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Estimation of the Degree of Self–incompatibility Reaction during Flower Bud Development and Production of Self–fertilized Seeds by Bud Pollination in Self–incompatible *Citrus* Cultivars

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Bud pollination in seven self–incompatible *Citrus* cultivars was carried out to produce self–fertilized seedlings with genotypes homozygous for the self–incompatibility gene. Flower buds of the seven self–incompatible *Citrus* cultivars, 'Banpeiyu' (*C. grandis*), 'Hirado Buntan' (*C. grandis*), 'Hassaku' (*C. hassaku*), 'Shishiyuzu' (*C. pseudogulgul*), 'Clementine' (*C. clementina*), 'Tosa Buntan' (*C. otachibana*) and 'Hyuganatsu' (*C. tamurana*), were emasculated at various stages of flower bud development, and self–pollinated with their fresh pollen from flower buds collected just before anthesis. Seeded fruits were obtained from flower buds of 6–8 mm length for 'Clementine', 7–11 mm for 'Hyuganatsu', 11–15 mm for 'Shishiyuzu', 6–14 mm for 'Hassaku', 12–19 mm for 'Hirado Buntan' and 12–22 mm for 'Banpeiyu'. The optimum length of flower buds for self–fertilized seed production was 6–8 mm for 'Clementine', 8–12 mm for 'Hassaku', 9–13 mm for 'Hyuganatsu', 11–14 mm for 'Shishiyuzu', 12–17 mm for 'Hirado' and 14–19 mm for 'Banpeiyu'. Flower buds producing the largest number of perfect seeds in each self–pollination were about half the length of those just before anthesis. At or just after this stage of flower bud development, stigmatic exudate production occurred. The largest number of perfect seeds in self–pollinated fruits was almost the same as the average number of perfect seeds in open– or cross–pollinated fruits in six cultivars except for 'Shishiyuzu' in which self–pollinated fruits contained a few seeds. These results suggested that half–sized flower buds in their development were the most optimum stage for the production of self–fertilized seeds by bud pollination in self–incompatible *Citrus* cultivars, and that self–incompatibility reaction increased after this stage. It was also suggested that in 'Shishiyuzu' the self–incompatibility reaction is different from that in the other six *Citrus* cultivars. No difference was found in size between self–fertilized and cross–fertilized seeds. The self–fertilized seeds germinated and grew normally without any inbreeding depression.

INTRODUCTION

Self–incompatibility distributes widely in fruit trees (Sedgley and Griffin, 1989). It has been reported, in self–pollination, that inhibition of pollen tube growth occurs in the

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stigma and style of self-incompatible *Citrus* cultivars (Yamashita, 1980; Khan and DeMason, 1988; Ngo *et al.*, 2001) as well as in those of the other gametophytic self-incompatible species of angiosperm (Nettancourt, 2001). The presence of self-incompatibility in citrus presents problems for the breeder as well as the opportunity to produce seedless cultivars (Soost, 1987). Pummelo is known to be self-incompatible *Citrus* species (Soost, 1964; Iwamasa, 1976). Self-incompatibility is also known to exist among *Citrus* cultivars with *C. grandis* in their pedigrees and some mandarin cultivars (Soost, 1956, 1965; Iwamasa, 1976; Yamashita, 1978). The self-incompatibility system in *Citrus* is gametophytic and controlled by one gene (*S* gene; Soost, 1969) or two genes (*S* and *Z* genes; Wakana *et al.*, 1998). Based on the result of segregation of self-incompatibility in the progenies of self-compatible *Citrus* cultivars such as grapefruit, Soost (1969) inferred that at least eight *S* alleles exist in *Citrus*, while Wakana *et al.* (1998) proposed the presence of more than eight *S* alleles based on the result of segregation distortion of GOT isozymes in F_1 progenies of self-incompatible *Citrus* cultivars cultivated in Japan. Because of the barrier such as long generation time and nucellar embryony in *Citrus*, however, identification of *S* genotypes in many *Citrus* cultivars is very difficult, even if the above-mentioned methods are applied.

One of the superior methods to identify the self-incompatibility genotypes in *Citrus* cultivars is usage of a series of plants with homozygous genotypes for the *S* gene. To establish the homozygous genotypes, production of haploid plants and self-fertilized seeds is important. Haploid plants were established in monoembryonic 'Banpeiyu' (Toolapong *et al.*, 1996) and 'Clementine' (Oiyama and Kobayashi, 1993), and doubled form of haploid plants from 'Clementine' was reported (Germana *et al.*, 2000). However, haploid plants scarcely occur and, so far, are not available except for the two monoembryonic cultivars.

On the other hand, self-fertilized seeds have been obtained from self-incompatible plants by various methods such as bud pollination, delayed pollination, irradiation, high temperature, application of CO_2, hormones and inhibitors, pistil grafting and mentor pollen (Nettancourt, 2001). It has been reported that in self-incompatible *Citrus* cultivars self-fertilized seeds were produced by bud pollination, decapitation of style and mentor pollen (Yamashita, 1978, 1980; Yamashita *et al.*, 1990). Delayed or old flower pollination has been reported to be effective for breakdown of self-incompatibility in *Lilium* and some fruit trees such as *Malus* (Ascher, 1966; Haniuda *et al.*, 1985). In *Citrus*, however, Ngo *et al.* (2001) demonstrated that self-pollination at 6 and 8 days after anthesis has no effect on seeded fruits production. It is suggested from these reports that bud pollination is most convenient and effective method for the production of self-fertilized seeds in self-incompatible *Citrus* cultivars. Therefore, to know the degree of self-incompatibility reaction during flower bud development is essential for the production of self-fertilized seeds. However, there are no reports on the relation between flower bud development and the rate of fertilized seed production by bud pollination.

The aim of this study is to demonstrate the degree of self-incompatibility reaction in the pistils during flower bud development and to find the optimum stage of flower bud development for producing self-fertilized seedlings homozygous for self-incompatibility gene(s) in *Citrus* cultivars.

MATERIALS AND METHODS

Six self-incompatible *Citrus* cultivars, 'Clementine' (*C. clementina* hort. *ex* Tanaka), 'Banpeiyu' (*C. grandis* Osbeck), 'Hirado Buntan' (*C. grandis* Osbeck), 'Hassaku' (*C. hassaku* hort. *ex* Tanaka), 'Hyuganatsu' (*C. tamurana* hort. *ex* Tanaka) and 'Shishiyuzu' (*C. pseudogulgul* hort. *ex* Shirai) were used as plant materials to produce self-fertilized seeds and to certify the degree of the self-incompatibility reaction in various stages of flower bud development. Based on these results, self-incompatible cultivar 'Tosa Buntan' (*C. otachibana* hort. *ex* Y. Tanaka) was also used to produce self-fertilized seeds. These cultivars were grown in the Sasaguri orchard of University Farm, Kyushu University, Fukuoka.

Flower buds of the seven cultivars were collected just before anthesis, removed the petals and dehisced under room conditions. Fresh pollen on the dehisced anthers was immediately and directly used for self-pollination. The self-pollination was made at the various stages of flower development. Namely, about ten flower buds each were chosen every 1 mm level in length from 5 mm to anthesis in each cultivar. The flower buds chosen in each level were emasculated and self-pollinated with the fresh pollen and were bagged to prevent out-cross. The bags were removed about one month after self-pollination and number of setting fruits was scored and tagged. As a control for the self-pollination, cross-pollination was also made in 'Shishiyuzu' with pollen of 'Tosa Buntan' and 'Yugehyokan' (*C. yugehyokan* hort. *ex* Y. Tanaka).

The self-pollinated and cross-pollinated fruits were harvested at November and examined for their weights and seed numbers. In addition, ten open-pollinated fruits of 'Clementine' were also harvested at the same time to compare the characteristics of seeds. The seeds derived were divided into three categories, i.e., perfectly developed, imperfectly developed and empty, based the degree of their embryo development. The perfect seeds from self-pollination were sown to produce seedlings homozygous for the self-incompatibility gene in each cultivar. The self-pollination experiments were carried out three times with two replications, i.e., 2001, 2002 and 2003, and the data were pooled to estimate the effect of bud pollination on the production of self-fertilized seeds.

RESULTS

Fruit set rates in self-pollinated flower buds

The rates of fruits setting one month after bud pollination in the six *Citrus* cultivars were listed in Table 1 and Fig. 1 together with the range of length of flower buds resulting in seeded fruit production. Generally, fruit set rates in the self-pollinated flower buds increased with the development of flower buds, and after leaching the maximum rate in each cultivar they decreased with the development of flower bud up to the flowering. The range of length of flower buds with wet stigma was also listed at Table 1. Exudate production in the stigmas was obvious when the length of young flower buds leached 8 mm for 'Clementine', 12 for 'Hassaku', 13 for 'Hyuganatsu', 14 for 'Shishiyuzu', 16 for 'Hirado' and 17 for 'Banpeiyu' (Table 1).

Self-pollination of $\geqq 6$ and < 9 mm flower buds of 'Clementine' resulted in high rates of fruit set and production of seeded fruits, whereas < 6 mm flower buds and $\geqq 9$ mm

Table 1. Number of fruits setting one month after self-pollination in six self-incompatible *Citrus* cultivars. The horizontal shaded bars indicate the range of length of flower buds resulting in seeded fruit production. The horizontal white bars indicate the range of length of flower buds producing exudate on the stigmas.

No. of self-pollinated flower buds (lower) and No. of fruits setting one months after self-pollination (upper) in indicated stage of flower bud development (bud length in mm level)

	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	>30
Clementine																										
	0	6	13	11	0	0	0	0																		
	10	12	15	19	11	14	12	9	☆[a]																	
Hassaku																										
	0	2	2	7	3	6	7	10	4	4	4	2	1	0	4	0										
	4	5	5	7	5	10	11	13	5	9	4	5	4	4	6	2	☆									
Hyuganatsu																										
	0	0	1	4	7	6	6	3	6	2	2	0	1	1	0	0										
	10	9	10	14	16	15	15	10	12	9	10	10	10	10	9	11	☆									
Shishiyuzu																										
		0	1	2	5	7	6	7	10	9	4	0	6	0	5	4										
		10	9	9	10	10	10	10	10	10	10	9	10	10	10	8	☆									
Hirado																										
					1	1	1	5	4	7	2	6	3	4	3	0	3	0	0	0	0					
					9	6	5	8	8	8	6	9	6	8	6	8	9	6	7	7	5	☆				
Banpeiyu																										
			0	1	1	4	1	1	6	4	4	6	5	5	6	4	2	4	1	1	1	1	1	0	1	0
			5	8	7	9	6	6	8	5	6	7	8	8	7	9	6	8	6	5	7	5	6	5	7	6 ☆

[a] The symbol ☆ indicates anthesis.

ones did not. Self-pollination of flower buds of 7 mm level showed the highest rate of fruit set (86.7%). Generally, flower buds developing beyond 8 mm had wet stigma, those leaching 7 mm level had semi-wet, and those before leaching 7 mm had dry.

In 'Hassaku', self-pollination of ≧6 mm flower buds resulted in fruit set, whereas < 6 mm flower buds did not. The fruit set rates were more than 40% up to 16 mm level. Self-pollination of ≧ 8 and < 15 mm flower buds showed high rates of fruit set (44–100%). Seeded fruits were found among fruits derived from the self-pollinated flower buds below 17 mm. However, fruits obtained from self-pollination of ≧17 mm flower buds did not contain seeds and were smaller than the seeded ones. Flower buds developing beyond 12 mm produced exudate in the stigma, those leaching 8–12 mm level had semi-wet stigmas, and those under 8 mm had dry stigmas.

Self-pollination of ≧7 mm flower buds of 'Hyuganatsu' resulted in fruit set, whereas <7 mm and ≧19 mm flower buds did not. Self-pollination of ≧8 and <14 mm flower buds showed high fruit set rates (29–50%). Seeded fruits were found among fruits derived from the self-pollinated flower buds below 18 mm. The flower buds over 13 mm had wet stigmas, those of 13 mm level had semi-wet and those under 13 mm had dry.

Self-pollination of ≧7 mm flower buds of 'Shishiyuzu' resulted in fruit set. The fruit set rates were relatively high throughout the developmental stage of flower bud as was

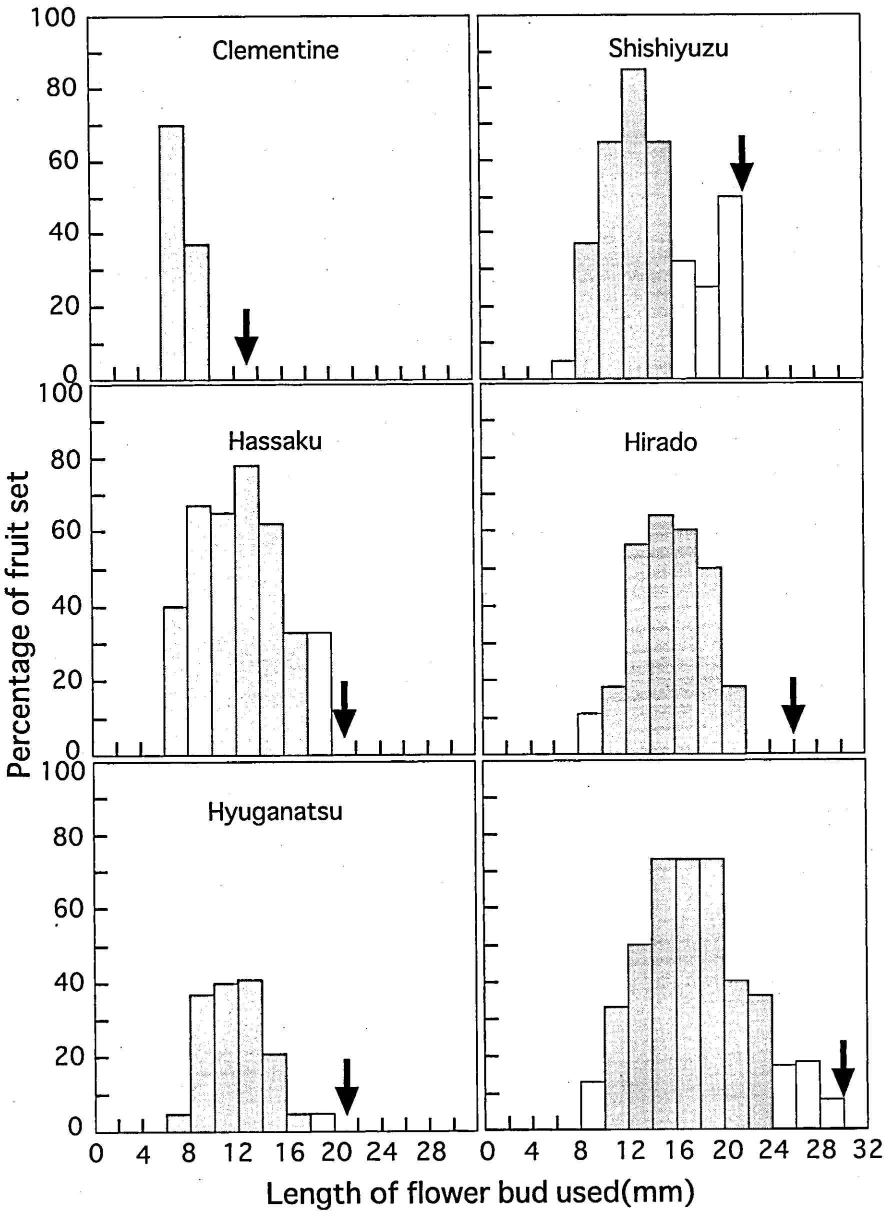

Fig. 1. Percentage of fruits setting one month after self-pollination in each level of flower bud development. The arrows indicate the length of flower buds just before anthesis. The shadded bars indicate that at least one of harvested fruits was seeded, and white bars indicate all of harvested fruits were seedless.

observed in 'Hassaku', and even in the flower buds just before anthesis the rate was relatively high (50%). Seeded fruits were found in fruits derived from the self–pollination of 9–15 mm flower buds. The flower buds over 15 mm had wet stigma, those leaching 14 mm level had semi–wet, and those under 14 mm had dry.

In 'Hirado Buntan', self–pollination of 9–21 mm flower buds resulted in fruit set, whereas ≧22 flower buds did not. Self–pollination of ≧12 and <19 mm flower buds showed more than 50% of the fruit set except for that of 15 mm level flower buds. The highest rate of fruit set was observed in the flowers of 14 and 15 mm levels. Seeded fruits were produced when the length of flower bud was between 10 and 21 mm. Stigmatic exudate observed at flower buds beyond 16 mm in length.

Except for flower buds of 7 and 28 mm levels and >30 mm flower buds, self–pollination of all levels of flower buds of 'Banpeiyu' resulted in fruit set. Self–pollination of ≧ 13 and <19 mm flower buds showed more than 60% of fruit set. The highest rate of fruit set was observed in 14–20 mm flower buds. Seeded fruits were produced from flower buds whose lengths were between 10 and 22 mm. Wet, semi–wet and dry stigmas were detected at the flower buds developing beyond 17 mm, those leaching 15 and 16 mm levels and those under 15 mm, respectively.

Effect of flower bud stage on self–fertilized seed production

Clementine

The rate of harvested fruits to self–pollinated flower buds was 50% for flower buds of 6 mm level, 67% for 7 mm level and 42% for 8 mm level. All the harvested fruits were seeded and the number of seeds per fruit ranged from 20.7 for 7 mm level to 24.7 for 6 mm level with the average of 22.2 (Table 2). More than 92% of the seeds were perfect ones with perfectly developed embryos. Average number and average weight of perfect seeds from self–pollinated fruits were almost the same as those from open–pollinated fruits, although the small number of imperfect seeds was produced in open–pollinated fruits (Table 3).

Hassaku

Among the harvested fruits, those derived from self–pollination of flower buds of

Table 2. Self–fertilized seed production by bud pollination in self–incompatible 'Clementine'.

Level of flower bud length (mm)	No. of fruits obtained		Number of seeds in indicated categories of seed development			
	Seeded (%[a])	Seedless	Perfect	Imperfect	Empty	Total (average)
Self–pollination						
6	6 (50)	0	138	3	7	148 (24.7)
7	10 (67)	0	187	7	13	207 (20.7)
8	8 (42)	0	166	8	4	178 (22.3)
Open–pollination						
–	10	0	263	1	8	272 (27.2)

[a] Percentage of flower buds resulting in seeded fruits production.

Table 3. Comparison of perfect seeds derived from self- and open-pollinated fruits of self-incompatible 'Clementine'.

Pollination	No. of fruits examined	No. of perfect seeds	Average No. of perfect seeds	Average weight of perfect seeds (mg)
Self-pollination	24	491	20.5	139
Open-pollination	10	263	26.3	132

Table 4. Self-fertilized seed production by bud-pollination in self-incompatible 'Hassaku'.

Level of flower bud length (mm)	No. of fruits obtained		Number of seeds in indicated categories of seed development			
	Seeded (%[a])	Seedless	Perfect	Imperfect	Empty	Total (average)
6	1 (20)	0	11	5	3	19 (19.0)
7	2 (40)	0	8	1	2	11 (5.5)
8	5 (71)	0	71	27	24	122 (24.4)
9	2 (40)	0	28	7	12	47 (23.5)
10	4 (40)	0	35	27	16	78 (19.5)
11	7 (64)	0	86	13	24	123 (17.6)
12	10 (77)	1	145	28	41	214 (19.5)
13	3 (60)	1	17	0	10	27 (6.8)
14	3 (33)	2	11	8	4	23 (4.6)
15	1 (25)	3	5	1	2	8 (2.0)
16	2 (40)	0	0	0	2	2 (1.0)
17	0 (0)	2	0	0	0	0 (0)
19	0 (0)	1	0	0	0	0 (0)

[a] Percentage of flower buds resulting in seeded fruits production.

8–12 mm levels produced many seeds with the range from 18 to 24 seeds per fruit (Table 4). Two fruits derived from self-pollination of 7 mm level flower buds contained small number of seeds, probably because of the low fertility of pistils in the flower buds, although one fruit from self-pollination of 6 mm level flower bud contained relatively large number of seeds, i.e., 19 seeds. In fruits from self-pollination of $\geqq$12 mm flower buds, number of seeds decreased as the flower bud length increased. In this case, conversely, the rate of seedless fruits increased with increase of the flower bud length, and all fruits derived from self-pollination of flower buds of 17 and 19 mm levels were seedless.

In each length level of self-pollinated flower buds, the rates of perfect seeds in the resultant seeded fruits were the highest and relatively constant (45–70%).

Hyuganatsu

Fruits derived from self-pollination of flower buds of 7–17 mm levels contained seeds with the range from 5 to 25 seeds per fruit (Table 5). One fruit derived from self-pollination of 15 mm level flower bud was seedless probably because of the low parthenocarpic ability in 'Hyuganatsu'. The largest number of seeds per fruit, i.e., 25 seeds, was produced

Table 5. Self-fertilized seed production by bud pollination in self-incompatible 'Hyuganatsu'.

Level of flower bud length (mm)	No. of fruits obtained		Number of seeds in indicated categories of seed development			
	Seeded (%[a])	Seedless	Perfect	Imperfect	Empty	Total (average)
7	1 (10)	0	10	2	1	13 (13)
9	3 (19)	0	69	3	3	75 (25)
10	4 (27)	0	43	11	11	65 (16.3)
11	2 (13)	0	24	2	7	33 (16.5)
13	1 (8)	0	14	3	2	19 (19)
14	1 (11)	0	9	0	2	11 (11)
15	1 (10)	1	15	0	2	17 (8.5)
17	1 (10)	0	4	0	1	5 (5)

[a] Percentage of flower buds resulting in seeded fruits production.

in the fruits from self-pollination of 9 mm level flower buds. One fruit obtained from self-pollination of 7 mm level flower bud contained half the largest number of seeds. The fruits from self-pollination of flower buds of 9–13 mm levels produced relatively large number of seeds, i.e., 16–25 seeds per fruit. In fruits from self-pollination of ≧14 mm flower buds, number of seeds decreased as the flower bud length increased. The rates of perfect seeds were very high in the resultant seeded fruits and relatively constant (66–92%).

Shishiyuzu

Some fruits derived from self-pollination of flower buds of 9 mm and 11–15 mm levels were seeded and the others were seedless. The seeded fruits contained a small number of seeds with the narrow range from 0.5 to 1 seed per fruit in each level of flower bud length (Table 6). Fruits derived from self-pollination of 8, 10 and ≧16 mm flower buds were seedless in any levels of flower bud length, partly because of the very high parthenocarpic ability in 'Shishiyuzu'. Of a total of 23 seeds obtained, 15 seeds (65%) were perfect, five were imperfect and three were empty.

In cross-pollination with 'Tosa Buntan' used as a pollen parent, one fruit obtained from pollinated flower bud of 10 mm level contained only one empty seed probably due to immaturity and underdevelopment of the pistil (Table 7). However, fruits from ≧11 mm flower buds contained 25–38 seeds per fruit. In cross-pollination with 'Yugehyokan', fruits from ≧12 mm flower buds also contained 28–35 seeds per fruit. Exudate production in the stigmas was observed in flower buds beyond 14 mm in length.

Hirado Buntan

All fruits derived from 10–21 mm self-pollinated flower buds were seeded and produced seeds with the range from 10 to 87 seeds per fruit (Table 8). The rates of perfect seeds were more than 50% in each length level of flower bud development. Self-pollinated flower buds of 17 mm level produced the largest number of seeds, followed by those of 12 mm, 16 mm and 15 mm levels. On the other hand, fruits from self-pollinated flower buds of 10, 11 and 23 mm levels contained small number of seeds, i.e., less than 30 seeds per fruit. Exudate production in the stigmas was observed in flower buds beyond

Table 6. Self-fertilized seed production by bud pollination in self-incompatible 'Shishiyuzu'.

Level of flower bud length (mm)	No. of fruits obtained		Number of seeds in indicated categories of seed development			
	Seeded (%[a])	Seedless	Perfect	Imperfect	Empty	Total (average)
8	0 (0)	1	0	0	0	0 (0)
9	2 (20)	2	4	0	0	4 (1.0)
10	0 (0)	3	0	0	0	0 (0)
11	3 (30)	4	3	3	0	6 (0.9)
12	2 (20)	2	0	2	1	3 (0.8)
13	1 (10)	4	4	0	0	4 (0.8)
14	1 (10)	6	4	0	0	4 (0.8)
15	2 (20)	3	0	0	2	2 (0.5)
16	0 (0)	4	0	0	0	0 (0)
17	0 (0)	5	0	0	0	0 (0)
18	0 (0)	3	0	0	0	0 (0)
19	0 (0)	8	0	0	0	0 (0)
20	0 (0)	5	0	0	0	0 (0)

[a] Percentage of flower buds resulting in seeded fruits production.

Table 7. Seed production in self-incompatible 'Shishiyuzu' crossed with self-incompatible 'Tosa Buntan' and 'Yugehyokan'.

Level of flower bud length (mm)	No. of fruits obtained		Number of seeds in indicated categories of seed development			
	Seeded	Seedless	Perfect	Imperfect	Empty	Total (average)
×Tosa Buntan						
10	1	0	0	0	1	1 (1.0)
11	3	0	101	8	6	115 (38.3)
12	3	0	68	3	5	76 (25.3)
13	2	0	54	8	1	63 (36.5)
14	6	0	178	3	9	190 (36.7)
×Yugehyokan						
12	4	0	128	0	6	134 (33.5)
13	6	0	156	1	11	168 (28.0)
14	3	0	104	1	1	106 (35.3)

16 mm in length.

Banpeiyu

Self-pollinated flower buds of 14–19 mm levels produced seeded fruits with high frequencies (29–59%). Self-pollination of flower buds of 10–22 mm levels resulted in seed production with the range from 2 to 71 seeds per fruit (Table 9). Two fruits derived from self-pollination of flower buds of 10 and 13 mm levels contained only 2 and 3 seeds

Table 8. Self-fertilized seed production by bud pollination in self-incompatible 'Hirado Buntan'.

Level of flower bud length (mm)	No. of fruits obtained		Number of seeds in indicated categories of seed development			
	Seeded (%[a])	Seedless	Perfect	Imperfect	Empty	Total (average)
10	1 (17)	0	19	0	5	24 (24.0)
11	1 (20)	0	5	0	5	10 (10.0)
12	1 (13)	0	44	26	15	85 (85.0)
13	3 (38)	0	71	2	25	98 (32.7)
14	5 (63)	0	144	63	38	245 (49.0)
15	3 (50)	0	144	13	47	204 (68.0)
16	2 (22)	0	83	40	32	155 (77.5)
17	4 (67)	0	281	1	65	347 (86.8)
18	2 (40)	0	88	0	31	119 (59.5)
19	2 (33)	0	23	22	11	56 (28.0)
21	3 (33)	0	113	0	46	159 (53.0)

[a] Percentage of flower buds resulting in seeded fruits production.

Table 9. Self-fertilized seed production by bud pollination in self-incompatible 'Banpeiyu'.

Level of flower bud length (mm)	No. of fruits obtained		Number of seeds in indicated categories of seed development			
	Seeded (%[a])	Seedless	Perfect	Imperfect	Empty	Total (average)
×Banpeiyu						
8	0 (0)	1	0	0	0	0 (0)
9	0 (0)	2	0	0	0	0 (0)
10	1 (11)	2	6	0	0	6 (2.0)
11	1 (16)	0	58	0	0	58 (58.0)
12	1 (16)	0	50	0	5	55 (55.0)
13	1 (13)	0	3	0	0	3 (3.0)
14	2 (40)	0	112	1	4	117 (58.5)
15	2 (33)	0	53	1	2	56 (28.0)
16	2 (29)	0	109	3	2	114 (57.0)
17	3 (38)	0	185	1	7	193 (64.3)
18	4 (50)	0	280	0	5	285 (71.2)
19	4 (57)	0	141	3	7	151 (37.8)
20	1 (11)	0	69	0	1	70 (70.0)
21	1 (17)	0	49	0	2	51 (51.0)
22	2 (25)	0	89	1	0	90 (45.0)
27	0 (0)	1	0	0	0	0 (0)
29	0 (0)	1	0	0	0	0 (0)
×Tosa Buntan						
29	3	0	189	3	57	248 (82.7)

[a] Percentage of flower buds resulting in seeded fruits production.

per fruit respectively. Fruits from self-pollination of flower buds of 17, 18 and 20 mm levels contained large number of perfect seeds as same as those from cross-pollination with 'Tosa Buntan' just before anthesis (flower bud of 29 mm length level).

In each 1mm length level of self-pollinated flower buds, the resultant fruits contained perfect seeds with very high rates, i.e., more than 91%. These results were sharply contrasted to that of cross-pollination with 'Tosa Buntan', i.e., the rate of perfect seeds was 76.2%. The difference in the rates between self- and cross-pollination was mainly due to the existence of empty seeds in the fruits from cross-pollination.

Exudate production in the stigmas was observed in flower buds beyond 17 mm in length.

Tosa Buntan

Based on the above results, self-pollination was carried out in flower buds of 13–16 mm levels that were expected to produce self-fertilized seeds in 'Tosa Buntan'. The self-pollination in all levels of the flower buds resulted in production of seeded fruits containing 15–48 seeds per fruit (Table 10). The highest number of seeds per fruit was obtained from self-pollination of 14 mm level flower buds. The rates of perfect seeds ranged from 31 to 58% with relatively high rates of imperfect seed production.

Growth of plants derived from self-pollination

Table 10. Self-fertilized seed production by bud pollination in self-incompatible 'Tosa Buntan'.

Level of flower bud length (mm)	No. of fruits obtained		Number of seeds in indicated categories of seed development			
	Seeded	Seedless	Perfect	Imperfect	Empty	Total (average)
13	2	0	20	14	0	34 (17)
14	1	0	15	16	17	48 (48)
15	1	0	8	7	0	15 (15)
16	3	0	37	22	21	80 (26.7)

About 100 self-fertilized seeds chosen in each cultivar germinated and grew normally. No inbreeding depression was observed among the seedlings. About 20 of the 100 seedlings in each cultivar were top-grafted on satsuma mandarin trees. These top-grafted seedlings have so far been growing normally.

DISCUSSION

Optimum stage of flower bud development

It is concluded from the present results of the fruit set rate and number of fertilized seeds that optimum stage of flower bud development for self-fertilized seed production is 6–8 mm for Clementine, 8–12 mm for 'Hassaku', 9–13 mm for 'Hyuganatsu', 11–14 mm for 'Shishiyuzu', 12–17 mm for 'Hirado' and 14–19 mm for 'Banpeiyu'. It is inferred from these results in the representative *Citrus* cultivars that size of flower buds suitable for self-fertilized seed production in self-incompatible *Citrus* cultivars is about half the length of flower buds just before anthesis.

Self-incompatibility reaction in pistil of developing flower bud

Ngo *et al.* (2001) reported that incompatible pollen tube growth ceased in the stigmas and styles of self-incompatible *Citrus* cultivars self-pollinated 2 days before or after anthesis, whereas many pollen tubes leached the ovaries in those pollinated 4 to 6 days before anthesis. In this study, we used flower bud length as an index of flower bud development instead of the days before anthesis that Ngo *et al.* (2001) used, mainly because flower bud length is superior to the days before anthesis for practical use to produce self-fertilized seeds. The present results of fruit set rates one month after bud pollination in each millimeter level of flower bud development (Table 1 and Fig. 1) indicate that when the flower bud is about half the length of those just before anthesis, the highest rate of fruit set occurs. This may almost correspond with 6 days before anthesis that Ngo *et al.* (2001) indicated. Furthermore, all fruits developing from bud pollination of the half-sized flower buds were seeded ones with almost the same number of perfect seeds as those developing from open- or cross-pollinations. This suggests that no self-incompatibility reaction occurs by this stage of flower bud development in self-incompatible *Citrus* cultivars and plants.

The fact that decrease of the fruit set rates and the number of seeds per fruit occurred with decrease of flower bud size before this stage is related to the degree of immaturity of the pistil. The length of flower buds that the immature pistils did not have reproductive capability is estimated to be <6 mm for 'Clementine' and 'Hassaku', <7 mm for 'Hyuganatsu', <9 mm for 'Shishiyuzu' and <10 mm for 'Hirado' and 'Banpeiyu'.

In each cultivar, the minimum length level of flower buds showing stigmatic exudate production well corresponds with the length level of flower buds showing the highest rates of fruit set. This fact indicates close relationship between self-incompatibility reaction and exudate production in the stigma, i.e., after the stigmatic exudate production activated, self-incompatibility reaction increases. Our data of self-fertilized seed production suggest that the increase rate of self-incompatibility reaction in the developing pistil is different in different cultivars and is related to the flower size in a given cultivar, since the increase rate was the fastest in 'Clementine' mandarin with small flowers, whereas it was the slowest in 'Banpeiyu' with the largest flowers.

Wakana and Uemoto (1987) reported that it took about 8–13 days from pollination to fertilization in *Citrus unshiu*. Thus, in natural conditions, embryo sacs of *Citrus* develop into mature ones about one week after anthesis. The success of bud-pollination in early stage of flower bud development in self-incompatible *Citrus* cultivars suggests that pollen germination and subsequent tube growth occurs at this stage and enhances the embryo sac development in the immature pistils. Pimienta and Polito (1983) have reported also that pollination enhanced the development of embryo sac in almond. In bud pollination at the very early stage of flower bud development as mentioned above, however, either pollen germination or subsequent pollen tube growth is unable because of lacking of function such as pistil exudate production in the immature pistils.

Examination of fruit set one month after pollination is important to evaluate the occurrence of self-fertilization in bud pollination. When fertilization occurs in the flower buds they undoubtedly set, but they may drop thereafter with high rates when they contain a few fertilized seeds. Thus, we may conclude that 'Clementine' and 'Hirado Buntan' flower buds dropping by one month after pollination do not fertilize. However, the other cultivars with various degree of parthenocarpic ability are considered to include seedless fruits one month after pollination as observed in the harvested mature fruits. The rate of seedless fruits appearing among the self-pollinated fruits in each cultivar indicates that the parthenocarpic ability is high in 'Shishiyuzu', followed by 'Hassaku', 'Banpeiyu' and 'Hyuganatsu', and does not exist in 'Hirado Buntan' and 'Clementine' mandarin.

Difference in self-incompatibility system among cultivars

Of the seven cultivars used for self-pollination, 'Shishiyuzu' showed very different self-incompatibility reaction in bud pollination. Presence of a large number of seeds in fruits from cross-pollinated flower buds of 11–14 mm length levels indicates that the pistils at these flower bud stages are functional. As indicated in the previous study (Ngo. *et al.*, 2001), the pollen tubes of 'Shishiyuzu' behave normally in the pistils of the other citrus cultivars. These facts and the presence of less than one seed in self-pollinated fruits from flower buds of 11–15 mm length levels indicate that 'Shishiyuzu' has different self-incompatibility system from the other six cultivars, although the detailed mechanism are not known at present.

Growth of plants derived from self-pollination

Since the average weight of self-fertilized seeds was almost the same as that of cross-fertilized seeds, it is concluded that there is no difference in seed development between self-pollination and cross-pollination in *Citrus*. Since the seedlings developing from self-fertilized seeds did not show the inbreeding depression, they are useful for future analysis of self-incompatibility genes in *Citrus*. One-half of the seedlings top-grafted on 15-year-old satsuma mandarin trees will be homozygous for the self-incompatible gene, and become markers to identify *S* genotypes in *Citrus* cultivars and their progenies.

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