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Selective inhibition of the growth of incompatible pollen tubes by S-protein in the Japanese pear

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Abstract The S-allele-associated proteins (S-proteins) in the styles of the Japanese pear (*Pyrus serotina* Rehd. var. *culta* Rehd.) were purified by cation exchange chromatography. Their inhibitory action on the growth of incompatible pollen tubes (pollen tubes bearing the same *S*-allele as in the style from which the S-proteins were prepared) was characterized in vitro. Germination and tube growth of self-pollen (pollen from the same cultivar from which the S-proteins were prepared) decreased dose-dependently when the S-protein was added to the medium. Tube length was reduced to 10% that of compatible pollen tubes (pollen tubes bearing the *S*-allele different from that in the style from which the S-proteins were prepared) at 1.5 μg μl^{-1}. S-proteins from Shinsui (S_4S_5) also inhibited growth of cross-incompatible Kosui (S_4S_5) pollen tubes, but not of compatible Chojuro (S_2S_3) pollen tubes. After inactivation of RNase of the S-protein, the inhibitory action of the S-protein disappeared. These results indicate that the S-protein acts directly to inhibit growth of incompatible pollen tubes in Japanese pear styles, and that the RNase activity of the protein is essential for the biological function. However, small amounts of proteins that co-migrated with the S-protein may also play some roles in the inhibition. This is the first report on the selective inhibitory action of S-proteins in Rosaceae.

Keywords Japanese pear · Pollen inhibition · *Pyrus serotina* · RNase · S-protein

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Introduction

Gametophytic self-incompatibility is a system by which growth of self-pollen tubes is selectively prevented, resulting in promotion of outcrossing. This system is controlled by a single polymorphic locus, the *S*-locus. The Japanese pear, one of the most important fruit crops in Japan, has a strong gametophytic self-incompatibility mechanism, and sometimes shows cross-incompatibility among the cultivars. Seven alleles at the *S*-locus (*S*-alleles) have been established using the paterclinal incompatibility phenomenon (Terami et al. 1946) and, recently, proteins associated with the respective *S*-alleles (S-proteins) have been identified (Sassa et al. 1992, 1993; Hiratsuka et al. 1995; Ishimizu et al. 1996). All of these proteins are RNases. However, the physiological role of these S-proteins in a self-incompatibility mechanism is still unclear.

The S-proteins from Solanaceae have been well studied. They have RNase activity and the purified *Nicotiana* S-protein significantly inhibits the growth of pollen tubes bearing the same *S*-allele as the style from which the *S*-protein was prepared (Harris et al. 1989; Jahnen et al. 1989; Gray et al. 1991). Thus, possible participation of S-proteins in self-incompatibility reactions was suggested. By utilizing transgenic plants, RNase activity of the protein has been shown to be essential for recognition and/or rejection of the incompatible pollen (Huang et al. 1994), and McCubbin et al. (1997) strongly suggested that the RNase activity is essential not for recognition, but for rejection. The similarities between *Nicotiana* S-protein and *Pyrus* S-protein imply that the physiological mechanisms are similar in the two families.

This paper reports the selective inhibitory action of pear S-proteins on the growth of self- and cross-incompatible pollen tubes in vitro, and the indispensability of RNase activity in inhibition of pollen-tube growth.

Materials and methods

Plant materials

Adult trees of the Japanese pear *Pyrus serotina* Rehd. var. *culta* Rehd. planted in the orchards of Mie University, Tsu, Japan, were used. The cultivars and their S-genotypes were: Chojuro (S_2S_3), Osa-Nijisseiki ($S_sS_4^{SM}$), Nijisseiki (S_2S_4), Shinsui (S_4S_5) and Kosui (S_4S_5). Osa-Nijisseiki is a self-compatible mutant and its S_4^{SM} allele is designated by progeny analysis of the cultivar (Sato et al. 1988). SM means stylar-part mutant, and S_4^{SM}-protein is the same as S_4-protein (Hiratsuka et al. 1999). Flowers of each cultivar were collected immediately before anthesis, and styles were detached, weighed, and stored in liquid nitrogen until use. Anthers were also collected, dehisced, dried in a bottle containing desiccants, and stored at –30°C.

Preparation of S-proteins

All procedures were carried out at 4°C or lower unless indicated otherwise. Procedures were based on the method of Ishimizu (1998) with some modifications. Four grams of styles were homogenized in a mortar containing liquid nitrogen. To the homogenate, 200 ml of 50 mM MES/NaOH buffer (pH 6.5) containing 5 mM Na_2EDTA, 1.5% sodium ascorbate, and 3% polyvinylpolypyrrolidone (Polyclar-AT, Sigma Chemical, St. Louis, Mo., USA) were added and the mixture was stirred for 30 min. It was then centrifuged at 16,000 g for 10 min and the supernatant was collected (protein sample). The resulting pellet was washed again with the same buffer, centrifuged, and the supernatant was combined with the protein sample. The sample was then filtered through a millipore membrane (0.45 µm, Millipore, Mass., USA) and passed through a corboxymethyl (CM) Sepharose column (20×1.6 cm) to adsorb S-proteins. The column was washed with 50 mM MES/NaOH buffer and the adsorbed S-proteins were eluted with 350 mM NaCl dissolved in 50 mM MES/NaOH buffer. The eluate was then diluted four-fold with 50 mM MES/NaOH buffer and passed through a sulfopropyl (SP) Sepharose column (20×1.6 cm). The column was first washed with more than two column volumes of 180 mM NaCl containing 50 mM MES/NaOH, and S-proteins were eluted with 300 mM NaCl containing 50 mM MES/NaOH. The eluate was saturated with $(NH_4)_2SO_4$ and proteins were precipitated by centrifugation at 30,000 g for 10 min. The sediment was redissolved with 10 µl of 50 mM MES/NaOH buffer and dialyzed overnight against the same buffer to remove $(NH_4)_2SO_4$. This S-protein sample was stored in Eppendorf tubes at –80°C until use. The protein concentration in the sample was determined by the method of Bradford (1976).

Purity check of S-protein

S-protein samples were analyzed by isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) according to Hiratsuka et al. (1999). The separated protein bands were visualized by Coomassie brilliant blue (CBB) or silver staining (Silver Stain Kit, Wako, Osaka, Japan). The gel was photographed and scanned with an image scanner (Epson, Nagano, Japan) to calculate the purity of S-protein as reported previously (Hiratsuka et al. 1999), i.e., each protein band on the gel was quantified with image software from the National Institutes of Health (NIH) using different amounts of bovine serum albumin (BSA) run on the same gel as standards. Each S-protein sample (0.5 µg) was also subjected to RNase stain following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by a modified version of the method of Yen and Green (1991). The 17.5% polyacrylamide gel was used. After electrophoresis, the gel was washed, incubated, stained with 0.2% toluidin blue (Merck, Darmstadt, Germany), and destained according to the method of Yen and Green (1991).

Preparation of liquid medium and bioassay

Based on the results of the S-protein assay with solanaceous plants (Harris et al. 1989; Jahnen et al. 1989; Gray et al. 1991) and our previous investigations with Japanese pear (Hiratsuka et al. 1987), several kinds of liquid media were prepared to determine the optimum medium for culturing Japanese pear pollen (Table 1). Among the five media tested, we selected medium E for the assay: 50 mM MES/NaOH buffer (pH 6.5) containing 10% sucrose, 15% polyethylene glycol 4000 (PEG), 0.01% H_3BO_3, 0.07% $Ca(NO_3)_2 \cdot 4H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.01% KNO_3. Because the concentration of S-protein in the medium was adjusted by the addition of 50 mM MES/NaOH buffer, the medium containing double-strength components was prepared first.

A piece of filter paper (5×5 mm), which had been washed with EtOH for 3 h to remove impurities, was placed in a petri dish (5 cm in diameter) and 7.5 µl of liquid medium containing S-proteins was added. Pollen grains were scattered uniformly onto the filter paper and bottled to imbibe all grains. Then the petri dish was placed in a wet chamber and maintained at 25°C in darkness. After incubation, the pollen grains were transferred onto a glass slide, stained with cotton blue dye (0.2% solution dissolved in a mixture of lactate: phenol:glycerin:H_2O=1:1:1:1) and sealed with a cover glass. Percentage of germination and the pollen tube length were determined under a light transmission microscope. More than 100 pollen grains were observed for pollen germination, and more than 30 tubes were measured for pollen tube length in each treatment. Each treatment was repeated five times.

Inactivation of RNase activity

The S-protein was suspended in 0.1 M NaOH solution for 5 min to inactivate RNase activity. After this treatment, proteins were gathered by centrifugation at 20,000 g for 5 min and subjected to the assay. More than 95% of the RNase activity disappeared following treatment.

Results

Table 1 shows pollen germination and pollen-tube growth of Japanese pear in various media. Medium A, which has been conventionally used to check for pollen viability of Japanese pear, performed much better than medium C, in which the distilled water of medium A

Table 1 Pollen germination and pollen-tube growth in the Japanese pear on various media. Values are means of 300 pollen grains on 35 pollen tubes

Medium	Pollen germination (%) ±SE	Pollen tube length (µm) ±SE
A	38.8±6.5	168.8±7.5
B	30.0±5.0	312.5±13.8
C	6.3±1.9	68.8±6.3
D	17.5±3.8	50.0±5.0
E	51.3±5.3	368.8±13.8

A Distilled water containing 10% sucrose and 0.01% H_3BO_3.
B 50 mM MES (pH 6.5) containing 10% sucrose, 0.07% $Ca(NO_3)_2 \cdot 4H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% KNO_3, and 0.01% H_3BO_3.
C 50 mM MES (pH 6.5) containing 10% sucrose and 0.01% H_3BO_3.
D 50 mM MES (pH 6.5) containing 10% sucrose and 15% PEG.
E 50 mM MES (pH 6.5) containing 10% sucrose, 0.07% $Ca(NO_3)_2 \cdot 4H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% KNO_3, 0.01% H_3BO_3, and 15% PEG.

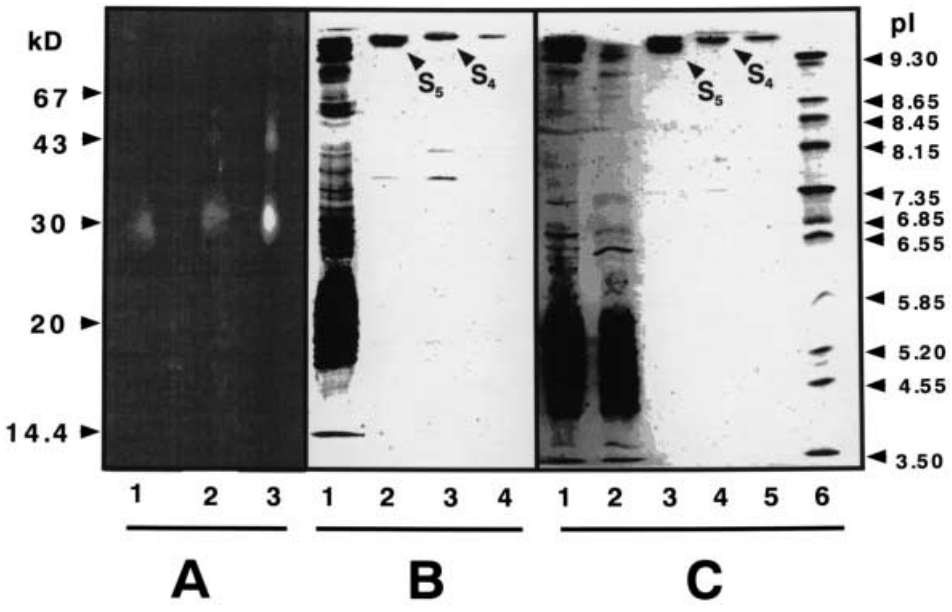

Fig. 1A–C Electrophoretic patterns of S-proteins purified from styles of three Japanese pear cultivars. **A** RNase stain profiles following SDS-PAGE. *Lane 1* Purified S-proteins (0.5 µg) from Shinsui style (S_4S_5). *Lane 2* Purified S-proteins (0.5 µg) from Nijisseiki style (S_2S_4). *Lane 3* Purified S-proteins (0.5 µg) from Osa-Nijisseiki style ($S_2S_4^{SM}$). Note the strong activity spot at about 30 kDa and the weak one at about 40 kDa in each cultivar. **B** Silver stain profiles following IEF-PAGE. *Lane 1* Crude proteins (100 µg) from Shinsui style (S_4S_5). *Lane 2* Purified S-proteins (4 µg) from Shinsui style (S_4S_5). *Lane 3* Purified S-proteins (2 µg) from Nijisseiki style (S_2S_4). *Lane 4* Purified S-proteins (2µg) from Osa-Nijisseiki style ($S_2S_4^{SM}$). Note the several faint bands in neutral zones in each cultivar. **C** CBB stain profiles following IEF-PAGE. *Lane 1* Crude proteins (100 µg) from Shinsui style (S_4S_5). *Lane 2* Crude proteins (100 µg) from Shinsui style (S_4S_5), from which S-proteins were removed. *Lane 3* Purified S-proteins (4 µg) from Shinsui style (S_4S_5). *Lane 4* Purified S-proteins (2 µg) form Nijisseiki style (S_2S_4). *Lane 5* Purified S-proteins (2 µg) from Osa-Nijisseiki style ($S_2S_4^{SM}$). S_2-protein bands of Nijisseiki and Osa-Nijisseiki are not clear because of its small amount

was replaced by MES/NaOH buffer for both pollen germination and pollen-tube growth. Addition of inorganic components such as $Ca(NO_3)_2$ and $MgSO_4$ were necessary for better germination when MES/NaOH buffer was used (media B and E gave better results than medium D). We concluded that medium E is the best for culture of Japanese pear pollen.

The purified S-proteins from each cultivar showed two RNase spots on the SDS gel. One showed strong activity and about 30 kDa; the other was weak and about 40 kDa (Fig. 1A). These separation profiles may be reasonable, because Japanese pear S-protein has sugar residues of different sizes and a major molecule is about 30 kDa in all S-proteins (Sassa et al. 1993; Okada and Hiratsuka 1994; Ishimizu 1998). At least two types of heterogeneity of sugar moieties have been detected in S_2-, S_3-, S_4-, and S_5-proteins (Ishimizu 1998). Stylar S-proteins from three cultivars were purified to near homogeneity by cation exchange chromatography (Fig. 1C). Shinsui (S_4S_5), Nijisseiki (S_2S_4), and Osa-Nijisseiki ($S_2S_4^{SM}$) involved S_4-/S_5-, S_2/S_4-, and S_2-S_4^{SM}-proteins, respectively. Each S-protein band was identified based on Hiratsuka et al. 1995. In these S-protein fractions, however, small amounts of contaminants were detected when the electrophoresed gel was stained with silver (Fig. 1B). The purities of these S-protein fractions monitored by NIH image software were: Shinsui 94.3%, Nijisseiki 88.2%, and Osa-Nijisseiki 80.2%. Based on these percentages, we determined the S-protein concentration in each sample.

The relationship between pollen and S-protein is defined hereafter as follows: self pollen is pollen from the same cultivar as that from which the S-proteins were prepared, incompatible pollen is pollen bearing the same *S*-allele as that in the style from which the S-proteins were prepared, and compatible pollen is pollen bearing an *S*-allele different from that in the style from which the S-proteins were prepared.

Germination of incompatible pollen was not inhibited by S-proteins at 0.4 and 0.8 µg µl^{-1}, but was reduced at concentrations higher than 0.8 µg µl^{-1} (Fig. 2A). The inhibitory effect increased gradually with increasing concentration of the S-proteins from 0.8 to 1.2 µg l^{-1}, and rapidly at higher concentrations. In contrast germination of compatible pollen was not affected by the S-proteins at any concentration tested. The elongation of incompatible pollen tubes was significantly inhibited by the S-proteins at 0.4 µg µl^{-1}; the inhibitory effect gradually increased with increasing protein concentration up to 1.2 µg µl^{-1} and rapidly at concentrations higher than 1.25 µg µl^{-1} (Fig. 2B). Growth of compatible pollen tubes was inhibited gradually with increasing S-protein concentration in the medium, but the inhibition was significantly weaker than that in incompatible pollen tubes.

The time line of the effects of S-proteins on pollen germination and elongation of pollen tubes was studied (Fig. 3A, B). Percentage of pollen germination increased

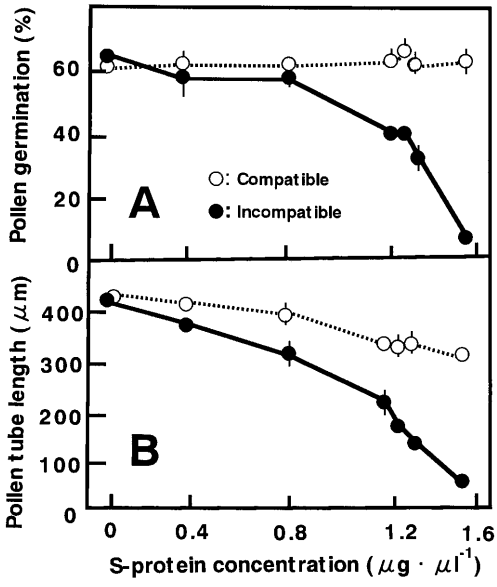

Fig. 2A,B Effect of S-protein concentration on pollen germination (**A**) and growth of pollen tubes (**B**) after 24 h of culture. Compatible: Chojuro (S_2S_3) pollen in the medium with Shinsui (S_4S_5) S-proteins. Incompatible: Shinsui (S_4S_5) pollen in the medium with Shinsui (S_4S_5) S-protein. *Vertical bars* indicate±SE. Each data point represents the mean of five replications with 100-pollen grains each, or five replications with 30 pollen tubes each

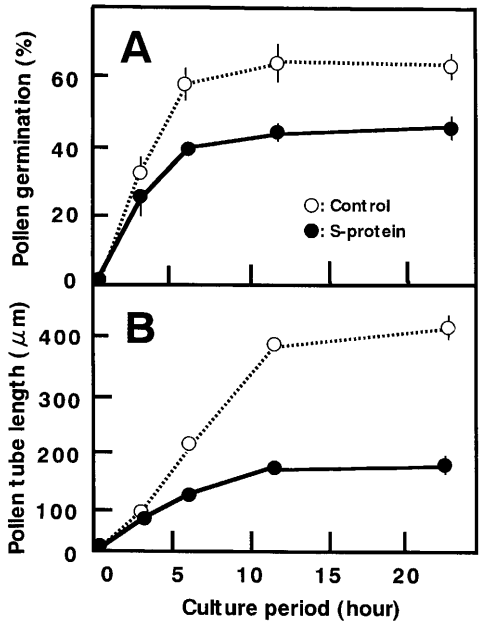

Fig. 3A,B Time line of pollen germination (**A**) and the growth of pollen tubes (**B**) in the medium with or without S-proteins (1.25 μg μl-1). S-protein: Nijisseiki (S_2S_4) pollen in the medium with Nijisseiki (S_2S_4) S-protein. Control: Nijisseiki (S_2S_4) pollen in the medium without S-protein. *Vertical bars* indicate±SE. Each data point represents the mean as given in Fig. 2

early during the culture period and reached nearly the maximum level at 6 h. Addition of S-proteins (1.25 μg μl-1) lowered germination at 3 h and reduced the maximum percentage to about 70% of the control. Elongation of pollen

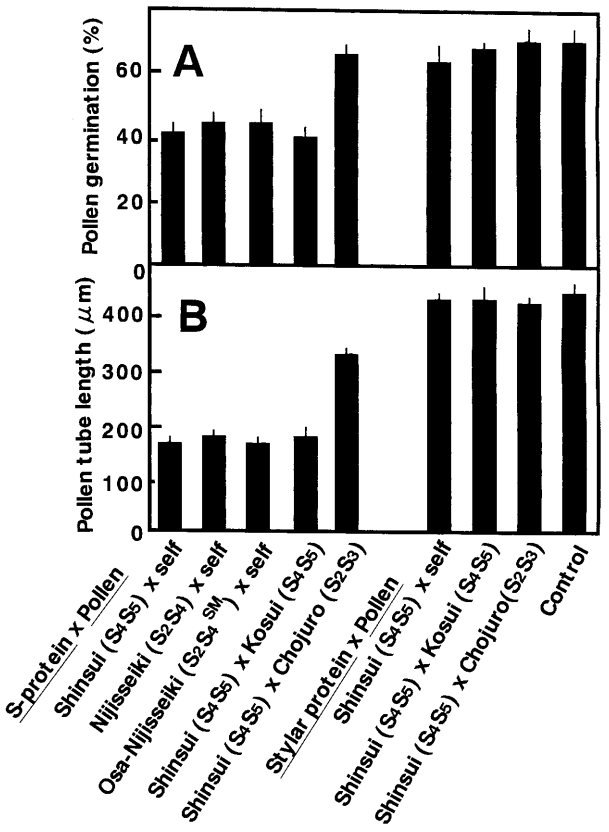

Fig. 4A,B Effect of S-proteins (1.25 μg μl-1) and stylar crude proteins (1.25 μg μl-1) with S-proteins removed (proteins of lane 2 in Fig. 1C) on pollen germination (**A**) and the growth of pollen tubes (**B**) after 24 h of culture. Control: Shinsui (S_4S_5) pollen in the medium without S-protein. *Vertical bars* indicate±SE. Each data point represents the mean as given in Fig. 2

tubes was strongly inhibited by S-proteins; growth continued for 12 h as in the control, but the length of the pollen tubes was at most 40% of the control.

Both pollen germination and elongation of pollen tubes were significantly inhibited by the S-proteins prepared from either self styles or cross-incompatible styles (Fig. 4A,B, Fig. 5A–F). The degree of inhibition was similar in self-pollen from three cultivars and cross-incompatible pollen at a concentration of 1.25 μg μl-1. Although the growth of compatible pollen tubes was slightly suppressed, germination was not affected by the S-proteins. On the other hand, stylar proteins with S-proteins removed (proteins of lane 2 in Fig. 1C) did not show any inhibitory effect on either incompatible or compatible pollen.

After heating, the S-proteins strongly inhibited pollen germination and growth of the pollen tubes irrespective of compatibility (data not shown), as reported by Jahnen et al. (1989) in *Nicotiana*. In this experiment, therefore, we treated the S-proteins with 0.1 M NaOH for 5 min, which inactivated the S-protein RNase more than 95%. The S-protein inactivated by this treatment had almost no activity on either pollen germination or growth of pollen tubes of incompatible pollen (Table 2).

Table 2 Effect of RNase activity of S-proteins on pollen germination and growth of pollen tubes, Unit=1 increase in A_{260} min^{-1} $protein^{-1}$ mg. *Control* = liquid medium. *S-proteins* and pollen from Nijisseiki (S_2S_4) were used. Each value represents the mean of five replicications with 100 pollen grains or five replications with 30 pollen tubes

Proteins	RNase activity (unit)	Pollen germination (%)±SE	Pollen tube length (μm)±SE
Inactivated S-protein	4.6±4.6	59.4±1.7	411.2±7.8
S-protein	280.6±3.7	44.1±2.1	164.5±12.9
Control	0.0±0.0	61.2±2.7	419.8±12.9

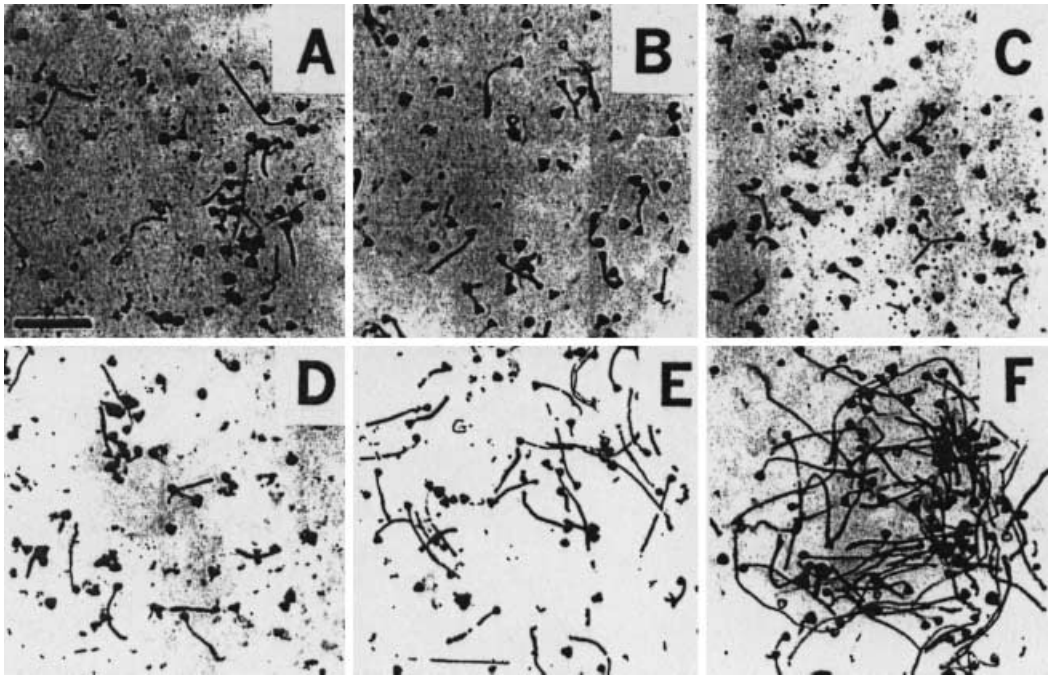

Fig. 5A–F Effect of S-proteins (1.25 μg $μl^{-1}$) on pollen germination and growth of pollen tubes after 24 h of culture. *Bar* in **A** indicates 200 μm. **A** Shinsui (S_4S_5) pollen in the medium with Shinsui (S_4S_5) S-proteins. **B** Nijisseiki (S_2S_4) pollen in the medium with Nijisseiki (S_2S_4) S-proteins. **C** Osa-Nijisseiki ($S_2S_4^{SM}$) pollen in the medium with Osa-Nijisseiki ($S_2S_4^{SM}$) S-proteins. **D** Kosui (S_4S_5) pollen in the medium with Shinsui (S_4S_5) S-proteins. **E** Chojuro (S_2S_3) pollen in the medium with Shinsui (S_4S_5) S-proteins. **F** Control: Shinsui (S_4S_5) pollen in the medium without S-proteins

Discussion

The present assay system for pollen germination of the Japanese pear seems to be much more efficient than other assays reported in the past. This may result from the use of wet filter paper on which pollen grains are sprinkled. Because part of a pollen grain is exposed to ambient air, pollen can utilize oxygen in the air far more easily than in liquid medium. This consideration can be partly supported by the fact that pollen germination and subsequent tube growth on agar medium are much greater than in liquid medium (unpublished data).

The present results strongly suggest that the S-proteins from Japanese pear function directly on recognation and rejection of incompatible pollen. This selective inhibition for incompatible pollen, however, was induced at concentrations higher than 0.4 μg $μl^{-1}$ of S-proteins for pollen-tube growth and 1.2 μg $μl^{-1}$ for pollen germination. This indicates that the S-protein at a concentration higher than the threshold functions to induce incompatibility. The rate of self-pollen inhibition in self-compatible Osa-Nijisseiki was almost the same as in self-incompatible Nijisseii when S-proteins were applied at 1.25 μg $μl^{-1}$ (Figs. 4, 5). The former cultivar contains a smaller amount of S_4-protein than the latter. If the ratio of S_4-protein level to total S-protein (S_2 + S_4) level decreases, the inhibitory effect on self pollen may become weaker. However, the amount of Osa-Nijisseiki S_2-protein is also less than the Nijisseiki S_2-protein, as reported earlier (Zhang and Hiratsuka 1999). Thus, S-proteins from Osa-Nijisseiki may show inhibitory functions similar to those from Nijisseiki when the assay is performed at the same S-protein concentration.

Jahnen et al. (1989) observed the inhibitory effect of S-proteins at 0.3 to 0.6 μg $μl^{-1}$ in *Nicotiana*. Harris et al. (1989) observed inhibitory effects of S-protein at 0.0031 μg $μl^{-1}$, and Gray et al. (1991) investigated the effect of S-protein at 2.0 μg $μl^{-1}$ on amino acid incorporation into pollen tubes. One factor that could cause the

differences in the effective concentration of S-protein might be the purity of the S-proteins. Since S-protein fractions utilized in this study had from 5.7% (Shinsui) to 19.8% (Osa-Nijisseiki) contaminants, we calculated the S-protein concentrations based on their purity. Other factors that could result in the differences in effectiveness might be difference in the assay conditions and loss of biological activity during purification or bioassay. We utilized two S-protein samples simultaneously for the assay because homozygous plants are not available in Japanese pears. Thus, we assume that the threshold concentration of S-protein for pollen tube inhibition is 0.2 μg μl^{-1} or lower in the Japanese pear.

However, we do not yet exclude the possibility that contaminants in the S-protein fraction play a role, together with S-proteins, in selective inhibition of the growth of incompatible pollen tubes. As shown in Fig. 1B, each S-protein fraction contained several neutral proteins, though the amounts were small. Further, the S-protein band separated by IEF-PAGE in this study actually showed from two to eight protein spots on the SDS gel (Okada and Hiratsuka, unpublished data); S_3- and S_5-bands show protein spots greater than S_2- and S_4-bands. One major spot of about 30 kDa, and one or two relatively major spots may be heterogeneity of sugar moieties in the S-protein as described, but other minor proteins have not yet been identified. Both the number and molecular weight of these minor proteins, however, were almost the same among the S-proteins produced from the same S-allele of different cultivars (Okada and Hiratsuka 1994). These unidentified minor proteins might play some roles in expressing selective inhibition of incompatible pollen-tube growth in vitro. This hypothesis is partly supported by the unilateral incompatibility in *Nicotiana*, in which pollen rejection requires non-S-RNase stylar factors (Beecher et al. 1998; Murfett et al. 1996).

The inhibitory effect of S-proteins was much stronger on growth of the pollen tubes than on pollen germination, except at extremely high concentrations (1.5 μg μl^{-1}) (Figs. 2, 3, 4). Although the percentage of germination was reduced only to about 70% of the control, elongation of the pollen tube was reduced to 40% of the control at 24 h after the start of culture (Fig. 3). In *Nicotiana alata*, the growth of incompatible pollen tubes was inhibited by S-protein 4–6 h after germination in vitro (Jahnen et al. 1989), but pollen germination was not affected (Harris et al. 1989). Our earlier study also suggested that the style cannot discriminate incompatible pollen for several hours after pollination in *Lilium longiflorum* (Hiratsuka et al. 1989). S-protein in gametophytically controlled self-incompatible plants may inhibit predominantly the growth of pollen tubes rather than pollen germination. This is probable because pollen germination is physiologically different from the growth of the pollen tubes (Mascarenhas 1993).

We showed that RNase activity of S-protein is necessary to inhibit the growth of incompatible pollen tubes in vitro by using S-proteins treated with NaOH, though the treatment may also cause structural changes in the S-protein. However, we do not yet understand the relationship between the tertiary structure of the S-protein and its biological function. These issues are currently being investigated. As an example, investigations using transgenic plants may be useful to obtain more direct evidence about the role of RNase activity in pollen tube inhibition in the Japanese pear. To create transgenic plants of the Japanese pear, first of all an efficient regeneration system should be established. In solanaceous plants, RNase activity was proved to be necessary for rejection of incompatible pollen in experiments using transgenic plants of *Petunia* (Huang et al. 1994; McCubbin et al. 1997) and *Lycopersicon* (Kowyama et al. 1994; Royo et al. 1994). However, one contradictory phenomenon is that S-protein similarly catalyses RNAs extracted from both compatible and incompatible pollen tubes in vitro, although it can discriminate incompatible from compatible pollen and degrades RNAs of incompatible pollen in vivo (McClure et al. 1990). In the in vitro assay for RNA degradation, a similar phenomenon was confirmed in the Japanese pear by using the S-proteins reported here (unpublished data). It is possible that the S-protein has strict substrate specificity. At present, however, we have no evidence to support this hypothesis. Two proposals presented by Kao and McCubbin (1996) may explain the contradiction: (1) the pollen tube has a transmembrane receptor to discriminate different S-proteins, or (2) the pollen tube has an RNase inhibitor specific to the S-proteins produced from a compatible style.

In conclusion, the S-protein of the Japanese pear selectively inhibits growth of incompatible pollen tubes, and the inhibition is caused by RNase activity of the protein. The inhibition is concentration dependent, and more than 0.4 μg μl^{-1} is necessary to appreciably slow the growth of incompatible pollen tubes. However, we do not exclude the possible role of a few minor proteins that co-migrated with S-proteins in the selective inhibition of incompatible pollen-tube growth.

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