSERK1ST→A-CFP and KAPP-YFP did not change the pattern seen for the AtSERK1ST→A-CFP mutant alone [Fig. 5Q–T]. However, the amount of internalized AtSERK1ST→A-CFP protein was increased compared with the AtSERK1T463A-CFP alone.

We conclude that KAPP and AtSERK1 colocalize to a certain extent. Preventing a biochemical or physical interaction by removing the potential KAPP substrate threonines in the A-loop of AtSERK1 resulted in a marked intracellular localization of the receptor. Coexpressing the mutant fusion proteins along with KAPP protein enhanced the fraction of internalized receptors. This suggests that KAPP-mediated dephosphorylation of the receptor is an essential step in the internalization of the entire receptor complex.

To verify that the punctate localization pattern of the two AtSERK1 mutants and of AtSERK1 when coexpressed with KAPP protein represents true endosomal localization, we simultaneously labeled the cells with FM4-64. FM4-64 is a lipophilic dye that is taken up by endocytosis when added to yeast [Vida and Emr 1995] or plant cells [Ueda et al. 2002]. Protoplasts transiently expressing AtSERK1T463A-YFP protein were incubated with FM4-64 and viewed by confocal microscopy after 15 min. At this timepoint, FM4-64 stained only the plasma membrane [Fig. 6A–D], where it overlaps with AtSERK1T463A-YFP localization. When incubated for 3 h, FM4-64 was internalized and localized in small punctate structures, similar to the early endosomal structures reported by Ueda et al. [2002]. As shown in Figure 6E–H, nearly all structures stained by FM4-64 also contained AtSERK1T463A-YFP. This suggests that the AtSERK1T463A-YFP protein is internalized via an endocytotic pathway.

Fluorescence resonance energy transfer studies

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

For FRET to be detected reliably using FSPIM, certain prerequisites must be satisfied. These are that donor and acceptor fluorophores must be present in a ratio close to 1:1; the absorption of the CFP fluorescence by chlorophyll should be minimized, fusion proteins should be properly targeted, and finally the fluorophores must be oriented so as to allow close proximity when the target proteins fused to the CFP/YFP interact. We performed FRET experiments with the AtSERK1, AtSERK1kin, KAPP, and KAPPSt fusion proteins fused to either the donor (CFP) or the acceptor (YFP) molecules. In all experiments where FRET was observed, similar changes in the fluorescence intensity ratios were observed regardless of the donor CFP being fused to AtSERK1 or to the KAPP [Figs. 7, 8]. This indicates that the observed increased donor/acceptor ratio is due to the energy transfer between the CFP and the YFP molecules fused to AtSERK1 or KAPP proteins and not due to an unbalanced expression ratio. To avoid interference by chlorophyll absorption, FRET measurements were performed exclusively over the regions lacking chloroplasts. Because the fluorophore orientation factor cannot be corrected for, any results showing absence of FRET may not completely guarantee that no interaction occurred between those proteins.

Protoplasts were first cotransfected with either AtSERK1kin-CFP and KAPPSt-YFP or AtSERK1kin-YFP...
and KAPPK constructs. The equal expression of the CFP and YFP fusion proteins in protoplasts was demonstrated by immunoblotting of the solubilized proteins using anti-GFP antisera [Fig. 7A]. The spectral images were recorded from the different regions of the protoplasts coexpressing AtSERK1kin-CFP and KAPPK-YFP fusion proteins. The YFP/CFP fluorescence intensity ratio deduced from these spectra is over 1.5 in every 3 of 5 measurements (Fig. 7B). The YFP/CFP intensity ratio obtained from AtSERK1kin-YFP and KAPPK-YFP cotransfected protoplasts was similar to the YFP/CFP intensity ratio shown by AtSERK1kin-CFP and KAPPK-YFP coexpressing protoplasts [Fig. 7C]. When the spectra were recorded from 30 protoplasts, each coexpressing AtSERK1kin-CFP and KAPPK-YFP or AtSERK1kin-YFP and KAPPK-CFP, the increased YFP/CFP fluorescence intensity ratio of more than 1.5 was seen in about 80% of the measurements (data not shown). These results show that when the AtSERK1kin and KAPPK proteins are in the cytoplasm, they interact physically, supporting the in vitro experiments performed on bacterially expressed proteins [see Fig. 2A]

Figure 7. Immunoblot and FSPIM analysis of fluorescent AtSERK1kin-CFP/YFP and KAPPK-CFP/YFP fusion proteins. (A) Immunoblot of proteins extracted from the protoplasts cotransfected with AtSERK1kin-CFP and KAPPK-YFP [lane 1] and AtSERK1kin-YFP and KAPPK-CFP [lane 2]. (B) Emission spectra of the AtSERK1kin-CFP and KAPPK-YFP proteins coexpressed in protoplasts. The spectra were recorded at a position where CFP/YFP colocalization was observed in the cytoplasm. The x-axis gives the wavelengths of the CFP and YFP fluorophores, and the y-axis shows their intensities. The emission wavelengths of both CFP and YFP are shown below the graph. (C) The same as B, for AtSERK1kin-YFP and KAPPK-CFP-expressing protoplasts.

Figure 6. FM4-64 labeling. [A–D] Protoplasts expressing AtSERK1T463-YFP [A] were labeled with FM4-64 and viewed after 15 min [B]. Chlorophyll autofluorescence is shown in red [C], and the superimposed images of A and B result in D. [E–H] Protoplasts expressing AtSERK1T463-YFP [E] were labeled with FM4-64 and viewed after 3 h [F]. Chlorophyll autofluorescence is shown in red [G], and the superimposed images of E and F result in H. Bars, 10 µm.
To determine whether physical interaction occurs between KAPP-CFP/YFP and the AtSERK1-CFP/YFP proteins, we cotransfected protoplasts with either KAPP-CFP and AtSERK1-YFP or KAPP-YFP and AtSERK1-CFP constructs. The immunoblot of the coexpressed proteins using anti-GFP antisera is shown in Figure 8A. The near identical size of the AtSERK1-CFP/YFP and KAPP-CFP/YFP fusion proteins prevents a clear distinction between two proteins on the blot. However, no gross imbalance in expression ratios is evident. We separated the FRET experiments performed on the proteins that were coexpressed at the plasma membrane and the proteins that were present intracellularly. An example of an area that was selected for recording is shown in Figure 8B, where AtSERK1 and KAPP are colocalized in the plasma membrane [shown in Fig. 8B by an arrowhead] and in the area where vesicular structures are seen [shown in Fig. 7B by an arrow]. These vesicular structures are present in the cytoplasm and are distinct from the ER [as shown in Fig. 5B] and may represent endosomes as suggested by FM4-64 staining. In Figure 8C, spectra are shown that were obtained from protoplasts coexpressing KAPP-YFP and AtSERK1-CFP at the plasma membrane. The YFP/CFP fluorescence intensity ratio deduced from these spectra was close to 1.0 in all of the measurements, suggesting that no interaction occurs between AtSERK1 and KAPP at the plasma membrane. The same fluorescence intensity ratios of 1.0 were obtained when protoplasts were cotransfected with KAPP-CFP and AtSERK1-YFP [Fig. 8D]. In about 95% of the protoplasts, the YFP/CFP fluorescence intensity ratio was close to 1.0 when the spectra were recorded at the plasma membrane from 30 protoplasts coexpressing either AtSERK1-CFP and KAPP-CFP proteins or AtSERK1-YFP and KAPP-CFP proteins [data not shown]. However, when the spectra were recorded on the AtSERK1-CFP and KAPP-YFP proteins [Fig. 8E] and AtSERK1-YFP and KAPP-CFP proteins [Fig. 8F] present intracellularly, there was a clear shift in the YFP/CFP fluorescence intensity ratio to above 1.3 and extending to 2.0 in 2 of 5 measurements. About 80% of the protoplasts showed an increased YFP/CFP fluorescence intensity ratio of about 1.4 when the spectra were recorded from 30 protoplasts coexpressing either AtSERK1-CFP and KAPP-YFP proteins or AtSERK1-

Figure 8. Immunoblot and FSPIM analysis of fluorescent AtSERK1-CFP/YFP and KAPP-CFP/YFP fusion proteins. [A] Immunoblot of proteins extracted from the protoplasts cotransfected with AtSERK1-CFP and KAPP-YFP (lane 1) and AtSERK1-YFP and KAPP-CFP (lane 2). [B] Emission spectra of the AtSERK1-CFP and KAPP-YFP proteins obtained from the plasma membrane of coexpressed protoplasts. The spectra were recorded at a position where CFP/YFP colocalization was observed in the plasma membrane. The x-axis presents the wavelengths of the CFP and YFP fluorophores, and the y-axis gives their intensities. The emission wavelengths of both CFP and YFP are shown below the graph. [C] The same as $B$, for AtSERK1-CFP and KAPP-CFP, expressing protoplasts. [D] Emission spectra of the AtSERK1-CFP and KAPP-YFP proteins obtained from the coexpressed proteins in the intracellular vesicles of protoplasts. The spectra were recorded at a position where CFP/YFP colocalization was observed intracellularly. [E] The same as $D$, for AtSERK1-CFP and KAPP-CFP-expressing protoplasts.
YFP and KAPP-CFP proteins intracellularly (data not shown).

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

**Discussion**

Our results show that the kinase domain of AtSERK1 and the kinase interaction domain of KAPP interact in a phosphorylation-dependent manner. KAPP and AtSERK1 proteins are colocalized in plant cells at the plasma membrane and in the intracellular vesicles, but only interact physically intracellularly. Either physical or biochemical interaction between KAPP and AtSERK1 in vivo is required for receptor internalization. This finding suggests that the proposed activity of receptor-associated phosphatases such as KAPP in receptor inactivation may be tightly coupled to an internalization mechanism dependent on the A-loop phosphorylation status of the receptor. Alternatively, the presence of KAPP could reduce the extent of plasma membrane localization of the receptor by KAPP-induced blocking of the exocytotic pathway for AtSERK1. Given the reported mechanisms of down-regulation of other receptor kinases, we consider the latter explanation to be unlikely.

In animals it has been shown that a common feature of protein–protein interactions is the binding to consensus motifs containing phosphorylated residues [Yaffe et al. 1997]. Phosphorylation/dephosphorylation events on plant RLKs are likely to be an important element in determining physical interaction. The KAPP protein was originally found by screening for proteins interacting with the catalytic domain of RLK5 (HAESA) [Stone et al. 1994]. Subsequently it was postulated to function as a negative regulator of the CLV1 signal transduction pathway [Williams et al. 1997]. KAPP is expressed throughout the shoot apical meristem (SAM) and in stage 1–2 flowers but not in stage 3 flowers, thus encompassing the region of CLV1 expression in the central cells of the SAM and young floral meristems [Williams et al. 1997]. KAPP has been shown to interact with many, but not all phosphorylated RLKs [Braun et al. 1997], which implies that it recognizes a particular sequence on the target kinases. In our previous studies with the AtSERK1 protein, we showed that AtSERK1 activation requires intermolecular autophosphorylation. This presumably results in movement of the AtSERK1 A-loop, followed by release of transphosphorylation activity and protein substrate binding. In this model, A-loop phosphorylation of Thr 462 and Thr 468 of one AtSERK1 monomer is also essential for releasing catalytic activity of that same monomer. The results show that the phosphorylation status of Thr 468 is essential for AtSERK1 transphosphorylation activity on casein and MBP [Shah et al. 2001a]. The failure of the KAPP protein to bind to the AtSERK1 Thr463→E mutant shows that the conformational change of the AtSERK1 A-loop is also a prerequisite for KAPP binding and subsequent transphosphorylation. In contrast to the studies on casein and MBP, Thr 459 and Thr 463 also influence KAPP binding and transphosphorylation, which suggests that the A-loop is itself actively involved in the interaction with protein substrates. It is clear however that Thr 468 is the most important residue that influences not only auto- and transphosphorylation activities but also interaction with KAPP. In vivo, Thr 463 is important for internalization, presumably for its interaction with KAPP. However, it is clearly not the only residue because the AtSERK1T463A mutant still responds to KAPP protein by acceleration of internalization.

In our studies with the KAPP−SA protein we showed that when both KAPP−SA and AtSERK1KIN proteins are incubated together, AtSERK1 phosphorylation is inhibited to a large extent. This implies that in vivo, KAPP may function by directly dephosphorylating AtSERK1, thus preventing other downstream components from receiving the AtSERK1-mediated signal. As the KAPP protein can be phosphorylated by other RLKs and also by AtSERK1, it is possible that KAPP may be modulated in response to an activated AtSERK1 receptor. However, the role of PTP1B-type phosphatases and their interaction with RLKs in plants is not fully understood. The observation that binding of RLKs with KAPP is very stable and is only weakened when dephosphorylation of the RLKs by the phosphatase is completed [Li et al. 1999] suggests an intimate interaction in controlling receptor phosphorylation status.

In situ hybridization and coimmunoprecipitation studies of CLV1 and KAPP have shown that they can interact physically and biochemically. Our studies using AtSERK1 and KAPP-CFP/YFP fusion proteins have refined these observations and may have more general applications as well. The coexpressed KAPP-CFP/YFP and AtSERK1-YFP/CFP proteins were clearly colocalized in the intracellular vesicles and in the plasma membrane. However, only the KAPP-CFP/YFP and AtSERK1-YFP/CFP protein in the intracellular vesicles interacted, whereas they did not in the plasma membrane.

In animals, receptor kinases have been shown to oligomerize, activate, autophosphorylate, and rapidly endocytose [Ullrich and Schlessinger 1990]. Receptor internalization has been shown to play an important role in signaling. For example, after activation of epidermal growth factor receptor (EGFR), the receptor is internalized into acidified endosomal compartments where most of the EGFR is degraded and the excess recycled back to the plasma membrane [Wells 1999]. It was recently shown that the ER-localized protein tyrosine phosphatase-1B (PTP1B) regulates dephosphorylation-dependent endocytosis of EGFR after ligand binding [Haj et al. 2002]. Other PTPs may function at the plasma membrane to prevent unscheduled receptor activation. The EGFR kinase domain remains active during endocytosis [Lai et al. 1989], and certain substrates are in fact phosphorylated following internalization. To date, receptor endocytosis has not
been described for plant RLKs, but our studies of AtSERK1 and KAPP proteins provide evidence of a novel role of the KAPP protein. Given that the IK domain of KAPP can only bind to the phosphorylated AtSERK1 protein, the observed FRET in the intracellular vesicles must derive from an interaction with the phosphorylated receptor. Notably no interaction is seen when KAPP and AtSERK1 proteins are present at the plasma membrane of the protoplasts, suggesting that the majority of the receptor molecules is in the nonphosphorylated state. At any one time, a minority of AtSERK1 protein is in the dimer state [Shah et al. 2001b]. This may equate to a small number of active (phosphorylated) receptors. The phosphorylated AtSERK1 receptor may then get internalized in intracellular vesicles, reminiscent of endosomes in animal cells, a process that is clearly very much enhanced by the simultaneous presence of KAPP. Most of the AtSERK1-KAPP complex may then be degraded, while some of the AtSERK1 molecules, dephosphorylated by KAPP, can be recycled to the plasma membrane. Thus it seems plausible that internalization might play a role in extending and/or initiating signaling events at a cellular location other than the plasma membrane in plant cells as well. Studies of the phosphorylation and dephosphorylation of the receptors in living cells such as that carried out for the EGF receptor [Verveer et al. 2000] are required to reveal possible spatial selectivity in the signal transduction cascade mediated by AtSERK1.

Materials and methods

GST-fusion proteins and their expression in E. coli

The cDNA sequence encoding the AtSERK1 kinase catalytic domain, corresponding to nucleotides 1000–2068 (GenBank accession no. A67827) was amplified by PCR using the 2.1-kb cDNA cloned in vector pBluescriptSK as a template. All of the primers used for PCR have tailored restriction sites added. Primer Small1000 (5'-TCCGCCGGCTTTCCTTCTGATGTCCTG-3') and primer NotI2068 (5'-TCCCCCGGGTATTTTCTTCGATGTCGGAAATACT-3') amplified a PCR fragment of 1.1 kb that was cloned into a Smal- and NotI-cleaved pGEX-4T1 vector (Pharmacia) resulting in AtSERK1::GST construct. Site-directed mutagenesis of the highly conserved Lys 330 essential for phosphotransfer in protein kinases to glutamic acid was performed according to the manufacturer’s instructions (Stratagene), resulting in the AtSERK1::GST-A construct. Site-directed mutagenesis of three threonine residues in the AtSERK1 A-loop, that is, Thr 462, Thr 463, and Thr 468 to glutamic acid residues resulting in an AtSERK1::GST-A construct. Single amino acid mutations ofThr 459, Thr 462, Thr 463, and Thr 468 to alanine residues resulted in AtSERK1::GST-A459A, AtSERK1::GST-A462A, and AtSERK1::GST-A463A constructs. The cDNA sequence encoding the Arabidopsis thaliana IK domain of KAPP and the KAPP cDNA missing only the N-terminal signal anchor was amplified by PCR using the KAPP cDNA in pBluescriptSK provided kindly by John C. Walker [University of Missouri–Columbia]. A forward primer (5'-GGAAAGATCTGGGATTTGGCAGAGACCA-3') and the reverse primer (5'-GGGAATCTTCAGGAATCTAACG-3') were used, and the 1.6-kb PCR fragment was cloned into the BamHI and XhoI sites of the pGEX-4T1 vector, resulting in the KAPP::GST construct. All plasmids were verified by restriction endonuclease digestion and DNA sequencing before being transformed into theE. coli BL21 strain for protein expression. A 2-mL overnight culture was transferred to 200 mL of LB medium, and the cells were grown at 37°C to an OD600 of 0.6 and then induced with 0.1 mM isopropyl-D-thiogalactopyranoside (IPTG) for 3–4 h at 30°C. The cells were collected by centrifugation, resuspended in 6 mL of phosphate-buffered saline (PBS) lysis buffer containing protease inhibitor cocktail [Mini, Boehringer Mannheim], sonicated, and cleared by centrifugation at 12,000g. The soluble GST-fusion proteins were purified from the supernatant by Glutathione Sepharose 4B (Pharmacia) chromatography according to the manufacturer’s instructions.

Phosphorylation and dephosphorylation assays

Transphosphorylation assays were performed by incubating 500 ng (100 µg/mL) of either AtSERK1 mutants or AtSERK1 mutants proteins with 500 ng of KAPP::GST protein in 20 mM Tris [pH 7.5], 50 mM NaCl, 0.01% Triton X-100, 10 mM MgCl2, 1 mM dithiothreitol [DTT], 50 µM unlabelled ATP, and 10 µCi of [γ-32P]ATP in a final volume of 30 µL. 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 by 10% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue (CBB) to verify equal loading and then dried. For dephosphorylation assays, 5 µg of AtSERK1::GST was phosphorylated on Glutathione Sepharose 4B (matrix), and the unbound label was washed away. The matrix-bound phosphorylated AtSERK1::GST was treated with 5 µg of KAPP::GST and incubated at 30°C. The aliquots were taken at time intervals of 30, 60, 90, 120, and 240 min, and the reaction was quenched, proteins separated and stained, and gels were dried as described above. The radioactivity was quantified with a PhosphorImager using the ImageQuant program (Molecular Dynamics).

In vitro binding assays

Immobilized KAPP::GST was phosphorylated with 10 units of bovine heart muscle kinase (Sigma) in 20 mM Tris [pH 7.5], 50 mM NaCl, 10 mM MgCl2, 10 µCi of [γ-32P]ATP and then eluted as described above. The resulting eluent was used to probe the nitrocellulose filter prepared as described below. AtSERK1::GST, AtSERK1 mutants, and AtSERK1 mutants treated with 25 units of alkaline phosphatase (AP) were incubated with [32P]ATP-labeled KAPP::GST probe in BB buffer (50 mM Hepes at pH 7.5, 2.5 mM MgCl2, 7.5 mM KCl, 0.1 mM EDTA and 1% nonfat dry milk). In 2 h at 37°C, the reaction was quenched by adding Laemmli SDS-PAGE sample buffer, boiled at 95°C for 5 min, and separated by 10% SDS-PAGE. The proteins were transferred to a nitrocellulose filter, blocked in HBB buffer (25 mM HEPES at pH 7.5, 5 mM MgCl2, 1 mM KCl, and 5% nonfat dry milk) at 4°C for 4 h, and probed overnight with [γ-32P]ATP-labeled KAPP::GST probe in BB buffer (25 mM HEPES at pH 7.5, 2.5 mM MgCl2, 7.5 mM KCl, 0.1 mM EDTA and 1% nonfat dry milk). After washes in BB buffer, the radioactivity of the filter was quantified with a PhosphorImager using the ImageQuant program.
Phosphoaminoacid analysis

KAPP\(^{KI}\) was phosphorylated by AtSERK\(^{KIN}\) in the presence of [\(^{γ}-^3\)P]ATP and separated by SDS-PAGE as described above. The CBM-stained phosphorylated bands of KAPP\(^{KI}\) were excised from the gel, rehydrated in 50 mM NH\(_4\)CO\(_3\), 0.1% SDS, and 0.25% β-mercaptoethanol (2-ME), ground with a small pestle, boiled for 5 min, and extracted by agitation at 37°C for 90 min. The proteins were precipitated by adding 20 μg of BSA and 20% [w/v] solid trichloroacetic acid (TCA) and incubated overnight at -20°C. The precipitate was collected by centrifugation, and the TCA was removed by lyophilization for 30 min. The sample was hydrolyzed in 50 μL 6M HCl for 1 h at 120°C. The HCl was evaporated, and the pellet was resuspended in the ascending solvent of acetic acid, ethanol, and water at 8:4:2. Phosphoaminoacid standardswere mixed with phosphopeptide buffer containing isobutyl alcohol, glacial acetic acid, and water at 8:4:2. Phosphoaminoacid standards were visualized by spraying the plate with 0.25% ninhydrin in acetone and then heating it at 65°C for 30 min. The radiofilm was exposed to the TLC plate for 12 h, and the radioactivity was quantified with ImageQuant program (Molecular Dynamics).

Construction of YFP/CFP tagged vectors

The entire open reading frame (ORF) of the AtSERK1 cDNA was amplified by polymerase chain reaction (PCR) from an AtSERK1 full-length cDNA and cloned downstream of the 35S promoter into the NcoI site of pMON999-CFP and pMON999-YFP using primers NcoI215f (5'-CATGGATCCATGCATCGATTG-3') and NcoI2068r. This resulted in the NcoI full-length cDNA and cloned downstream of the 35S promoter constructswas also individually mutated into alanine, resulting in the AtSERK1-CFP and AtSERK1-YFP constructs. Thr 462, Thr 463, and Thr 468 residues in the AtSERK1-CFP and AtSERK1-YFP constructs were mutated into alanines by using a Site Directed Mutagenesis Kit (Stratagene) as described previously (Shah et al. 2001a), resulting in the AtSERK1-T462A-CFP and AtSERK1-T462A-YFP constructs. The Thr 463 residue in the AtSERK1-CFP and AtSERK1-YFP constructs was also individually mutated into alanine, resulting in the AtSERK1-T463A-CFP and AtSERK1-T463A-YFP fusions. The AtSERK1 intracellular kinase domain was similarly cloned into the NcoI site of pMON999-CFP and pMON999-YFP using primers nAtSERKC (5'-CATCATGGATCCATGCCAGTCAG-3') and NcoI2068r (5'-CATGGATCCATGCATCGATTG-3'). This resulted in the AtSERK1-CFP and AtSERK1-YFP constructs. The entire ORF of KAPP cDNA was amplified by PCR using primers BglKAP39f (5'-GGAGGATCCATATGGCCGATGATAGG-3') and BglTKPr (5'-GGAGGATCCAGGGAAGTATCGAAATCTCAA-3') and cloned into the BglII site of pMON999-CFP and pMON999-YFP. This resulted in the KAPP-CFP and KAPP-YFP constructs. A 1.6-kb cDNA fragment corresponding to the entire KAPP cDNA missing only the N-terminal signal anchor was amplified by PCR using primers BglKIKAPf and BglTKPr (5'-GGAGGATCCATATGGCCGATGATAGG-3') and cloned into the BglII site of pMON999-CFP and pMON999-YFP. This resulted in the KAPP-KIN-CFP and KAPP-KIN-YFP constructs. Ultimately, this fragment was amplified by PCR using primers BglKIKAPf and BglTKPr (5'-GGAGGATCCATATGGCCGATGATAGG-3') and cloned into the BglII site of pMON999-CFP and pMON999-YFP. This resulted in the KAPP-KIN-CFP and KAPP-KIN-YFP constructs. All constructs were verified by sequencing.

ER-YFP construct, provided kindly by Dr. Jan Carette and Dr. Joan Wellink [Wageningen University, The Netherlands], contains an N-terminal Arabidopsis thaliana basic chitinase signal sequence and a C-terminal HDEL ER retention signal cloned downstream of the 35S promoter of pMON999-YFP vector.

Transient expression in protoplasts

Cowpea mesophyll protoplasts were prepared essentially as described previously (Reselmann et al. 1989) except that 10 mM CaCl\(_2\) was added to the enzyme solution (0.5 M mannitol, 10 mM CaCl\(_2\), 0.1% cellulose, and 0.05% pectinase). Ten to fifteen micrograms of each purified plasmid DNA was added to 0.5-1 x 10\(^6\) protoplasts in 75-150 μL of ice-cold solution C [0.6 M mannitol, 10 mM CaCl\(_2\) at pH 5.3–5.8], mixed by gentle shaking and followed immediately by the addition of 0.5 mL of solution H [40% PEG Mw 6000, 0.6 M mannitol, 0.1 M Ca(NO\(_3\))\(_2\)]. The protoplast suspension was incubated for 5-25 sec with gentle shaking followed by the addition of 4.5 mL of solution M [0.5 M mannitol, 15 mM MgCl\(_2\) and 0.1% MES at pH 5.3–5.7] and incubated at room temperature for 20 min. Protoplasts were then washed 3 times with solution C, resuspended in protoplast medium containing 0.6 M mannitol, and incubated for 8–24 h as described (Rottier et al. 1979).

Double labeling with AtSERK\(^{T462A,YFP}\) and FM4-64

Cowpea mesophyll protoplasts 12 h after transfection with AtSERK\(^{T462A,YFP}\) were incubated on ice for 10 min with 50 μM FM4-64 (Molecular Probes). After labeling, the cells were washed twice and resuspended in protoplast medium. The protoplasts were incubated for 15 min to 3 h at 26°C and viewed by confocal laser microscopy.

SDS-PAGE and Western blotting

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

Colocalization studies using confocal microscopy

The intracellular fluorescence and the membrane fluorescence were analyzed with a CLSM 510 confocal laser scanning microscope [Carl-Zeiss]. Excitation was provided by the 458 and 514 nm Ar laser lines controlled by an acousto-optical tuneable filter (AOTF). Three dichroic beam splitters were used to separate excitation from emission and to divide the fluorescence emis-
Interaction between AtSERK1 and KAPP proteins

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

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

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

Carey, K.L., Richards, S.A., Lounsbery, K.M., and Macara, I.G. 1996. Evidence using a green fluorescent protein-glucocorticoid receptor chimera that the Ran/TC4 GTPase mediates an essential function independent of nuclear protein import. J. Cell Biol. 133:985–996.

Clark, S.E., Williams, R.W., and Meyerowitz, E.M. 1997. The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. Cell 89:575–585.

Cohen, P. 1989. Structure and regulation of protein phosphatases: Variety is the spice of life. Trends Biochem. Sci. 22:245–251.

Gadella, Jr., T.W.J., Van der Kogt, G.N.M., and Bisseling, T. 1999. GFP-based FRET microscopy in living plant cells. Trends Plant Sci. 4:287–291.

Gerdes, H.H. and Kaether, C. 1996. Green fluorescent protein: Applications in cell biology. FEBS Lett. 389:44–47.

Goedhart, J. and Gadella, Jr., T.W.J. 2000 Advanced fluorescence microspectroscopic methods for the study of single living root hairs. In Root hairs: Cell and molecular biology (eds. R.W. Ridge and A.M.C. Emmons), pp. 65–94. Springer-Verlag, Tokyo.

Haj, F.G., Vermeer, P.J., Squire, A., Neel, B.G., and Bastianens, P.H.I. 2002. Imaging sites of receptor dephosphorylation by FFP1IB on the surface of the endoplasmic reticulum. Science 295:1708–1711.

Hecht, V., Vielle-Calzada, J.P., von Recklinghausen, I., Hartog, M.V., Schmidt, E.D.L., Boutillier, K., Grossniklaus, U., and de Vries S.C. 2001. The Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. Plant Physiol. 127:803–816.

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

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

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

Li, J., Smith, G.P., and Walker, J.C. 1999. Kinase interaction domain of kinase-associated protein phosphatase, a phospho-protein-binding domain. Proc. Natl. Acad. Sci. 96:7821–7826.

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

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

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

Rottier, P.J, Rezelman, G., and van Kammen, A. 1979. The inhibition of cowpea mosaic virus replication by actinomycin D. Virology 92: 299–309.

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

Shah, K., Vervoort, J., and de Vries S.C. 2001a. Role of threonines in the AtSERK1 activation loop in phosphorylation. J. Biol. Chem. 276: 41263–41269.

Shah, K., Gadella, T.W.J., van Erp, H., Hecht, V., and de Vries S.C. 2001b. Subcellular localization and dimerization of the A. thaliana somatic embryogenesis receptor kinase 1 protein. J. Mol. Biol. 309: 641–655.

Shenolikar, S. and Nairn, A.C. 1991. Protein phosphatases: Recent progress. Adv. Sec. Messenger Phosphoprotein Res. 23: 1–121.

Sorkin, A., McClure, M., Huang, F., and Carter, R. 2000. Interaction of EGF receptor and Grb2 in living cells visualized by fluorescence resonance energy transfer FRET microscopy Curr. Biol. 10: 1395–1398.

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

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

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

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

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

Ueda, T., Yamaguchi, M., Uchimiya, H., and Nakano, A. 2002. Ara6, a plant-unique novel type Rab GTPase, functions in the endocytic pathway of Arabidopsis thaliana. EMBO J. 21: 4730–4741.

Ullrich, A. and Schlessinger, J. 1990. Signal transduction by receptors with tyrosine kinase activity Cell 61: 203–212.

Vervoer, P.J., Wouters, F.S., Reynolds, A.R., and Bastiaens, P.I. 2000. Quantitative imaging of lateral ErbB1 receptor signal propagation in the plasma membrane. Science 290: 1567–1570.

Vida, T.A. and Emr, S.D. 1995. A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. J. Cell Biol. 128: 779–792.

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

Williams, R.W., Wilson, J.M., and Meyerowitz, E.M. 1997. A possible role for kinase associated protein phosphatase in the
The *Arabidopsis* kinase-associated protein phosphatase controls internalization of the somatic embryogenesis receptor kinase 1

Khalid Shah, Eugenia Russinova, Theodorus W.J. Gadella, Jr., et al.

*Genes Dev.* 2002, 16: 2002, Genes Dev. 16:
Access the most recent version at doi:10.1101/gad.220402

References
This article cites 34 articles, 15 of which can be accessed free at: http://genesdev.cshlp.org/content/16/13/1707.full.html#ref-list-1

License

Email Alerting Service
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.