Variations in S-protein Levels in Styles of Japanese Pears and the Expression of Self-incompatibility

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Summary

When excised styles of the Japanese pear were self-pollinated and cultured on an agar medium, the percentage of styles with protruding pollen tubes onto the medium from cut ends varied significantly among different Japanese pear cultivars. Based on these data, the cultivars were classified into the following groups; strongly, intermediately, and weakly self-incompatible (SI), and their levels of S-protein in the style were compared.

The levels of S-proteins which were separated by isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) and quantified by an image analyzer differed considerably among each of S-proteins (S$_3$ > S$_1$ > S$_2$ > S$_4$ > S$_5$ > S$_7$), even when they were produced from the same S-allele.

The sum of two allelic S-proteins correlated positively with the strength of SI in the cultivars (r=0.876). The cultivars which were strongly, intermediately, and weakly SI contained 1.51, 1.23, and 0.67ng of S-protein per mg of buffer extractable protein, respectively. But the level of individual S-proteins was not directly related to the strength of SI in the cultivars. The total amount of S-proteins was mainly due to the combination of 2 S-proteins in the cultivar, i.e. the cultivars with S$_1$- and S$_3$- and/or S$_5$-allele generally contained high level of S-proteins. The distribution pattern of the S-proteins in the style was unrelated to the SI strength of the cultivar.

Thus, each Japanese pear cultivar may possess different strengths of SI system which may be the function of the total S-proteins in the style.

Key Words: Japanese pear cultivar, pollen tube growth, strength of self-incompatibility, stylar S-protein level.

Introduction

Japanese pears have a gametophytic SI system which is controlled by a single locus with multiple alleles, seven of which (S$_1$ to S$_7$) have been identified (Sato, 1992). However, the fruit set by self-pollination differs considerably among Japanese pear cultivars (Kikuchi, 1927; Nagai, 1925). Recently, we found that the growth of self-pollen tubes in the style varies significantly among different Japanese pear cultivars, and that there is a strong correlation between the degree of the tube elongation and percentage of fruit set after self-pollination (Zhang and Hiratsuka, 1999). We ascribed this phenomenon to the different functions of SI system in each cultivar, because the main tissue for arresting selfed pollen tube growth of Japanese pears is the style itself (Hiratsuka et al., 1982).

Recently, Lee et al. (1994) and McCubbin et al. (1997) obtained several transgenic plants of petunia with an intermediate SI system; their styles produce much less S-protein, whereas styles of the self-compatible one produce almost no S-proteins. This condition is likely natural among self-incompatible cultivars, although the transgenic plants were obtained from tissue culture manipulations. Several radish cultivars with an intermediately strong SI system were found recently (Hawlader and Mian, 1997).

S-allele associated proteins (S-proteins) in Japanese pears have been detected by several workers; all of the S-proteins possess RNase activity (Sassa et al., 1992, 1993; Hiratsuka et al., 1995a; Ishimizu et al., 1996). During the S-protein analysis, we found significant quantitative variations among the respective S-proteins. The S$_1$- and S$_3$-proteins are major components of the style, S$_4$- and S$_5$-proteins are moderately abundant, the S$_2$-protein is extremely scarce (Hiratsuka et al., 1995a).

In addition, the amount of S-protein produced from the same S-allele varied among cultivars (Hiratsuka and Okada, 1995).

In this study, we tried to establish the relationship between S-protein levels in the style and the strength of the SI response in the Japanese pear cultivars.

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Materials and Methods

Plant materials

Adult trees of the Japanese pear (Pyrus serotina Rehd. culta Rehd.) from the orchards of Mie University, Tottori University, Mie Agricultural Research Center, and Mr. T. Kuromiya, Hisai city, Japan were used. The cultivars were: strongly SI cultivars; ‘Yakumo’ (S₁S₁), ‘Imamuraaki’ (S₁S₂), ‘Kohsui’ (S₂S₂), and ‘Chojuuro’ (S₁S₂), intermediately SI cultivars; ‘Okusankitsu’ (S₁S₂), ‘Kikusui’ (S₁S₁), ‘Nijisseiki’ (S₂S₂), and ‘Kisu’ (S₁S₂), weakly SI cultivar; ‘Osa-Nijisseiki’ (S₁S₂SMI, SM means stilar - part mutant). This classification was made according to the data by Zhang and Hiratsuka (1999).

Pollen tube growth in excised styles

Procedures were based on the methods of Hiratsuka et al. (1982) in that pistils from each cultivar were self-pollinated 1–2 days before anthesis. The styles were excised at the top of the ovary, half, and one-third the distance from the stigmatic surface immediately after the pollination (referred to 1/1-, 1/2-, and 1/3-length style, respectively). The excised styles were then placed carefully onto an agar medium containing 0.8 % agar, 10% sucrose, and 100 ppm H₃BO₃ in a petri dish (9 cm in diameter) and cultured at 25 °C for 48h in the dark. Pollen was prepared 1 day before the experiment by allowing the anthers to dehisce in a bottle with silica gel. After the 48h culture period, pollen tubes protruding from the cut ends were stained with cotton blue solution and observed under a microscope. The number of styles with protruding pollen tubes from the stilar cut-end and the number of protruding pollen tubes per style were recorded.

Preparation of the styles for protein analysis

Styles from each cultivar were collected 1–2 days before anthesis, weighed and stored in liquid nitrogen until analyzed.

Extraction of stylar proteins and electrophoresis

Proteins were extracted from the whole styles (with stigmas) of the above 9 cultivars, and from the stigmas, the upper and lower halves of the styles of ‘Kohsui’, ‘Chojuuro’, ‘Kikusui’, ‘Nijisseiki’, and ‘Osa-Nijisseiki’ to check the distribution pattern of S-proteins in the style. Proteins were extracted according to Hiratsuka et al. (1995a) by homogenizing 500mg of the styles in a chilled glass homogenizer containing 5ml of 0.05M Tris-HCl buffer (pH 8.4) with 150mM NaCl, 10mM L-cysteine, 1mM L-ascorbic acid, 1mM CaCl₂, 1mM Na₂EDTA and polyclor-AT. The homogenate was centrifuged at 20,000g for 10 min and the supernatant was immediately passed through a Sephadex G-25 gel column to remove phenolic compounds. Protein fractions were precipitated with saturated (NH₄)₂SO₄ solution and the mixture centrifuged at 20,000g for 10 min. The resulting pellet was then redissolved in approximately 1.5ml of Tris-HCl buffer (pH 8.4), dialyzed against the same buffer overnight; an aliquot of the purified concentrate was subjected to electrophoresis. The protein concentration of the samples was determined according to Bradford (1976).

A polyacrylamide gel plate, containing pH 3.5–9.5 amophines, was purchased from Pharmacia Ltd. and used for electrophoresis. One hundred μg of proteins was electrophoresed at 10 °C for 90 min. Running conditions were 1,200 V, 30 mA, and 25 W for a 200 x 110 x 1 mm gel. After electrophoresis, proteins were visualized by staining with a Silver Stain Kit (Wako Ltd., Japan). The amount of S-proteins was estimated by comparison to a standard amount of bovine serum albumin on the same gel.

Determination of S-protein amount

The photograph of a stained gel was incorporated into a computer through an image scanner (Epson Ltd., Japan) and each S-protein band was quantified by utilizing NIH software. The procedures were based on the manufacture’s manual.

Results

The growth of self-pollinated pollen tubes in excised styles of 9 Japanese pear cultivars was tested by using 1/1-, 1/2-, and 1/3-length styles to estimate their inhibitory zones (Table 1). The pollen tube growth differed significantly among the Japanese pear cultivars in 1/1-length styles; about 40% of the ‘Osa-Nijisseiki’ styles had protruding pollen tubes. The average number of the tubes was more than 1.0 per style; whereas in ‘Yakumo’, ‘Imamuraaki’, ‘Kohsui’, and ‘Chojuuro’, less than 10% of the styles had protruding tubes, and the number of protruded tubes was smaller than 0.1 per style. On the other hand, pollen tube growth of ‘Okusankitsu’, ‘Kikusui’, ‘Nijisseiki’, and ‘Kisu’ was intermediate between ‘Osa-Nijisseiki’ and 4 others. On the basis of the style lengths and the percentages of styles with protruding pollen tubes, the styles were divided into 4 groups: 1) 1/1- and 1/2-length styles had similar values, but 1/3-length styles had a significantly higher percentage (‘Osa-Nijisseiki’, ‘Imamuraaki’, ‘Kohsui’, ‘Chojuuro’); 2) the percentage became higher as styles were shortened (‘Okusankitsu’); 3) pollen tubes protruded consistently, independent of the length of the style (‘Kikusui’, ‘Kisu’, ‘Yakumo’); and 4) many pollen tubes protruded from the 1/2- and 1/3-length styles (‘Nijisseiki’).

The electrophoretic profiles of stylar S-proteins in the 9 cultivars (Fig. 1) reveal several protein bands unrelated to S-alleles. The S-protein bands sometimes overlap each other on the IEF gel, which made identifying each S-protein band difficult. However, we could identify it by repeated analysis. The S₀ and S₁-protein
Table 1. The growth of selfed pollen tubes in excised styles of Japanese pear cultivars.

<table>
<thead>
<tr>
<th>SI intensity</th>
<th>Cultivar</th>
<th>S-genotype</th>
<th>1/1 style</th>
<th>1/2 style</th>
<th>1/3 style</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No. of styles</td>
<td>% of styles</td>
<td>No. of tubes</td>
</tr>
<tr>
<td>Weak</td>
<td>Osa-Nijisseiki</td>
<td>S2S4sn</td>
<td>65</td>
<td>38.5 ± 6.5</td>
<td>1.07 ± 0.46</td>
</tr>
<tr>
<td>Intermediate</td>
<td>Okusankitsu</td>
<td>S5S7</td>
<td>49</td>
<td>30.6 ± 6.7</td>
<td>0.51 ± 0.13</td>
</tr>
<tr>
<td>&quot;</td>
<td>Kikusui</td>
<td>S2S4</td>
<td>62</td>
<td>29.0 ± 7.4</td>
<td>0.34 ± 0.07</td>
</tr>
<tr>
<td>&quot;</td>
<td>Nijisseiki</td>
<td>S2S4</td>
<td>29</td>
<td>27.6 ± 3.7</td>
<td>0.51 ± 0.21</td>
</tr>
<tr>
<td>&quot;</td>
<td>Kisui</td>
<td>S3S4</td>
<td>30</td>
<td>26.7 ± 4.9</td>
<td>0.40 ± 0.16</td>
</tr>
<tr>
<td>Strong</td>
<td>Yakumo</td>
<td>S1S4</td>
<td>38</td>
<td>7.9 ± 3.7</td>
<td>0.08 ± 0.04</td>
</tr>
<tr>
<td>&quot;</td>
<td>Imamuraaki</td>
<td>S1S6</td>
<td>30</td>
<td>6.7 ± 4.2</td>
<td>0.13 ± 0.10</td>
</tr>
<tr>
<td>&quot;</td>
<td>Kohsui</td>
<td>S4S5</td>
<td>97</td>
<td>5.2 ± 2.8</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>&quot;</td>
<td>Chojuro</td>
<td>S3S2</td>
<td>30</td>
<td>3.3 ± 3.3</td>
<td>0.03 ± 0.03</td>
</tr>
<tr>
<td>Control (cross-compatible combination)</td>
<td>Kisuix</td>
<td>S1S3</td>
<td>28</td>
<td>85.7 ± 6.7</td>
<td>7.04 ± 1.35</td>
</tr>
<tr>
<td>&quot;</td>
<td>Kikusui</td>
<td>S3S4</td>
<td>30</td>
<td>85.7 ± 6.7</td>
<td>7.04 ± 1.35</td>
</tr>
</tbody>
</table>

![Diagram](image)

Fig. 1. Silver stained gel of stylar S- proteins in Japanese pear cultivars which are different in strength of self-incompatibility response. One hundred micrograms of protein were applied to each lane of an IEF-polyacrylamide gel containing pH 3.5-9.5 ampholines. Arrows in the figure indicate 2 S- protein bands in each cultivar. Common band: the band found in all cultivars. S=strong SI cultivar, I=intermediate SI cultivar, W=weak SI cultivar.
bands were identified by comparing their mobilities on the IEF gel with those of other S-proteins, because only a single cultivar with the S_6 or S_7 allele was available (Sato, 1992). The bands which did not correspond to the S_1, S_8-protein bands and were not found in other cultivars were designated as the S_2-protein in 'Imamuraaki' (S_3S_0) and S_7-protein in 'Okusankitsu' (S_0S_7).

The staining intensity of each S-protein band was strikingly different; the intensity order was S_0 > S_1 > S_8 > S_4 > S_5 > S_2 > S_7. For example, the intensity of S_2-protein band was less than half the level of the S_1-protein band. In addition, the quantity of S-proteins produced from the same S-allele varied among cultivars. For example, 'Yakumo' (S_1S_1) had less S_4-protein than had 'Kikusui' (S_2S_2), whereas 'Kikusui' had more S_2-protein than had 'Chojuro' (S_2S_3).

The sum of two allelic S-proteins and the amount of each S-protein in the respective cultivars were calculated (Figs. 2 and 3). There exists a clear relation between the amount of S-proteins and the self-incompatibility reaction.

![Fig. 2](image.png)

**Fig. 2.** Comparison of the amount of total S-protein in styles of Japanese pear cultivars which are different in strength of self-incompatibility response. Vertical bars indicate SE. S=strong SI cultivar, I=intermediate SI cultivar, W=weak SI cultivar.

![Fig. 3](image.png)

**Fig. 3.** Comparison of respective S-protein amounts in styles of Japanese pear cultivars which are different in strength of self-incompatibility response. Vertical bars indicate SE. S=strong SI cultivar, I=intermediate SI cultivar, W=weak SI cultivar.

![Fig. 4](image.png)

**Fig. 4.** Gels of S-proteins in styles of Japanese pear cultivars with different strengths of self-incompatibility. One hundred micrograms of protein were applied to each lane of an IEF-polyacrylamide gel containing pH 3.5-9.5 ampholines. Common band; the band found in all cultivars. S=strong SI cultivar, I=intermediate SI cultivar, W=weak SI cultivar. L=lower part of style, U=upper part of style, S=stigma.
between pollen tube growth and total amount of S-protein; the cultivars with better pollen tube growth contained less total S-protein (Fig. 2). This relationship became obvious among the 3 groups described above; 'Kohsui' and 'Chojuro', the strongly SI cultivars exhibited significantly larger amount of total S-proteins compared to the intermediate or weak ones such as 'Kikusui', 'Okusankitsu', and 'Osa-Nijisseiki'. However, the level of protein in an individual S-allele appears to be unrelated to the pollen tube growth (Fig. 3).

Distribution patterns of S-proteins in the style of the 5 strongly, intermediate, and weakly SI cultivars (Figs. 4 and 5) revealed that they were similar in all cultivars. But the concentration was highest in the upper style, followed by the stigma and the lower style. This was not the case of self-compatible 'Osa-Nijisseiki', which had the highest concentration in the stigma. Accordingly, strongly SI cultivars contained more S-proteins than the intermediate or weakly SI ones in all parts of the style. On the other hand, each S-protein was distributed similarly to the total S-proteins except for the case of S2-protein in 'Chojuro' which contained a significantly smaller amount in the lower style.

Figure 6 shows the relationships between the amount of total S-protein and the growth of selfed pollen tubes in 1/3 (A), 1/2 (B) or 1/1-length style (C). The data indicate that at any style length, tube growth correlated negatively with the amount of S-protein in the style and stigma, but that the 1/1-length style had the highest coefficient of correlation (r = 0.876, n=9).

Discussion

The concentration of S-proteins (sum of the 2 S-proteins/extractable stylar protein) in the style has a negative correlation with the growth of selfed pollen tubes, independent of style length (Fig. 6A, B, C). The varietal differences in S-protein concentration are mainly due to the combination of 2 S-proteins; cultivars
with $S_1^-, S_3^-$ and/or $S_5^-$ allele contain a relatively large amount of $S$-proteins. Thus, the amount of $S$-proteins in the cultivar seems heritable. The quantities of respective $S$-proteins also differ among the cultivars (Fig. 3). However, they do not correlate well with the growth of selfed pollen tubes in each cultivar. For example, the amounts of $S_1^-$ proteins from ‘Nijisseiki’ and ‘Kikusui’ exceed significantly that of $S_1^-$ protein from ‘Yakumo’, even though the former 2 cultivars belong to immediately SI group, and the latter to a strong one.

Varietal differences in the amount of respective $S$-proteins suggest the presence of other modifier(s) unlinked with the $S$-locus which provides a new idea on $S$-gene expression in the Japanese pear. On the other hand, several proteins partially associated with the $S$-allele have been previously reported in Japanese pears (Nakanishi et al., 1992). Thus, SI systems in the Japanese pear seem to be considerably complex.

Concerning the role of $S$-RNase ($S$-protein with RNase activity) in allele-specific inhibition of pollen, 2 models have been proposed by Kao and McCubbin (1996): 1) only the incompatible RNase (RNase produced from the same $S$-allele as the pollen) can enter into the pollen tube through transmembrane receptors and degrade RNAs in the tube; 2) Both incompatible and compatible (RNase produced by the different $S$-allele from the pollen) RNases can enter into the pollen tube, but compatible RNase is inactivated by an RNase inhibitor in the tube. In both cases, enough concentration of the RNase will be required to accomplish the complete inhibition of pollen tube growth. If the RNase concentration in and/or around the pollen tubes is below the complete inhibition level, the tube growth inhibition would be concentration dependent. The levels of $S_1^-, S_3^-, S_6^-$, and $S_5^-$ proteins in the Japanese pear might be near the threshold value, resulting in some fruit set after self-pollination in the cultivars possessing these $S$-alleles. This idea is partially supported because self-pollination yields some inbred seeds in young buds of Japanese pears, during which $S$-protein synthesis in the style is not fully completed (Hiratsuka et al., 1985; Hiratsuka and Okada, 1995).

The question is whether the specific activity of RNase varies or not among respective S-proteins. If a particular $S$-protein is a very active RNase, it may not need to be present in high quantities which would explain why the difference in SI strength does not correlate with the $S$-protein concentration. However, the stability of each $S$-protein band on the IEF gel by toluidine blue dye does not reveal the any clear differences in specific RNase activity (Hiratsuka et al., 1995a). Precise investigations focusing on this point are now in progress in our laboratory.

The $S$-proteins are distributed throughout the style in all cultivars, except for the $S_1^-$ protein in the self-compatible ‘Osa-Nijisseiki’ which is localized only in the stigmas. The concentration of the $S$-protein, however, is different in each part; upper part of the style contained the highest amount, followed by the stigmas, and the lower part of the style. This gradient was almost the same in all cultivars and may be attributed to the increasing volume of the conducting tissue (Hiratsuka and Okada, 1995). Accordingly, we could not find a direct relationship between the probable inhibitory site for the selfed pollen tubes in the style (Table 1) and $S$-protein distribution (Fig. 5).

‘Osa-Nijisseiki’ is a bud mutant of ’Nijisseiki’ and is considered self-compatible by a modification of $S_1^-$ allele (Sato et al., 1988; Hiratsuka et al., 1995b). This proposal has been strengthened because the amount of $S_1^-$ protein is significantly smaller in ‘Osa-Nijisseiki’ than that of ’Nijisseiki’ (Sassa et al., 1992; Hiratsuka et al., 1995b). Furthermore, in this study we found that almost all $S_1^-$ protein of ‘Osa-Nijisseiki’ existed in the stigmas (Fig. 5); this distribution pattern may cause the cultivar to be self-compatible. In the lily with gametophytic self-incompatibility systems (Hiratsuka et al., 1989), the selfed pollen tubes are unaffected by the incompatibility system for several hours after pollination. The longest selfed pollen tubes of ‘Osa-Nijisseiki’ could pass through the stigmatic zone for the first several hours after pollination and might not interact with the $S_1^-$ proteins. This argument is partly based on the growth of selfed pollen tubes in ‘Osa-Nijisseiki’, which is inferior to that of cross-pollinated ‘Osa-Nijisseiki’ × ‘Kikusui’ (Zhang and Hiratsuka, 1999); i.e., ‘Osa-Nijisseiki’ possesses some extent of self-incompatibility. Concerning the modified $S_1^-$ allele in ‘Osa-Nijisseiki’, Norioka et al. (1996) reported the absence of the allele itself, and Sassa et al. (1997) recently detected a small amount of the $S_1^-$ gene fragment by PCR technique. The solution on the mutation of ‘Osa-Nijisseiki’ from a molecular biological standpoint seems to be incomplete but our finding on the specific localization of the $S_1^-$ protein may give a strong clue to the solution. Meanwhile, the amount of $S_2^-$ protein in ‘Osa-Nijisseiki’ is significantly smaller than those in ‘Nijisseiki’ and ‘Kikusui’ (Fig. 3). Varietal differences in $S$-protein amounts may be affected either by a combination of $S$-alleles in the style or some modifier genes which could function in the style.

We conclude that each Japanese pear cultivar involves the different strengths of the SI system, and that the amount of total $S$-proteins in the style itself may be one of the main determinants of SI system in a cultivar.

**Literature Cited**


ニホンナシの花柱内Sタンパク質レベルと自家不和合性発現の変動

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摘 要

自家受粉したニホンナシ花柱を寒天培地上で培養すると、花柱切断面から出現する花粉管の数、またその花柱の比率は品種によって著しく異なった。この比率は、圃場での自家受粉後の着果率と対応するので、この値によってニホンナシ品種を自家不和合性の強いグループ、中間グループおよび弱いグループに分類し、これらのグループの代表的品種を用いて花柱内のSタンパク質含量を比較した。

等電点電気泳動法によって分離し、イメージスキャナーで定量したタンパク質含有量は、各Sタンパク質間で有意に異なり(S₁>S₃>S₄>S₅>S₆>S₇)。同じ連対立遺伝子から生産されたSタンパク質含有量も異った。例えば、'辛水'のS₁タンパク質含有量は'八雲', '喜水'のそれより有意に高かった。

このことは、ニホンナシにはS遺伝子座と連鎖せずにS遺伝子発現を制御する遺伝子が存在することを示唆している。

二種類のSタンパク質含量の和として示した総Sタンパク質含量は、品種の不和合性の強さと正の相関を示し(r = 0.876, n = 9)。強い、中間および弱いグループの平均総Sタンパク質含量はそれぞれ1.51, 1.23および0.67 ng (1μg可溶性タンパク質当り) であった。しかし、各S遺伝子に対応したタンパク質含量の多少と不和合性の強さとの直接的な関係は認められなかった。すなわち、強い品種はSタンパク質の多いS₁, S₃およびS₅遺伝子を有し、弱い品種は含量の少ないS₂, S₇遺伝子などをもっていた。

Sタンパク質は花柱上部で最も濃度が高く、次いで柱頭、花柱下部の順となり、不和合性の強い品種ではどの部分においても中間および弱い品種より濃度が高かった。このように、Sタンパク質の花柱内の分布と不和合性の強弱との関係は認められなかった。

以上の結果から、ニホンナシにおける自家不和合性の強さの品種間差を引き起こす要因の一つに、花柱内の総Sタンパク質含量が考えられた。