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Pollen tube behaviors in self–incompatible and self–compatible *Citrus* cultivars

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To observe pollen tube behavior in the pistils of ten self–incompatible *Citrus* cultivars, bud pollination, delayed pollination, self–pollination and cross pollination were carried out. In self–pollinations of self–incompatible cultivars, pollen tubes were perfectly arrested in the stigmas and styles by 6 days after pollination, while in three self–compatible and 19 cross–compatible pollinations pollen tubes penetrated into the ovaries by 4 to 8 days after pollination. Various abnormal behavior and morphology of pollen tubes were observed in both incompatible and compatible pollinations, but were less common in the compatible ones. In the pistils of self–incompatible cultivars self–pollinated two days before anthesis and two days after anthesis, the self–incompatible pollen tubes ceased to grow in the stigmas and styles, while in the pistils self–pollinated 4 and 6 days before anthesis many pollen tubes reached the ovaries. However, numbers of pollen tubes penetrating the ovaries were different in different cultivars. In the pistils of self–incompatible cultivars self–pollinated 4 days after anthesis, pollen tube growth was enhanced to some extent, while in those self–pollinated 6 and 8 days after anthesis pollen tubes could not detected probably because of the degeneration of the styles. These studies suggested that in the pistil of self–incompatible *Citrus* self–incompatibility initiates 6 days before anthesis and increases up to the day of anthesis and that some of the cultivars have a common *S* allele that controls self–incompatibility.

INTRODUCTION

In most cases, self–incompatibility operates via the inhibition of pollen tube growth in the pistil, and is under the control of one or more genes with multiple alleles. Genetic control may be either gametophytic or sporophytic. In the sporophytic self–incompatibility system, the pollen grain is tricellular at anthesis, and the site of the incompatibility reaction is on or just below the stigma surface. In the gametophytic self–incompatibility system, however, the pollen grain is bicellular at anthesis, and the incompatibility reaction occurs after pollen tube has grown some distance through the gynoecium (Nettancourt, 1977 and 1997; Sedgley and Griffin, 1989; Williams *et al.*, 1994). This fact has led us to more difficulty to study the mechanism of the gametophytic self–incompatibility.

In *Citrus*, the occurrence of gametophytic self–incompatibility has been well documented in several cultivars (Soost, 1965). The self–incompatibility is a crucial economic problem in certain commercial cultivars without high parthenocarpic ability such as those in pummelos, pummelo relatives, grapefruit relatives, mandarins and mandarin relatives. Self–incompatibility in *Citrus* has been studied by many investigators such as Nagai and Tanigawa (1928), Soost (1956 and 1965), Ton and Krezdorn (1966), Yamashita

(1978 and 1980), Kahn and DeMason (1986 and 1988). These investigations have concentrated on the examination of seed formation after self–pollination, the growth of pollen tubes in incompatible gynoecium and the overcome of self–incompatibility in self–incompatible cultivars. However, these studies did not determine accurately the variation of self–incompatibility during flower development and the difference of the degree of self–incompatibility among cultivars.

In this study, the site of pollen tube arrest in incompatible styles and pollen tube behaviors in both incompatible and compatible pistils were investigated using seven self–incompatible cultivars to demonstrate the degree of the self–incompatibility in the cultivars. In addition, pollen tube growth in incompatible pistils was investigated after bud pollination and delayed pollination to determine the relationship between the degree of self–incompatibility and the aging of flower in *Citrus* cultivars.

MATERIALS AND METHODS

Plant materials

More than ten–year–old trees of ten *Citrus* cultivars grown at the Sasaguri orchard of the University Farm, Kyushu University, Fukuoka, were used for this study. These cultivars consisted of seven self–incompatible cultivars and accessions and three self–compatible cultivars. The self–incompatible cultivars and accessions were 'Banpeiyu' (*C. grandis*), Ipoh No. 1 (*C. grandis*), Nagashima No. 12 (*C. grandis*), 'Hassaku' (*C. hassaku*), 'Yuge–hyokan' (*C. ampullacea*), 'Shishiyuzu' (*C. pseudogulgul*) and 'Tosa Buntan' (*C. otachibana*). The three self–compatible cultivars were 'Kabosu' (*C. sphaerocarpa*), 'Seminole' (*C. paradisi*×*C. reticulata*) and 'Zadaidai' (*C. aurantium*).

Bud pollination and delayed pollination

To obtain the pollen, mature flower buds just before anthesis were collected, and their petals were removed. The anthers were dehisced under sunlight or under incubator conditions maintained at 25 °C, and the fresh pollen was immediately used for pollination. For bud pollination, the fresh pollen was applied to the stigma surface of emasculated flower bud of the same cultivar 2, 4 and 6 days before anthesis and just before anthesis. The age of flower buds used as pistilate parents was determined as follows: Two same–sized flower buds in a flower cluster were chosen and one of the two flower buds was used for pollination. The other one was used to determine the time from the bud stage to flowering. For delayed pollination, the flower buds just before anthesis were emasculated and immediately bagged with paraffin wax paper to prevent outcross. The bagged flowers were self–pollinated with fresh pollen of the same cultivar 2, 4, 6 and 8 days after the emasculation. In both bud pollinations and delayed pollinations, one or two flowers on each bearing shoot were used for pollination and immediately covered again with paraffin wax paper bags to prevent outcross. After the self–pollinations, all remaining untreated flowers were removed to prevent abscission of the self–pollinated flowers. Three to ten self–pollinated gynoecia were collected randomly for each cultivar and for each collecting time. In 'Banpeiyu' and 'Tosa Buntan', five flower buds self–pollinated 6 days before anthesis were bagged and allow to grow up to their mature stages for the examination of seed number in the mature fruits.

Self- and cross-pollinations

Fresh pollen grains were applied to the stigma surface of emasculated flower buds at a balloon stage or one day before anthesis for both self- and cross-pollinations. One or two flowers on each bearing shoot were pollinated and immediately bagged as mentioned above. The pollinated flowers were randomly collected 1, 2, 4, 6 and 8 days after pollination. A minimum of six gynoecia were collected for each cultivar and each collection time.

Pollen tube observation

All of the collected samples were immediately fixed in a solution of acetic acid alcohol (1:3 V/V) for 1 day, and then stored in 70% ethanol at 4°C until use. The sample was cut into five sections, i.e., stigma, upper style, middle style, lower style and ovary. These sections were softened and cleaned with 0.8 N sodium hydroxide for more than 24 hours at room temperature, then rinsed in distilled water, stained with 0.1% aniline blue dissolved in 0.1 M tribasic potassium phosphate (Martin, 1959) for 18–24 hours at room temperature. The softened samples were placed on glass slides and the cuticle or epidermis emitting strong fluorescence under a fluorescence microscope was removed from the samples. The samples were then slightly pressed under a cover glass. Pollen tubes in the pressed samples were observed using the epifluorescence microscope at wave length of 450–500 nm. The number of pollen tubes was counted and recorded for each section. In addition, pollen tubes showing abnormal behavior and morphology were observed and photographed on the epifluorescense microscope equipped with camera.

RESULTS

Pollen tube growth in self- and cross-pollinations

In 1997, pollen tubes did not exist beyond stigmas by one day after pollination in all self-pollinations and cross pollinations except for 'Banpeiyu'×'Yuge-hyokan' and 'Hassaku'×'Yuge-hyokan' in which pollen tubes penetrated the upper one-third portion of the styles (Table 1). In self-pollination of 'Hassaku', all the pollen tubes could not grow beyond the stigma. In self-pollination of 'Yuge-hyokan', a few pollen tubes reached upper one-third portion of the style 2 days after pollination but could not grow beyond the portion by day 8. In self-pollinations of 'Banpeiyu' and 'Tosa Buntan', about 10–20 and 1–10 pollen tubes penetrated the middle portion of styles by 8 days after pollination respectively, while in self-pollination of 'Shishiyuzu' a few pollen tubes penetrated the lower style by day 6 and 8.

Cross incompatibility was not found in all of 19 cross combinations examined in 1997 (Tabe 1) and 10 cross combinations in 1999 (Table 2). In the 19 crosses, compatible pollen tubes penetrated into the ovaries by 4, 6 or 8 days after pollination. Among the pollen tubes of five cultivars, those of 'Yuge-hyokan' showed the fastest growth rate and reached 'Banpeiyu' and 'Hassaku' ovaries by day 4. In 8 days after pollination, the number of compatible pollen tubes penetrating into the ovaries increased greatly in all cross pollinations.

To reconfirm these results obtained in 1997, additional ten cross pollinations and eight self-pollinations were carried out in 1999. The results were summarized at Table 2.

Table 1. Growth of pollen tubes in the styles of five self- incompatible cultivars in self- and cross-pollinations (1997).

Cross	Site of pollen tube tip in the pistil in indicated days after pollination (N[a])					Presence of pollen tubes with abnormal morphology in stigma
	1	2	4	6	8	
Banpeiyu ×						
Hassaku	stigma	stigma	middle style (A)	lower style (B)	ovary (D)	–
Shishiyuzu	stigma	stigma	upper style (B)	ovary (A)	ovary (D)	yes
Tosa Buntan	stigma	upper style (A)	lower style (C)	ovary (D)	ovary (D)	yes
Yuge-hyokan	upper style (B)	middle style (D)	ovary (C)	ovary (D)	ovary (D)	yes
Hassaku ×						
Banpeiyu	stigma	upper style (A)	lower style (A)	ovary (A)	ovary (D)	–
Shishiyuzu	stigma	upper style (A)	middle style (A)	lower style (C)	ovary (D)	–
Tosa Buntan	stigma	upper style (A)	middle style (A)	lower style (C)	ovary (D)	–
Yuge-hyokan	upper style (A)	middle style (D)	ovary (D)	ovary (D)	ovary (D)	–
Shishiyuzu ×						
Banpeiyu	stigma	upper style (B)	lower style (C)	ovary (D)	ovary (D)	yes
Hassaku	stigma	upper style (B)	middle style (C)	ovary (C)	ovary (D)	–
Tosa Buntan	stigma	upper style (A)	lower style (A)	ovary (C)	ovary (D)	yes
Tosa Buntan ×						
Banpeiyu	stigma	upper style (B)	lower style (D)	ovary (D)	ovary (D)	yes
Hassaku	stigma	upper style (A)	middle style (B)	ovary (A)	ovary (D)	–
Shishiyuzu	stigma	upper style (A)	middle style (A)	ovary (C)	ovary (D)	yes
Yuge-hyokan	stigma	upper style (A)	lower style (B)	ovary (D)	ovary (D)	yes
Yuge-hyokan ×						
Banpeiyu	stigma	upper style (A)	lower style (C)	ovary (C)	ovary (D)	yes
Hassaku	stigma	upper style (A)	lower style (B)	ovary (C)	ovary (D)	–
Shishiyuzu	stigma	upper style (B)	lower style (C)	ovary (D)	ovary (D)	yes
Tosa Buntan	stigma	upper style (A)	lower style (C)	ovary (D)	ovary (D)	yes
Self-pollination						
Banpeiyu	stigma	upper style (A)	middle style (B)	middle style (B)	middle style (B)	yes
Hassaku	stigma	stigma	stigma	stigma	stigma	yes
Shishiyuzu	stigma	stigma	middle style (A)	lower style (A)	lower style (A)	yes
Tosa Buntan	stigma	upper style (A)	middle style (A)	middle style (A)	middle style (A)	yes
Yuge-hyokan	stigma	upper style (A)	upper style (A)	upper style (A)	upper style (A)	yes

[a] Number of pollen tubes observed: 1-10 (A); 11-20 (B); 21-50 (C) and more than 51 (D).

In self-pollinations of the five self-incompatible cultivars, the situation of pollen tube arrest was almost similar to that observed in 1997, but the time required for the penetration of pollen tubes into the ovary delayed for two days in 'Hassaku'×'Yuge-hyokan' and its reciprocal cross and four days in 'Banpeiyu'×'Yuge-hyokan'. In self-pollinations of three self-compatible cultivars, the pollen tubes penetrated into the ovaries by day 4 for 'Seminole' and 'Kabosu' and by day 6 for 'Zadaidai'. In the ten cross combinations, pollen tubes were found in the ovaries by day 4, 6, or 8. When 'Hassaku' was pollinated

Table 2. Growth of pollen tubes in the styles of five self-incompatible and three self-compatible cultivars in self- and cross-pollinations (1999).

Cross	Site of pollen tube tip in the pistil in indicated days after pollination (N[a])					Presence of pollen tubes with abnormal morphology in stigma
	1	2	4	6	8	
Banpeiyu ×						
Hassaku	stigma	stigma	upper style (A)	lower style (A)	ovary (C)	–
Yuge-hyokan	stigma	upper style (A)	middle style (A)	lower style (B)	ovary (C)	yes
Zadaidai	stigma	upper style (C)	lower style (C)	ovary (D)	ovary (D)	yes
Hassaku ×						
Banpeiyu	stigma	upper style (A)	middle style (A)	ovary (A)	ovary (D)	–
Kabosu	upper style (A)	lower style (B)	ovary (B)	ovary (D)	ovary (D)	–
Seminole	upper style (A)	lower style (C)	ovary (C)	ovary (D)	ovary (D)	–
Yuge-hyokan	stigma	upper style (A)	middle style (B)	ovary (A)	ovary (D)	–
Yuge-hyokan ×						
Banpeiyu	stigma	upper style (A)	lower style (C)	ovary (D)	ovary (D)	yes
Hassaku	stigma	upper style (A)	middle style (A)	lower style (B)	ovary (C)	–
Zadaidai	stigma	upper style (C)	lower style (B)	ovary (C)	ovary (D)	yes
Self-pollination						
Banpeiyu	stigma	upper style (A)	middle style (B)	middle style (B)	middle style (A)	yes
Hassaku	stigma	stigma	stigma	stigma	stigma	yes
Shishiyuzu	stigma	upper style (A)	middle style (A)	lower style (A)	lower style (A)	yes
Tosa Buntan	stigma	stigma	upper style (A)	middle style (A)	middle style (A)	yes
Yuge-hyokan	stigma	stigma	upper style (A)	upper style (A)	upper style (A)	yes
Kabosu	stigma	upper style (B)	ovary (A)	ovary (D)	ovary (D)	–
Seminole	upper style (A)	middle style (B)	ovary (B)	ovary (D)	ovary (D)	yes
Zadaidai	stigma	upper style (B)	lower style (B)	ovary (B)	ovary (D)	yes

[a] Number of pollen tubes observed: 1–10 (A); 11–20 (B); 21–50 (C) and more than 51 (D).

with 'Seminole' and 'Kabosu', the pollen tubes penetrated into the ovaries by day 4 in the both crosses as observed in self-pollinations of the two cultivars. The pollen tubes of the two cultivars showed the highest growth rate in this study.

Not only slow growth rates and arrest of pollen tubes but also quite abnormal behaviors of pollen tubes were observed in incompatible styles. In compatible styles, pollen tubes with thin wall grew straightly without twisting and swelling and formed regular-sized callose plugs at regular intervals (Fig. 1). In the incompatible styles, however, the pollen tubes often twisted (Fig. 2) and deposition of callose in the pollen tubes was quite irregular (Fig. 3–11). The heavy deposition of callose (Fig. 5–7) and serial formation of irregular callose plugs (Fig. 3, 5 and 9) were also observed in pollen tube tip and tube colse to the tip. The other abnormal behaviors of incompatible pollen tubes were strong fluorescence from the pollen tube with a very slow growth rate (Fig. 10), spiral form of swelled pollen tube tip with protrude wall (Fig. 8), formation of balloon-shaped pollen tube Fig. 4), various degree of branching of pollen tube (Fig. 3, 4 and

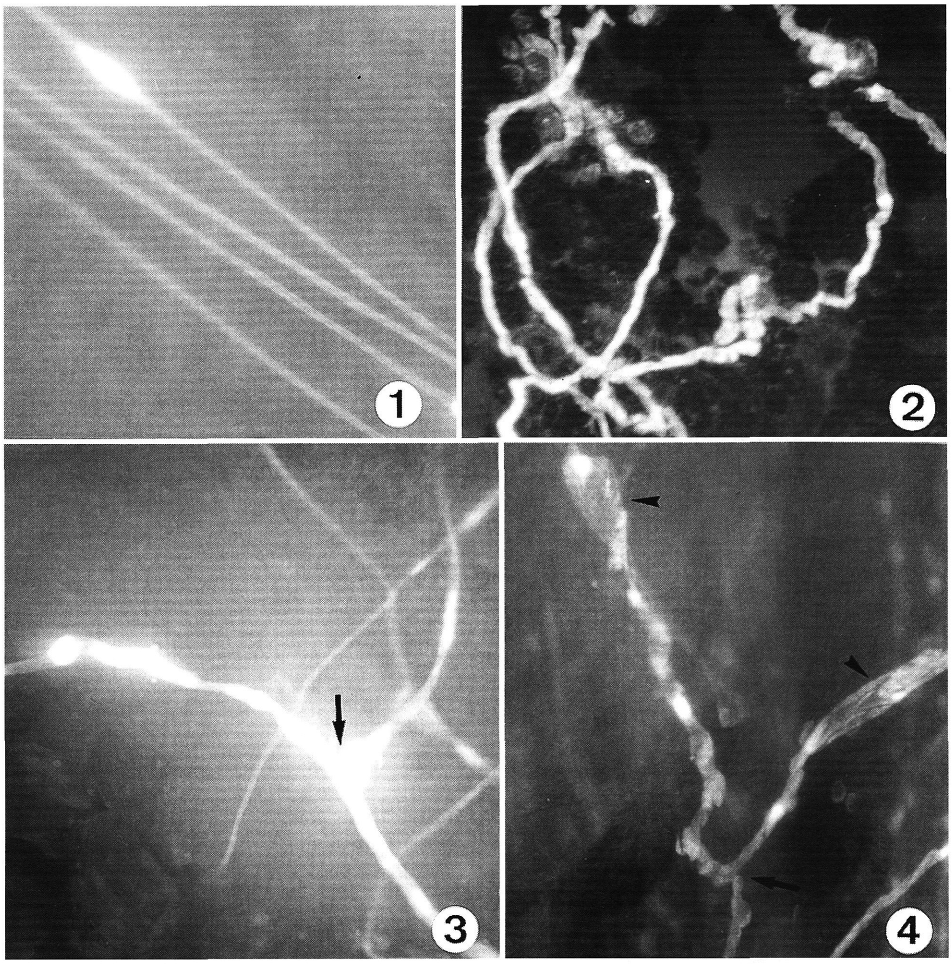

Fig. 1–4. Pollen tube behaviors in the pistils. Fig. 1. Pollen tubes in the lower portion of style of 'Zadaidai' 8 days after self–pollination. Note the formation of a normal callose plug emitting strong fluorescence and normal straight form of pollen tubes. ×150. Fig. 2. Spiral–shaped pollen tubes observed in the upper portion of style of 'Banpeiyu' 4 days after self–pollination. ×150. Fig. 3. Branching (arrow) and serial heavy deposition of callose in a pollen tube observed in the upper potion of style of 'Tosa Buntan' 8 days after self–pollination. ×300. Fig. 4. Pollen tube with branching (arrow) and balloon–shaped parts (arrow heads) observed in the upper portion of style of 'Shishiyuzu' 8 days after self–pollination. ×300.

7), and irregular swelling and heavy deposition of callose in spiral pollen tube tip (Fig. 6).

Some of these abnormal behaviors of pollen tubes were observed not only in incompatible stigmas and styles but also in compatible stigmas and styles (Table 1 and 2). However the abnormal shapes of pollen tubes seen in Fig. 5–9 and 11 were difficult to find in these compatible styles probably because of the presence of many normal pollen tubes.

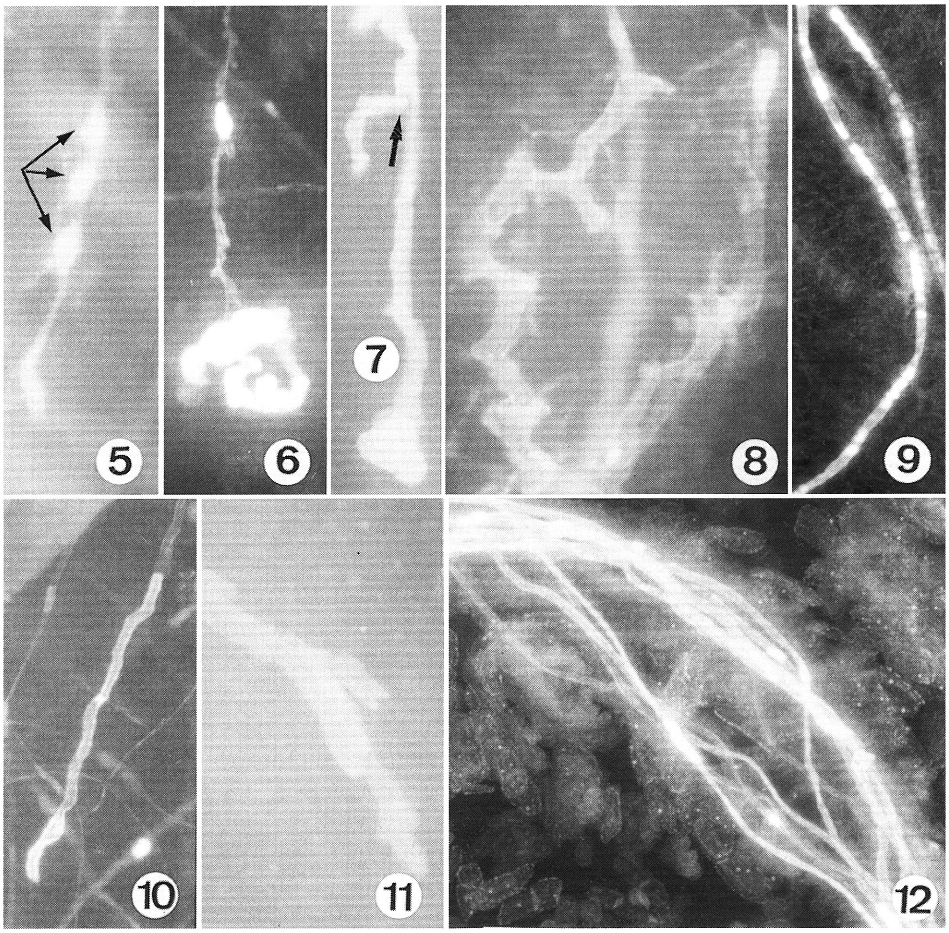

Fig. 5–12. Pollen tube behaviors in the pistil. Fig. 5. A serial formation of callose plugs (arrow) in the pollen tube wall and heavy callose deposition in pollen tube tip in the upper style of 'Yuge-hyokan' 4 days after self-pollination (×150). Fig. 6. A swelled and spiral pollen tube with heavy deposition of callose on the tip in the upper style of 'Banpeiyu' 8 days after self-pollination (×150). Fig. 7. Branching (arrow) of a pollen tube and swelling and heavy callose deposition in the tube tip observed in upper style of Nagashima No. 12 8 days after self-pollination (×300). Fig. 8. A zigzag-shaped pollen tube with wall protrusion in the upper style of 'Tosa Buntan' 6 days after self-pollination. Note also irregular deposition of callose in the tube wall (×300). Fig. 9. Pollen tube with irregular deposition of the callose at the pollen tube wall in the upper style of 'Shishiyuzu' 6 days after self-pollination (×150). Fig. 10. An abnormal pollen tube of 'Zadaidai' growing in the upper style of 'Zadaidai' 4 days after self-pollination, showing a very slow growth rate, twisted shape and heavy callose deposition at the tube tip (×150). Fig. 11. An abnormal pollen tube observed in the upper style of 'Shishiyuzu' 6 days after self-pollination. Note the swelling and irregular small protrusion of the tube wall (× 300). Fig. 12. Normal pollen tubes of 'Banpeiyu' self-pollinated 6 days before anthesis and observed 8 days after pollination, showing normal morphology and growth (×150).

In self–pollinations of two self–compatible cultivars 'Zadaidai' and 'Seminole', pollen tubes with abnormal morphology were observed in the stigmas and rarely in the upper styles together with those with normal morphology, whereas in the middle and lower styles pollen tubes with normal morphology were exclusively observed. The same situation was observed also in many cross pollinations. However, when 'Hassaku', 'Kabosu' and 'Seminole' were used as pistilate or pollen parents, no abnormal behaviors of pollen tubes were detected in stigmas and styles.

Pollen tube growth in bud pollination

Results of bud pollinations and delayed pollinations in six self–incompatible *Citrus* cultivars were summarized at Table 3. In the flowers self–pollinated at anthesis and observed 8 days after pollination, the number of pollen tubes was greatest in the stigma and decreased dramatically in the upper style. Pollen tubes did not exist beyond the lower stylses for Nagashima No. 12 and 'Shishiyuzu', the middle styles for 'Banpeiyu', Ipoh No. 1 and 'Tosa Buntan', and the upper style for 'Yuge–hyokan'. In these cases, less than three pollen tubes penetrated the lower portion of styles in 'Shishiyuzu' and Nagashima No. 12, while about eight and four pollen tubes penetrated the middle portion of the styles of 'Tosa Buntan' and 'Banpeiyu' respectively. On the other hand, about ten pollen tubes penetrated the middle style of Ipoh No. 1 and the upper style of 'Yuge–hyokan' which showed the highest degree of inhibition of pollen tube growth.

In the flowers self–pollinated 2 days before anthesis, results of pollen tube growth were almost the same as those of pollen tube growth in the flowers self–pollinated at anthesis. In the flowers self–pollinated 4 days before anthesis, pollen tubes penetrated into the ovaries of 'Tosa Buntan', 'Banpeiyu', Ipoh No. 1 and Nagashima No. 12, whereas pollen tubes of 'Shishiyuzu' and 'Yuge–hyokan' were unable to grow beyond the lower styles. In the flowers self–pollinated 6 days before anthesis, more than 50 pollen tubes penetrated into the ovaries of 'Banpeiyu', Ipoh No. 1, Nagashima No. 12 and 'Tosa Buntan', and about ten pollen tubes penetrated into the ovaries of 'Shishiyuzu' and 'Yuge–hyokan'. The mean number of pollen tubes penetrating the styles and ovaries was

Table 3. Effect of the age of pistil on the growth of pollen tubes in self–incompatible *Citrus* cultivars and accessions 8 days after self–pollination.

Cultivar and accession No.	Part penetrated by pollen tubes in the pistil self–pollinated in indicated days after anthesis (N[a])							
	−6	−4	−2	0	+2	+4	+6	+8
Banpeiyu	ovary (D)	ovary (A)	middle style (A)	middle style (A)	middle style (B)	lower style (A)	nc[b]	nc
Ipoh No. 1	ovary (D)	ovary (B)	middle style (B)	middle style (B)	middle style (B)	middle style (B)	nc	nc
Nagashima No. 12	ovary (D)	ovary (C)	middle style (A)	lower style (A)	middle style (B)	middle style (B)	nc	nc
Shishiyuzu	ovary (A)	lower style (A)	lower style (A)	lower style (A)	lower style (A)	lower style (A)	lower style (B)	nc
Tosa Buntan	ovary (D)	ovary (A)	middle style (A)	middle style (A)	middle style (A)	lower style (A)	nc	nc
Yuge–hyokan	ovary (B)	lower style (A)	upper style (A)	upper style (A)	upper style (A)	middle style (A)	–	nc

[a] Number of pollen tubes: 1–10 (A), 11–20 (B), 21–50 (C) and more than 51 (D). [b] nc: pollen tubes were not observed clearly.

Table 4. Comparison of the pollen tube growth between the pistils self-pollinated 6 days before anthesis and those self-pollinated at anthesis in six self-incompatible *Citrus* cultivars and accessions 8 days after self-pollination (1999)

Cultivar and accession No.	Number of pollen tubes in indicated position of the pistils self-pollinated							
	Six days before anthesis				At anthesis			
	Upper style	Middle style	Lower style	Ovary	Upper style	Middle style	Lower style	Ovary
Banpeiyu	>250	>200	>200	>150	89.0	3.7	0	0
Ipoh No.1	>250	>200	>200	>100	36.1	11.3	0	0
Nagashima No. 12	>200	>150	>150	>100	67.7	7.3	2.0	0
Shishiyuzu	97.5	23.5	12.3	6.0	22.0	3.0	3.0	0
Tosa Buntan	>200	>200	>100	>50	150.0	8.3	0	0
Yuge-hyokan	60.5	31.0	19.5	12.5	9.3	0	0	0

listed in Table 4. These pollen tubes showed almost normal growth and morphology in the stigmas, styles, and ovaries (Fig. 12). In bud pollinations made 6 days before anthesis, 109 and 69 perfect seeds were derived from two fruits of 'Banpeiyu' and two fruits of 'Tosa Buntan' respectively.

Pollen tube growth in delayed pollination

In the flowers self-pollinated 2 days after anthesis, growth of pollen tubes was similar as that in the flowers self-pollinated at anthesis. In the flowers self-pollinated 4 days after anthesis, pollen tubes of 'Banpeiyu', 'Shishiyuzu' and 'Tosa Buntan' did not grow beyond the lower styles. A small difference of pollen tube growth between the flowers self-pollinated 2 days after anthesis and those self-pollinated 4 days after anthesis were found in 'Banpeiyu', 'Tosa Buntan', and 'Yuge-hyokan'. In 'Tosa Buntan' and 'Banpeiyu' self-pollinated 4 days after anthesis, the pollen tubes reached the lower styles, while in 'Yuge-hyokan' self-pollinated 4 days after anthesis pollen tubes reached the middle portion of styles. In the flowers self-pollinated 6 days after anthesis, 5.3 pollen tubes were observed in the lower style of 'Shishiyuzu'. In the flowers self-pollinated 6 and 8 days after anthesis, however, the intensity of fluorescence emitting from the pollen tubes was extremely low. Hence, the data could not obtained in all cultivars and accessions except for 'Shishiyuzu' self-pollinated 6 days after anthesis (Table 3).

DISCUSSION

Degree of pollen tube arrest in self-incompatible citrus

The pollen barrier usually operates on or just below the stigma in species characterized by a dry stigma and within the style or, more rarely, the ovary in species with a wet stigma (Nettancourt, 1997). In *Citrus* with a wet stigma or a gametophytic self-incompatibility system (Soost, 1965 and 1969), the process of pollen tube rejection appears to be similar in most self-incompatible species with wet stigma, i.e., it occurs in the style at various distance from the stigma. As suggested by many workers in various species (e.g., Brewbaker, 1957; Nettancourt, 1977 and 1997), the capacity of *Citrus* flower to reject incompatible pollen tubes at any place on the pistil was often incomplete and supplemented by a sequence of barrier from stigma to style.

In the self–pollinated 'Hassaku', the stigma is the site of pollen tube arrest, since no pollen tubes penetrated the upper style beyond the stigma. Yamashita (1980) reported that in the same pollination about ten pollen tubes penetrated the upper style and that a few pollen tubes rarely penetrated the middle style 5 to 9 days after pollination. However, his result does not negate our conclusion that the stigma is the site of pollen tube arrest, since almost all pollen tubes were arrested also within the stigma in his study. The difference between our and his results may be due to the factors such as a temperature condition and number of pollen grains on the stigma which is known to affect the growth of pollen tubes (Sedgley, 1977; Ter–Avanesian, 1978), or may be simply due to the difference in number of samples: eight pistils in his and three to five pistils in our studies. The fact that growth rates of pollen tubes in some of the cross– and self–pollinations were higher in 1997 than in 1999 (Table 1 and 2) may also suggest that the differences of the growth rates in the same pollinations attribute to the difference of environmental conditions in the two years. In self–pollinations, however, the site of pollen tube arrest was almost invariable in the two years in all cultivars (Table 1 and 2). This result suggests that exogenous factors affecting pollen tube growth do not greatly affect the self–incompatible reactions.

Even in the cultivars that incompatible pollen tubes penetrated the styles, the stigmas are the primary site that pollen tubes are rejected strongly, since in spite of the observations of more than 500 pollen tubes in all the stigmas the mean number of pollen tubes penetrating the upper styles was 150 for 'Tosa Buntan', 72 for 'Banpeiyu', 68 for Nagashima No. 12, 53 for 'Yuge–hyokan', 37 for Ipoh No. 1, 22 for 'Shshiyuzu' in 1999 (Table 4). The difference in these numbers of pollen tubes growing beyond the stigma indicates that the degree of self–incompatibility reaction in the stigma is different in different cultivars. However, drastic reduction of the pollen tube numbers in the middle and lower styles (Table 4) indicates that the upper and middle styles show high degree of self–incompatible reaction in all cultivars.

The numbers of pollen grains placed on the stigmas of these cultivars may be different because of the difference in the sizes of the stigmas, and pollen germination rates on the stigmas may be different to some extent in different cultivars. Hence, it is difficult to estimate exact degree of self–incompatibility from the present results obtained in these cultivars. However, quantitative comparison of pollen tube numbers in each part of the self–pollinated pistils of the six cultivars (Table 4) and 'Hassaku' (Table 1 and 2) at hand suggests that these cultivars are highly self–incompatible plants with different degree of self–incompatibility in the stigmas and styles, i.e., 'Hassaku' shows the highest degree of self–incompatibility followed by 'Yuge–hyokan', 'Shishiyuzu', 'Banpeiyu', 'Tosa Buntan', Ipoh No. 1 and Nagashima No. 12 (Table 5). This estimation of the degree of self–incompatibility in each cultivar is well correspond with the result of bud pollination listed in Table 3, 4 and 5 and the result of bud pollination of 'Hassaku' reported by Yamashita (1980) who observed a few (0.4 to 1.0 in average) pollen tubes in the lower styles 5 to 9 days after self–pollination. Namely, the higher the degree of self–incompatibility in a given plant, the lower the effect of but pollination is. Furthermore, this also

Table 5. The degree of factors affecting pollen tube growth in three self-compatible and seven self-incompatible *Citrus* cultivars and accessions [a].

Cultivar and accession No. of collected plants	Degree of Self-incompatibility		Degree of growth ability of pollen tube itself	Effect of pistil on the growth of compatible pollen tubes	Effect of bud pollination on the growth of self-incompatible pollen tubes
	Stigma	Style			
Self-incompatible					
Banpeiyu	* *	* * * * *	* * *	* *	* * *
Hassaku	* * * * *	* * * * * [b]	*	* *	* [b]
Ipoh No. 1	* * *	* *	ne	ne	* * * *
Nagashima No. 12	* *	* * *	ne	ne	* * * * *
Shishiyuzu	* * *	* * * *	* *	* * *	*
Tosa Buntan	*	* * *	* *	* * *	* * *
Yuge-hyokan	* * * *	* * * * *	* * *	* * * *	* *
Self-compatible					
Kabosu	–	–	* * * * *	*	–
Seminole	–	–	* * * * *	*	–
Zadaidai	–	–	* * * *	*	–

[a] The degree of each item was divided into five categories: very high * * * * * ; high * * * * ; moderate * * * ; slightly low * * ; low * ; ne: not examined. [b] Based on the report by Yamashita (1980).

suggests that the higher the degree of self-incompatibility of the plant is, the longer the plant have the term of self-incompatibility reaction during the aging of flowers.

Degree of the growth ability of compatible pollen tubes

Comparison of pollen tube growth rates between cultivars in the compatible pistil of a given cultivar makes us possible to estimate the difference of compatible pollen tube growth ability in them. From the result of various reciprocal and cross pollinations of the six self-incompatible and three self-compatible cultivars (Table 1 and 2), it is apparent that the degree of compatible pollen tube growth ability is different in different cultivars, i.e., the growth ability is the highest in 'Seminole', followed by 'Kabotsu', 'Zadaidai' ', 'Yuge-hyokan', 'Banpeiyu', 'Tosa Buntan', 'Shishiyuzu', and the lowest in 'Hassaku' (Table 5). With regard to the pollen tube penetration into the self-incompatible style, no clear relationship is detected between the degree of self-incompatibility and the degree of the pollen tube growth ability. However, the lowest pollen tube growth ability and the highest degree of self-incompatibility in 'Hassaku' are seemed to result in complete rejection of pollen tube penetration into the incompatible style. It is concluded from the present results that the pollen tube growth ability itself is a minor factor affecting the penetration of self-incompatible pollen tubes into the pistil.

Effect of pistil on the growth rate of compatible pollen tubes

Comparison of growth rates of pollen tubes of a given cultivar in the compatible pistils of various cultivars makes us possible to estimate the degree of promotive effect of the pistils on pollen tube growth. From the results of pollen tube growth in various compatible cross pollinations and self–pollinations (Table 1 and 2), it is suggested that there are a small difference among cultivars as to the effect of the pistils on the compatible pollen tube growth. The effect of the pistil is estimated to be highest in 'Yuge–hyokan' and the low in three self–compatible cultivars (Table 5). However, the effect of the pistils on the self–incompatible pollen tube growth is not recognized. This indicates that in the pistils self–incompatible reaction is greatly higher than the nutritional effect of the pistils.

Relation between abnormalities of pollen tubes and incompatibility

The abnormalities of the pollen tubes found in self–incompatible gynoecia are similar to those reported in *Citrus* (Kahn and DeMason, 1986) and *Rhododendron* (Williams and Knox, 1982). Pollen tubes with abnormal morphology were also found in some of the compatible crosses between self–incompatible cultivars and in self–pollinations of the two self–compatible cultivars 'Seminole' and 'Zadaidai'. A similar result has been reported by Kahn and DeMason (1986) in a self–pollination of self–incompatible 'Orlando' and a compatible cross between self–incompatible 'Orlando' and self–incompatible 'Dancy' being a pollen parent of 'Orlando'. They reported that in the compatible cross many 'Orlando' pollen tubes penetrated 'Dancy' ovules but significant numbers of tubes were arrested in the stigma, and they considered from this result that 'Dancy' could possibly shares incompatibility alleles with 'Orlando' tangelo. We also consider that in the compatible crosses between self–incompatible cultivars both parents have a common incompatibility allele, since in the reciprocal crosses abnormalities of the pollen tubes were also detected in the stigma and upper style (Table 5). On the other hand, 'Seminole' must have inherited at least one incompatible allele from its pollen parent 'Dancy' if the self–incompatibility is controlled by more than one gene, since self–compatible 'Seminole' is a hybrid between self–incompatible 'Dancy' and self–semicompatible grapefruit (Soost, 1969). Sour orange (*C. aurantium*) is considered to be a hybrid with self–incompatible pummelo in its pedigree. Hence, 'Zadaidai' may have inherited at least one self–incompatible allele. Recently, based on the genetic analysis of glutamate oxaloacetate transaminase (GOT) isozyme genes linking to self–incompatible genes, Ngo *et al.* (1997) have been reported that 'Zadaidai' is a semicompatible (SnSf) cultivars and that 'Banpeiyu', 'Shishiyuzu', 'Tosa Buntan' and 'Yuge–hyokan' have one or two common alleles controlling self–incompatibility. These may be the reasons for the occurrence of pollen tubes with abnormal morphology in the stigmas and rarely in the styles in the two compatible self–pollinations and some of the cross pollinations between self–incompatible cultivars.

Pollen tube growth in bud pollination

Effect of bud pollination on the production of seeds from incompatible crosses and self–pollinations of self–incompatible plants have been reported by many workers (e.g., Lewis, 1951; Pandey, 1959; Shivana and Rangaswamy, 1969). This method is now routine for establishing the required inbred line through self–fertilized seed production in many species (e.g., Nettancourt, 1977; Shivana, 1969).

In *Citrus*, however, detailed works related to bud pollination were not performed probably because of the importance of hybrid breeding. A few works have been made by Soost (1964) who reported that many self–fertilized seeds were obtained from bud pollination made 3–5 days prior to anthesis in 'Kaopan' (*C. grandis*), while Yamashita (1980) reported that in 'Hassaku' four self-fertilized seeds were obtained per fruit from one time of and repeated self-pollination of immature flower buds. These results support our conclusion that in self-pollinations of six self-incompatible *Citrus* cultivars arrest of pollen tube growth by pistils, or self-incompatible reaction, was the highest between 2 days before anthesis and 2 days after anthesis and rapidly decreased before and after this period.

In bud pollinations, the growth rates of pollen tubes were the fastest in the pistil self-pollinated 6 days before anthesis and more than 51 pollen tubes penetrated into most of the ovaries 8 days after self-pollination (Table 3). In this case, the growth rates of pollen tubes of 'Tosa Buntan' and 'Banpeiyu' in their pistils are well correspond to those in the pistils of cross compatible cultivars (Table 1 and 3). This suggests that self-incompatibility reaction between the pollen tubes and the pistils does not occur or is very low up to 4 days before anthesis in the upper styles and 2 days before anthesis in the lower styles, since pollen tubes of 'Tosa Buntan' and 'Banpeiyu' penetrated these positions of styles of the other cross compatible cultivars up to 2 days after pollination for the upper styles and 4 days after pollination for the lower styles.

In bud pollinations of 'Yuge-hyokan' and 'Shishiyuzu', relatively small numbers of pollen tubes penetrated into the ovaries of flowers self-pollinated 6 days before anthesis and none of pollen tubes penetrated the ovaries of flowers self-pollinated 4 days before anthesis (Table 3). These results suggest that self-incompatible reaction in the pistils of the two cultivars initiated about 2 days earlier than that in 'Tosa Buntan' and 'Banpeiyu'. On the other hand, in the pistils of Ipoh No. 1 and Nagashima No. 12 self-pollinated 4 days before anthesis, relatively many pollen tubes penetrated into the ovaries as compared with the other self-pollinations. Thus, it is concluded from these results that the duration of the self-incompatibility reaction before anthesis is the longest in 'Shishiyuzu' follow by 'Yuge-hyokan', 'Banpeiyu', 'Tosa Buntan', Ipoh No. 1 and the shortest in Nagashima No. 12 (Table 5). The present results also suggest that, irrespective of the difference of the duration and the degree of pollen tube arrest, bud pollination carried out 6 days before anthesis may be effective for overcoming self-incompatibility in *Citrus*. This suggestion is partially probed by the facts that in bud pollinations made 6 days before anthesis 109 and 69 perfect seeds were derived from two fruits of 'Banpeiyu' and two fruits of 'Tosa Buntan' respectively.

Pollen tube growth in delayed pollination

There are no reports on the delayed pollination in self-incompatible *Citrus* species and cultivars. The present result suggests that delayed pollination is not effective for the production of self-fertilized seeds. In some of the pistils self-pollinated 4 and 6 days after anthesis, pollen tube growth was slightly enhanced as compared with that in the pistils self-pollinated 2 days after anthesis (Table 3). In most of the pistils self-pollinated 6 and 8 days after anthesis, however, pollen tubes could not be detected because of the loss of fluorescence emitting from the pollen tubes. In addition, the stigmas and styles were difficult to soften sufficiently with a solution of NaOH for the preparation of well-spread specimen suitable for pollen tube observation. These phenomena are considered to be related to the formation of abscision layer in the style base and subsequent degeneration of the style. Since the abscision layer was visible in the pistils about 12 days after anthesis, and since the longest pollen tubes reached the style base up to 4–6 days after pollination in the compatible crosses (Table 1 and 2), it is concluded that pollen tubes in the pistils self–pollinated 8 days after anthesis could not reach the ovaries. Furthermore, these pollen tubes were disintegrated with decrease of the function of the style in which abscision layer formation initiated several days before the day 12. Thus, in the delayed pollination, self–incompatible interaction between pollen tubes and style decreases to some extent with aging of the pistils, but the loss of the function and physical separation of the style from the ovary due to the abscision layer formation result in failure of normal pollen tube growth and subsequent fertilization.

Conclusion remarks

It has been obvious from the results of the present studies that in self–incompatible *Citrus* cultivars growth of the pollen tubes in the pistils is inhibited in various extent in the stigmas and styles before they reach the ovaries, and that difference in the degree of self–incompatibility reaction among the cultivars is the major reason of the various extend of pollen tube growth. However, the degree of growth ability of pollen tube itself may have very small effect on the pollen tube growth in self–incompatible pistils. Furthermore, it has been also obvious from the result of bud pollination of self–incompatible cultivars and accessions that self–pollination of flower buds 6 days before anthesis results in almost normal growth of pollen tubes in the pistils. In an additional experiment, we obtained many perfect seeds from self–incompatible ‘Banpeiyu’ and ‘Tosa Buntan’ self–pollinated 6 days before anthesis. Among seedlings established from these seeds, segregation of self–incompatible seedlings with homozygous self–incompatibility gene (*S*) is highly expected. The homozygous seedling for the *S* gene may play an important role for the genetic analyses of self–incompatible genotypes in full self–incompatible (SnSn) and self–semicompatible (SnSf) plants and may help us to understand the mechanism and evolution of a self–incompatibility system in *Citrus*.

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