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Subcellular Localization of the *S* Locus F-box Protein AhSLF-S_2 in Pollen and Pollen Tubes of Self-Incompatible *Antirrhinum*

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Abstract: The distribution of the *S* locus F-box (SLF) protein was examined by immunocytochemistry and Western blot techniques using an antibody against the C-terminal part of AhSLF-S_2 in self-incompatible lines of *Antirrhinum*. Abundant gold particles were found where pollen tubes emerge *in vitro*. With the elongation of pollen tubes, binding sites for the antibody were found in the cytoplasm of the pollen tubes, including the peripheral part of the endoplasmic reticulum. After germination *in vitro* for 16 h, the product of AhSLF-S_2 and possibly its allelic products could still be detectable, implying that the SLF protein has a role in the elongating process of pollen tubes. The present study provides evidence at the protein level that the SLF protein is present in pollen cytoplasm during pollen tube growth. These findings are discussed, as is their potential role in the self-incompatible response in *Antirrhinum*.

Key words: *Antirrhinum*; pollen tube; self-incompatibility; *S* locus F-box protein; subcellular localization.

Self-incompatibility (SI) is a widespread mechanism in flowering plants that prevents self-fertilization and promotes out-crossing and genetic studies have shown that most SI systems are controlled by a single multi-allele locus called the *S* locus (de Nettancourt 2001). In most cases, the *S* locus contains at least two polymorphic genes. One is expressed in pollen and the other in pistil. Self/non-self pollen discrimination occurs as a result of the interaction between the *S*-determinants of pollen and pistil (McCubbin and Kao 2000). In the gametophytic SI system found in Scrophulariaceae, Solanaceae, and Rosaceae, self-incompatibility locus RNases (S-RNases) are known to encode the female *S* component (Anderson *et al*. 1989; McClure *et al*. 1989; Ai *et al*. 1990; Lee *et al*. 1994; Murfett *et al*. 1994; Sassa *et al*. 1996; Xue *et al*. 1996). However, the pollen *S* (*Sp*) gene remains to be identified. Considerable effort has been directed towards the identification of the *Sp* gene (Li *et al*. 2000; McCubbin *et al*. 2000; Lai *et al*. 2002; Entani *et al*. 2003; Ushijima *et al*. 2003). Recently, we have cloned an F-box gene in *Antirrhinum*, named *AhSLF-S_2* (*S* locus F-box), which is located approximately 9 kb downstream of the *S_2-RNase* gene and specially expressed in pollen and tapetum (Lai *et al*. 2002). The *AhSLF-S_2* gene and its allelic products appear to be the closest pollen-expressed gene next to the *S-RNase* and, as such, represent a good candidate for the *Sp* (Zhou *et al*. 2003). Similar F-box genes have also been found in the *S* locus of several rosaceous species (Entani *et al*. 2003; Ushijima *et al*. 2003), implying that the *S* locus F-box proteins are expected to play a role in the SI process.

The F-box proteins are a known component of E3 ubiquitin ligase (SCF (Skp1/Cullin or CDC53/F-box) complex) and are involved in ubiquitin-mediated proteolysis (Bai *et al*. 1996; Craig and Tyers 1999). In plants, this proteolysis pathway has been shown to control a large number of events, such as cell cycle progression, stress response, hormone signaling, senescence, and floral differentiation (Vierstra 2003).

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To date, the authentic protein encoded by *AhSLF-S2* in the pollen of *Antirrhinum* has not been documented and the subcellular location of this protein is unknown, which is a prerequisite for understanding its physiological function. Antibodies are very convenient reagents for detecting the location of a target protein. Thus, to begin the characterization of the F-box protein AhSLF-S_2, it was first expressed in *Escherichia coli* and the resulting recombinant protein was used as an antigen for immunizing rabbits to generate an antibody. Using this antibody, we conducted immunolocolization studies for AhSLF-S_2 in pollen and pollen tubes germinated *in vitro*.

1 Materials and Methods

1.1 Plant materials

Self-incompatible *Antirrhinum* lines have been described previously (Xue *et al*. 1996). For the *in vitro* germination of pollen, matured pollen was cultured in a medium containing 0.44 mol/L sucrose, 1 mmol/L $CaCl_2$, 1 mmol/L H_3BO_3, 1 mmol/L $MgSO_4$, and 2 mmol/L citrate-phosphate buffer, pH 5.8, in small petri dishes at (25 ± 1) °C in a saturated atmosphere (100% relative humidity).

1.2 Production of antiserum against AhSLF-S_2

To examine the localization of AhSLF-S_2, the pET-28b recombinant expression vector (Novagen, CA, USA) was used to produce a fusion protein containing the C-terminal of AhSLF-S_2 (299 amino acids, missing the F-box domain) with His-tag fused to the N terminus. The protein was expressed in *E. coli* strain BL21 (DE3) with the induction of 1 mmol/L IPTG (isopropylthio-β-D-galactoside). The recombinant protein was purified with Ni-NTA resin (Pharmacia Biotech, Uppsalla, Sweden) according to the manufacturer's instructions. The purified protein was used for the production of antiserum in rabbits. Western blotting analysis was performed to detect the specificity of the antibody. A sample of rabbit serum (pre-immune serum) was taken before immunization and used as a control in all experiments. The antibody was purified using saturated $(NH_4)_2SO_4$.

1.3 Protein extraction

To test the specificity of the antibody, various plant tissues were ground in liquid nitrogen and extracted in 50 mmol/L Tris buffer (pH 8.0) with 300 mmol/L NaCl, 10 mmol/L EDTA, 10 mmol/L dithiothreitol (DTT), and a protease inhibitor cocktail (Sigma, St Louis, MO, USA) at a dilution of 1∶100. The extraction mixtures were kept on ice for 15 min and then centrifuged for 10 min at 10 000*g* to pellet cellular debris.

For pollen protein extraction, after the supernatant and pellet had been separated, the pellet was extracted further by the addition of homogenization buffer supplemented with 1% (w/v) sodium dodecyl sulfate (SDS) and incubation at room temperature for 15 min. After centrifugation (2000*g*, 8 min, room temperature) the supernatant was collected and termed the "pellet extract".

To extract the soluble proteins of pollen tubes, after germinating *in vitro* for 3 or 16 h, pollen tubes were separated from the medium by mild centrifugation (150*g*, 10 min) and the soluble proteins were extracted as described above. The protein concentrations of the different fractions were estimated according to the method of Bradford (1976).

1.4 Western blotting analysis

Protein extracts were separated on 10% SDS — polyacrylamide gels and blotted to nitrocellulose (Pharmacia) using the Bio-Rad Mini-Protein II apparatus (Bio-Rad, Hercules, CA, USA). After blotting, the filters were blocked with 8% skimmed milk power in TBS (10 mmol/L Tris-HCl, pH 7.4, and 150 mmol/L NaCl) for at least 8 h at room temperature. Then, the membranes were incubated with the antibody against AhSLF-S_2 diluted to 1∶200 for 6–7 h at room temperature. The pre-immune serum served as a control. The secondary antibodies were alkaline phosphatase-conjugated anti-rabbit secondary antibody (Sigma) diluted 1∶10 000 in TBS. Signals were detected with 0.33 mg/mL 4-nitro blue tetrazolium (NBT) and 0.165 mg/mL 5-bromo-4chloro-3indoyl phosphate (BCIP) in alkaline phosphate buffer (100 mmol/L Tris, pH 9.5, 100 mmol/L NaCl, and 5 mmol/L $MgCl_2$).

1.5 Immunogold detection of AhSLF-S_2 by transmission electron microscopy

Mature pollens of *Antirrhinum* were germinated *in vitro* for 3 h and fixed overnight in 0.1 mol/L phosphate buffer (pH 7.0) containing 3% glutaraldehyde. They were then washed in the same buffer, dehydrated in a graded ethanol series, embedded in LR-White, and polymerized by heat. Immunogold labeling was performed on ultra-thin sections. Copper grids with sections were incubated with a 1:50 dilution of the antiserum in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.05% gelatin overnight at 4 °C. After washing twice on a drop of PBS, grids with sections were transferred to a colloidal 10 nm gold-conjugated anti-rabbit IgG diluted 1:20 in the same buffer and incubated for 2 h at room temperature. Samples were washed again and subsequently stained with uranyl acetate and lead citrate, or only stained with uranyl acetate. Preparations were examined and photographed in a Hitachi (Tokyo, Japan) H-7500 transmission electron microscope (TEM).

2 Results

2.1 Production of antiserum against AhSLF-S_2

To examine the localization of AhSLF-S_2, the pET-28b recombinant expression vector was used to produce a fusion protein containing the C-terminal of AhSLF-S_2 with His-tag fused at the N terminus. With the induction of 1 mmol/L IPTG, a prominent band with the expected size was produced (Fig. 1A, lane 2). After purification (Fig. 1A, lane 3), the protein was used for the production of a polyclonal antibody in rabbits. By immunoblotting analysis, we found that the antibody had a high degree of specificity (Fig. 1B); a strong signal at approximately 35 kDa was specifically detected in the proteins extract from the *E. coli* strain expressing the fusion protein.

2.2 Immunoblot detection of AhSLF-S_2 in pollen and pollen tubes

The *AhSLF-S_2* gene and its allelic products have more than 95% identity at the amino acid level (Zhou *et al*. 2003) and may share putative antigenic regions within their C-terminal domains and cross-react with the antibody. Therefore, Western blotting analysis was performed on different *S* genotypes of *Antirrhinum*. The results showed that the antibody was unable to discriminate different alleles of AhSLF-S_2 (data not shown). Thus, the signals in our experiment may be derived from both AhSLF-S_2 and its allelic products.

To examine the specificity of the antibody, proteins from different tissues were extracted and the supernatant was subject to SDS — Polyacrylamide Gel Electrophoresis (SDS-PAGE). After Western blotting, a strong signal at approximately 40 kDa was only found in the pollen extract (Fig. 2). No signal was found with the protein extracts from other organs, including leaves, sepals, petals, and pistils (Fig. 2).

Using the antibody, we further conducted an immunoblotting experiment to detect the expression of AhSLF-S_2 in the supernatant and the pellet of pollen extract (the pellet was extracted with the extraction buffer containing SDS). The results showed that the protein was present predominantly in the supernatant and that no detectable protein was found in the extract of the pellet (Fig. 3), suggesting that AhSLF-S_2 is

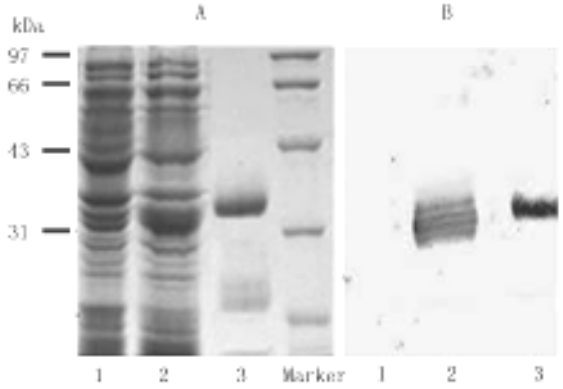

Fig. 1. Expression and immunoblot detection of the AhSLF-S_2 (*S* locus F-box) fusion protein in *Escherichia coli*. **A.** The staining with Coomassie blue of the proteins. lane 1, the proteins extracted from *E.coli* strain (not induced with isopropylthio-β-D-galactoside (IPTG)); lane 2, the proteins extracted from *E. coli* strain induced with 1 mmol/L IPTG; lane 3, the purified fusion protein from *E. coli*. **B.** Proteins from a gel identical to that shown in **A** were transferred to a nitrocellulose filter and detected with the antibody against the C-terminal region of AhSLF-S_2.

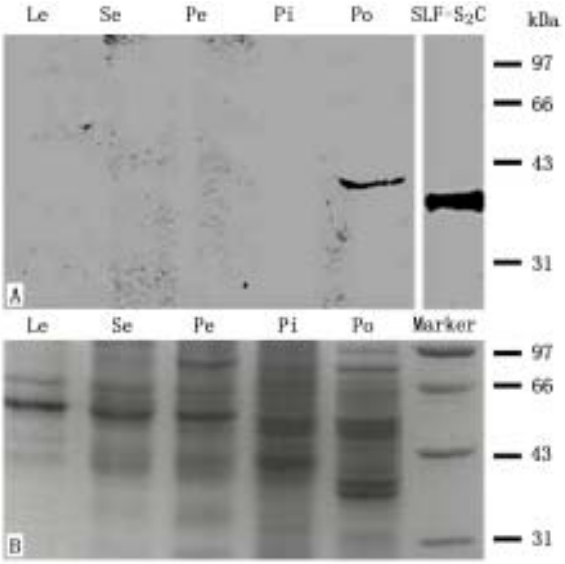

Fig. 2. Detection of AhSLF-S_2 (*S* locus F-box) in several tissues by the polyclonal antibody against its C-terminal region. Immunoblotting analysis of total proteins (40 µg per lane) from leaf, sepal, petal, pistil, pollen and *Escherichia coli* expressing AhSLF-S_2C is shown in **A** and the staining with Coomassie blue of the total proteins (the bacterial proteins were not shown) in **B**. Le, leaf; Pe, petal; Pi, pistil; Po, pollen; Se, sepal.

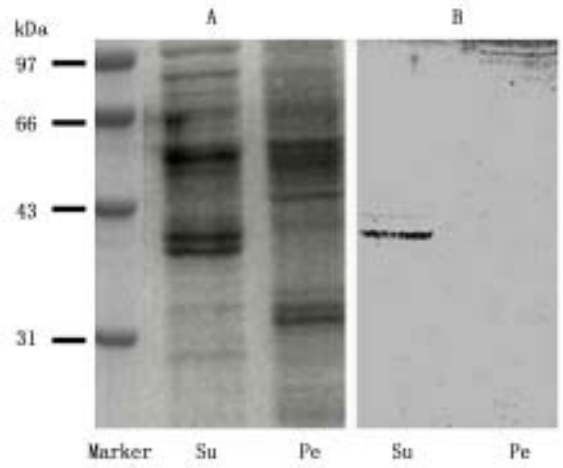

Fig. 3. Immunoblot detection of AhSLF-S_2 (*S* locus F-box) in pollen fractions. SDS-PAGE (**A**) and immunoblotting (**B**) of the supernatant and pellet of pollen extract were conducted using AhSLF-S_2 antibody. A strong signal was only found in the supernatant. Pe, pellet; Su, supernatant.

localized mainly in the pollen cytoplasm.

To determine the stability of the protein, we germinated pollens *in vitro* for 3 or 16 h and analyzed the protein extracts by SDS-PAGE and immunoblotting. The results show that after 16 h *in vitro* germination, the protein was still present (Fig. 4), indicting that the protein is persistent over the elongation period of the pollen tube.

2.3 Subcellular localization by immunoelectron microscopy of AhSLF-S_2 in pollen tubes germinated *in vitro*

To determine the subcellular location of the F-box protein, the immunogold method was used. We used the AhSLF-S_2 antibody as the primary antibody and a gold-conjugated secondary antibody to examine the location of the protein in the pollen tube of *Antirrhinum*. In our experiments, after 3 h germination *in vitro*, some pollen tubes had a length of more than 60 µm and some had just started to germinate. The results reveal that, following germination, abundant gold particles were detected around the area where pollen tubes emerged (Fig. 5A). With respect to other parts of the pollen, gold particles were dispersed randomly (Fig. 5B, C). In order to determine the degree of specificity of the immunogold reaction, a control reaction was performed. The control reaction, conducted by incubation with pre-immune serum, showed no traces of gold in the pollen

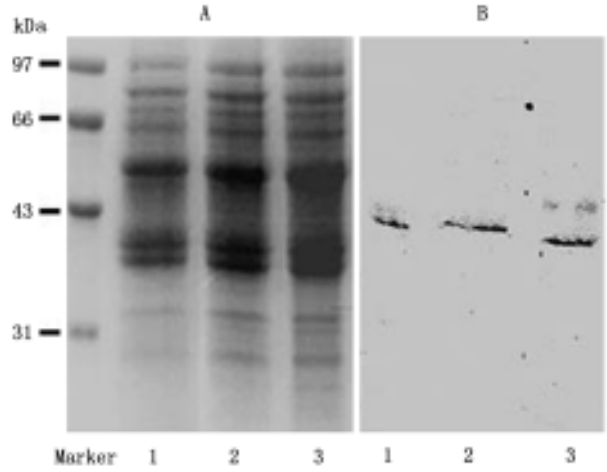

Fig. 4. Immunoblot detection of AhSLF-S_2 (*S* locus F-box) during pollen tube elongation. SDS-PAGE (**A**) and immunoblotting (**B**) of proteins extracted from mature pollen and pollen germinated *in vitro* were conducted. 1, mature pollen; 2, pollen germinated *in vitro* for 3 h; 3, pollen germinated *in vitro* for 16 h.

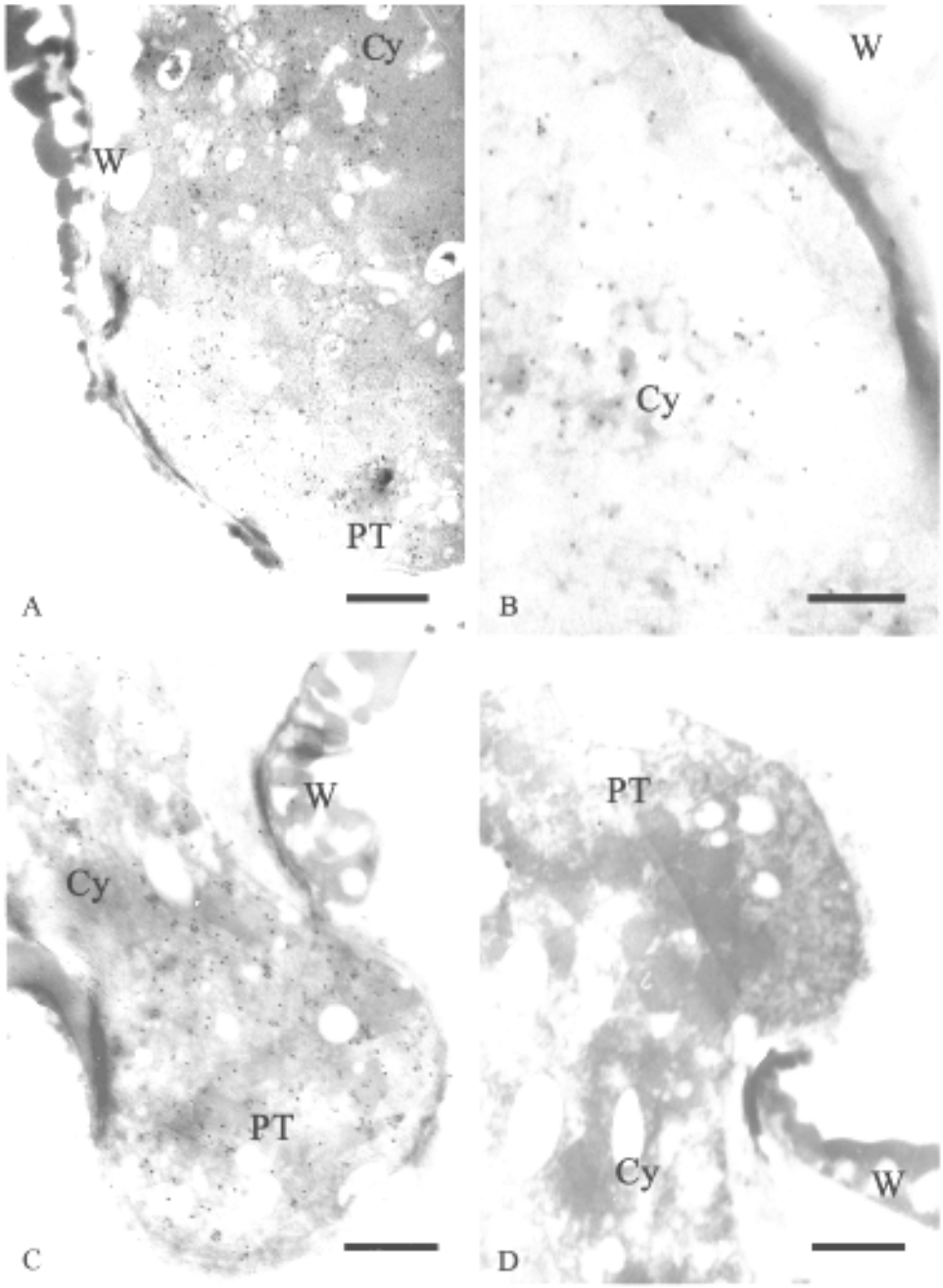

Fig. 5. Immunogold localization of AhSLF-S_2 (*S* locus F-box) in a germinating pollen. **A.** Gold particles dispersed at the germinating place of pollen. Bar = 667 nm. **B.** Gold particles scattered at the pollen cytoplasm. Bar = 333 nm. **C.** With the germination of pollen, gold particles moved toward the pollen tubes. Bar = 1 μm. **D.** The control reaction conducted by incubation with pre-immune serum showed no gold traces in the pollen and pollen tube. Bar = 667 nm. Cy, pollen cytoplasm; PT, pollen tube; W, pollen wall.

and pollen tubes (Fig. 5D). In the elongating pollen tubes, some gold particles appeared to be distributed randomly (Fig. 6). Furthermore, we found that some gold particles were gathered in the zones where the endoplasmic reticulum (ER) was present (Fig. 7).

3 Discussion

In the gametophytic SI system, the female determinant

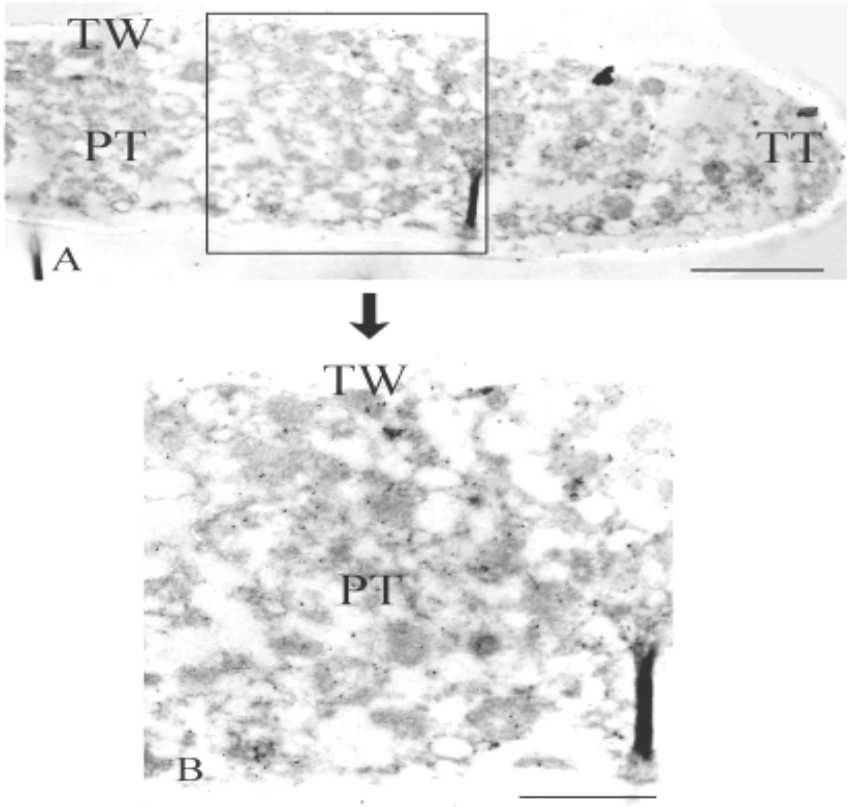

Fig. 6. Localization of AhSLF-S_2 (*S* locus F-box) in an elongating pollen tube. **A.** An elongating pollen tube. Bar = 2.5 μm. **B.** Box in **A** was magnified. Gold particles were dispersed randomly at the cytoplasm of pollen tubes. Bar = 1.25 μm. PT, pollen tube; TT, pollen tube tip; TW, pollen tube wall.

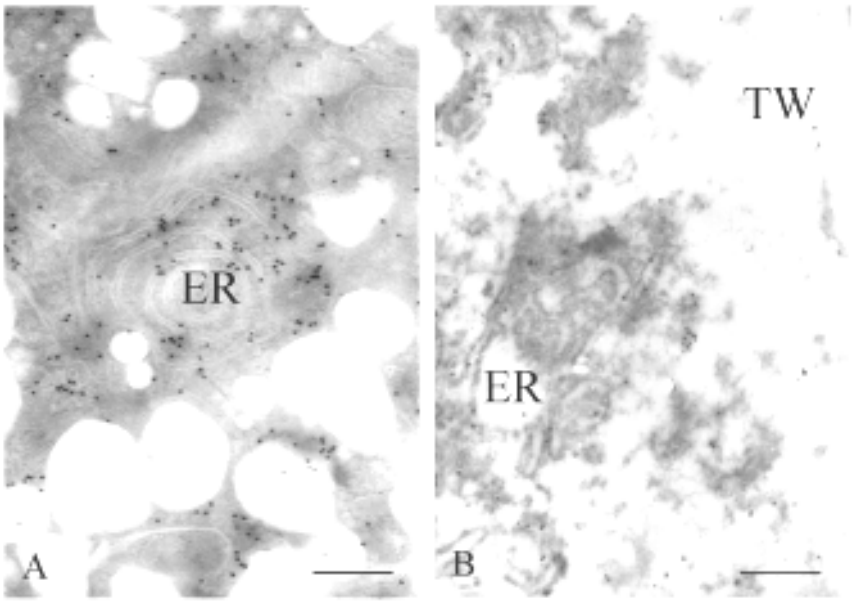

Fig. 7. Localization of AhSLF-S_2 (*S* locus F-box) at the endoplasmic reticulum (ER) of a germinating pollen. **A.** Some gold particles were found in close association with the ER of the germinating pollen. Bar = 333 nm. **B.** Gold particles were found at the ER of an elongating pollen tube. Bar = 500 nm. TW, pollen tube wall.

S-RNase is predicted to interact with the pollen determinant for self/non-self pollen discrimination (McCubbin and Kao 2000). Previously, immunogold localization with both *in vitro*- and *in vivo*-grown pollen tubes clearly demonstrated the non-allelic uptake of S-RNase by pollen and the S-RNase was found in the cytoplasm of the pollen tube (McFadden *et al*. 1992; Luu *et al*. 2000). In the present study, the data show that the F-box protein AhSLF-S_2 appears to be localized in the cytoplasm of pollen tubes germinated *in vitro*, consistent with the possibility that it encodes an *Sp* candidate (Lai *et al*. 2002; Zhou *et al*. 2003).

Our results also suggest that AhSLF-S_2 appears to be dispersed around the periphery of the ER in the germinating and elongating pollen tubes. AhSLF-S_2 has no known signal peptide (Lai *et al*. 2002), indicating that it is a cytoplasm protein. The immunoblotting experiment confirmed that AhSLF-S_2 is present in the cytoplasm of the pollen. The only known function of the F-box protein is to mediate protein degradation by the ubiquitin/proteasome system (Craig and Tyers 1999). Early data showed that the cytosolic face of the ER membranes is a major site for proteasomal protein degradation (Sommer *et al*. 2001; Stone *et al*. 2003). Perhaps the association of AhSLF-S_2 with the ER is likely to be connected to its possible role in proteasomal protein degradation.

Until now, no direct evidence linking the ubiquitin/proteasome system and the S-RNase-based gametophytic SI system has been reported. Using pistils of SI *Nicotiana alata*, Li *et al*. (1995) reported the localization of ubiquitin-conjugated proteins detected by immunofluorescence of antibody to ubiquitin and an intense beaded label was found in pollen intines and pollen tube walls. Recent studies in *Brassica*, which exhibit sporophytic SI, have shown that ubiquitin-mediated proteolysis plays an important function in the SI response (Stone *et al*. 2003). It is not clear whether a similar process occurs in the *Antirrhinum* SI response. Being an important factor in ubiquitin-mediated proteolysis, the F-box protein can specifically recognize the target protein that is to be ubiquitined. AhSLF-S_2 is localized in the elongating pollen tubes (Figs. 5–7) and, after 16 h *in vitro* germination, the protein was still present, indicating that the protein is persistent over the elongation period of the pollen tube. If it is indeed encoded by *Sp*, it would recognize S-RNases and mediate their degradation by the ubiquitin/proteasome system. Clearly, further experimental work involving *in vivo* pollen localization and functional studies of AhSLF-S_2 will help address these questions.

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