Patterns and Strength of Pollen Tube Arrest in Self-incompatible Citrus Accessions (Rutaceae)

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Patterns and Strength of Pollen Tube Arrest in Self–incompatible *Citrus* Accessions (Rutaceae)

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Pollen tube growth was examined to demonstrate the degree of self–incompatibility reaction in the pistils of 121 *Citrus* accessions including 77 pummelo accessions. One hundred of the 121 accessions were determined to be self–incompatible with the aid of an epifluorescent microscope. Based on the difference of self pollen tube growth between the self–incompatible and self–compatible accessions, the degree of self–incompatibility reaction in four portions of the pistils was roughly divided into three categories, i.e., high (H), moderate (M) and low (L). The degrees of the reaction in the stigmas, upper styles, middle styles and basal styles of these accessions were high–high–high–high (H–H–H–H), moderate–high–high–high (M–H–H–H), moderate–moderate–high–high (M–M–H–H), low–high–high–high (L–H–H–H), low–moderate–high–high (L–M–H–H) or low–low–high–high (L–L–H–H) in order from the stigma to lower style, and were designated as H, MH, MMH, LH, LMH and LLH types respectively. All of 77 pummelo accessions examined were self–incompatible and all of the seven types of self–incompatibility reaction were observed in the accessions of which about 80% showed either H or MH type of reaction. The degree of self–incompatibility reaction was more variable in the pummelo accessions originating in Southeast Asia than those in Japan. In three mandarin relatives and four yuzu relatives, the H and MH types of self–incompatibility reaction were exclusively found. These results suggested that the degree of self–incompatibility reaction in each part of style was different in different *Citrus* accessions and showed geographical diversity. The reason for this is discussed.

Key words: *Citrus*, pollen tube arrest, pummelo, self–incompatibility, stigma, style

INTRODUCTION

Self–incompatibility in parthenocarpic *Citrus* such as pummelo and its hybrid cultivars is an important character for production of seedless fruit that is one of the highly desirable characters for consumers. Characterizing pollen performance is especially relevant for some economically important genus like *Citrus*, in which failure of the sexual reproductive process resulting in parthenocarpic fruit development and seedlessness is a prized character. The self–incompatibility in *Citrus* is gametophytic (Soost, 1965, 1969). In a gametophytic self–incompatibility system, expression of self–incompatibility alleles involves an interaction between the phenotype of microgametophyte and the phenotype of diploid tissue of the gynoecium (Nettancourt, 2001; Sedgley and Griffin, 1989). The existence of the same alleles in both pollen and pistil results in self–incompatibility in the plant. In general, the site of incompatibility reaction has been reported to be species–specific and varies from within the stigma to within the ovary (Lewis, 1956; Brewbaker, 1957). Because of these reasons, observation of pollen tube behaviors in the pistils and examination of seed formation in self–pollinated *Citrus* plants are suitable method for identification of the degree of their self–incompatibility.

In *Citrus*, Nagai and Tanigawa (1928) examined the seed formation after self–pollinations and found that several *Citrus* varieties were self–incompatible. Soost (1965, 1969), Krezdorn and Robinson (1958), and Li (1980) reported that some *Citrus* varieties were seedless when they were self–pollinated. Ton and Krezdorn (1966) observed pollen tube growth in self–incompatible gynoecia of some *Citrus* varieties, while Kahn and DeMason (1986) observed pollen tube growth in both cross– and self–incompatible gynoecia of 'Orland' (*C. paradisi* × *C. reticulata*). In both reports, they pointed out that in the incompatible situations the growth of most pollen tubes was arrested in the upper portion of the styles and/or in the stigmas. However, the number of self–incompatible *Citrus* plants used in these studies was too small to conclude the site of pollen tube rejection and to describe the detailed behaviors of self–incompatible pollen tubes in the pistils of *Citrus*. Yamamoto *et al*. (2006) examined pollen tube behaviors in self–pollinated pistils of many self–incompatible and self–compatible *Citrus* cultivars and found for the first time that two pummelo cultivars, two pummelo hybrid cultivars and two mandarin cultivars were self–incompatible. Recently, Ngo *et al*. (2011) estimated eight self–incompatibility gene alleles from S_1 to S_8 based on the

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segregation distortion for *Got–3* isozyme gene alleles in F_1 families of many *Citrus* accessions. Kim *et al.* (2010) further estimated S9 and S10 alleles for 'Hirado–buntan' pummelo. Distribution of *S* alleles in *Citrus* accessions and *S* genotypes of *Citrus* accessions were reported in terms of S_1 and S_2 (Kim *et al.*, 2011), and S_4 and S_5 (Zhou *et al.*, 2018). Hiratsuka *et al.* (2012) reported that in Japanese pair, the strength of self–incompatibility reaction was different in different *S* genotype or *S* alleles. Thus, in self–incompatible *Citrus* accessions, the strength of self–incompatibility reaction was different in different *S* genotype or *S* alleles.

In this study, pollen tube growth in the pistils of self–pollinated flowers of 118 *Citrus* accessions including 77 pummelo accessions was quantitatively examined to demonstrate the site of pollen tube arrest, strength of self–incompatibility and patterns of self–incompatibility reaction between pollen tubes and the pistils.

MATERIALS AND METHODS

Plant materials

Citrus accessions chosen for this study were 78 for pummelo (*C. grandis* Osbeck), 23 for pummelo–relatives including two sour orange (*C. aurantium* Linn.) cultivars, nine for mandarin (*C. reticulata* Blanco) and its relatives including tachibana (*C. tachibana* Tanaka) and its relatives, and eight for yuzu (*C. junos* Sieb. *ex* Tanaka) relatives. The pummelo accessions consisted of 22 cultivars in Japan and Vietnam, 45 garden plants collected from various districts of Kyushu area in Japan and 10 plants establishing from seeds collected from various countries of Southeast Asia. The identification of whether or not pummelo cultivars, collected pummelo plants and pummelo seedlings belong to pure species of *C. grandis* was based not only on their morphology but also on their *Got–1*, *Got–2* and *Got–3* genotypes wherein number of pummelo–specific alleles is three for *Got–1*, one for *Got–2* and three for *Got–3* (Ngo, 2001); the plants or cultivars that had alleles other than these were determined to be *C. grandis* relatives with genes from species such as *C. reticulata* and *C. tachibana* in their pedigrees.

The garden pummelo plants were more than 100–year–old trees locating at Amami Island, Shimo Koshiki Island and Nagashima Island of Kagoshima Prefecture, three parts of Kagoshima proper (Ibusuki City, Akune City and Kawanabe City), Amakusa Islands of Kumamoto Prefecture, Yatsushiro City of Kumamoto proper and Hirado Island of Nagasaki Prefecture. Accession numbers were given to the garden plants and plants from Southeast Asia together with the name of districts or countries where they were collected. Kawanabe–Buntan, Taiwan–Buntan, Nejime–Buntan, Ibusuki–Buntan, Makurazaki–Buntan and Iriki–Buntan were old pummelo plants locating at Kagoshima Prefecture and were provided for the Kyushu University from the Fruit Tree Experiment Station of Kagoshima Prefecture. Some of the Southeast Asian pummelo plants and cultivars were also provided from the Fruit Tree Experiment Station of Kagoshima Prefecture and the National Institute of Fruit Tree Science in Japan. Most of Vietnamese pummelo cultivars were collected by the Thai Nguyen University of Agriculture and Forestry, Thai Nguyen, Vietnam. Except these Thai Nguyen University pummelo collection, all *Citrus* accessions were grown in the University Farm of Kyushu University, Fukuoka, Japan.

Pollen tube observation

Mature unopened flower buds were collected just before anthesis and their petals and pistils were removed to expose the anthers. Pollen was obtained by allowing the anthers to dehisce under the sunlight or under incubation conditions at 25°C. A large amount of the fresh pollen was immediately applied to the stigma surface of unopened flowers of the same plant just before anthesis or one day before anthesis until the stigma became deep yellow, since there was no difference in the degree of self–incompatibility reaction between pollen tubes and pistil by 2 days before anthesis in *Citrus* (Ngo *et al.*, 2001). The pollinated flowers were covered with paraffin bags to prevent outcross. All the remaining flowers were removed from the tree to prevent abscission of the self–pollinated flowers.

The self–pollinated flowers were collected 8 days after pollination, and their pistils were fixed in a solution of acetic acid alcohol (1:3 v/v) for one or two days, and then stored in 70% ethanol at 4°C until use. Three to six pistils were collected for each plant and cut into five segments, i.e., stigma, upper one–third portion of style, middle one–third portion of style, lower one–third portion of style and ovary. The segments were softened and cleaned in a solution of 0.6 N sodium hydroxide for more than 24 hours at room temperature, rinsed in distilled water, and stained with 0.2% aniline blue dissolved in 0.1 M potassium phosphate (Martin, 1959) for 18–24 hours at room temperature. The stained samples were gently squashed on microscope slides. Pollen tubes in each segment were observed using a Nikon epifluorescence microscope at wave lengths 450–500nm and photographed on the microscope. The number of pollen tubes in each segment was counted and the mean number of pollen tubes entering in each segment was calculated for each sampled gynoecium.

To compare the quantitative variation of pollen tube growth between years, two pummelo plants and three pummelo–relative cultivars with low to very high degree of self–incompatibility reaction in the stigmas were chosen. Self–pollination and subsequent observation of pollen tube growth were carried out for two or three years according to the procedure mentioned above. The mean number of pollen tubes entering each segment was calculated for each year and its variation was scored.

Seed formation

Nine self–incompatible cultivars and three self–compatible cultivars were chosen to examine the seed formation in the self–pollinated flowers. Twenty–five flowers each were self–pollinated as described above. The fruits

Table 1. Year–to–year variation of pollen tube growth in pistils of four self–incompatible and one self–compatible *Citrus* accessions eight days after self–pollination

Accession (Cultivar and plant No.)	Year	No. of flowers observed	Mean No. of pollen tubes in indicated part of pistil			
			Upper style	Middle style	Lower style	Ovary
Bangladesh No.49	1996	4	225.0	6.5	0	0
	1997	3	101.7	3.0	0	0
	1998	3	117.0	12.0	0	0
Hassaku	1996	3	0	0	0	0
	1997	3	0	0	0	0
	1999	3	0	0	0	0
Ipoh No.1	1998	3	9.7	3.0	0	0
	1999	3	36.1	11.3	0	0
	2000	3	17.3	13.0	0	0
Shishiyuzu	1996	4	7.0	4.0	2.5	0
	1998	3	7.0	0.7	0.7	0
Miyauchi Iyo	1996	3	76.0	53.0	44.3	30.0
	1999	3	107.3	78.7	41.3	41.3

set were collected at a mature stage and the number of developed seeds was counted for each cultivar.

RESULTS

Eight days after self–pollination, a great number of pollen tubes crowded in the stigmas of all accessions examined. Clear differences in the number of pollen tubes among accessions were observed in each segment of the styles of these plants.

Yearly variation of pollen tube growth

In four of five self–pollinated accessions, pollen tubes did not penetrate the ovaries 8 days after pollination, indicating that the four plants are self–incompatible (Table 1). Differences in pollen tube growth were found among these accessions in the two or three years of evaluation (Table 1), but year–to–year variation of pollen tube growth in each accession was small. No pollen tubes existed beyond the stigma for ‘Hassaku’, the middle one–third portion of styles for Bangladesh No. 49 and Ipoh No.1 and the lower one–third portion of styles for ‘Shishiyuzu’ in all the years. Hence, it was suggested from these results that the rejection site of pollen tubes is plant–specific and highly invariable in each plant.

Quantitative comparisons of pollen tube growth in these self–pollinations also suggested that in each accession the rates of reduction of pollen tube numbers from the upper to lower styles were similar in all the years (Table 1). The numbers of pollen tubes penetrating the upper styles were relatively invariant in all the years but different in different accessions, whereas those penetrating the middle styles were also relatively invariant but not greatly different between plants. Thus, the numbers of pollen tubes observed in each plant in each year were averaged and used for later analysis of self–incompatibility in the respective accession.

Pummelos and their relatives

Of 78 self–pollinated pummelo accessions including 22 cultivars, the pollen tubes did not exist beyond the stigmas for two plants, the upper one–third portion of styles for 16 accessions, the middle one–third portion of styles for 44 accessions and the lower one–third portion of styles for 68 plants (Table 2, Fig. 1). In three of the 78 accessions, a few pollen tubes penetrated the ovaries, i.e., three and four pollen tubes were detected in one of six ovaries of Kawanabe No.1 and one of three ovaries of Nagashima No.7 respectively, while a total of 11 pollen tubes existed in all of three ovaries in ‘Hirado–buntan’. In the 78 accessions, numbers of pollen tubes penetrating the upper style ranged from 0 to 433, and those penetrating the middle style ranged from 0 to 139 (Table 2). However, numbers of pollen tubes penetrating the lower styles ranged from 0 to 5.7. These pollen tubes showed abnormal behaviors such as slow growth rates, swelling, branching, twisting, spiraling, formation of callose plugs at irregular intervals and irregular deposition of callose on the walls (Fig. 1–3).

Of 23 self–pollinated pummelo–relative plants including two sour orange cultivars (Table 2), no pollen tubes existed beyond the stigmas for one accession, the upper styles for five accessions, the middle styles for 10 accessions and the lower styles for 13 accessions. In both of self–pollinated ‘Anseikan’ and ‘Itoshima Bankan’, two pollen tubes penetrated one of the two ovaries examined. All these pollen tubes also showed abnormalities in their growth and morphology. In ‘Hassaku’, pollen tubes did not grow beyond the stigma. Almost all these pollen tubes showed abnormalities in their morphology.

In the upper style and ovary of self–pollinated ‘Miyauchi Iyokan’, 92 and 36 pollen tubes were detected respectively, while in the remaining seven cultivars, 150 to 500 and 23 to more than 100 pollen tubes were

Table 2. Pollen tube growth in the pistils of 121 *Citrus* plants 8 days after self–pollination

Accession (Cultivar or plant No.)	Sampling cite, originating country, or species (Tanaka's classification No.)[a]	No. of flowers observed	Mean No. of pollen tubes in indicated part of pistil				Degree of self–incompatibility (SI) reaction in indicated part of pistil				Type of SI rea-tion [b]
			Upper style (US)	Middle style (MS)	Lower style (LS)	Ovary (OV)	STG	US	MS	LS	
Pummelo	*C. grandis* Osbeck (56)										
1. Koshiki No. 13	Koshiki Is., Japan	3	0.0	0.0	0.0	0.0	H	–	–	–	H
2. Koshiki No. 6	Koshiki Is., Japan	3	0.0	0.0	0.0	0.0	H	–	–	–	H
3. Yatsushiro No. 5	Yatsushiro, Japan	4	0.5	0.5	0.0	0.0	H	–	H	–	H
4. Amami No. 2	Amami Is., Japan	3	1.0	1.0	0.0	0.0	H	–	H	–	H
5. Amakusa No. 4	Amakusa Is., Japan	3	1.0	0.0	0.0	0.0	H	–	–	–	H
6. Kaopan	Thailand	3	3.0	1.0	0.0	0.0	H	H	–	–	H
7. Yatsushiro No. 3	Yatsushiro, Japan	3	4.0	0.0	0.0	0.0	H	–	–	–	H
8. Kawanabe Buntan	Kagoshima, Japan	4	4.8	0.5	0.0	0.0	H	H	–	–	H
9. Hirado No. 3	Hirado Is., Japan	4	5.3	0.5	0.0	0.0	H	H	–	–	H
10. Koshiki No. 1	Koshiki Is., Japan	3	6.0	5.7	1.7	0.0	H	H	H	–	H
11. Koshiki No. 12	Koshiki Is., Japan	3	7.0	3.7	0.0	0.0	H	H	–	–	H
12. Amakusa No. 2	Amakusa Is., Japan	3	9.3	0.3	0.0	0.0	H	H	–	–	H
13. Amami No. 8	Amami Is., Japan	3	10.0	0.0	0.0	0.0	H	H	–	–	H
14. Amakusa No. 13	Amakusa Is., Japan	5	10.0	0.0	0.0	0.0	H	H	–	–	H
15. Akune No. 5	Kagoshima, Japan	3	13.7	1.0	0.7	0.0	H	H	–	–	H
16. Amami No. 6	Amami Is., Japan	3	14.7	0.3	0.0	0.0	H	H	–	–	H
17. Akune No. 2	Kagoshima, Japan	3	15.7	11.0	0.3	0.0	H	H	H	–	H
18. Taiwan Buntan	Taiwan	3	16.7	0.7	0.0	0.0	H	H	–	–	H
19. Amami No. 7	Amami Is., Japan	3	18.0	0.0	0.0	0.0	H	H	–	–	H
20. Hirado No. 2	Hirado Is., Japan	3	18.0	9.0	2.0	0.0	H	H	H	–	H
21. Hirado No. 5	Hirado Is., Japan	3	18.7	0.3	0.0	0.0	H	H	–	–	H
22. Amakusa No. 17	Amakusa Is., Japan	3	20.3	0.0	0.0	0.0	H	H	–	–	H
23. Yatsushiro No. 1	Yatsushiro, Japan	3	22.0	0.0	0.0	0.0	H	H	–	–	H
24. Amakusa No. 7	Amakusa Is., Japan	3	22.7	0.0	0.0	0.0	H	H	–	–	H
25. Nagashima No. 1	Nagashima Is., Japan	3	24.0	1.0	0.0	0.0	H	H	–	–	H
26. Yatsushiro No. 8	Yatsushiro, Japan	3	25.3	0.3	0.0	0.0	H	H	–	–	H
27. Yatsushiro No. 6	Yatsushiro, Japan	3	25.3	0.0	0.0	0.0	H	H	–	–	H
28. Chandler	USA (counted as Thailand)	5	25.4	0.0	0.0	0.0	H	H	–	–	H
29. Ipoh No. 3	Malaysia	3	29.0	0.3	0.0	0.0	H	H	–	–	H
30. Nagashima No. 8	Nagashima Is., Japan	3	30.0	0.7	0.3	0.0	H	H	–	–	H
31. Da Xanh	Vietnam	3	33.3	1.0	0.3	0.0	H	H	–	–	H
32. Ipoh No. 5	Malaysia	3	36.7	7.0	0.3	0.0	H	H	H	–	H
33. Koshiki No. 9	Koshiki Is., Japan	3	36.7	9.3	0.7	0.0	H	H	H	–	H
34. Hoang Trach	Vietnam	3	37.3	15.0	0.0	0.0	H	H	–	–	H
35. Hirado No. 7	Hirado Is., Japan	5	37.6	0.0	0.0	0.0	H	H	–	–	H
36. Bangladesh No. 52	Bangladesh	3	40.0	3.7	0.0	0.0	H	H	–	–	H
37. Vietnam No. 2	Vietnam	3	40.0	9.5	0.0	0.0	H	H	H	–	H
38. Ibusuki No. 2	Kagoshima, Japan	3	45.0	0.0	0.0	0.0	H	H	–	–	H
39. Amami No. 1	Amami Is., Japan	4	50.5	5.0	1.0	0.0	H	H	H	–	H
40. Hirado No. 1	Hirado Is., Japan	5	53.2	5.0	0.0	0.0	H	H	H	–	H
41. Nejime Buntan	Kagoshima, Japan	5	58.6	0.0	0.0	0.0	H	H	–	–	H
42. Yatsushiro No. 2	Yatsushiro, Japan	3	63.0	0.0	0.0	0.0	H	H	–	–	H
43. Sekitoyu	Taiwan	3	73.3	1.7	0.3	0.0	M	H	H	–	MH

44. Nagashima No. 12	Nagashima Is., Japan	8	76.4	29.9	1.5	0.0	M	H	H	–	MH
45. Nagashima No. 2	Nagashima Is., Japan	3	81.3	56.0	1.3	0.0	M	M	H	–	MMH
46. Nagashima No. 10	Nagashima Is., Japan	3	82.0	0.7	0.0	0.0	M	H	–	–	MH
47. Honda Buntan	Kagoshima, Japan	3	83.3	25.0	0.7	0.0	M	H	H	–	MH
48. Buoi Do	Vietnam	3	87.0	2.0	0.0	0.0	M	H	H	–	MH
49. Banpeiyu	Vietnam	3	89.0	3.7	0.0	0.0	M	H	H	–	MH
50. Kanoya No.1	Kagoshima, Japan	3	95.3	2.0	0.0	0.0	M	H	H	–	MH
51. Amakusa No.8	Amakusa Is., Japan	6	100.0	17.3	0.5	0.0	M	H	H	–	MH
52. Kawanabe No. 2	Kagoshima, Japan	6	105.0	17.0	0.5	0.5	M	H	H	–	MH
53. Amakusa No.15	Amakusa Is., Japan	5	105.0	1.6	0.0	0.0	M	H	–	–	MH
54. Amakusa No.14	Amakusa Is., Japan	3	105.0	5.0	3.0	0.0	M	H	H	H	MH
55. Indonesia No. 2080	Indonesia	4	109.0	10.5	0.0	0.0	M	H	H	–	MH
56. Nagashima No. 7	Nagashima Is., Japan	3	118.8	14.7	1.3	1.3	M	H	H	–	MH
57. Doan Hung	Vietnam	3	125.3	12.0	0.0	0.0	M	H	H	–	MH
58. Amami No. 4	Amami Is., Japan	4	133.3	94.5	0.0	0.0	M	M	H	–	MMH
59. Yatsushiro No.7	Yatsushiro, Japan	3	133.3	13.7	0.7	0.0	M	H	H	–	MH
60. Ibusuki Buntan	Kagoshima, Japan	3	142.0	59.3	0.0	0.0	M	M	H	–	MMH
61. Bangladesh No. 49	Bangladesh	10	147.9	7.2	0.0	0.0	M	H	H	–	MH
62. Mato-anyu	Taiwan	3	148.7	1.3	1.0	0.0	M	H	–	–	MH
63. Amami No. 5	Amami Is., Japan	3	150.0	2.0	0.5	0.0	M	H	H	–	MH
64. Hirado Buntan	Hirado Is., Japan	3	150.0	61.3	5.7	3.7	M	M	H	–	MMH
65. Hirado No. 4	Hirado Is., Japan	3	150.0	0.3	0.0	0.0	M	H	–	–	MH
66. Makurazaki Buntan	Kagoshima, Japan	3	150.0	6.3	2.7	0.0	M	H	H	H	MH
67. Nagashima No. 4	Nagashima Is., Japan	3	151.0	57.3	0.3	0.0	M	M	H	–	MMH
68. Nagasaki Zabon	Nagasaki, Japan	3	150.0	59.7	0.0	0.0	M	M	H	–	MMH
69. Mato Buntan	Taiwan	4	180.0	27.5	4.5	0.0	M	H	H	H	MH
70. Nam Roi	Vietnam	3	185.0	6.0	0.0	0.0	M	H	H	H	MH
71. Phuc Trach	Vietnam	3	195.0	10.3	0.0	0.0	M	H	H	H	MH
72. Kaophuang	Thailand	6	241.7	4.5	0.0	0.0	L	H	H	–	LH
73. Nagashima No. 5	Nagashima Is., Japan	5	250.0	2.6	1.0	0.0	L	H	H	–	LH
74. Iriki Buntan	Kagoshima, Japan	3	266.7	84.3	1.0	0.0	L	M	H	–	LMH
75. Dien	Vietnam	3	305.3	22.0	1.0	0.0	L	H	H	–	LH
76. Shatienyu (NIFTS)	China	2	325.0	11.5	0.0	0.0	L	H	H	–	LH
77. Vietnam No. 22	Vietnam	3	383.2	139.0	0.0	0.0	L	L	H	–	LLH
78. Bangladesh No. 48	Bangladesh	3	433.3	136.0	0.7	0.0	L	L	H	–	LLH
Pummelo relatives											
79. Hassaku	*C. hassaku* hort. ex Tanaka (74)	9	0.0	0.0	0.0	0.0	H	-	–	–	H
80. Kawachi–bankan	*C. kawachiensis* hort. *ex* Y. Tanaka	4	0.5	0.0	0.0	0.0	H	-	–	–	H
81. Kochi–hakuyu	*C. grandis* complex	3	2.0	0.0	0.0	0.0	H	H	–	–	H
82. Otachibana	*C. otachibana* hort. *ex* Y. Tanaka (80)	3	4.0	0.0	0.0	0.0	H	H	–	–	H
83. Shishiyuzu	*C. pseudogulgul* hort. *ex* Shirai (59)	10	12.0	2.6	2.1	0.0	H	H	–	H	H
84. Hyokan	*C. ampullacea* hort. *ex* Tanaka (81)	4	15.8	7.5	1.0	0.0	H	H	H	–	H
85. Anseikan	*C. anseikan* hort. *ex* Tanaka	2	17.5	6.5	4.5	1.0	H	H	H	H	H
86. Tanikawa Buntan	*C. grandis* cv. Mato × *C. sulcata* ?	3	17.7	0.7	0.0	0.0	H	H	–	–	H
87. Yuge–hyokan	*C. yuge–hyokan* hort. *ex* Y. Tanaka (82)	9	20.1	1.3	0.3	0.0	H	H	–	–	H
88. Iwaikan	*C. iwaikan* hort. *ex* Y. Tanaka (75)	3	30.0	0.7	0.0	0.0	H	H	–	–	H
89. Kugatsukan	*C. grandis* complex	3	45.3	1.0	0.0	0.0	H	H	–	–	H
90. Kirapeiyu	*C. grandis* complex	3	46.7	0.0	0.0	0.0	H	H	–	–	H
91. Itoshima Bankan	*C. grandis* complex	2	50.0	4.5	4.5	1.0	H	H	–	H	H

92. Higo Pummelo	*C. grandis* complex	3	101.0	28.0	0.0	0.0	M	I	I	–	MH
93. Tosa–buntan	*C. otachibana* hort. *ex* Y. Tanaka (80)	3	150.0	8.3	0.0	0.0	M	I	I	–	MH
94. Suishyo–buntan	*C. grandis* complex	3	200.0	1.7	0.0	0.0	L	H	–	–	LH
95. Miyauchi Iyo	*C. iyo* hort. *ex* Tanaka (105)	6	91.7	65.9	42.8	35.7	C	C	C	C	C
96. Kinukawa	*C. glaberima* hort. *ex* Tanaka (63)	4	>200.0	92.5	36.5	22.5	C	C	C	C	C
97. Kaikokan	*C. truncata* hort. *ex* Tanaka (57)	3	>200.0	>150.0	40.7	ne	C	C	C	–	C
98. Tengu	*C. tengu* hort. *ex* Tanaka (76)	4	>200.0	>150.0	>100.0	30.0	C	C	C	C	C
99. Kawano–natsudaidai	*C. natsudaidai* Hayata (78)	4	>250.0	>200.0	>200.0	>100.0	C	C	C	C	C
100. Summer Fresh	*C. hassaku* × *C. natsudaidai*	3	>350.0	>300.0	>100.0	>100.0	C	C	C	C	C
101. Bangkok Buntan	*C. grandis* complex	3	>450.0	216.7	133.3	56.7	C	C	C	C	C
Sweet orange											
102. Xa Doai	*C. sinensis* Osbeck	3	125.7	124.0	122.0	120.0	C	C	C	C	C
Sour orange and relatives											
103. Zadaidai	*C. aurantium* Linn.	6	274.0	214.0	133.0	75.0	C	C	C	C	C
104. Bouquet	*C. aurantium* Linn.	3	>450.0	>250.0	>250.0	>100.0	C	C	C	C	C
105. Sanbokan	*C. sulcata* hort. *ex* Takahashi	3	>300.0	>200.0	>200.0	>150.0	C	C	C	C	C
Yuzu (*C. junos* Sieb. ex Tanaka) relatives											
106. Hyuganatsu	*C. tamurana* hort. *ex* Tanaka (107)	6	75.0	6.8	0.0	0.0	M	H	H	–	MH
107. Ujukitsu	*C. ujyukitsu* hort. *ex* Tanaka (102)	3	102.7	1.3	0.3	0.0	M	H	–	–	MH
108. Dada	*C. luteo–turgida* Tanaka (109)	3	106.7	2.3	0.7	0.7	M	H	H	–	MH
109. Shunkokan	*C. shunkokan* hort. *ex* Tanaka (111)	3	316.7	233.3	116.7	100.0	C	C	C	C	C
110. Hanayu	*C. hanajyu* hort. *ex* Shirai (114)	8	>300.0	>250.0	>150.0	>100.0	C	C	C	C	C
111. Kabosu	*C. sphaerocarpa* hort. *ex* Tanaka (121)	6	>300.0	>225.0	>200.0	>150.0	C	C	C	C	C
Mandarin and relatives											
112. Kunenbo	*C. nobilis* Lour. (123)	3	4.3	0.3	0.0	0.0	H	H	–	–	H
113. Keraji	*C. keraji* hort. *ex* Tanaka (126)	3	0.0	0.0	0.0	0.0	H	–	–	–	H
114. Kabuchi	*C. keraji* hort. *ex* Tanaka (126)	3	78.3	7.3	0.0	0.0	M	H	H	–	MH
115. Obeni-mikan	*C. tangerina* hort. *ex* Tanaka (133)	3	173.3	105.3	68.0	ne	C	C	C	–	C
116. Tachibana No.1	*C. tachibana* Tanaka (143)	3	91.0	71.0	>60.0	>60.0	C	C	C	C	C
117. Shiikwasha No.1	*C. depressa* Hayata (153)	3	>100.0	>50.0	>50.0	>50.0	C	C	C	C	C
118. Tokkunin	*C. nobilis* complex	3	>100.0	>50.0	40.3	ne	C	C	C	–	C
119. Yatsushiro	*C. yatsushiro* hort. *ex* Tanaka (125)	3	>100.0	>100.0	>50.0	>50.0	C	C	C	C	C
120. Koji	*C. leiocarpa* hort. *ex* Tanaka (154)	3	>200.0	>150.0	93.3	>50.0	C	C	C	C	C
121. Seminole	*C. clementina* × *C. paradisi*	8	>400.0	>300.0	>250.0	>150.0	C	C	C	C	C

[a] Tanaka, 1969.

[b] H: high degree of SI reaction; M: moderate degree of SI reaction between H and L; L: nearly self–compatibility reaction;
–: no penetration of pollen tubes.; C: self–compatibility reaction.

observed in the upper styles and ovaries respectively. Almost all the pollen tubes in the seven cultivars and ‘Miyauchi Iyokan’ were fine and straight and showed thin tube wall with callose plugs at regular intervals.

Mandarins and their relatives

In mandarin and its relatives, two cultivars ‘Dancy’ and ‘Kunenbo’ showed high degree of self–incompatibility, i.e., the pollen tubes did not exist beyond the middle styles (Table 2). About 80 pollen tubes penetrated the upper style of ‘Kabuchi’, but the pollen tubes did not penetrate the lower style. In the pistil of self–pollinated ‘Seminole’ a hybrid cultivar between self–incompatible ‘Clementine’ and semi–self-incompatible grapefruit, more than 400 pollen tubes existed in the upper style and more than 150 normal pollen tubes penetrated the ovary. In tachibana and its relatives being also members of a mandarin group, no pollen tubes penetrated the upper style of ‘Keraji’ and no pollen tubes existed beyond the middle style of ‘Kabuchi’. Pollen tubes of tachibana, Shiikuwasha No.1, ‘Yatsushiro’ and ‘Koji’ penetrated their styles and more than 50 pollen tubes existed in their ovaries. The pollen tubes of these cultivars and ‘Seminole’ showed normal behavior and morphology, but those of the other cultivars showed abnormality.

Yuzu relatives

No pollen tubes existed beyond the middle styles in self–pollinated 'Kugatsukan' and 'Hyuganatsu', although 45 and 75 pollen tubes with abnormal morphology existed in their upper styles respectively (Table 2). In self–pollinated 'Ujukitsu', about 100 pollen tubes penetrated the upper style, but a total of four and one pollen tubes penetrated the middle and lower styles of three flowers respectively. The upper style of self–pollinated 'Dada' contained 107 pollen tubes per style, but the number decreased dramatically in the middle style and only two pollen tubes penetrated into one of three ovaries examined. Abnormality was observed in these pollen tubes. In 'Kabosu', 'Sanbokan', 'Hanayu' and 'Shunkokan', more than 300 pollen tubes penetrated their upper styles and more than 10 normal pollen tubes penetrated their ovaries. No abnormality was detected in these pollen tubes.

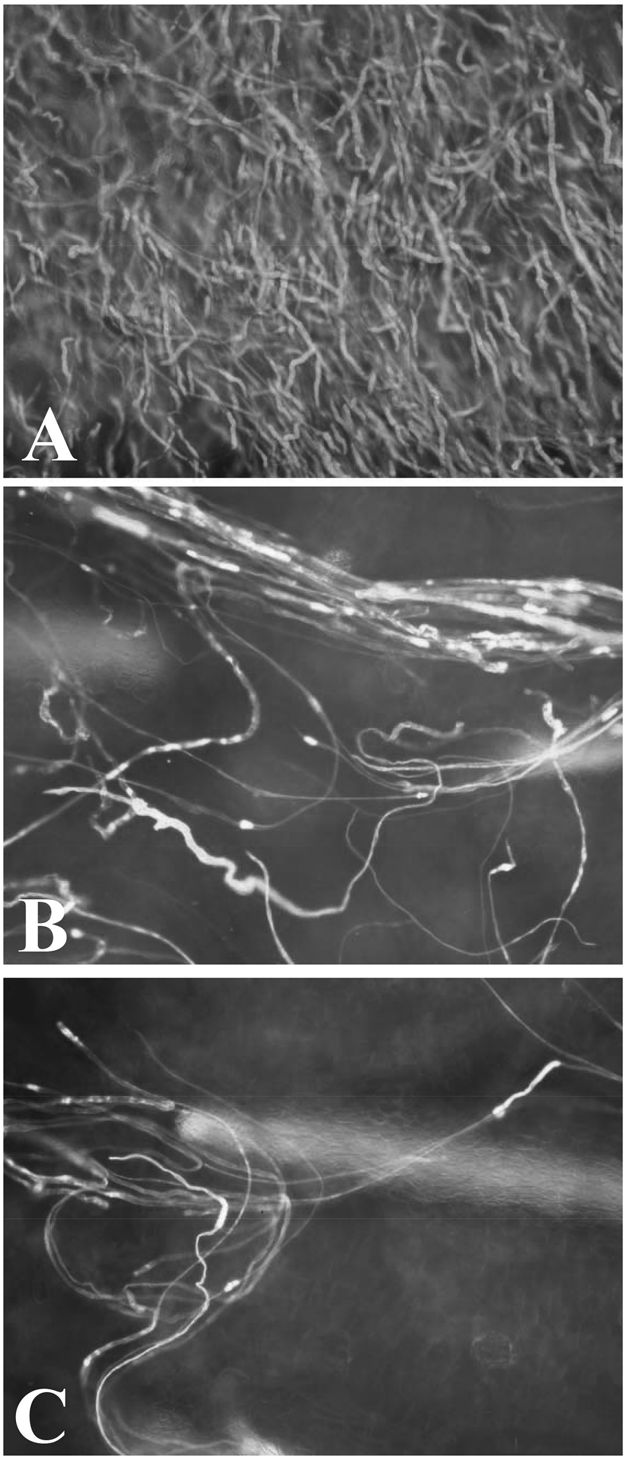

Fig. 1. Pollen tube behavior in the style of 'Nagasaki Zabon' pummelo 8 days after self–pollination. A: stigma; B: upper style; C: middle style. Pollen tube growth appeared to be normal in the stigma, but was highly abnormal in the upper and middle style in which most of pollen tubes twisted, swelled and deposited callose in their apices and produced irregular callose plugs within the tubes.

Seed formation in self–pollinated cultivars

Of the 12 cultivars self–pollinated just before anthesis, eight cultivars that showed no penetration of pollen tubes into the ovaries produced seedless fruits, while 'Hirado–buntan' pummelo that showed penetration of a few pollen tubes into the ovaries also produced seedless fruit (Table 3). Although the numbers of pollen tubes penetrating the ovaries were relatively small in two of the 12 cultivars, 'Kinukawa' and 'Miyauchi Iyokan', they showed high rates of fruit setting and produced fruits with 15 and 8 seeds per fruit respectively. These results suggested that the eight cultivars and 'Hirado–buntan' were self–incompatible and that the two cultivars were self–compatible.

DISCUSSION

In this study, pollen tube growth was quantified at various locations within the pistils of both self–compatible and self–incompatible pollinations to demonstrate the patterns and strength of pollen tube arrest for self–incompatibility in *Citrus*. The pollen tubes in their self–incompatible styles were characterized by their abnormal behavior, arrest in various distance from the stigma to ovary and failure of fertilization and subsequent seed formation as has been reported in some *Citrus* cultivars (Kahn and DeMason, 1986; Yamashita, 1978 and 1980; Ngo *et al.*, 2001) as well as in many other plants of angiosperm belonging to a dinucleate or gametophytic type of self–incompatibility (Nettancourt, 1977; Sedgley and Griffin, 1989). Thus, the plants with pistils in which almost all pollen tube arrest occurred are self–incompatible. In this study, 100 of the 118 plants are determined to be self–incompatible.

One of the most important environmental factors that could affect pollen performance is the temperature regime during the progamic phase spanning from pollination to fertilization. It has been shown that temperature affects pollen germination (Elgersma *et al.*, 1989; Shivanna *et al.*, 1991), and pollen tube kinetics in the style (Elgersma *et al.*, 1989; Hedhly *et al.*, 2005). All three true ancestral *Citrus* species plus clementine showed their maximum germination percentage at 25°C. It has been considered that these may be the factors

Table 3. Result of self–pollination in seven self–incompatible and two self–compatible *Citrus* cultivars

Cultivar	No. of flowers pollinated	No. of fruits obtained	No. of fruits with seeds	No. of seeds per fruit	Type of self–incompatibility
Banpeiyu	25	4	0	0	MH
Da Xanh	25	11	0	0	H
Hassaku	25	7	0	0	H
Hirado Buntan	25	2	0	0	MMH
Hyokan	25	5	0	0	H
Hyuganatsu	25	6	0	0	MH
Nam Roi	25	10	0	0	MH
Shishiyuzu	25	14	0	0	H
Tosa Buntan	25	9	0	0	MH
Kinukawa	20	14	14	15.3	SC
Miyauchi Iyo	20	14	14	7.6	SC
Xa Doai	30	15	15	16.1	SC

affecting year–to–year variation of pollen tube growth rate and the degree of pollen tube arrest. Because our observation for pollen tube growth has been carried out for more than ten years from 2001, we analyzed the influence of the environment (temperature) in each year on pollen performance; i.e., year–to–year variation for pollen tube growth was examined for two to three years. All the analyzed *Citrus* cultivars showed almost the same pollen tube growth rates and pollen tube arrest patterns in different years. This suggests that year–to–year variation for self–incompatible reaction is negligible in the present study.

In *Citrus* species, the incompatibility reaction was classified by the degree of self–incompatibility in different positions of the style (Ngo, 2001; Yamamoto, 2006). In some cases, pollen tubes are arrested very soon in the stigma (Ton and Krezdorn, 1966), while in the others they are arrested in the base of the style, the ovary or ovules.

Kahn and DeMason (1986) have reported that in self–pollinated 'Orland' gynoecia the stigma is the site of primary pollen tube arrest and suggested that the factors, whether biochemical or structural, that bring about the incompatibility reaction are localized in the stigma. However, the present result of self–pollinations of the 100 self–incompatible *Citrus* accessions suggested that the extent of pollen tube arrest in the stigmas varies from very low to very high, since 0 to 433 pollen tubes penetrated the upper one–third portion of styles of these self–incompatible cultivars.

Definition of the degree of self–incompatibility

In the self–compatible accessions except for mandarin and its relative accessions, the numbers of pollen tubes ranged from about 200 to 500 for the upper one–third portion of the styles, 92.5 to 300 for the middle one–third portion of the styles, 36.5 to 250 for the lower one–third portion of the styles and 22.5 to 150 for the ovaries (Table 2 and Fig. 2). On the basis of these ranges in each portion of the pistils of self–compatible cultivars, the degree of self–incompatibility reaction between pollen tubes and each part of pistils of self–incompatible plants is defined as follows (Fig. 2): (1) When a part of the pistil of a given plant contains many pollen tubes with the range seen in the corresponding part of self–compatible accessions, the part is defined to be low self–compatible and the strength of the reaction in the part is revealed with a symbol L. (2) When number of pollen tubes in a part of the pistil is less than the range in corresponding part in self–compatible accessions, the degree of self–incompatibility reaction is divided into three categories high (H) moderate (M) and low (L) based on the distribution frequencies of self–incompatible accessions in each part of pistils shown in Fig. 2, e.g., highly self–incompatible (H) is 0 to 70 pollen tubes for the upper one–third portion of the style and 0 to 40 pollen tubes for the middle one–third portion of style, whereas moderately self–incompatible (M) is 70 to 199 pollen tubes for the upper one–third portion of the style and 40 to 91 pollen tubes for the middle one–third of the style contained (Fig. 2).

In the cases of mandarins including tachibana and its relatives, numbers of pollen tubes penetrating each portion of pistils are considerably small as compared with the other *Citrus* accessions, certainly because of their small stigmas and styles with some exceptions. Thus, self–incompatibility reaction between pollen tubes and each part of pistils is defined by comparison of number of pollen tubes in the corresponding parts of the pistils of self–compatible mandarins and their relatives (Table 2), i.e., (a) when a part of pistil contains many pollen tubes close to, but less than, 91 pollen tubes, the degree of self–incompatibility reaction is defined to be moderately self–incompatible; (b) when a part of pistil contains a small number of pollen tubes close to zero, it is defined to be highly self–compatible; (c) when a part of pistil contains more than 91 pollen tubes, it is defined to be highly self–compatible.

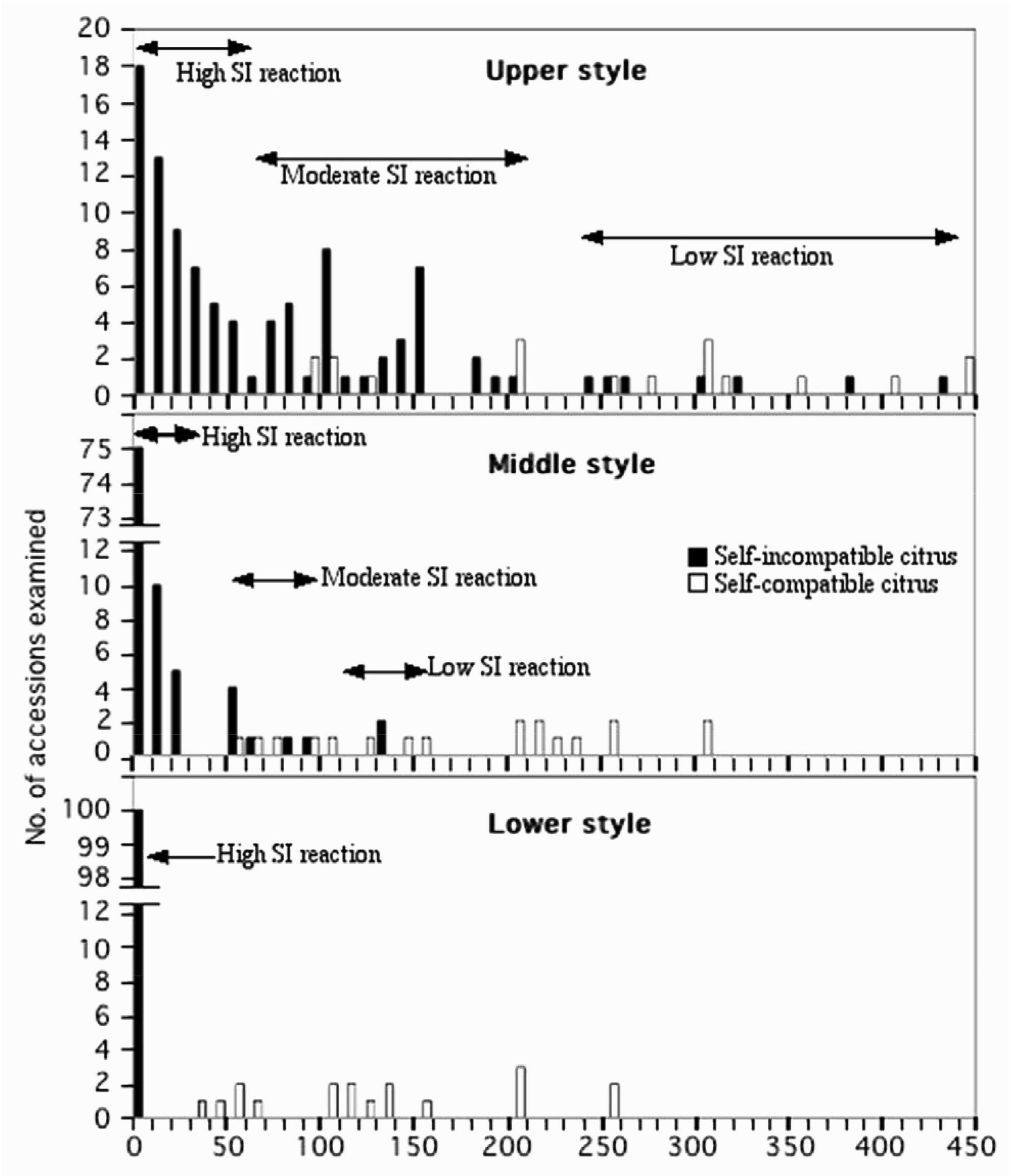

Fig. 2. Number of pollen tubes reaching upper, middle and lower portions of the styles of self–incompatible and self–compatible citrus accessions 8 days after self–pollination. Solid bars: self–incompatible citrus accessions; white bars: self–compatible citrus accessions.

Definition of the patterns of self-incompatibility

From the result of the present study, it became obvious that the degree of self–incompatibility reaction is very high in the middle and lower portions of styles in any self–incompatible *Citrus* accessions, but it varies from plant to plant in the stigmas and upper portions of styles of different accessions.

Of the 100 self–incompatible accessions examined in this study, seven pummelo accessions contained more than 200 pollen tubes in the upper one–third portions of the styles. Therefore, the degree of self–incompatibility reaction between pollen tubes and the stigmas of the seven accessions are determined to be almost compatible and give the symbol L. Similarly, the degree of self–

incompatibility in each part of the pistils of all plants was estimated and listed in Table 2.

In these plants, the degree of self–incompatibility reaction between pollen tubes and each portion of pistil (stigma – upper style – middle style – lower style) is classified into six types, i.e., H–H–H–H, M–H–H–H, M–M–H–H, L–H–H–H, L–M–H–H and L–L–H–H in order from stigma to lower style, and is designated here as H, MH, MMH, LH, LMH and LLH types respectively.

Self–incompatibility reaction in ovary

In self–pollinations of the self–incompatible plants, pollen tubes detected in the ovaries showed abnormal morphology. Furthermore, even in the case of self–pollinated fruit of 'Hirado–buntan' that contained a few pollen tubes in the self–pollinated ovaries, no seeds formed (Table 3). In addition, Yamashita (1978) reported that in 'Hyuganatsu' self–pollinations after decapitation of the styles from the ovaries did not result in fertilization and subsequent seed formation. These evidences suggest the strong possibility that the ovaries of self–incompatible *Citrus* accessions show high self–compatibility reaction. Among the other genera that showing gametophytic self–incompatibility, pollen tube arrest has been also detected in the ovaries (Cope, 1962).

Distribution of self–incompatibility types within group

Distributions of the plants with different self–incompatibility types are summarized in Table 4. All types of self–incompatibility are found in pummelos wherein a predominant type is H (58%), followed by MH (24%), MMH (7%), LH (7%), LLH (3%) and LMH (1%) types. On the other hand, only two types of self–incompatibility, H and MH, are exclusively found in pummelo relatives, mandarins and their relatives, and yuzu relatives. Since the plants belonging to the pummelo relatives and yuzu relatives are considered complex hybrids with pummelos in their pedigrees, small variation for patterns of self–incompatibility in these plants may suggest that a small number of pummelo plants are related to the differentiation of these groups.

Among the mandarin cultivars, only a few are self–incompatible. Some of the self–incompatible mandarin cultivars such as 'Clementine' and 'Kunenbo' have been suspected not to be a pure mandarin but considered to originate from hybrids between mandarin and plants of the other groups with pummelos in their pedigrees (Shimizu *et al*., 2016; Zhou *et al*., 2018). Yamamoto *et al*. (1993) carried out RFLP analysis in *Citrus* cultivars and also indicated that 'Kunenbo' originates from a hybrid between pummelo and mandarin. This indicates the possibility that the genes controlling self–incompatibility in 'Kunenbo' originate in pummelo.

'Keraji' and 'Kabuchi' are cultivars with 'Kunenbo'–flavor and are supposed to be complex hybrids with 'Kunenbo', tachibana and mandarins in their pedigrees. The present findings that 'Keraji' exhibits H type of self–incompatibility and that 'Kabuchi' exhibits MH type of self–incompatibility support this supposition.

Distribution of pummelo accessions with different types of self–incompatibility

Distribution of pummelo plants with different patterns of self–incompatibility reaction is summarized in each of districts and countries (Table 5). In Japan, plants with H type of self–incompatibility exist predominantly (63%), followed by MH (23%), MMH (10%), LH (2%) and LMH (2%) types, while in Southeast Asia plants with H(44%), MH (25%), LH (19%) and CLLH(13%) types of self–incompatibility reaction exist with relatively high frequencies. In addition, about 95% of the plants collected in Japan belong to H, MH and MMH types, whereas about 30% of the plants collected from Southeast Asia belong to LH and LLH types. These results suggest that the degree of self–incompatibility is more variable in the plants locating in Southeast Asia being an original center of pummelo than in those locating in Japan.

In Japan, relatively wide distribution of plants with different types of self–incompatibility exists in Kagoshima proper and Nagashima island of Kagoshima Prefecture, whereas in the other districts and islands plants with H types of self–incompatibility exist with very high frequencies. These may suggest that in Kagoshima Prefecture various kinds of pummelo plants

Table 4. Types of self–incompatibility reaction in four groups of self–incompatible *Citrus* plants

Type of self–incompatibility	No. of plants in indicated *Citrus* group[y]				Total
	Pummelo	Pummelo relatives	Mandarin and relatives	Yuzu relatives	
H	42	12	2	1	57
MH	22	3	1	3	29
MMH	4	0	0	0	4
LH	6	1	0	0	7
LMH	1	0	0	0	1
LLH	2	0	0	0	2
Total	77	16	3	4	100

[z] See Table 2.

[y] Tachibana and its relatives are included in mandarin and relatives.

Table 5. Frequency distribution of plants with different types of self–incompatibility reaction in pummelos collected from various districts and countries

Country and district	No. of plants in indicated types of self–incompatibility[z]						
	H	MH	MMH	LH	LMH	LLH	Total
Japan							
Amakusa island	5	3	0	0	0	0	8
Amami island	5	1	1	0	0	0	7
Hirado island	5	1	1	0	0	0	7
Kagashima proper	4	3	1	0	1	0	9
Koshiki island	5	0	0	0	0	0	5
Nagashima island	3	3	2	1	0	0	9
Yatsushiro	6	1	0	0	0	0	7
Total	33	12	5	1	1	0	52
Southeast Asia							
Bangladesh	1	0	0	1	0	1	3
China	0	0	0	1	0	0	1
Indonesia	0	1	0	0	0	0	1
Malaysia (Ipoh)	3	0	0	0	0	0	3
Thailand	2	0	0	1	0	0	3
Taiwan	0	3	0	0	0	0	3
Vietnam	3	4	1	1	0	1	10
Total	9	8	1	4	0	2	24

[z] See Table 00.

have been introduced from Southeast Asia.

Different types of self–incompatibility reaction are seen in pummelo plants from Bangladesh and Viet Nam. However, the plants collected from the Southeast Asian countries are too small to discuss about the distribution of plants with different types of self–incompatibility reaction. To solve this problem, further analysis of a large number of pummelo plants collected from these countries will be necessary.

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Supervision: Binh Xuan Ngo, Akira Wakana.
Validation: Binh Xuan Ngo, Akira Wakana.
Visualization: Binh Xuan Ngo, Akira Wakana
Writing – original draft: Binh Xuan Ngo
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