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Arabidopsis thaliana Somatic Embryogenesis Receptor Kinase 1 protein is present in sporophytic and gametophytic cells and undergoes endocytosis

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Summary. *Arabidopsis thaliana* plants expressing AtSERK1 fused to yellow-fluorescent protein were generated. Fluorescence was detected predominantly at the cell periphery, most likely the plasma membrane, of cells in ovules, embryo sacs, anthers, and embryos and in seedlings. The AtSERK1 protein was detected in diverse cell types including the epidermis and the vascular bundles. In some cells, fluorescent receptors were seen in small vesicle-like compartments. After application of the fungal toxin Brefeldin A, the fluorescent receptors were rapidly internalized in the root meristem and root vascular tissue. We conclude that the AtSERK1 receptor functions in a common signalling pathway employed in both sporophytic and gametophytic cells.

Keywords: AtSERK1 protein; Fluorescence; Gametophyte; Sporophyte; Endocytosis; Brefeldin A.

Abbreviations: BFA Brefeldin A; CHX cycloheximide; CLSM confocal laser scanning microscopy; LRR leucine-rich repeat; MMC megaspore mother cell; RLK receptor-like kinase; YFP yellow-fluorescent protein.

Introduction

Membrane-localized receptor-like kinases (RLKs) bind extracellular ligands and convey signals inside the cell by initiating signalling cascades that usually result in altered gene expression. Analysis of the sequenced *Arabidopsis thaliana* genome revealed that more than 400 genes are predicted to encode RLKs and that leucine-rich repeat (LRR)-containing RLKs are the largest class, containing over 200 genes (Arabidopsis Genome Initiative 2000; Shiu and Bleeker 2001, 2003). For only few members of the LRR-RLK family a biological function has been reported. For example, CLAVATA1 (Clark et al. 1997), BRI1 (Li and Chory 1997), and ERECTA (Yokoyama et al. 1998) have functions in shoot meristem maintenance, hormone perception, and regulation of organ size, respectively.

Arabidopsis thaliana Somatic Embryogenesis Receptor Kinases (AtSERKs) represent a small family of five closely related LRR-RLKs (AtSERK1 to -5). The extracellular domain of SERK receptors is predicted to contain an N-terminal leucine zipper, 5 LRRs, and a proline-rich domain (SPP), which is unique for this family of receptors. A single membrane-spanning domain links the extracellular part to the intracellular serine/threonine kinase domain (Hecht et al. 2001).

The AtSERK1 function is implemented in early embryo development as the expression of the gene marks cells competent to form embryos while over-expression of the protein increases the efficiency for initiation of somatic embryogenesis in culture (Hecht et al. 2001).

The AtSERK3 protein is also known as BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) and has a role in brassinosteroid (BR) signalling. Genetic and molecular data support the notion that AtSERK3 is a part of the BR receptor complex and functions as a coreceptor of BRASSINOSTEROID INSENSITIVE 1 (BRI1) (Li et al. 2002, Nam and Li 2002). Recently it was shown that in living plant cells, BRI1 and AtSERK3 undergo continuous endocytosis and form heterodimers mainly in endosomal compartments. Furthermore, coexpression of BRI1 and BAK1 in the same protoplast resulted in an accelerated internalization by endocytosis. Endocytic vesicles containing either one or both of the receptors were observed, suggesting receptor-specific sorting (Russinova et al. 2004).

In vitro, AtSERK1 interacts in a phosphorylation-dependent manner with a number of proteins including the

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kinase-associated protein phosphatase (KAPP) (Shah et al. 2002), CDC48 and 14-3-3λ (Rienties et al. 2005). KAPP was previously found to interact with other LRR-RLKs such as HEASA (Stone et al. 1994) and CLAVATA1 (Stone et al. 1998). The same interactions were demonstrated in live cells by using in-frame fusions of AtSERK1, KAPP, or 14-3-3λ with either cyan-fluorescent protein (CFP) or yellow-fluorescent protein (YFP) transiently expressed in cowpea protoplasts. Detection of Förster resonance energy transfer (FRET) between the CFP and YFP groups verified that AtSERK1 and KAPP interact after receptor internalization (Shah et al. 2002) and 14-3-3λ interaction occurs at the plasma membrane (Rienties et al. 2005). FRET studies also showed that AtSERK1 protein is present as a homodimer in the plasma membrane of living cells (Shah et al. 2001, Russinova et al. 2004).

In order to provide a background for studies of receptor homo- or heterodimerization and interaction with other components of the signalling complex in plants, a detailed knowledge of the localization of the receptor protein is essential. So far, the expression pattern of *AtSERK1* gene in *A. thaliana* has been studied by reverse transcriptase-polymerase chain reaction, by AtSERK1 promoter-β-glucuronidase (GUS) fusions and by in situ hybridization analysis (Hecht et al. 2001). The *AtSERK1* gene is expressed during megasporogenesis and in all cells of the embryo sac up to the stage of fertilization. After fertilization, *AtSERK1* promoter-driven GUS activity is found in all cells of the developing embryo up to the heart stage. Furthermore, a weak *AtSERK1* GUS expression is found in the vascular tissue of seedlings. In plant tissue culture, *AtSERK1* is expressed during embryogenic cell formation (Hecht et al. 2001).

This study presents a detailed analysis of the cellular and subcellular localization of the AtSERK1 protein fused in-frame with YFP and stably expressed in *A. thaliana* plants under the control of its own promoter. We used confocal laser scanning microscopy (CLSM) to localize the protein in different tissues and during plant development. The localization pattern of AtSERK1-YFP during ovule, anther, and lateral-root development is discussed. Our observations reveal a complex pattern of receptor protein localization in the plasma membrane and in intracellular vesicle-like compartments as a result of receptor endocytosis.

Material and methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Colombia (Col-0) was used in this study as a wild type. *Arabidopsis thaliana* seeds were sterilized for a few minutes in 70% ethanol followed by a 10 min incubation in 5× diluted commercial bleach solution containing 0.5% Tween 20 (Sigma) and finally washed 3 times with sterile water. Seeds were germinated on 0.5× Murashige and Skoog (MS) salt medium (Duchefa) supplemented with 0.8% (w/v) agar (Daishin) and 2% (w/v) sucrose and then transferred to a growth chamber. Plants were grown at 22 °C under a photoperiod of 16 h light and 8 h dark until flowering.

AtSERK1-YFP construct and plant transformation

The 2 kb promoter region of *AtSERK1* gene (Hecht et al. 2001) was cloned into pCAMBIA1390 vector (CAMBIA) by directional cloning using *Sal*I and *Nco*I restriction sites. The full-length *AtSERK1* cDNA fused to CFP or YFP was PCR amplified from the pMON999-AtSERK1-CFP or YFP constructs (Shah et al. 2001) using primers AtSERK1F-*Spe*I GACTAGTATGGAGT CGAGTTATGTGT and YFPR-*Spe*I GACTAGTCTTGTACAGCTCGTC CATG. The PCR fragment was then inserted into the *Spe*I site of pCAMBIA1390 containing the *AtSERK1* promoter. The construct was verified by DNA sequencing before being used for plant transformation.

Arabidopsis thaliana plants were transformed by vacuum infiltration and further selected on antibiotics as described in Hecht et al. (2001). T2 or T3 homozygous plants containing a single insertion were used for CLSM imaging.

Brefeldin A and cycloheximide treatments of seedlings

10-day-old seedlings were incubated for 1 h in 0.5× MS medium containing 50 μM cycloheximide (CHX) (Sigma) from a stock solution of 50 mM in water. Brefeldin A (BFA) (Sigma) was added to a final concentration of 50 μM from a stock solution of 18 mM in dimethyl sulfoxide. Seedlings were incubated for up to an hour with both chemicals. To wash out the BFA, the seedlings were incubated in 0.5× MS containing CHX for 1 to 2 h.

Plasmolysis

Ovules were isolated from the carpel, plasmolysed in 1 M NaCl, and directly imaged by CLSM.

Aniline blue staining

Aniline blue staining was essentially done as described by Bougourd et al. (2000).

Enzymatic digestion of ovules

Ovules after stage 3-II in development were removed from the pistil and transferred to 0.5× MS medium containing 1.5% cellulose R-10 (Yakult Honsha Co. Ltd) and 0.1% pectinase (Biochemica), after 30 min of vacuum infiltration the samples were incubated for 2 h at room temperature with mild agitation. For imaging, ovules were pressed between slides to release the embryo sac.

CLSM imaging

The live plant material was transferred into a drop of 0.5× MS medium containing 2% (w/v) sucrose on a microscope glass slide and covered with a coverslip. To image the ovules, the carpel was isolated from the flower and pressed between the slide and the cover to release the ovules. The expression of AtSERK1-YFP was studied with a Zeiss LSM510 CLSM. A 40× oil objective was used with a numerical aperture of 1.3. The YFP was excited using the 514 nm argon laser line, and the emitted light was filtered through a 535–590 nm bandpass filter. Pictures were edited by Adobe Photoshop 6.0 (Adobe).

Western blot analysis

Total protein extracts from seedlings were prepared by grinding seedlings in liquid nitrogen. Extraction buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail (1 tablet per 25 ml extraction buffer) (Roche) was added and the samples were incubated on ice for half an hour. The extract was centrifuged and the supernatant was analyzed further. The protein concentration was determined with Bio-Rad protein assay (Bio-Rad), and 20 to 30 μg of total protein was loaded per lane in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, in which an 8% gel was used. The Bio-Rad precision plus dual colour protein marker was used to estimate protein sizes. The proteins were analyzed by Western blot with anti-GFP (immunoglobulin G fraction, 1500× diluted; Molecular Probes) and a polyclonal antibody generated against the GST-AtSERK1 kinase domain fusion (5000× diluted); anti-rabbit horseradish peroxidase (immunoglobulin G purified, 10000× diluted; Rockland Laboratories) was the secondary antibody, and the horseradish peroxidase was detected with ECL plus (Amersham).

Results

Expression of AtSERK1 protein in planta

To examine the expression pattern and the subcellular localization of AtSERK1 in living cells by fluorescence microscopy, AtSERK1 was tagged at the C terminus with YFP or CFP and then stably expressed in *A. thaliana* wild-type plants under the control of the AtSERK1 promoter. Expression of AtSERK1-YFP or AtSERK1-CFP did not result in any unusual phenotype, suggesting that the fusion did not interfere with the endogenous protein. The CFP and YFP versions of the protein fusion showed an identical localization in *A. thaliana* plants. The data obtained with the YFP fusion of AtSERK1 will be presented, because of better image quality due to the higher brightness of YFP compared to CFP. To verify the presence of the AtSERK1-YFP protein in the transgenic lines, total protein was extracted from 10-day-old seedlings and compared with protein from wild-type seedlings on immunoblot (Fig. 1). The AtSERK1-YFP protein was detected with an anti-GFP antibody (Fig. 1, lane 1) and with an anti-AtSERK1-kinase domain antibody in a separate blot (Fig. 1, lane 3). A band above the 100 kDa marker band was present only in the AtSERK1-YFP expressing line and corresponded to the fusion protein, which was recognized by both antibodies (Fig. 1, lanes 1 and 3). The anti-AtSERK1-kinase domain antibody recognized two extra bands with a molecular mass of around 75 kDa in both the wild type and the transgenic line possibly representing more AtSERK family members or other related kinases (Fig. 1, lanes 3 and 4). The recognition of more than one protein also explains the higher intensity of the 75 kDa bands when compared with the >100 kDa AtSERK1-YFP band.

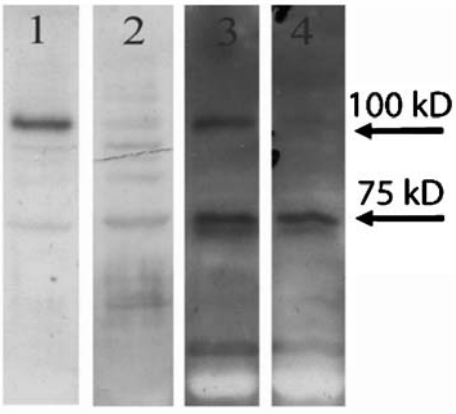

Fig. 1. Immunoblot analysis of AtSERK1 protein. Total protein extracts from AtSERK1-YFP-expressing seedlings (*1*) and wild-type seedlings (*2*) detected with anti-GFP antibody. In a separate blot, total protein extracts from AtSERK1-YFP-expressing seedlings (*3*) and wild-type seedlings (*4*) were detected with anti-AtSERK1-kinase domain antibody. The sizes of detected proteins are indicated by arrows

Localization of AtSERK1-YFP protein during ovule development

Northern blot, in situ hybridization, and AtSERK1 promoter GUS analysis have previously shown that in planta *AtSERK1* mRNA was highly abundant before meiosis during ovule development and later in both gametophytic and sporophytic tissues of the female gametophyte (Hecht et al. 2001). Therefore, the localization of the fluorescently labelled AtSERK1 protein was first imaged at different stages of ovule development according to Schneitz et al. (1995). The AtSERK1-YFP protein was first detected when the ovule primordia, visible as protrusions, extended from the placental tissue of the carpel (stage 1-II) (Fig. 2A) and later when the protrusions elongated and the nucellus, the chalaza, and the funiculus were distinguishable along the proximal-distal axis (stage 2-I) (Fig. 2B). The protein was present in all cells of stage 1-II ovules (Fig. 2A). AtSERK1-YFP was detected at stages 2-II and 2-III, when the inner and the outer integuments are initiated (Fig. 2C, D). At stages 2-I to 2-IV the chalazal end of the nucellus showed more AtSERK1-YFP fluorescence than the other tissues of the ovule (compare Fig. 2B, C, and D with Fig. 2A, F, and G). At stage 2-IV, when the nucellus enlarges and subsequently the first meiotic division of the megaspore mother cell (MMC) takes place, giving rise to four megaspore cells in stage 2-V, AtSERK1-YFP was seen in all cells including the functional megaspore (Fig. 2E, F). To demonstrate that the AtSERK1 receptor is located in the membrane of the MMC at stage 2-IV, we employed aniline blue staining

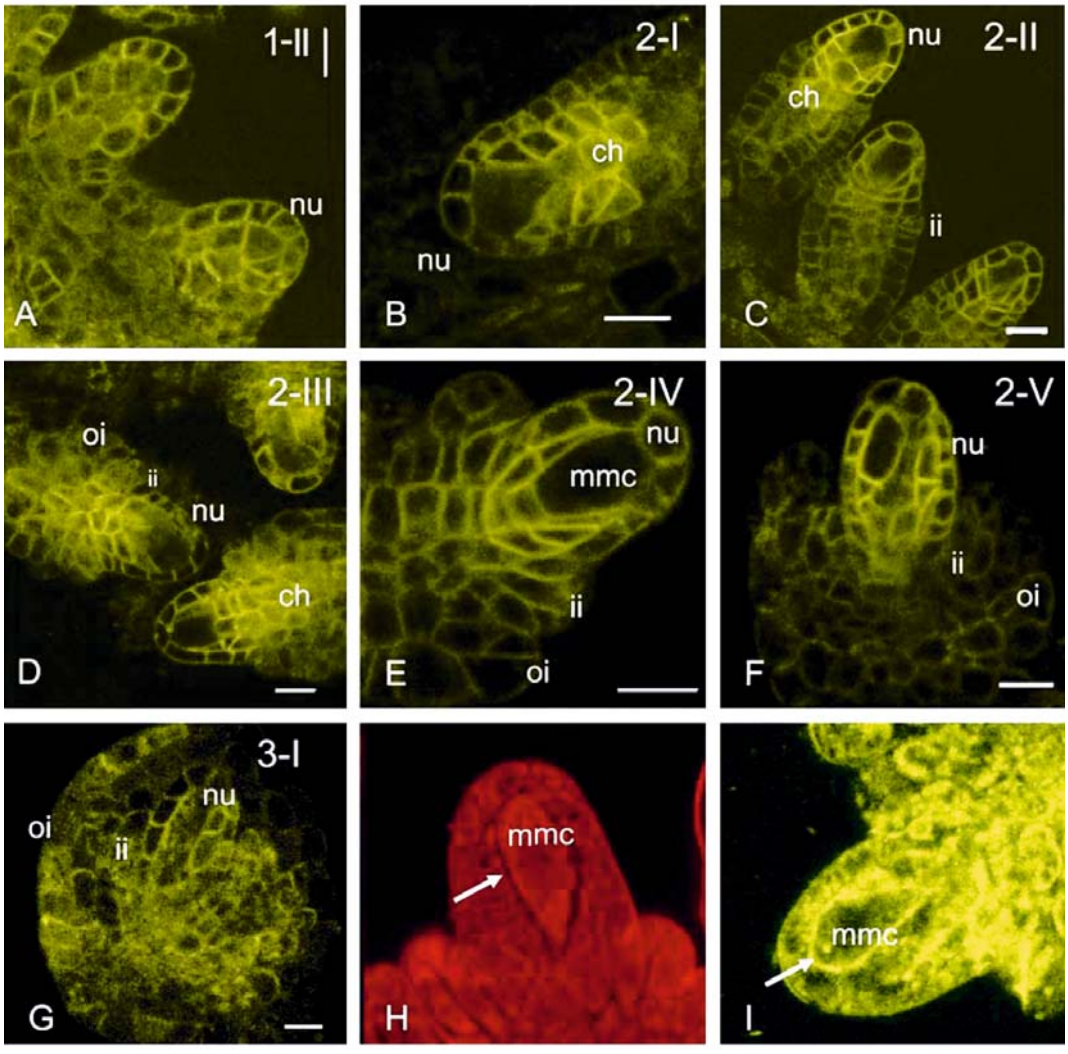

Fig. 2 A–I. Localization of AtSERK1-YFP during ovule development. Confocal images of developing ovules expressing AtSERK1-YFP at stage 1-II (**A**); stage 2-I (**B**); stage 2-II (**C**), at which the inner integument initiates; stage 2-III (**D**), at which the outer integument initiates; stage 2-IV (**E**), at which the megaspore mother cell enlarges; and stages 2-V (**F**) and 3-I (**G**). **H** Aniline blue staining of the megaspore mother cell (arrow). **I** Ovule after plasmolysis. Megaspore mother cell is indicated by an arrow. *oi* Outer integument; *ii* inner integument; *nu* nucellus; *ch* chalaza; *mmc* megaspore mother cell. Bars: 10 μm

(Fig. 2H) and mild plasmolysis (Fig. 2I). The anilin58e blue stained the MMC more intensely compared to rest of the ovule as seen in Fig. 2H. Figure 2I clearly shows AtSERK1-YFP fluorescence located to the cell periphery in the MMC. AtSERK1-YFP fluorescence was also detected in the outer and in the inner integuments at stage 3-I (Fig. 2F, G). However, the amount of fluorescence was lower when compared to that in the nucellus.

After stage 3-I, the outer integument completely envelops the embryo sac, making it impossible to image the structures within the embryo sac directly. In this case, the outer cell layers of the ovule were removed by mild enzymatic digestion and imaged directly by CLSM. Figure 3A shows an embryo sac containing three antipodal cells at the chalazal end of the embryo sac with AtSERK1-YFP fluorescence. The fluorescence visible in Fig. 3C represents the central cell as confirmed by the bright-field image in Fig. 3B. An embryo sac is also shown in Fig. 3D, wherein an AtSERK1-YFP fluorescently labelled central cell is visible. In parallel experiments, the amount of background fluorescence was imaged in wild-type ovules (Fig. 3E), illustrating that the fluorescence visible in the AtSERK1-YFP transgenic lines indeed originated from the fusion protein. The endothelium of wild-type ovules after stage 3-IV generated a substantial amount of autofluorescence as seen in Fig. 3F.

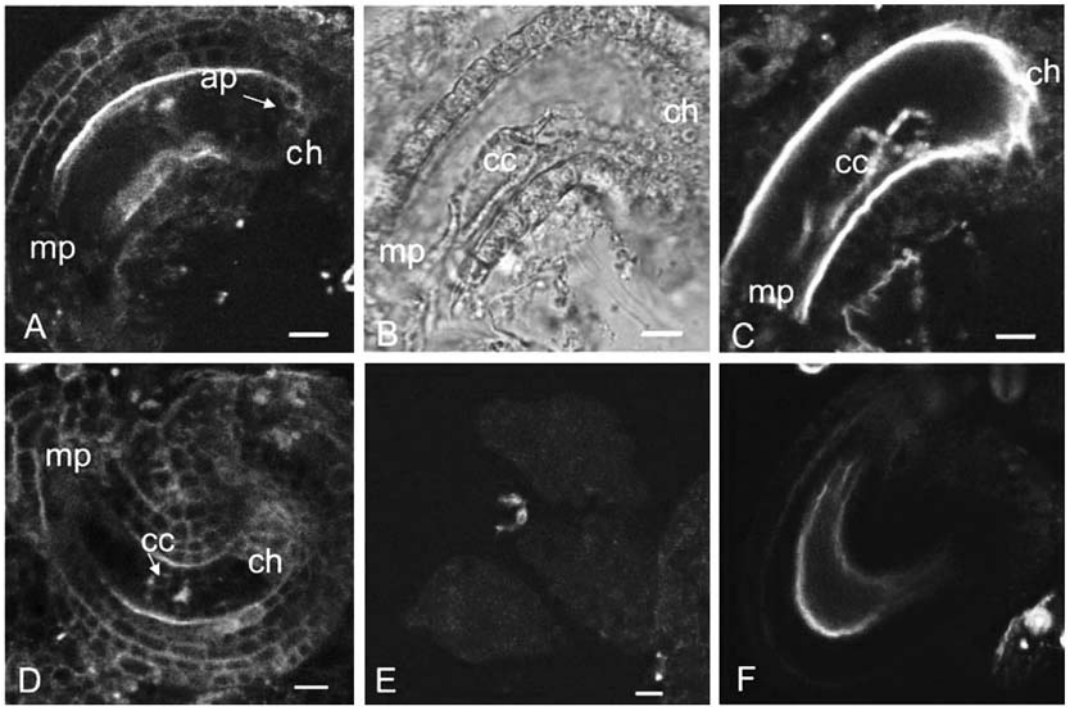

Fig. 3 A–F. Localization of AtSERK1-YFP during megagametogenesis. **A** Confocal image shows an embryo sac with fluorescent antipodal cells (*ap*) indicated by an arrow. **B** Confocal bright-field image of an embryo sac with visible central cell (*cc*) and the corresponding fluorescence image in **C**. **D** Confocal image of an embryo sac with a fluorescent central cell (*cc*) indicated by an arrow. **E** and **F** Background fluorescence in wild-type stage IV ovules (**E**) and autofluorescence of the endothelium of mature ovule (**F**). *mp* Micropyle; *ch* chalaza; *cc* central cell; *ap* antipodal cell. Bars: 10 μm

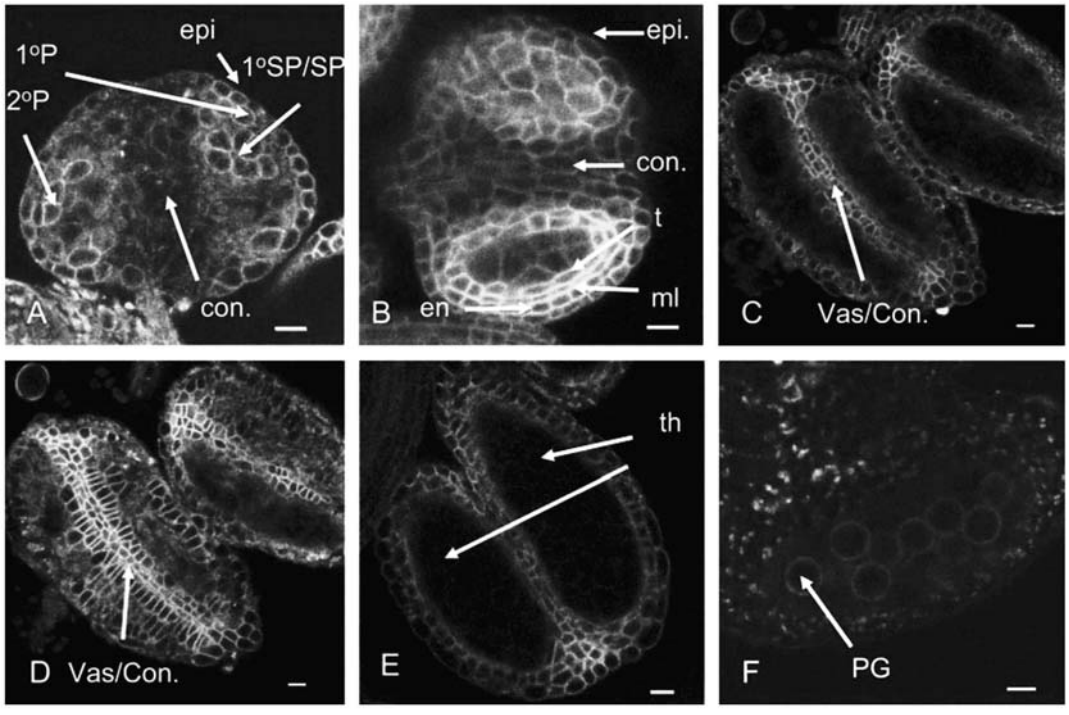

Fig. 4 A–F. Localization of AtSERK1-YFP during anther development. Confocal images of anthers at stage 3 (**A**), stage 4 (**B**), stage 6 (**C** and **D**), and stages 7 to 8 (**E**). **F** Autofluorescence from wild-type pollen grains is shown. *1°P* Primary parietal cell layer; *2°P* secondary parietal cell layer; *1°SP/SP* primary sporogenous layer and sporogenous cells; *con* connective tissue; *epi* epidermis; *t* tapetum; *ml* middle layer; *en* endothecium; *th* thecae; *PG* pollen grains. Bars: 10 μm

Localization of AtSERK1-YFP protein during anther development

AtSERK1 promoter GUS analysis showed expression of *AtSERK1* in the anther (Hecht et al. 2001). The AtSERK1-YFP protein was detected from at least stage 3 of anther development according to Sanders et al. (1999). Figure 4A shows a stage 3 anther with AtSERK1-YFP fluorescence in the epidermis, primary and secondary parietal cell layers, primary sporogenous layer, and possibly in the sporogenous cells; no protein was found in the connective tissue. At stage 4 to 5, AtSERK1-YFP fluorescence was brighter in the tapetum, the middle layer, and the endothecium compared to that in the other anther tissues (Fig. 4B). At later stages (Fig. 4C–E), the protein was present in all cells surrounding the thecae, but more fluorescence was always seen in the vascular and connective tissue separating the thecae, as indicated by arrows in Fig. 4C and D. When the amount of AtSERK1-YFP fluorescence in transgenic pollen grains was compared with the autofluorescence generated by the wild-type pollen grains, no difference in the fluorescence intensity was found, which indicates that AtSERK1-YFP is either not present in pollen grains or cannot be distinguished from autofluorescence (Fig. 4F).

Localization of AtSERK1-YFP during embryo development

AtSERK1-YFP protein localization was further determined during embryo development. Studies by Hecht et al. (2001) showed that *AtSERK1* gene expression continued after fertilization and persisted in all cells of the embryo and the endosperm nuclei. Figure 5A shows a single cell at the micropylar pole of the embryo sac with AtSERK1-YFP fluorescence at the periphery, most likely representing the zygote. Consecutive stacked images (z-stacks) through the embryo sac are shown in Fig. 5BI–III, where a globular stage embryo exhibiting AtSERK1-YFP fluorescence is visible. Interestingly, the protein only appears to be present in the outer cell layer of the embryo at this stage. We were not able to image embryos between late globular and heart stages due to limitations of the penetration depth of the CLSM in the immature seed. Figure 5C shows a late-torpedo-stage embryo removed manually from the developing seed. AtSERK1-YFP fluorescence is seen mainly in the epidermal cells of the embryonic root, excluding the columella root cap (Fig. 5D), and in the root vascular tissue (data not shown). AtSERK1-YFP fluorescence appears to be present in most cells of the cotyledons and to be

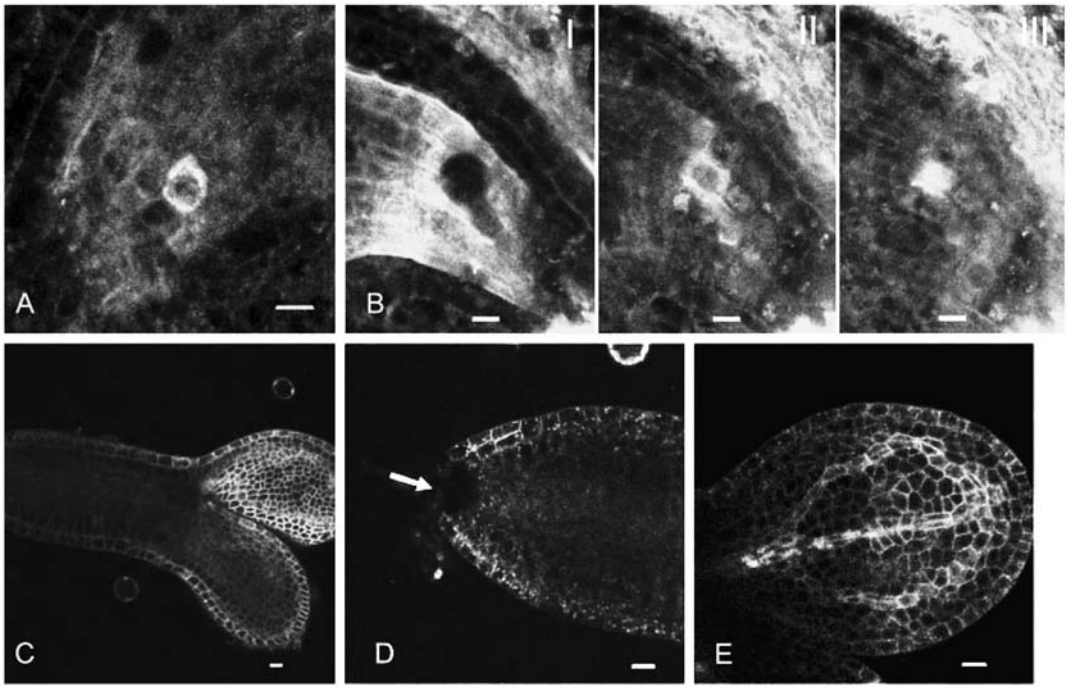

Fig. 5A–E. Localization of AtSERK1-YFP during embryo development. **A** Confocal image of an embryo sac containing most likely a fertilized zygote; **B** z-stack through an embryo sac containing globular stage embryo (I–III). **C–E** Confocal images of a late-torpedo-stage embryo (**C**), the root tip (**D**), and the cotyledon of late-torpedo-stage embryo (**E**). Bars: 10 μm

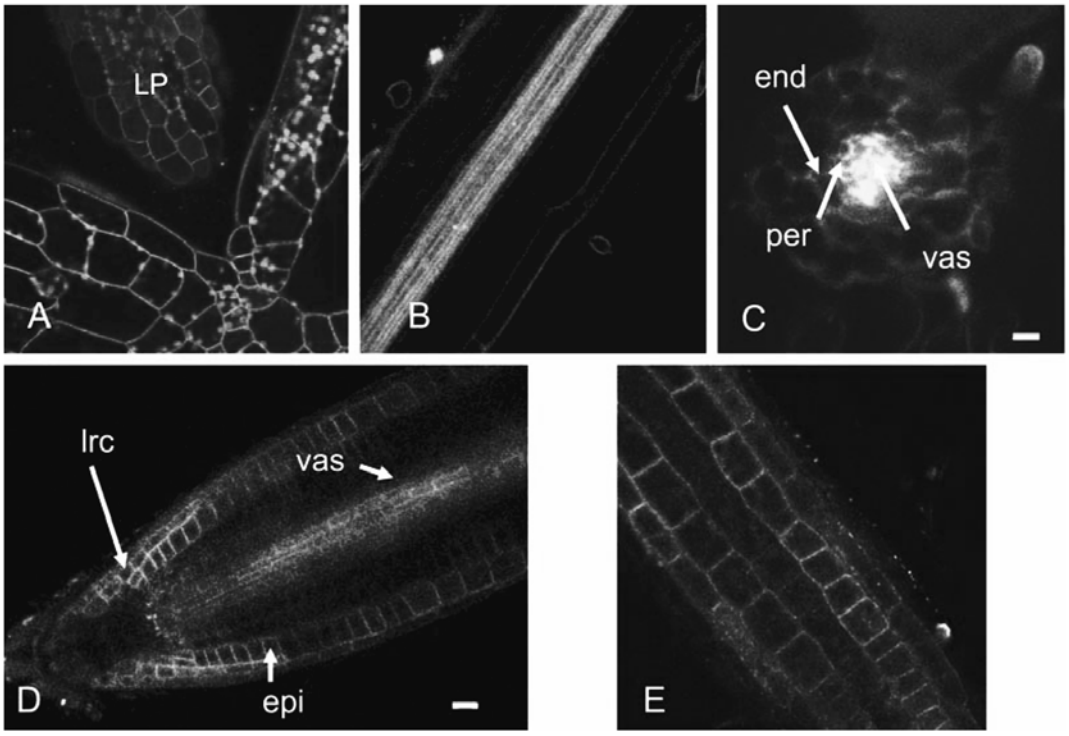

Fig. 6 A–E. Localization of AtSERK1-YFP in seedlings. Confocal images showing AtSERK1-YFP fluorescence in cotyledons and leaf primordia (**A**); in root vascular tissue shown in optical longitudinal (**B**) and in optical cross sections (**C**). **D** AtSERK1-YFP fluorescence in primary root tip. **E** AtSERK1-YFP fluorescence in the epidermis of a primary root. *LP* leaf primordium; *per* pericycle; *vas* vascular tissue; *epi* epidermis; *lrc* lateral-root cap; *end* endodermis. Bars: 10 μm

brighter in the epidermal cells and the embryonic vascular tissue (Fig. 5E).

Localization of AtSERK1-YFP protein during postembryonic development

In 7-day-old seedlings, AtSERK1-YFP fluorescence was observed in newly formed leaf primordia and at the adaxial side of the cotyledons (Fig. 6A). In the root, AtSERK1-YFP protein was present in both the vascular bundle and the pericycle as shown in Fig. 6B and C, respectively. In most cells of the vascular tissue the protein appeared to be located at the cell periphery as in Fig. 6D, in which an enlargement of the primary root tip is shown. In addition, AtSERK1-YFP is present in the epidermis and cells of the lateral-root cap. Figure 6E shows epidermal cells with AtSERK1-YFP fluorescence in the transition zone of the root, where the cells undergo both division and elongation. Fully elongated root cells generated a large amount of autofluorescence and this made it impossible to distinguish AtSERK1-YFP fluorescence from autofluorescence in the epidermis. The vascular tissue could not be clearly imaged in tissues other than the root.

AtSERK1-YFP fluorescence was also examined during lateral-root formation. Different developmental stages of the lateral root were determined according to Malamy and Benfey (1997) and Casimiro et al. (2003). AtSERK1-YFP was present in lateral roots from at least stage IV and the fluorescent signal was brighter in developing lateral roots when compared to that of the primary and mature lateral roots. AtSERK1-YFP fluorescence was present in all cells of the stage IV lateral-root primordium (Fig. 7A). Stronger signal was present in the vascular tissue of the lateral root compared to that in other lateral-root tissues (Fig. 7A). At stage VIII (Fig. 7B), after emergence of the lateral root through the epidermis of the primary root, AtSERK1-YFP fluorescence was found in the vascular bundle, the epidermis, and the initials of the lateral-root cap and epidermis. Fluorescence intensity was less in the root cap compared to that in the surrounding tissues. No or little AtSERK1-YFP fluorescence was found in the quiescent center, cortex, and endodermis. After emergence of the lateral root, the lateral-root meristem is activated and cells at the tip start to divide. Cells at the base enlarge only and their number stays essentially the same. Figure 7C shows a lateral root after activation of the lateral-root meristem. In the

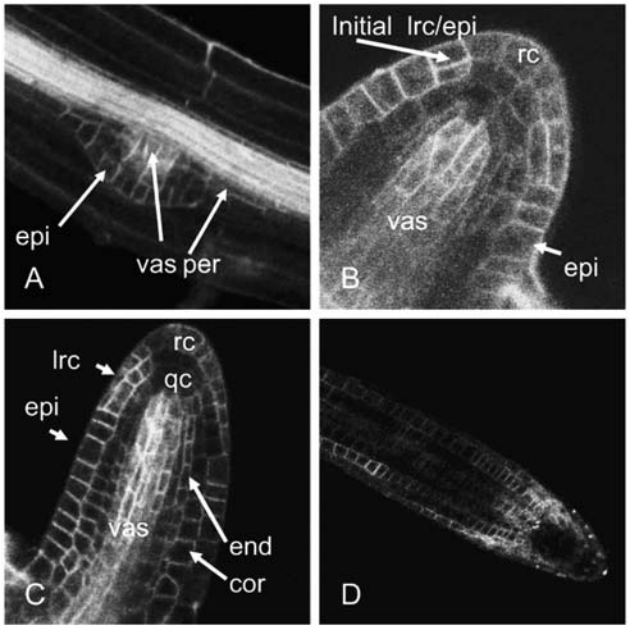

Fig. 7 A–D. Localization of AtSERK1-YFP during lateral-root formation. Confocal images of stage IV preemergence of the lateral-root primordium (**A**), emerged lateral root (**B**), lateral-root formation after meristem activation (**C**), and mature lateral root (**D**). *qc* quiescent center; *epi* epidermis; *vas* vascular tissue; *cor* cortex; *rc* root cap; *lrc* lateral root cap; *per* pericycle; *end* endodermis

lower part of the lateral root, AtSERK1-YFP fluorescence was essentially present in all cells visible. Near the tip AtSERK1-YFP was absent from the quiescent center, the root cap initials, and the cortex. Later in lateral-root development, the localization of AtSERK1-YFP became increasingly similar to that in the primary root (Fig. 7D).

Internalized AtSERK1-YFP protein in roots

In a number of cells expressing the AtSERK1-YFP construct, intracellular, rapidly moving vesicle-like compartments containing the fluorescent receptor were observed. This occurred in ovules (Fig. 8A) as well as in roots (Fig. 8B, C) and was similar to the previously described endosomal localization of the BRI1-GFP receptor in *A. thaliana* (Russinova et al. 2004). To show that AtSERK1-YFP also localizes to the endosomal compartments, roots were treated with the fungal toxin BFA. When exogenously applied to *A. thaliana* roots, BFA reversibly inhibits a number of large ADP ribosylation factor-GTP exchange factors (ARF-GEFs) that regulate vesicle trafficking to the plasma membrane. This results in rapid internalization of plasma membrane proteins into so-called BFA compartments (reviewed by Geldner 2004). AtSERK1-YFP expressing seedlings were treated with BFA in the presence of CHX to prevent de novo protein synthesis and the primary roots were imaged by CLSM. Figure 9A shows meristematic and Fig. 9D shows differentiated parts of a root in the presence of CHX, where the AtSERK1-YFP fluorescence was localized to the periphery of the epidermal and vascular cells, respectively. After BFA treatment, AtSERK1-YFP fluorescence was found in internal aggregates corresponding to the BFA compartments in both tissue types (Fig. 9B, E), suggesting that AtSERK1 undergoes recycling possibly via endosomal compartments. The BFA effect was completely reversible as the original situation was restored in both epidermal (Fig. 9C) and vascular (Fig. 9F) tissues of the root after the BFA was washed out in the presence of CHX.

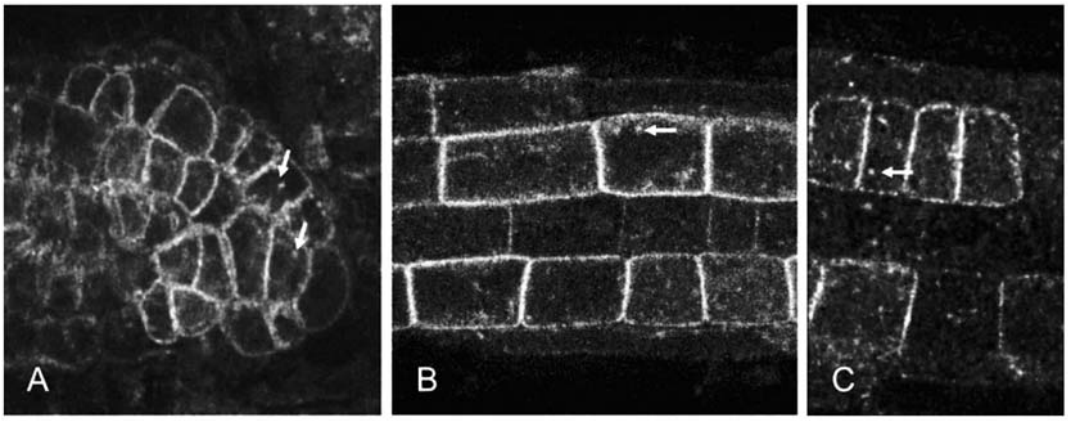

Fig. 8 A–C. Internalized AtSERK1-YFP in vesicle-like compartments. Confocal images of AtSERK1-YFP fluorescence in vesicle-like structures in stage 2-I ovules (**A**) and in the epidermal layer in the transition zone of the root meristem (**B** and **C**). Arrows point to the vesicle compartments

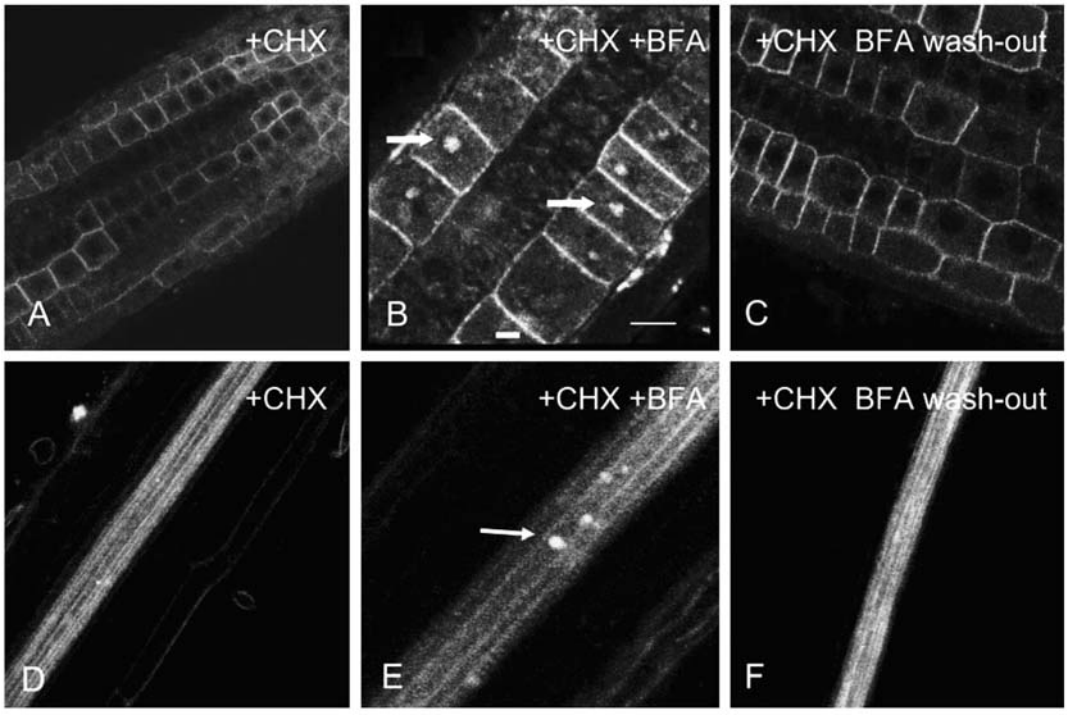

Fig. 9A–F. CHX and BFA treatment of AtSERK1-YFP transgenic roots. Confocal images of the epidermal layer of the root meristem (**A–C**) and the root vascular tissue (**D–F**) in the presence of CHX (**A** and **D**), after treatment with BFA (**B** and **C**), and after washout of the BFA (**C** and **F**). Note the BFA compartment indicated by arrows

Discussion

AtSERK1-YFP localization during plant development

In this study we used CLSM to localize the fluorescently tagged AtSERK1 protein in living *A. thaliana* cells. Because the AtSERK1-YFP construct was expressed under the control of the *AtSERK1* promoter, we considered that the localization of the fluorescent receptor represented the endogenous AtSERK1 protein. Previously, the expression pattern of the *AtSERK1* gene was determined by promoter GUS studies and in situ mRNA localization (Hecht et al. 2001). In general, the same tissue types and developmental stages at which *AtSERK1* mRNA was detected accumulated the corresponding AtSERK1-YFP protein. Due to the much improved cellular and subcellular resolution of the fluorescent-protein localization techniques, we could further refine and extend the previous observations.

AtSERK1-YFP protein was detected during both ovule and pollen development. At early stages of ovule development, all sporophytic cells accumulated AtSERK1-YFP protein. Following the enlargement of the MMC in the nucellus, AtSERK1-YFP accumulated more in the chalaza when compared to its accumulation in the nucellus. The protein was clearly present at the periphery, most likely the plasma membrane, of the enlarged MMC before and possibly during the onset of meiosis. In mature ovules, AtSERK1-YFP was detected in the central cell of the embryo sac. Due to the performed enzymatic digestion and the subsequent tissue damage, we cannot state with certainty whether the synergids and egg cell contain AtSERK1-YFP protein.

During early pollen development, AtSERK1-YFP was detected in both primary parietal and sporogenous tissues. AtSERK1-YFP fluorescence was also observed when the MMC appeared and the surrounding tissue differentiated into the endothecium, middle layer, and the tapetum of the locules. During later stages of anther development, AtSERK1-YFP protein was found exclusively in the vascular and the connective tissue of the anther and it was low or absent in the pollen grains.

After fertilization, the AtSERK1-YFP accumulated in the globular embryo, and surprisingly, fluorescent protein was found in epidermal and vascular cells of the late torpedo and cotyledon stages embryos. The presence of *AtSERK1* promoter GUS activity or accumulation of *AtSERK1* mRNA at those stages of embryo development was not reported previously by Hecht et al. (2001).

During seedling development, AtSERK1-YFP was detected in the root meristem. In the primary root, AtSERK1-YFP fluorescence was mainly present in the epidermis and

lateral root cap, the vascular bundle, and the root pericycle. As the root matured and differentiated, the fluorescence was restricted entirely to the root vascular tissue. AtSERK1-YFP fluorescence was higher during early stages of lateral root formation and predominantly found in the vascular tissue.

Our observations showed that AtSERK1 protein is present in both sporophytic and gametophytic tissues of the female and male reproductive organs. Interestingly, the AtSERK1 protein accumulation always precedes the first differentiation steps of the progenitor cells of the gametes. Therefore, it is likely that the signalling mediated by the AtSERK1 receptor is not restricted to either the sporophytic or the gametophytic phase of development. Similar observations were made for the expression pattern of *Hieracium* spp. (Tucker et al. 2003) and *Medicago truncatula* (Nolan et al. 2003) *AtSERK1* orthologous genes.

During embryo formation and postembryonicly, AtSERK1 again does not appear to be cell-specific as the protein was detected in embryos and in seedlings. We can therefore conclude that the role of the AtSERK1 signalling is not restricted to embryogenic cell formation (Schmidt et al. 1997, Hecht et al. 2001) but appears to be of a more general nature.

AtSERK1-YFP localization at the cellular level

At the cellular level, AtSERK1-YFP was localized mainly at the cell periphery in agreement with previous experiments showing that AtSERK1-YFP is localized to the plasma membrane in plant protoplasts (Shah et al. 2001). In a restricted number of cells in the ovule (Fig. 8 A) and in the root (Fig. 8 B, C), we have observed intracellular vesicle-like compartments containing AtSERK1 receptor protein. In our hands it was not possible to determine if AtSERK1-YFP colocalizes with the endocytic marker FM 4-64 (Vida and Emr 1995, Ueda et al. 2001) due to the low intensity of AtSERK1-YFP fluorescence. We do, however, propose that the vesicles observed in the intact roots represent endocytic compartments rather than protein in transit from the endoplasmic reticulum and Golgi to the plasma membrane. This proposition is mainly supported by the observed effect of BFA on AtSERK1-YFP localization. In plants, BFA acts as an inhibitor of the endosomal recycling due to interfering with the large ARF-GEFs such as GNOM, resulting in accumulation of plasma membrane proteins inside the cell in BFA-induced compartments (reviewed by Geldner 2004). AtSERK-YFP receptors were quickly and reversibly accumulated into similar intracellular compartments as a result of the BFA application. Previous studies have shown that BRI1 receptor fused to GFP also showed a reversible accumulation in BFA compartments as a result of BFA treatment. BRI1-GFP was consistently found in vesicles corresponding to the compartments accumulating the AtSERK1-YFP protein. In the case of BRI1-GFP localization, the vesicle-like structures were colocalized with the endocytic tracer FM 4-64 and concluded to be endosomes, suggesting that BRI1 receptor undergoes constant recycling in live cells (Russinova et al. 2004). A protein recycling via endosomes was also reported for auxin efflux carrier PIN1 (Geldner et al. 2001), cell wall pectins (Baluška et al. 2002), and plant sterols (Grebe et al. 2003).

The importance of the endosomal trafficking of the plasma membrane-localized LRR class of receptors for signalling and plant development has not been demonstrated yet. Recently, Shah et al. (2002) have shown that the AtSERK1 trafficking is influenced by the phosphorylation status of the receptor and by its interaction with KAPP when transiently coexpressed in protoplasts. Furthermore, when dephosphorylated or in complex with KAPP, AtSERK1 receptors localized to FM 4-64–positive compartments. So far, endocytosis has been demonstrated not only as a mechanism for receptor down-regulation but also as a prerequisite for signalling through animal receptor tyrosine kinases and TGF-β receptors (reviewed by Gonzáles-Gaitán 2003). It is not excluded that similar mechanisms exist in plant cells and the internalization of the receptors is essential for signalling. Although not demonstrated in intact plants yet, an accelerated endocytosis of the putatively active BRI1/BAK1 heterodimers was observed in transient protoplast expression system by Russinova et al. (2004). If this situation also holds true for the AtSERK1-YFP receptor, we can propose that only cells showing endocytosis of the receptors are actively engaged in AtSERK1-mediated signalling.

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