Endocytosis and Endosomal Regulation of the *S*-Receptor Kinase during the Self-Incompatibility Response in *Brassica oleracea* [W]

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Intracellular trafficking of plant receptor kinases (PRKs) is a key step in regulation of cellular signaling. Our current knowledge in this field is based on systems that address signaling pathways affecting the whole cell. There are, however, signaling phenomena that add a further layer of complexity. In the *Brassica* self-incompatibility response, a single cell can adequately respond to two opposite stimuli: accepting cross-pollen and rejecting self-pollen simultaneously. To understand how PRK signaling can influence the coexistence of two seemingly exclusive states of the cell, we investigated the subcellular localization and internalization of the *S*-receptor kinase (SRK) involved in the self-incompatibility response of *Brassica oleracea*. Here, we describe the unusual subcellular distribution of SRK$_3$, which localizes predominantly to intracellular compartments and to a much lesser extent to the plasma membrane. Using an anti-SRK antibody that fully substitutes for the natural ligand, we demonstrate that the interaction with the receptor takes place at the plasma membrane and is followed by SRK internalization in endosomes that are enriched in the SRK negative regulator Thioredoxin-h-like1.

INTRODUCTION

The key role played by the intracellular trafficking of receptor kinases in the regulation of signaling pathways has been described in numerous studies. Upon ligand binding, activated receptors are internalized in endosomal compartments, where they are either routed to lysosomes for degradation and down-regulation or reset and recycled back to the plasma membrane (PM) (Wiley, 2003). During plant development, plant receptor kinases (PRKs) are required for correct differentiation of organs and normal growth (Haffani et al., 2004a). The role of their localization in correct signaling is still poorly understood. Expression studies have shown an almost exclusive PM localization for most of the investigated PRKs (Jinn et al., 2000; Shah et al., 2001; Hematy et al., 2007), with several notable exceptions (Russinova et al., 2004; Gifford et al., 2005; Geldner et al., 2007). The L1 layer-specific ARABIDOPSIS CRINKLY4 receptor localizes to the PM and at least two distinct compartments in the cytoplasm, corresponding to export and endocytic vesicles (Gifford et al., 2005). The brassinosteroid receptor, BRI1, localizes both to the PM and to so far unidentified cytoplasmic compartments, and its localization and trafficking are not dependent on the presence of its ligand. A recent study by Geldner et al. (2007) demonstrated the ability of BRI1 to transmit signal from these cytosolic compart-

ments, a major step in understanding receptor signaling in plants. Interestingly, a different type of subcellular distribution was reported for the FLS2 receptor, which is required for rare but massive signaling in response to pathogen infection (Gomez-Gomez and Boller, 2000). In its inactive state, FLS2 localizes to the PM, whereas it is rapidly internalized and degraded upon binding to the flg22 protein (Robatzek et al., 2006).

In both pathways, activation of the receptor triggers a signaling cascade and causes the reaction of the whole cell. However, there are cases in which cells are required to adequately respond to two opposite simultaneous stimuli. An example of this is the self-incompatibility (SI) response in *Brassica*, a reaction preventing self-fertilization by rejection of self-pollen grains that have fallen upon the stigma. Micromanipulator experiments have demonstrated that single stigma papilla cells are able to simultaneously reject self-pollen and accept cross-pollen (Sarker et al., 1988). Genetically, the SI response is regulated by the highly polymorphic *S*-locus, containing the genes of the two key SI components (Gaude et al., 2006). Those are the stigma papilla cell-expressed *S* Receptor Kinase (SRK) and its ligand, the *S*-locus Cysteine-Rich protein (SCR), whose product is expressed in anthers and adheres to the pollen coat. The high degree of polymorphism at the *S*-locus has led to the formation of interaction partners of the type SRKn-SCRn, in which a given SRK will recognize only SCR from the same *S*-haplotype. If a self-pollination event occurs, the ligand (SCR) will interact with SRK, resulting in SRK phosphorylation and triggering of the SI response (Cabrillac et al., 2001). SCR from a different haplotype will not be recognized by the receptor kinase and will not cause an SI reaction. Searches for SRK regulators led to the identification of several interacting proteins (Bower et al., 1996; Gu et al.,

1998; Vanoosthuyse et al., 2003; Kakita et al., 2007). Three of these, ARC1, THL1, and MLPK, have been shown to be involved in the SI response (Stone et al., 1999; Haffani et al., 2004b; Murase et al., 2004). THL1 has been demonstrated to negatively modulate the kinase activity of SRK_3 (Cabrillac et al., 2001).

The current model of *Brassica* SI assumes that SRK, which has a canonical PRK structure (Stein et al., 1991), localizes to the PM of the stigma papilla cells where the interaction with SCR occurs (Goring and Walker, 2004). Indeed, by cell fractionation in transgenic tobacco (*Nicotiana tabacum*), Stein et al. (1996) demonstrated that *Brassica oleracea* SRK_6 can localize to the PM, but the studies in native papilla cells were hampered by the complexity of *Brassica* as a model system. A fractionation performed on SRK_{15} (Cabrillac et al., 1999) resulted in a clearer signal in PM-enriched fractions, but because the line used is self-compatible due to an unknown cause, the possibility of receptor mislocalization cannot be excluded. Despite the difficulties, however, *Brassica* remains a preferred model for studying SI due to the large amount of acquired physiological and biochemical data and well-characterized genetic and molecular interactions.

To address the question of how SRK triggers a local negative response to self-pollen, while the rest of the cell remains competent for a possible positive interaction with cross-pollen, we analyzed the subcellular localization of SRK and investigated the importance of its internalization for the SI response. Applying an immunocytochemical approach, we found that in stigma papilla cells of S_3-haplotype, SRK_3 localizes preferentially to intracellular compartments and to a lower extent to the PM. By colocalization with the known markers VPS29 and SYP21, we managed to identify the compartments as sorting endosomes. Using an anti-SRK_3 antibody as a ligand, we demonstrated that recognition occurs at the PM during the SI response and is followed by ligand internalization. SRK_3 colocalizes with its inhibitor THL1 in the endosomes, but not at the PM, indicating that receptor signaling might occur at the PM.

RESULTS

SRK_3 Localizes Predominantly to Intracellular Compartments

To investigate the localization of SRK in papilla cells, we employed a direct immunocytochemical approach using the monoclonal antibody mAb85-36-71 (designated anti-SRK_3-N-ter for clarity), which recognizes the first nine–amino acid sequence of the N terminus of SRK_3 (Gaude et al., 1993; Delorme et al., 1995) (see Supplemental Figure 1A online). This peptide is also found in the N termini of SRK_2, SRK_5, SRK_{15}, and the respective related secreted *S*-locus glycoproteins (SLGs) but is absent in SLG_3 and all other SRK and SLG sequences analyzed so far in *B. oleracea* (Cabrillac et al., 1999; Miege et al., 2001). On protein gel blots of total S_3 protein extracts, this antibody recognizes the full-length SRK_3 receptor as well as its splice variant designated $eSRK_3$. This protein, which makes up the whole extracellular domain of the receptor, has four detectable glycosylation forms: two weak at 65.2 and 56.7 kD and two abundant at 62.8 and 59.5 kD (see Supplemental Figure 1A online) (Giranton et al., 1995).

In S_3-stigmas, we detected immunolabeling in the papilla cells and to a lower extent in the underlying cell layers (Figure 1A). The signal intensity was much lower compared with that from S_{15}-stigmas (see Supplemental Figure 1C online), which showed a strong signal due to the sum of SRK_{15} and SLG_{15} immunolabeling, as previously observed in immunohistochemistry experiments (Gaude et al., 1993). S_{29}-stigmas exhibited no signal above background level (Figure 1B). A closer inspection of the immunofluorescent signal in S_3-papilla cells revealed that, rather unexpectedly, the majority of the signal was concentrated in intracellular compartments and some diffuse regions, whereas only a small portion of the signal could be detected in patches around the periphery of the cell (Figure 1C). To see whether the peripheral labeling corresponds to the PM, we performed an immunolocalization of SRK_3 by electron microscopy. As expected, the amount of SRK_3 signal was low, but nevertheless, the labeling was in close proximity to the PM. The signals were often grouped together, leaving zones completely devoid of signal (Figure 1D). Additionally, signals probably corresponding to $eSRK_3$ could be seen on the cell wall. Labeling was also detected in multivesicular bodies, Golgi apparatus, small vesicles, and the endoplasmic reticulum (ER) (Figures 1E to 1H).

Such a localization pattern is unique among the known PRKs, all of which seem to be predominantly localized at the PM (Jinn et al., 2000; Robatzek et al., 2006; Hematy et al., 2007), even if actively internalized (Gifford et al., 2005; Geldner et al., 2007).

SRK_3 Localizes to Sorting Endosomes

Since the majority of SRK_3 is localized in intracellular compartments, and not at the PM, we attempted to identify the nature of these compartments by colocalization studies with known markers for endomembrane compartments. First, colocalization was tested between SRK_3 and the endosome marker VPS29. VPS29 is a key member of the plant retromer complex involved in the trafficking of certain proteins between the sorting endosome and the PM (Jaillais et al., 2007). Partial colocalization was observed in the cytosolic compartments as seen in Figures 2A to 2C. This result might be significant; VPS29 participates in the retromer together with SORTING NEXIN1, a protein previously isolated as a SRK_3 interactor (Vanoosthuyse et al., 2003). The amorphous regions positive for SRK_3 but not labeled by the anti-VPS29 antibody could correspond to ER, since considerable amounts of SRK_3 are located there, as suggested by the electron microscopy immunolocalization. To verify the observed endosomal localization of SRK_3, we used a second marker, the SYP21 protein, a membrane-anchored syntaxin localized in the prevacuolar compartment of root cells (da Silva Conceicao et al., 1997). This compartment was recently identified as the plant sorting endosome (Jaillais et al., 2008); therefore, SYP21 was selected for an independent confirmation of SRK_3's endosomal localization. The immunodetection signals for the two proteins showed good colocalization (Figures 2D to 2F). Another member of the syntaxin family, SYP61, was also used as a marker of the *trans*-Golgi network, but only isolated colocalizing signals could be detected (Figures 2G to 2I). The event of cross- or self-pollination did not change the localization of SRK_3 and its colocalization with the investigated markers (see Supplemental
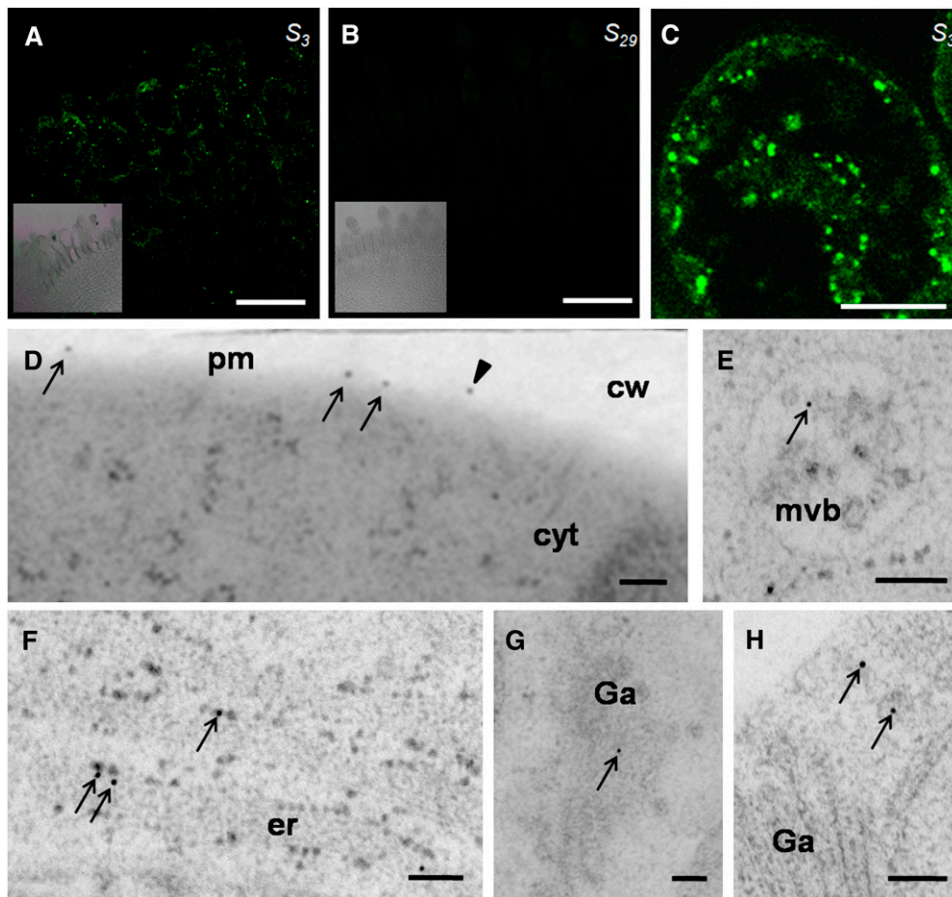
Figure 1. SRK$_3$ Localizes to PM and Sorting Endosomes in *B. oleracea* Stigmas.

(A) Signal recognition by anti-SRK$_3$-N-ter in S_3-papilla cells. Inset presents the bright-field image. Bar = 50 μm.

(B) Signal recognition by anti-SRK$_3$-N-ter in S_{29}-papilla cells. No specific signal can be detected. Inset presents the bright-field image. Bar = 50 μm.

(C) Higher magnification of a S_3-stigma papilla cell shows fluorescent signals in the periphery of the cell and intracellular compartments. The experiment was reproduced five times. Bar = 10 μm.

(D) to **(H)** Immunolocalization on electron microscopy sections. Bars = 100 nm.

(D) SRK$_3$ signal at the PM (arrows) and in the cell wall (arrowhead). Signals visible in the cell wall probably correspond to the soluble splice variant eSRK$_3$.

(E) SRK$_3$ signal in the multivesicular bodies (arrow).

(F) SRK$_3$ signal in the ER (arrows).

(G) SRK$_3$ signal in the Golgi apparatus (arrow).

(H) SRK$_3$ signal in different small vesicles in the vicinity of the Golgi apparatus (arrows).

cw, cell wall; cyt, cytoplasm; er, endoplasmic reticulum; Ga, Golgi apparatus; pm, plasma membrane; mvb, multivesicular body.

Figure 2 online), similar to what was demonstrated for the brassinosteroid receptor BRI1 (Geldner et al., 2007). These results indicate that cytoplasmic SRK$_3$ is likely to accumulate in sorting endosomes, revealing that this compartment is a major step in receptor intracellular trafficking.

SRK$_3$ Is Degraded upon Activation

In an attempt to follow SRK$_3$ turnover, we investigated its stability over a 2-h time course of self- or cross-pollination. Protein gel blot analysis showed that SRK$_3$ levels do not change under these conditions (Figures 3A and 3B), suggesting either the potential high stability of SRK$_3$ independently of its activation or a com-

pensatory mechanism, in which a new receptor is synthesized to replace the degraded one. To test which of these hypotheses is correct, we treated stigmas with the protein biosynthesis inhibitor cycloheximide (CHX) prior to self-pollination. To avoid unwanted secondary effects, the treatment was done directly on the stigma undetached from the plant. We observed a 25 to 30% decrease in SRK$_3$ levels (Figure 3C), which demonstrates that during self-pollination, SRK$_3$ is degraded, while de novo protein synthesis allows restoration of its basal level. To see whether the effect is specific to self-pollination, we performed a similar experiment, in which stigmas were self-, cross-, or nonpollinated after the CHX treatment (Figure 3D). The reduction of SRK$_3$ levels was specific to the self-pollination situation, showing that once
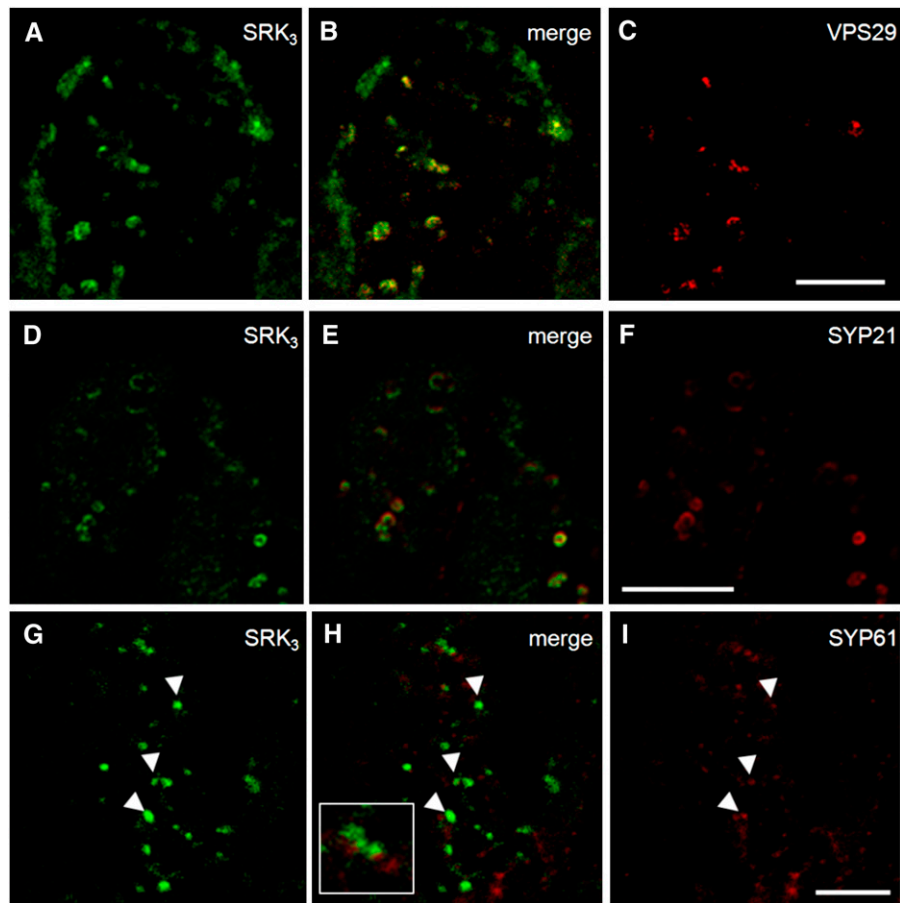
Figure 2. SRK$_3$ Colocalizes with Sorting Endosome Markers in Papilla Cells.

(A) to **(C)** Colocalization between SRK$_3$ **(A)** and sorting endosome marker VPS29 **(C)**. Merged image is presented in **(B)**. Images shown are representative of five independent experiments. Bar = 10 μm.

(D) to **(F)** Colocalization between SRK$_3$ **(D)** and sorting endosome marker SYP21 **(F)**. Merged image is presented in **(E)**. Images shown are representative of three independent experiments. Bar = 10 μm.

(G) to **(I)** Colocalization between SRK$_3$ **(G)** and trans-Golgi network marker SYP61 **(I)**. Merged image is presented in **(H)**. Cases of colocalization are rare and are indicated with arrowheads. Inset represents a case where signals are closely associated but do not colocalize. Images shown are representative of three independent experiments. Bar = 10 μm.

the SI response is triggered, the activated receptor undergoes degradation. The 62.8-kD eSRK$_3$ form showed no sensitivity to CHX, while the 59.4-kD form was affected under all conditions, irrespective of the type of pollination. This result suggests higher stability of the 62.8-kD eSRK$_3$ or could alternatively mean that the degradation of the 62.8-kD form triggers the conversion of the 59.4-kD form to the 62.8-kD one to maintain the required levels. The physiological relevance of the CHX treatment to the SI response was confirmed by pollen tube counts. We observed a breakdown of SI after the treatment as already described by Sarker et al. (1988) (see Supplemental Figure 3 online).

Receptor–Ligand Interaction during SI Happens at the PM

Current models of the SI response postulate the ligand–receptor interaction to occur at the PM in analogy with other mammalian and plant systems (Goring and Walker, 2004). Now, this idea is

challenged by the low availability of SRK$_3$ at the PM. To identify the topology of the interaction, we designed an experiment in which the anti-SRK$_3$-N-ter antibody was used as a ligand, and its internalization was followed in the stigmas of different S haplotypes. This approach was chosen due to the lack of a reliable detection method for the native ligand, SCR$_3$. Anti-SRK$_3$-N-ter antibody has two important functional properties: first, it is able to pass through the cell wall of stigma papilla cells (Luu et al., 1999); and second, like the native ligand SCR$_3$, its interaction with SRK$_3$ leads to activation of the receptor (Cabrillac et al., 2001). This theoretically makes it a suitable replacement for SCR$_3$ and brings the advantage of easy detection. To be sure of the physiological relevance of the substitution, we performed a pollination assay using compatible S$_{15}$-pollen on S$_3$- or S$_{29}$-stigmas. The stigmas were pretreated with either an anti-SRK$_3$-N-ter antibody solution or a mock solution for 1.5 h. The antibody treatment led to a strong rejection reaction of the otherwise
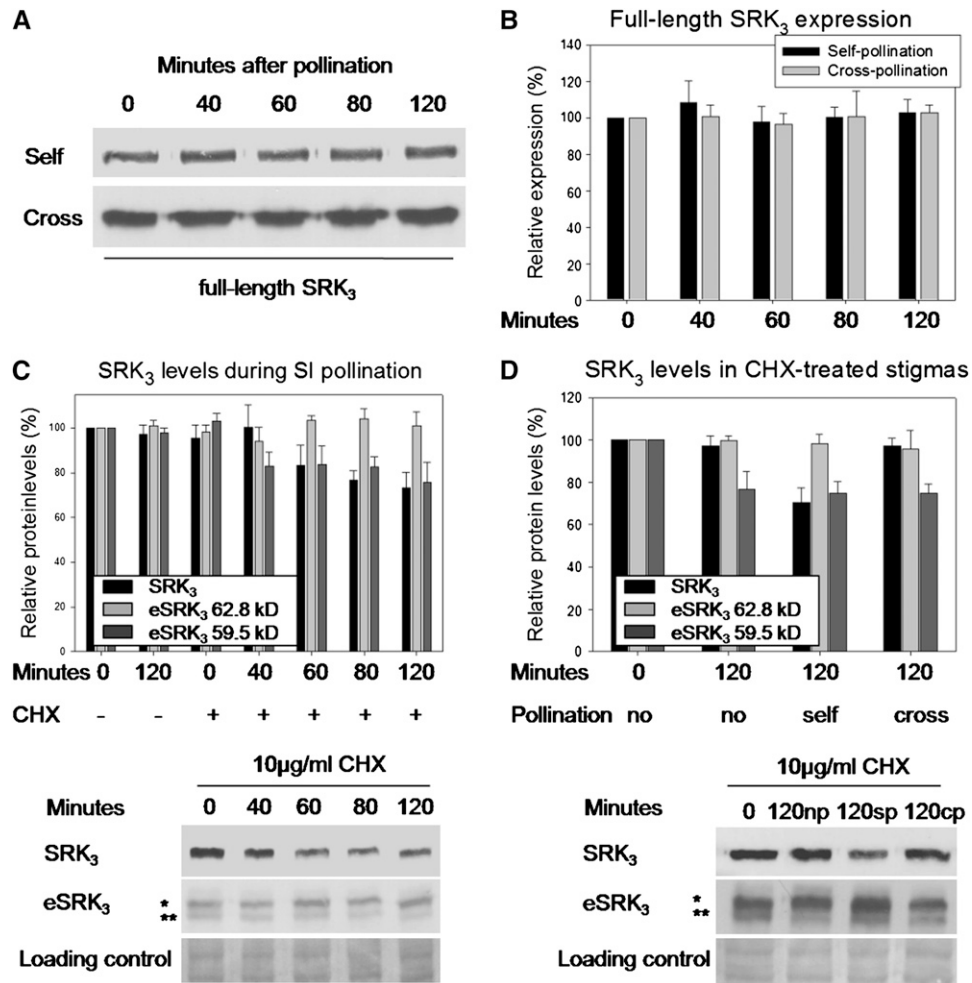
Figure 3. SRK$_3$ Undergoes Degradation upon Self-Pollination.

(A) Protein gel blot showing stability of full-length SRK$_3$ between 0 and 120 min following self- or cross-pollination.

(B) Quantification of **(A)**. No significant change in signal intensity was observed. The bars represent the means (+SD) of three independent experiments.

(C) Degradation of SRK$_3$ after self-pollination of stigmas treated with CHX (+) or a mock solution (−) between 0 and 120 min . The bars represent the means (+SD) of three independent experiments. The 62.8-kD splice variant eSRK$_3$ (*) is not sensitive to the CHX treatment, while the 59.4 kD variant (**) and the full-length receptor are sensitive. A typical protein gel blot is shown as an example below the graph.

(D) CHX treatment affects SRK$_3$ specifically upon self-pollination. The bars represent the means (+ SD) of three independent experiments. The 62.8 kD eSRK$_3$ form (*) is not sensitive to the CHX treatment, while the 59.4-kD eSRK$_3$ (**) is sensitive irrespective of the pollination type. A typical protein gel blot is shown as an example below the graph. np, nonpollinated; sp, self-pollination; cp, cross-pollination.

compatible S_{15}-pollen on the S_3-stigma surface but not on the control S_{29}-stigma (Figures 4A to 4D). This result indicates that anti-SRK$_3$-N-ter is capable of specifically inducing SI reaction upon interaction with SRK$_3$, probably by mimicking the effect of the ligand and provoking receptor dimerization as previously suggested (Cabrillac et al., 2001). In the following experiments, we used this antibody as a ligand for SRK$_3$.

SRK$_3$ Is Routed to Sorting Endosomes upon Ligand Recognition

To investigate whether anti-SRK$_3$-N-ter was internalized during the SI reaction, we treated S_3- or S_{29}-stigmas with antibody/

ligand. After 1.5 h, the stigmas were pollinated with S_{15}-pollen and then fixed for immunolocalization 1 h after the pollination. In S_3, we observed signals in cytoplasmic compartments of the papilla cells, indicating that antibody/ligand internalization had taken place (Figure 4E). No such internalized signals were observed in S_{29}-papilla cells. Instead, a continuous fluorescent halo around the cell was visible, suggesting antibody/ligand retention at the cell surface (Figure 4F). As a control, the internalization of the secondary anti-mouse antibody was investigated in S_3-stigmas. No internalization was observed (Figure 4G), proving that the internalization in this haplotype requires an interaction with a specific PM component and that the interaction between SRK$_3$ and its ligand occurs first at the PM and not in the endosomes. To
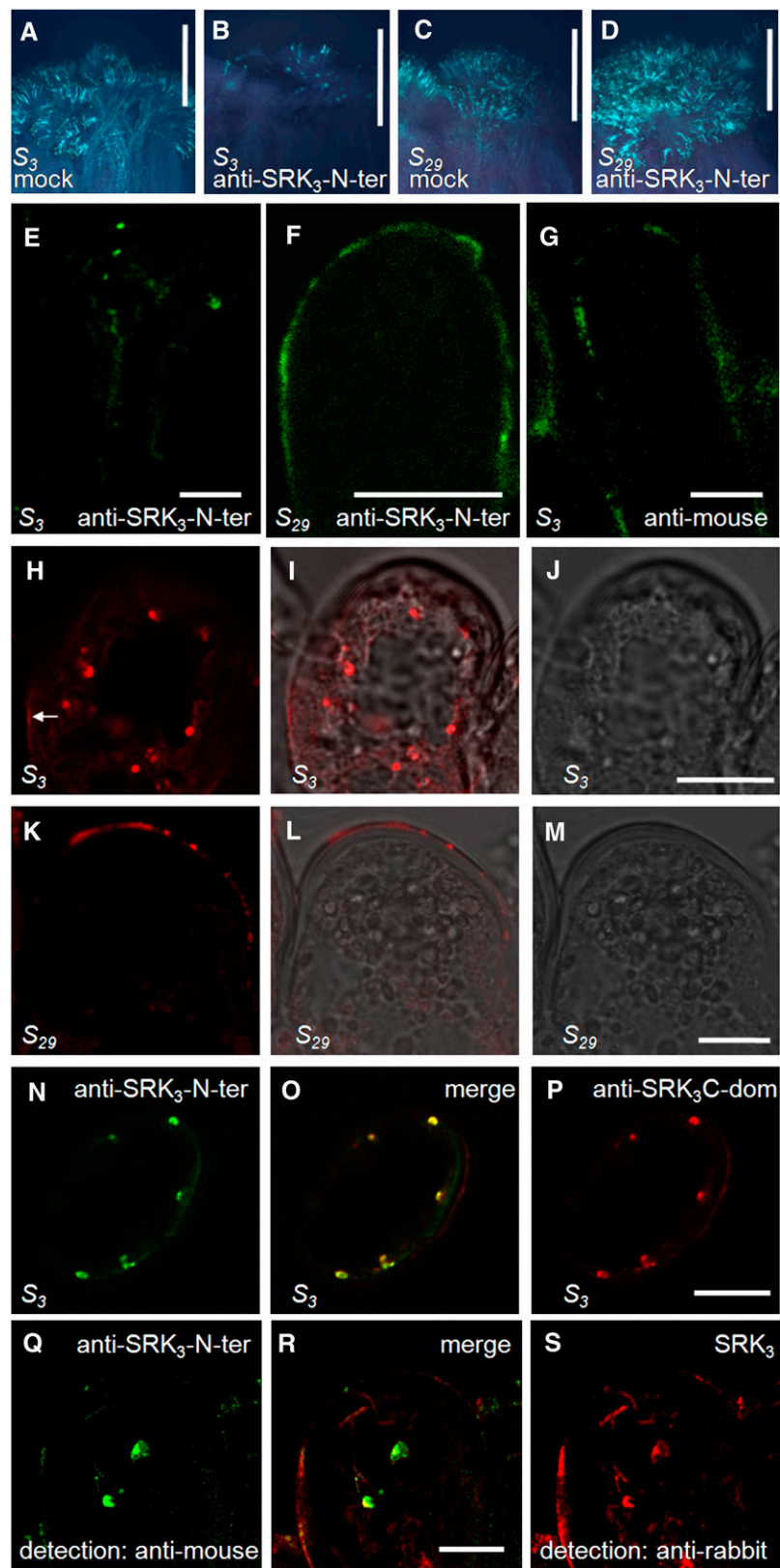
Figure 4. Ligand-Induced Internalization of SRK₃.

ascertain that antibody/ligand internalization correlates with SRK_3 endocytosis, we looked at whether the internalized monoclonal antibody and SRK_3 colocalize to the same cytoplasmic compartments. To detect SRK_3, we used a polyclonal anti-SRK_3 antibody (anti-SRK_3-C-dom) raised against an 11–amino acid sequence within the C terminus of the kinase domain (Delorme et al., 1995). On protein gel blots, this antibody recognizes SRK_3 but not any other SRK protein tested (see Supplemental Figure 1A online), and it also cross-reacts with several bands irrespective of the *S*-haplotype. In control immunocytochemistry experiments, the cross-reactivity was limited to the cell wall (Figures 4H to 4M), and the antibody was not able to recognize the PM-localized SRK_3. Nevertheless, anti-SRK_3-C-dom antibody was suitable to detect the intracellular SRK_3 and showed perfect colocalization with anti-SRK_3-N-ter in the endosomes (Figures 4N to 4P). The signal of the endocytosed antibody colocalized well with the position of the receptor in the cell (Figures 4Q to 4S). Furthermore, we found that the anti-SRK_3-N-ter–containing compartments colocalize with VPS29 (Figures 5A to 5C), suggesting that following internalization, the complex is routed to sorting endosomes before SRK_3 is sent for degradation. The presence of activated receptor complexes in endosomal compartments implies that SRK_3 signaling might happen there, in a way similar to the brassinosteroid receptor BRI1 (Geldner et al., 2007).

SRK_3 Colocalizes with Negative Regulator THL1 Both before and after Activation

To investigate how the subcellular localization of SRK_3 relates to that of its negative regulator, THL1, a stigma thioredoxin h-like protein (Cabrillac et al., 2001), we generated an anti-THL1 antibody (see Supplemental Figure 1 online). Both THL1-only compartments and SRK_3/THL1 endosomal compartments could be detected, suggesting additional cell functions of THL1 (Figures 5D to 5F). No THL1 signal was detected at the PM (Figures 5D to 5F, insets) in any of the six repetitions of the experiment. This might indicate that the negative regulation of SRK_3 at the PM is relaxed, which might lead to a high reactivity upon ligand recognition. The low amounts of SRK_3 at the PM support this hypothesis, as this reduces the probability of an unspecific autophosphorylation. The need for a strict regulation in the endosomes is reasonable because the abundance of the receptor there might lead to unspecific activation, as demonstrated by Giranton et al. (2000). However, it is still possible that undetectable low amounts of THL1 are present at the PM or that THL1 is substituted by another inhibitor, for example, THL2 (Bower et al., 1996).

Because upon internalization the receptor/ligand complex is sorted to VPS29-containing endosomes, we tried to see whether some of the compartments are depleted of THL1 and therefore may allow signaling to occur. As seen in Figures 5G to 5I, activated SRK_3 colocalized with THL1 (similarly to what was shown above for SRK_3/VPS29). Thus, the routing of the active receptor to sorting endosomes might lead to immediate signal attenuation by THL1, which precedes the degradation of the receptor in the lytic vacuoles.

DISCUSSION

Activity of SRK is required for self/nonself-pollen recognition during the fertilization process in *Brassicaceae*. Over the last 50

Figure 4. (continued).

(A) to **(D)** Anti-SRK_3-N-ter can functionally substitute for the natural SRK_3 ligand in vivo. *B. oleracea* S_3- and S_{29}- stigmas were treated with anti-SRK_3-N-ter or mock solution and pollinated with S_{15}-pollen, which is normally compatible for both haplotypes. Pollen germination was visualized under UV light after aniline blue staining. Five independent experiments were performed, yielding similar results.

(A) Germination of S_{15}-pollen on S_3-stigma treated with mock solution. Bar = 500 μm.

(B) Rejection of S_{15}-pollen on S_3-stigma treated with anti-SRK_3-N-ter solution. Bar = 500 μm.

(C) Germination of S_{15}-pollen on S_{29}-stigma treated with mock solution. Bar = 500 μm.

(D) Germination of S_{15}-pollen on S_{29}-stigma treated with anti-SRK_3-N-ter solution. No rejection can be observed. Bar = 500 μm.

(E) and **(F)** *B. oleracea* S_3- and S_{29}-stigmas were treated with anti-SRK_3-N-ter solution and pollinated with S_{15}-pollen (normally compatible for both haplotypes). Antibody internalization was followed by immunofluorescence on fixed sections. The results could be reproduced in three independent experiments.

(E) Internalization in S_3-papilla cells. Anti-SRK_3-N-ter is found in intracellular compartments after 2.5 h, detected directly by the secondary anti-mouse antibody. Bar = 10 μm.

(F) No internalization is observed in S_{29}-papilla cells. Bar = 10 μm.

(G) *B. oleracea* S_3-stigmas were treated with secondary donkey anti-mouse Alexa555 solution and pollinated with S_{15}-pollen. No internalization is observed in S_{29}-papilla cells after 2.5 h. Bar = 10 μm.

(H) to **(J)** Detection of SRK_3 on S_3 stigma papilla sections. SRK_3 is detected in intracellular compartments and unspecifically on the cell wall (arrow) by anti-SRK_3C-dom antibody. Bright-field image is presented in **(J)** and the merge image in **(I)**. Similar results were observed in three independent experiments. Bar = 10 μm.

(K) to **(M)** Absence of SRK_3 from S_{29}-stigma papilla sections (control experiment). The unspecific cell wall staining probably corresponds to the bands detected on protein gel blot with the same antibody. Bright-field image is presented in **(M)** and the merge image in **(L)**. Similar results were observed in three independent experiments Bar = 10 μm.

(N) to **(P)** Simultaneous detection of SRK_3 in S_3-papilla cells using anti-SRK_3-N-ter (recognizing the N terminus of SRK_3) and anti-SRK_3C-dom antibody (recognizing the C terminus of SRK_3). Anti-SRK_3C-dom does not recognize the PM-localized SRK_3, possibly due to an interaction blocking the antibody epitope and shows a weak unspecific cell wall signal. Similar results were observed in three independent experiments. Bar = 10 μm.

(Q) to **(S)** Colocalization of internalized anti-SRK_3-N-ter (detected directly by the secondary anti-mouse antibody) and SRK_3 (detected by anti-SRK_3C-dom and secondary anti rabbit antibody) in S_3-papilla cells. Similar results were observed in three independent experiments. Bar = 10 μm.
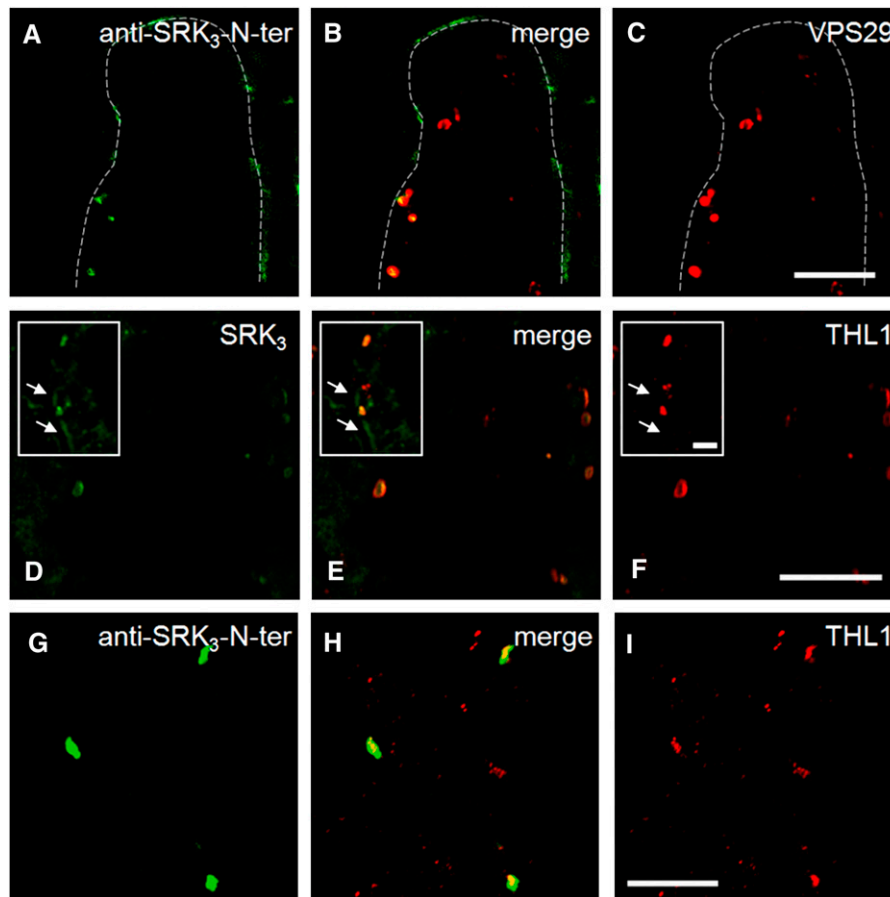
Figure 5. Internalized SRK$_3$ Localizes in Sorting Endosomes with THL1.

(A) to **(C)** Colocalization of internalized anti-SRK$_3$-N-ter **(A)** and VPS29 **(C)**. Merged picture is shown in **(B)**. Similar results were observed in three independent experiments. Bar = 10 μm.

(D) to **(F)** THL1 **(F)** colocalizes partially with SRK$_3$ **(D)** in endosomes, but not at the PM. Merged picture is shown in **(E)**. Arrows in the inserts indicate the PM-localized SRK$_3$. Similar results were observed in six independent experiments. Bar = 10 μm in the panels and 5 μm in the insets.

(G) to **(I)** Colocalization of internalized anti-SRK$_3$-N-ter **(G)** and THL1 **(I)**. Merged picture is presented in **(H)**. Similar results were observed in three independent experiments Bar = 10 μm.

years, physiological and genetic studies have suggested the complexity of the underlying signaling process. One of the manifestations of this complexity is the possibility of a cell to accept cross-pollen while successfully rejecting self-pollen at the same time (Sarker et al., 1988). The advancement in knowledge of receptor kinase trafficking in plants raises the question of how SRK can govern this complex interaction and what the role of SRK intracellular distribution and trafficking is in SI. Previous attempts to localize SRK within the cell have lacked sufficient precision due to the methods available or were relying on heterologous systems due to the limitations of the *Brassica* stigma papilla cells (Gaude et al., 1993; Stein et al., 1996; Giranton et al., 2000). In this work, we applied a direct immunocytochemical approach.

We found that SRK$_3$ localizes preferentially to cytoplasmic compartments and to a lower extent to the PM. This is a unique case of PRK subcellular distribution and is probably the basis of the great specificity of self-pollen rejection. The only known case

of similar localization in plant cells was observed for the human transferrin receptor when expressed in *Arabidopsis thaliana* protoplasts (Ortiz-Zapater et al., 2006). The transferrin receptor accumulates predominantly in cytoplasmic compartments, resembling the situation in its native human cells. Interestingly, expression of SRK$_3$ in *Sf21* insect cells resulted in a subcellular distribution similar to the one in papilla cells, with small amounts of signal at the PM and predominant localization in cytoplasmic structures (Giranton et al., 2000). In papilla cells, the PM labeling seemed uneven with regions completely devoid of signal. This was confirmed by our electron microscopy study, indicating the presence of SRK in discreet domains on the PM.

As SRK is predominantly intracellular, it was of great interest to reveal the functional identity of the compartments where SRK$_3$ localizes. We used antibodies against known marker proteins and demonstrated that SRK$_3$ localizes to the sorting endosomes. Such an observation is not entirely unexpected, as it is known that the animal receptor kinases are internalized from the PM to

sorting endosomes, where their fate is decided depending on their activation state. In plants, BRI1 has also been demonstrated to undergo recycling from endosomes to the PM, suggesting a regulatory mechanism conserved between plants and animals (Geldner et al., 2007). It is tempting to imagine a similar scenario for SRK, but most of the drugs we used to disrupt different stages of intracellular trafficking in root cells, such as the Brefeldin A (100 μM, up to 60 min), Wortmannin (33 μM, up to 120 min), and Tyrphostin A23 (30 μM, up to 60 min), are unable to cause any of their respective effects in papilla cells, thus limiting the possibilities for an extensive functional analysis (data not shown).

SRK has the typical structure of a receptor kinase, so it is considered that the interaction with its ligand occurs at the PM (Goring and Walker, 2004). However, this notion is challenged by our finding that the amounts of SRK_3 at the PM might be insufficient to induce a response. Moreover, the endosomes contain large quantities of the receptor, suggesting that the ligand might well be first internalized and routed to endosomes, where it could then interact with SRK. However, the exclusive internalization of the artificial ligand anti-SRK_3-N-ter in S_3 papilla cells demonstrates that the PM is the place of ligand recognition. We propose that SRK_3 activation occurs within ready-to-be-activated domains at the PM upon ligand binding. SRK_3 has the ability to perform transphosphorylation (Giranton et al., 2000), and due to the higher SRK density within these ready-to-be-activated domains, one or several neighboring domains could be activated following recognition. The low amounts of SRK outside the domains would prevent activation from spreading along the whole PM, thus creating a localized response to the self-pollen grain and leaving the rest of the cell in a naive state for a possible simultaneous interaction with compatible pollen. The dual localization of SRK at the PM and in endosomes implies that the subsequent steps of SI signal transduction can take place in any of these compartments. Indeed, PRKs can signal from endosomes, as demonstrated recently for BRI1 (Geldner et al., 2007), and in the case of SRK_3, this is suggested by its internalization to sorting endosomes after ligand binding. On the other hand, the endosomes are motile structures, and it is highly probable that the event of signaling and all subsequent steps of SI response happen directly in the domain responsible for the initial recognition at the PM. This view is supported by the colocalization experiments with THL1, a thioredoxin with well-documented negative effects on SRK_3 signaling and the SI response (Cabrillac et al., 2001; Haffani et al., 2004b), suggesting that SRK_3 is inhibited in the endosomal compartments both before and after the receptor–ligand interaction. An independent observation that supports this hypothesis is the recent demonstration of a direct interaction between SRK and the M-Locus Protein Kinase (MLPK) in BY-2 protoplasts (Kakita et al., 2007). MLPK is a positive regulator of the SI response, as evidenced by the loss of self-pollen rejection in plants altered in MLPK function (Murase et al., 2004). The interaction between SRK and MLPK was reported to occur at the PM, thus supporting the idea of the formation of an active signaling complex at the PM.

The role of endosomes in SI signaling is not clear. In the case of SRK_3, our data are in favor of the classic view of endosomes as a step required for downregulation of the receptor complex and routing it for degradation in the vacuole. This hypothesis is supported by the enhanced degradation rate of SRK_3 upon self-pollination. However, we cannot rule out that SRK signaling occurs in the endosomes, for instance, if a specific component needs to be recruited. Our data support an endocytosed-based regulatory mechanism for SRK signaling, in which the receptive papilla cells display only reduced amounts of the receptor at the PM, while the majority of it is sequestered in sorting endosomes. This differs from the reported BRI1 signaling pathway and is more similar to that proposed for FLS2. However, unlike SRK, both these receptors are exclusively or predominantly localized at the surface of the cell. It is likely that this peculiar distribution and regulation of SRK is related to the very unique feature of SI signaling, where a single papilla cell can react simultaneously to self- and cross-pollen grains.

METHODS

Plant Material and Treatments

Brassica oleracea haplotypes S_3 and S_{15} were described by Gaude et al. (1993), and S_{29} was a kind gift from D.J. Ockendon (Ockendon and Currah, 1977). Stigmas from either S_3- or S_{29}-haplotypes were pollinated with S_3- or S_{15}-pollen. Chemical treatments were done directly on undetached stigmas with the following solutions: 10 μM CHX in 150 mM NaCl, 10 mM Tris, pH 7.5, and 0.05% Triton X-100 for 2 × 20 min; 1:10 diluted anti-SRK_3-N-ter antibody in 150 mM NaCl, 10 mM Tris, pH 7.5, and 0.05% Triton X-100 for 2.5 h; 150 mM NaCl, 10 mM Tris, pH 7.5, and 0.05% Triton X-100.

Pollen Germination Assays

Stigmas were collected 24 h after pollination and fixed in solution containing 70% ethanol, 3.7% formaldehyde (Sigma-Aldrich), and 10% acetic acid (Sigma-Aldrich) for 24 h. The fixed material was then briefly washed and incubated for 24 h in 10 M NaOH and for another 24 h in destained aniline blue solution (0.1% [w/v] aniline blue in 0.1 M K_3PO_4, pH 7.0). Pollen tube growth was observed under UV light on a Nikon Optiphot-2 microscope.

Antibodies

Anti-SRK_3-N-ter monoclonal antibody (mAb85-36-71) was described by Gaude et al. (1993). Anti-SRK_3-C terminus rabbit polyclonal antiserum (anti-SRK_3-C-dom) was described previously (Delorme et al., 1995). Polyclonal antiserum against the peptides CRGEFDEDARYPENKT and DKIEFKKPPTTSSGP (Eurogentec) of *Arabidopsis thaliana* VPS29 protein was raised in rabbit (Covalab). Rabbit polyclonal antiserum against *B. oleracea* THL1 was raised against the peptides CEDWNNKLKAAKESNK and CKGEEKLDKVVGAAK (Covalab). Polyclonal antiserums against *Arabidopsis* SYP21 and SYP61 proteins are a kind gift from Natasha Raikhel (da Silva Conceicao et al., 1997; Sanderfoot et al., 2001). All antibodies were tested on protein gel blots of total extracts from *B. oleracea* S_3 haplotype stigmas (see Supplemental Figure 1A online)

Immunolocalization on *B. oleracea* Stigmas

S_3-, S_{15}-, or S_{29}-haplotype stigmas were either directly harvested or pretreated, as described above, before fixation in 4% paraformaldehyde (Sigma-Aldrich) and DPBS (Gibco), pH 7.0. Samples were washed in D-PBS (Dulbecco's Phosphate-Buffered Saline; Gibco), and dehydrated in an ethanol series (30, 50, 70, 90, 96, and 100%). The samples were embedded in Steedman wax (PEG 400 distearate [Sigma-Aldrich]:

1-hexadecanol [Sigma-Aldrich] = 9:1) as previously described (Baluska et al., 1992). Ten-micrometer-thick sections were placed on ProbeOn Plus microscope slides (Fisher Scientific). Following rehydration in an ethanol series (100, 96, 90, 70, 50, and 30%), the slides were washed in DPBS and used for immunolocalization. The incubations were 2 h for the primary and 1 h for the secondary antibodies, with DPBS washes after each incubation. Slides were analyzed on a LSM-510 laser scanning confocal microscope (Zeiss).

Dilutions of the antibodies were as follows: anti-SRK$_3$-N-ter, 1:500; anti-SRK$_3$-C-dom, 1:250; anti-VPS29, 1:250; anti-THL1, 1:200; anti-SYP21, 1:100; anti-SYP61, 1:100; Alexa555 donkey anti-mouse, 1:500 (Invitrogen); Alexa488 goat anti-rabbit IgG, 1:500 (Invitrogen).

Immunolocalization on Electron Microscopy Sections

Sections (200 μm) of *B. oleracea* stigmas were placed in hexadecene and frozen in a Leica EM PACT high-pressure freezer (Leica Microsystems) under 2000 bars and subsequently placed in freeze-substitution medium (0.25% uranyl acetate in anhydrous acetone) in a Leica automated freeze-substitution system. Freeze-substitution was performed as follows: 36 h at −90°C, 15 h of progressive increase of temperature up to −60°C at the rate of 2°C/h; 8 h at −60°C, 15 h of progressive increase of temperature up to −30°C at the rate of 2°C/h; 14 h at −30°C, 5 h of progressive increase of temperature up to −20°C at the rate of 2°C/h. At −20°C, the medium was replaced by acetone, which was subsequently progressively replaced by LR Gold resin. The samples were polymerized under UV light at −20°C for 48 h and then at +20°C for 36 h.

Immunodetection was performed on 50-nm sections using standard procedures. Dilution for anti-SRK$_3$-N-ter was 1:250 and for the secondary 10-nm gold goat anti-mouse antibody (Nanoprobes), 1:40.

SDS-PAGE and Protein Gel Blots

Stigma extracts and electrophoretic analyses were performed as described (Cabrillac et al., 2001). Antibody dilutions used for protein gel blots were as follows: anti-SRK$_3$-N-ter, 1:1000; anti- SRK$_3$-C-dom, 1:1000; anti-VPS29, 1:500; anti-THL1, 1:200; anti-SYP21, 1:500; anti-SYP61, 1:500; goat anti-mouse IgG HRP, 1:5000 (Promega); anti-rabbit IgG HRP, 1:5000 (Amersham). Densitometry on membranes obtained after protein gel blotting (Amersham ECL protein gel blotting detection reagents; GE Healthcare) was done using the ImageJ software (http://rsb.info.nih.gov/ij).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: THL1, AF273844; SRK$_3$, X79432; SCR3, UniProt Q9FNT3_BRAOL.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Specificity Test for Antibodies on *Brassica oleracea* Stigma Proteins.

Supplemental Figure 2. SRK3 Colocalizes with Sorting Endosome Markers after Pollination.

Supplemental Figure 3. Effect of Cycloheximide Treatment on Self-Incompatibility.

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