

## Estimation of Self-incompatibility S Genotypes of Citrus Cultivars and Plants Based on Controlled Pollination with Restricted Number of Pollen Grains

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## Estimation of Self-incompatibility *S* Genotypes of *Citrus* Cultivars and Plants Based on Controlled Pollination with Restricted Number of Pollen Grains

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Self-incompatibility *S* genotypes of 29 *Citrus* cultivars were estimated by the observation of pollen tube behavior in the lower one-third of styles with the aid of an epifluorescent microscope 7 or 8 days after controlled pollination with restricted number of pollen grains (about 100) on their stigmas. In eight crosses considered to be fully compatible on the basis of segregation distortion of glutamate oxaloacetate transaminase (GOT) isozymes in the progenies of cultivars used for the crosses, the rate of pollen tubes reaching the style base ranged from 7.7% to 24.6% of pollen grains putting on the stigmas, while in twelve crosses considered to be semi-compatible the rate of pollen tubes reaching the style base ranged from 1.6% to 19.3% of pollen grains on the stigmas. The rates were slightly different in different pistillate parents. Twenty cultivars whose genotypes were not determined were pollinated with restricted number of 'Banpeiyu' ( $S_1S_2$ ) pollen grains and their genotypes were predicted by the pollen tube behaviors. Similarly, 13 controlled crosses were carried out with restricted number of pollen grains and their *S* genotypes were determined based on the number of pollen tubes reaching the style base. From these results, it became clear that the predicted *S* genotypes of the cultivars are well correspond with pollen tube behaviors in full or semi compatible crosses and, eventually, it was estimated that 15 cultivars have either  $S_1$  or  $S_2$  allele that 'Banpeiyu' pummelo has.

### INTRODUCTION

Seedlessness in *Citrus* cultivars is one of the desirable characters for consumers. Many mandarin and mandarin relative cultivars released recently were seedless ones with male sterile Satsuma mandarins in their pedigrees. Breeding seedless cultivars with self-incompatibility is another method to expand and strengthen the breeding possibility.

There are two systems for self-incompatibility in angiosperms, i.e., sporophytic and gametophytic. In a gametophytic self-incompatibility system, rejection of pollen tube occurs when an *S* allele carried by the haploid pollen matches either of two *S* alleles existing in the diploid tissue of the pistil. Successful pollen tube growth occurs when the *S* allele carried by the haploid pollen is different from both *S* alleles expressing in the diploid pistil. The interaction between pollen tubes and the pistil in a gametophytic system controlled by single gene is divided into three types, i.e., full compatible, semi compatible (or semi incompatible) and incompatible crosses. By pollination tests, these incompatibility reactions were

defined in cherries (Cane and Brown, 1938). In apple, Manganaris and Alston (1987) observed the difference in pollen tube behaviors in incompatible, semi compatible and full compatible crosses.

In *Citrus*, Nagai and Tanikawa (1928) firstly reported several self-incompatible cultivars, and then many authors did (e.g., Miwa, 1951; Nuriyal, 1952; Soost, 1956 and 1964; Iwamasa and Oba, 1980). Soost (1969) proposed a self-incompatibility *S* gene system in *Citrus* on the basis of the segregation of hybrid seedlings with self-incompatibility. There was one report (Khan and De Mason, 1986) on the pollen tube behavior in semi compatible crosses. In the previous study (Ngo *et al.*, 2001), not only normal but also abnormal pollen tube growth was detected in the stigmas and styles of self-compatible cultivars after self-pollination. These results suggest the possibility that *S* genotypes predicted for the cultivars (Wakana *et al.*, 1998) can be certified directly by the reaction between pistil and pollen tubes even in semi compatible pollinations. In this study, therefore, pollen tube behaviors were analyzed to certify the cultivar *S* genotypes in probably semi compatible and full compatible crosses that were estimated on the basis of predicted *S* genotypes of several cultivars (Wakana *et al.*, 1998).

### MATERIALS AND METHODS

#### Plant materials

Thirty cultivars and plants including self-incompatible ( $S_nS_n$ ) and self-semi-compatible ( $S_nS_j$ ) plants predicted in previous study (Wakana *et al.*, 1998; Ngo, *et al.*, 2001) were chosen for this experiment. Cultivar name, accession number, scientific name, self-incompat-

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**Table 1.** *Citrus* cultivars used for controlled and restricted pollination

Cultivar or Accession No.	Scientific name	Self-incompatibility <sup>a</sup>	Poly-embryony
Banpeiyu	<i>C. grandis</i> Osbeck	SI (Ngo <i>et al.</i> , 2001)	mono
Bansei Siam Buntan	<i>C. grandis</i> Osbeck	SI	mono
Bangladesh No. 49	<i>C. grandis</i> Osbeck	SI (Ngo, 2001)	mono
Honda Buntan	<i>C. grandis</i> Osbeck	SI (Ngo, 2001)	mono
Kawanabe Buntan	<i>C. grandis</i> Osbeck	SI (Ngo, 2001)	mono
Sekitoyu	<i>C. grandis</i> Osbeck	SI (Ngo, 2001)	mono
Shatienyu	<i>C. grandis</i> Osbeck	SI (Ngo, 2001)	mono
Vietnam No. 2	<i>C. grandis</i> Osbeck	SI (Ngo, 2001)	mono
Vietnam No. 22	<i>C. grandis</i> Osbeck	SI (Ngo, 2001)	mono
Mato Buntan	<i>C. grandis</i> Osbeck	SI (Ngo, 2001)	mono
Nagashima No. 1	<i>C. grandis</i> Osbeck	SI (Ngo, 2001)	mono
Nagashima No. 2	<i>C. grandis</i> Osbeck	SI (Ngo, 2001)	mono
Nagashima No. 4	<i>C. grandis</i> Osbeck	SI (Ngo, 2001)	mono
Nagashima No. 7	<i>C. grandis</i> Osbeck	SI (Ngo, 2001)	mono
Nagashima No. 8	<i>C. grandis</i> Osbeck	SI (Ngo, 2001)	mono
Nagashima No.12	<i>C. grandis</i> Osbeck	SI (Ngo, 2001)	mono
Ipoh No. 1	<i>C. grandis</i> Osbeck	SI (Ngo, 2001)	mono
Ipoh No. 5	<i>C. grandis</i> Osbeck	SI (Ngo, 2001)	mono
Taiwan Buntan	<i>C. grandis</i> Osbeck	SI (Ngo, 2001)	mono
Tanikawa Buntan	<i>C. grandis</i> Osbeck	SI (Ngo, 2001)	mono
Hino Buntan	<i>C. grandis</i> x <i>C. reticulata</i> ?	SI (Ngo, 2001)	mono
Hassaku	<i>C. hassaku</i> Hort. <i>ex</i> Tanaka	SI (Nishiura,1963)	mono
Hyokan	<i>C. ampullacea</i> Hort. <i>ex</i> Tanaka	SI (Ngo, 2001)	mono
Yugehyokan	<i>C. yuge-hyokana</i> Hort. <i>ex</i> Y. Tanaka	SI (Ngo <i>et al.</i> , 2001)	mono
Kawano Natsudaikai	<i>C. natsudaikai</i> Hayata	SSC (Ngo, 2001)	poly
Tosa Buntan	<i>C. otachibana</i> Hort. <i>ex</i> Y. Tanaka	SI (Ngo <i>et al.</i> , 2001)	mono
Zadaikai	<i>C. aurantium</i> L.	SSC (Ngo, 2001)	poly
Shishiyuzu	<i>C. pseudogulgul</i> Hort. <i>ex</i> Shirai	SI (Ngo <i>et al.</i> , 2001)	mono
Sweet Spring	<i>C. hassaku</i> x <i>C. unshiu</i>	? <sup>b</sup>	mono
Tachibaba No. 1	<i>C. tachibana</i> Tanaka	SSC (Ngo, 2001)	poly

<sup>a</sup> SI: self-incompatibility; SSC: semi self-compatibility. <sup>b</sup> Male sterile; either SI or SSC because of *S<sub>1</sub>S<sub>2</sub>* genotype of *C. unshiu* (Valdi *et al.*, 1995).

ibility and polyembryony were listed in Table 1.

### Controlled and restricted pollination

Based on cultivar *S* genotypes predicted from the segregation distortion for GOT isozyme genes in their progenies (Wakana *et al.*, 1998; Ngo, 2001), presumably semi compatible and full compatible crosses were carried out with controlled and restricted number of pollen grains of about 100 per stigma in 1997, 1998 and 1999. Flower buds were collected one day before anthesis. The anthers were dehisced under sunlight conditions and the pollen obtained was scattered about on both-sided adhesive tape attached on the glass slide. The tape containing pollen was sectioned into small area (1×1 mm<sup>2</sup> to 2×2 mm<sup>2</sup>) by cutting with a razor blade. One sectioned area or a small piece of the tape was almost equal to two-third of the stigma surface of the tested cultivars or plants. Each of the sectioned area that contained about 100–200 pollen grains with normal morphology was detected under a light microscope, and was taken off from the glass slide using a razor blade. In this case, the abnormal pollen grains that were not counted were small-sized pollen grains and those without cytoplasm

(empty or imperfect pollen grains). Pollination was carried out to apply the sectioned tape on the stigma surface of mature emasculated gynoecia with the pollen grain side so that the pollen grains attach to the stigma surface. The gynoecia were prepared by emasculating the flower buds just before anthesis and bagged to prevent outcrosses after the controlled and restricted pollination.

### Pollen tube observation

About three to six gynoecia were collected for each cultivar 7 or 8 days after pollination in each of the three years. The methods of staining and observation of pollen tubes have been described previously (Ngo *et al.*, 2001). In this experiment, number of pollen tubes in the lower one-third portion of style (style base) was counted in three to six gynoecia per one cross, recorded for each sampled gynoecium and averaged in each restricted cross pollination.

## RESULTS

### Year-to-year variation in pollen tube growth

In several crosses, the average number of pollen tubes

**Table 2.** Year-to-year variation for pollen tube–pistil interaction in *Citrus* cultivars

Cross	Year	No. of pollen grains on stigma	No. of pollen tubes in style base	% pollen grains reaching style base
Banpeiyu × Hassaku	1998	145.3	14.2	9.8
Banpeiyu × Hassaku	1999	195.0	55.3	28.4
Banpeiyu × Kawano Natsudaikai	1997	188.8	18.0	9.6
Banpeiyu × Kawano Natsudaikai	1999	229.5	33.6	14.6
Banpeiyu × Shishiyuzu	1998	116.6	10.7	9.1
Banpeiyu × Shishiyuzu	1999	157.8	4.0	2.5
Banpeiyu × Zadaikai	1997	201.5	14.5	7.2
Banpeiyu × Zadaikai	1998	166.0	7.5	4.5
Banpeiyu × Zadaikai	1999	248.3	26.6	10.7
Hassaku × Banpeiyu	1998	124.7	33.8	27.1
Hassaku × Banpeiyu	1999	151.8	45.5	29.9
Kawano Natsudaikai × Kawano Natsudaikai	1998	106.8	13.2	12.4
Kawano Natsudaikai × Kawano Natsudaikai	1999	124.8	15.2	12.2
Shishiyuzu × Kawano Natsudaikai	1997	202.5	16.5	8.1
Shishiyuzu × Kawano Natsudaikai	1999	162.0	11.4	7.0
Sweet Spring × Kawano Natsudaikai	1997	220.0	24.5	11.1
Sweet Spring × Kawano Natsudaikai	1998	62.2	15.3	24.6

**Table 3.** Pollen tube-pistil interaction in crosses between *Citrus* cultivars with predicted *S* genotypes

Cross (Predicted <i>S</i> genotypes)	Mean No. of pollen grains on stigma	Mean No. of pollen tubes in style base	% of pollen grains germinating and reaching style base	
			Observed	Expected (Type of Compatibility <sup>a</sup> )
Banpeiyu ( <i>S<sub>i</sub>S<sub>2</sub></i> ) × Hyokan ( <i>S<sub>i</sub>S<sub>2</sub></i> )	159.1	2.6	1.6	50 (SCC)
Banpeiyu ( <i>S<sub>i</sub>S<sub>2</sub></i> ) × Zadaikai ( <i>S<sub>i</sub>S<sub>7</sub></i> )	205.3	16.2	7.9	50 (SCC)
Banpeiyu ( <i>S<sub>i</sub>S<sub>2</sub></i> ) × Shishiyuzu ( <i>S<sub>i</sub>S<sub>8</sub></i> )	116.6	10.7	9.1	50 (SCC)
Banpeiyu( <i>S<sub>i</sub>S<sub>2</sub></i> ) × Kawano Natsudaikai ( <i>S<sub>s</sub>S<sub>7</sub></i> ) <sup>b</sup>	209.2	25.8	12.1	50 (SCC)
Banpeiyu ( <i>S<sub>i</sub>S<sub>2</sub></i> ) × Tosa Buntan ( <i>S<sub>i</sub>S<sub>3</sub></i> )	108.8	17.0	15.6	50 (SCC)
Banpeiyu ( <i>S<sub>i</sub>S<sub>2</sub></i> ) × Hassaku ( <i>S<sub>s</sub>S<sub>5</sub></i> )	170.2	34.8	20.4	100 (FCC)
Banpeiyu ( <i>S<sub>i</sub>S<sub>2</sub></i> ) × Yugehyokan ( <i>S<sub>s</sub>S<sub>7</sub></i> )	86.6	19.3	22.2	100 (FCC)
Tosa Buntan ( <i>S<sub>i</sub>S<sub>3</sub></i> ) × Zadaikai ( <i>S<sub>i</sub>S<sub>7</sub></i> )	183.8	6.0	3.3	50 (SCC)
Tosa Buntan ( <i>S<sub>i</sub>S<sub>3</sub></i> ) × Shishiyuzu ( <i>S<sub>i</sub>S<sub>8</sub></i> )	120.3	6.0	5.0	50 (SCC)
Tosa Buntan ( <i>S<sub>i</sub>S<sub>3</sub></i> ) × Banpeiyu ( <i>S<sub>i</sub>S<sub>2</sub></i> )	95.8	18.5	19.3	50 (SCC)
Tosa Buntan ( <i>S<sub>i</sub>S<sub>3</sub></i> ) × Hassaku ( <i>S<sub>s</sub>S<sub>5</sub></i> )	161.0	24.0	14.9	100 (FCC)
Hassaku ( <i>S<sub>s</sub>S<sub>5</sub></i> ) × tachibana No.1( <i>S<sub>s</sub>S<sub>7</sub></i> )	86.6	7.0	8.1	50 (SCC)
Hassaku ( <i>S<sub>s</sub>S<sub>5</sub></i> ) × Kawano Natsudaikai ( <i>S<sub>s</sub>S<sub>7</sub></i> )	124.5	15.8	12.7	100 (FCC)
Hassaku ( <i>S<sub>s</sub>S<sub>5</sub></i> ) × Banpeiyu ( <i>S<sub>i</sub>S<sub>2</sub></i> )	124.7	33.8	27.1	100 (FCC)
Shishiyuzu ( <i>S<sub>i</sub>S<sub>8</sub></i> ) × Zadaikai ( <i>S<sub>i</sub>S<sub>7</sub></i> )	232.0	8.3	3.6	50 (SCC)
Shishiyuzu ( <i>S<sub>i</sub>S<sub>8</sub></i> ) × Tosa Buntan ( <i>S<sub>i</sub>S<sub>3</sub></i> )	95.5	7.5	7.9	50 (SCC)
Shishiyuzu ( <i>S<sub>i</sub>S<sub>8</sub></i> ) × Banpeiyu ( <i>S<sub>i</sub>S<sub>2</sub></i> )	96.5	8.5	8.8	50 (SCC)
Shishiyuzu ( <i>S<sub>i</sub>S<sub>8</sub></i> ) × Kawano Natsudaikai ( <i>S<sub>s</sub>S<sub>7</sub></i> )	182.5	14.0	7.7	100 (FCC)
Sweet Spring ( <i>S<sub>s</sub>S<sub>7</sub></i> ) <sup>c</sup> × Hassaku ( <i>S<sub>s</sub>S<sub>5</sub></i> )	130.0	11.5	8.8	50 (SCC)
Sweet Spring ( <i>S<sub>s</sub>S<sub>7</sub></i> ) <sup>c</sup> × Kawano Natsudaikai ( <i>S<sub>s</sub>S<sub>7</sub></i> )	62.2	15.3	24.6	100 (FCC)
Zadaikai ( <i>S<sub>i</sub>S<sub>7</sub></i> ) × Zadaikai ( <i>S<sub>i</sub>S<sub>7</sub></i> )	147.8	2.3	1.6	50 (SCC)
Zadaikai ( <i>S<sub>i</sub>S<sub>7</sub></i> ) × Kawano Natsudaikai ( <i>S<sub>s</sub>S<sub>7</sub></i> )	121.4	11.8	9.7	100 (FCC)
Kawano Natsudaikai ( <i>S<sub>s</sub>S<sub>7</sub></i> ) × Kawano Natsudaikai ( <i>S<sub>s</sub>S<sub>7</sub></i> )	115.8	14.2	12.3	50 (SCC)

<sup>a</sup> SCC: semi cross compatibility; FCC: full cross compatibility. <sup>b</sup> Mori *et al.*, 2009. <sup>c</sup> Either *S<sub>s</sub>S<sub>7</sub>* or *S<sub>s</sub>S<sub>5</sub>*.

**Table 4.** Estimation of *S* genotypes of cultivars by pollen-pistil interaction in controlled and restricted pollination with 'Banpeiyu' ( $S_iS_2$ )

Cross (Estimated <i>S</i> genotype)	Mean No. of pollen grains on stigma	Mean No. of pollen tubes in style base	% pollen grains reaching style base (Compatibility <sup>b</sup> )
Taiwan Buntan ( $S_iS_2$ ) <sup>b</sup> × Banpeiyu ( $S_iS_2$ )	137.7	4.3	3.1 (SCC)
Nagashima No.12 ( $S_iS_2$ ) <sup>b</sup> × Banpeiyu ( $S_iS_2$ )	139.0	9.0	6.5 (SCC)
Ipoh No.1 ( $S_2S_2$ ) <sup>b</sup> × Banpeiyu ( $S_iS_2$ )	129.3	13.0	10.1 (SCC)
Nagashima No.8 ( $S_iS_2$ ) <sup>b</sup> × Banpeiyu ( $S_iS_2$ )	99.7	11.0	11.0 (SCC)
Bansei Siam Buntan ( $S_iS_2$ ) <sup>b</sup> × Banpeiyu ( $S_iS_2$ )	145.0	19.0	13.1 (SCC)
Nagashima No.7 ( $S_iS_2$ ) <sup>b</sup> × Banpeiyu ( $S_iS_2$ )	129.0	17.7	13.7 (SCC)
Hino Buntan ( $S_iS_2$ ) <sup>b</sup> × Banpeiyu ( $S_iS_2$ )	105.0	19.0	18.1 (SCC)
Kaophuang ( $S_iS_2$ ) <sup>b</sup> × Banpeiyu ( $S_iS_2$ )	138.0	26.0	18.8 (SCC)
Nagashima No.4 ( $S_iS_2$ ) <sup>b</sup> × Banpeiyu ( $S_iS_2$ )	147.0	30.5	20.7 (SCC)
Ipoh No.5 ( $S_iS_2$ ) <sup>b</sup> × Banpeiyu ( $S_iS_2$ )	139.3	30.7	22.0 (SCC)
Nagashima No.1 ( $S_iS_2$ ) × Banpeiyu ( $S_iS_2$ )	130.0	33.0	25.3 (FCC)
Nejime Buntan ( $S_iS_2$ ) × Banpeiyu ( $S_iS_2$ )	130.3	36.3	27.9 (FCC)
Sekitoyu ( $S_iS_2$ ) × Banpeiyu ( $S_iS_2$ )	104.0	32.0	30.8 (FCC)
Kawanabe Buntan ( $S_iS_2$ ) × Banpeiyu ( $S_iS_2$ )	150.3	48.0	31.9 (FCC)
Bangladesh No.49 ( $S_iS_2$ ) <sup>c</sup> × Banpeiyu ( $S_iS_2$ )	173.0	56.0	32.4 (FCC)
Nagashima No.2 ( $S_iS_2$ ) × Banpeiyu ( $S_iS_2$ )	168.0	68.5	40.8 (FCC)
Ipoh No.4 ( $S_iS_2$ ) × Banpeiyu ( $S_iS_2$ )	85.3	35.3	41.4 (FCC)
Banpeiyu ( $S_iS_2$ ) × Mato Buntan ( $S_iS_2$ ) <sup>c</sup>	213.7	42.0	19.7 (FCC)
Banpeiyu ( $S_iS_2$ ) × Bangladesh No.49 ( $S_iS_2$ ) <sup>c</sup>	229.5	63.0	27.5 (FCC)
Banpeiyu ( $S_iS_2$ ) × Vietnam No.2 ( $S_iS_2$ ) <sup>d</sup>	221.3	72.3	32.7 (FCC)

<sup>a</sup> Estimation of cross compatibility. FCC: full compatible cross; SCC: semi compatible cross. <sup>b</sup> Either ( $S_iS_2$ ) or ( $S_2S_2$ ).

<sup>c</sup>  $S_i$  is one of *S* alleles except  $S_x$ ,  $S_1$  and  $S_2$ . <sup>d</sup>  $S_x$  is one of *S* alleles except  $S_y$ ,  $S_1$  and  $S_2$ .

penetrating into the style base varied to some extent not only between crosses but also between year and year (Table 2). The year-to-year variation was seen in some presumably full compatible crosses such as 'Banpeiyu' × 'Hassaku' and 'Sweet Spring' × 'Kawano Natsudaikai' and some presumably semi compatible crosses such as 'Banpeiyu' × 'Shishiyuzu'. Generally, the year-to-year variation was more prominent in presumably full compatible crosses than semi compatible crosses. Thus, repeated observation of pollen tube growth for two or three years is seemed to be important for exact estimation of full or semi cross compatibility in the cross pollinations.

### Crosses between cultivars with predicted *S* genotypes

Between the cultivars whose *S* genotypes were prospected on the basis of the results of the segregation distortion for GOT isozyme genes in their progenies, three kinds of crosses were expected, i.e., full compatible cross (FCC), semi compatible cross (SCC) or semi incompatible cross (SIC) and incompatible cross (IC). In the semi compatible or semi incompatible cross, pistillate and pollen parents have one of  $S_n$  alleles except a self-compatible  $S_j$  allele in common, whereas in full compatible crosses neither have  $S_n$  alleles except the  $S_j$  allele in common.

When 'Banpeiyu' that was defined to have  $S_iS_2$  genotype (Wakana *et al.*, 1998) was used as a pistillate parent, the rate of pollen grains germinating and penetrating into the style base ranged from 1.6% to 15.6% for

predicted semi compatible crosses and from 20.4% to 22.2% for predicted full compatible crosses (Table 3), suggesting the predicted *S* genotypes of these cultivars used as pollen parents were well correspond with the pollen-pistil interaction. When 'Tosa Buntan' with a predicted  $S_iS_2$  genotype was used as a pistillate parent, the rate of pollen grains germinating and reaching the style base ranged from 1.6% to 5.0% for semi compatible crosses except for 'Banpeiyu' pollen grains of which 19.5% reached there, and was 14.9% for a full compatible cross with 'Hassaku'. When 'Hassaku' with  $S_iS_2$  genotype was used as a pistillate parent, the rate of pollen grains germinating and penetrating into the style base was 8.1% for a predicted semi compatible cross with tachibana No. 1, and was 12.7% and 27.1% for predicted full compatible crosses. When 'Shishiyuzu' with predicted  $S_iS_2$  genotype was used as a pistillate parent, the rate of pollen grains reaching the style base ranged from 3.6% to 8.8% for predicted semi compatible crosses. However, a predicted full compatible cross with 'Kawano Natsudaikai' ( $S_2S_j$ ; Mori *et al.*, 2009) also showed a low rate of 7.7% (Table 3). When 'Sweet Spring' ( $S_iS_2$  or  $S_2S_j$ ) was backcrossed with 'Hassaku' ( $S_iS_2$ ), the rate of pollen grains germinating and reaching the style base was 8.8%, while the rate was 24.6% for a predicted full compatible cross with 'Kawano Natsudaikai' ( $S_2S_j$ ). When 'Zadaikai' ( $S_iS_j$ ) was self-pollinated, the rate of pollen grains reaching the style base was 1.6%, while the rate increased to 9.7% for a predicted full compatible cross with 'Kawano Natsudaikai' ( $S_2S_j$ ). When 'Kawano Natsudaikai' ( $S_2S_j$ )

**Table 5.** Estimation of *S* genotypes of cultivars by pollen-pistil interaction in controlled and restricted pollinations with twelve *Citrus grandis* cultivars and plants

Cross (Estimated <i>S</i> genotype)	Mean No. of pollen grains on stigma	Mean No. of pollen tubes in style base	% pollen grains reaching style base (Compatibility <sup>a</sup> )
Nagashima No.12 ( <i>S<sub>1</sub>S<sub>2</sub></i> ) × Honda Buntan ( <i>S<sub>1</sub>S<sub>2</sub></i> )	134.0	8.0	5.9 (SCC)
Shatienyu ( <i>S<sub>2</sub>S<sub>3</sub></i> ) <sup>b</sup> × Vietnam No.2 ( <i>S<sub>2</sub>S<sub>3</sub></i> )	198.0	17.0	8.6 (SCC)
Mato Buntan ( <i>S<sub>1</sub>S<sub>2</sub></i> ) <sup>c</sup> × Bangladesh No.49 ( <i>S<sub>1</sub>S<sub>2</sub></i> )	210.0	21.0	10.0 (SCC)
Tosa Buntan ( <i>S<sub>1</sub>S<sub>2</sub></i> ) <sup>d</sup> × Honda Buntan ( <i>S<sub>1</sub>S<sub>2</sub></i> )	67.7	7.7	11.3 (SCC)
Shishiyuzu ( <i>S<sub>1</sub>S<sub>2</sub></i> ) <sup>d</sup> × Honda Buntan ( <i>S<sub>1</sub>S<sub>2</sub></i> )	144.3	17.3	12.1 (SCC)
Mato Buntan ( <i>S<sub>1</sub>S<sub>2</sub></i> ) × Tanikawa Buntan ( <i>S<sub>1</sub>S<sub>2</sub></i> )	122.5	12.5	14.8 (SCC)
Nagashima No.12 ( <i>S<sub>1</sub>S<sub>2</sub></i> ) × Tosa Buntan ( <i>S<sub>1</sub>S<sub>2</sub></i> )	81.0	12.0	14.8 (SCC)
Nagashima No.12 ( <i>S<sub>1</sub>S<sub>2</sub></i> ) × Hyokan ( <i>S<sub>1</sub>S<sub>2</sub></i> )	83.3	14.3	17.2 (SCC)
Bangladesh No. 49 ( <i>S<sub>1</sub>S<sub>2</sub></i> ) × Vietnam No. 2 ( <i>S<sub>2</sub>S<sub>3</sub></i> )	213.0	40.0	18.8 (FCC)
Mato Buntan ( <i>S<sub>1</sub>S<sub>2</sub></i> ) × Vietnam No. 22 ( <i>S<sub>2</sub>S<sub>3</sub></i> )	223.5	44.0	18.8 (FCC)
Nagashima No. 12 ( <i>S<sub>1</sub>S<sub>2</sub></i> ) × Shishiyuzu ( <i>S<sub>1</sub>S<sub>2</sub></i> )	147.0	31.3	21.4 (SCC)
Mato Buntan ( <i>S<sub>1</sub>S<sub>2</sub></i> ) × Shatienyu ( <i>S<sub>2</sub>S<sub>3</sub></i> )	250.0	55.5	22.0 (FCC)
Bangladesh No. 49 ( <i>S<sub>1</sub>S<sub>2</sub></i> ) × Vietnam No. 22 ( <i>S<sub>2</sub>S<sub>3</sub></i> )	223.0	60.5	27.1 (FCC)

<sup>a</sup> Estimation of cross compatibility. FCC: full compatible cross; SCC: semi compatible cross. <sup>b</sup> *S<sub>2</sub>* is an *S* allele except *S<sub>1</sub>*, *S<sub>2</sub>*, and *S<sub>3</sub>*. <sup>c</sup> *S<sub>1</sub>* is an *S* allele except *S<sub>2</sub>*, *S<sub>1</sub>* and *S<sub>2</sub>*. <sup>d</sup> Mori *et al.* (2009).

was self-pollinated, the rate of pollen grains reaching the style base was 12.3%.

These results suggest that the rates in semi compatible crosses and full compatible crosses are slightly different in different cross combinations, pistillate parents and pollen parents, i.e., the rates were relatively low in the crosses with ‘Zadaidai’ and/or ‘Shishiyuzu’, whereas the rates were relatively high in the crosses with ‘Banpeiyu’ and/or ‘Tosa Buntan’. Generally, the rate of pollen grains reaching the style base after germination was less than 12% for semi compatible crosses and more than that for full compatible crosses. In the crosses with ‘Banpeiyu’, however, the rate is considered to be less than 20% for semi compatible crosses.

#### Crosses with self-incompatible cultivars with unknown *S* genotypes

In 20 crosses between ‘Banpeiyu’ (*S<sub>1</sub>S<sub>2</sub>*) and self-incompatible pummelo cultivars and plants without predicted *S* genotypes, the rates of the pollen tubes germinating and reaching the lower one-third portion of the style ranged from 3.1% to 41.4% for the crosses with ‘Banpeiyu’ used as a pistillate parent and from 19.7% to 32.7% for the crosses with ‘Banpeiyu’ used as a pollen parent (Table 4). Based on the results of the pollen tube behavior in the cross pollinations between cultivars with the predicted *S* genotypes (Table 3) and those in the 20 crosses between ‘Banpeiyu’ (*S<sub>1</sub>S<sub>2</sub>*) and self-incompatible pummelo cultivars, nine pummelo plants showing the rates lower than the 22.0% of ‘Ipoh No. 5’ were estimated to be the plants with either *S<sub>1</sub>* or *S<sub>2</sub>* allele (Table 4).

In 13 crosses between self-incompatible pummelo plants whose parental *S* genotypes were not predicted except ‘Shishiyuzu’ (*S<sub>1</sub>S<sub>2</sub>*) and ‘Tosa Buntan’ (*S<sub>1</sub>S<sub>2</sub>*), the rates of the pollen tubes germinating and reaching the lower one-third portion of the style ranged from 5.9% to 27.1% (Table 5). Based on the results of the pollen tube

behavior in the crosses between cultivars with the predicted *S* genotypes (Table 4) and those in the crosses predicted to be semi compatible (Table 5), eight crosses showing the rates lower than 17.2% were predicted to be semi compatible.

## DISCUSSION

Although year-to-year variation in the rates of pollen grains germinating and reaching lower one-third of style was sometimes observed in controlled and restricted pollination of several *Citrus* cultivars and plants, the present results are considered to be enough for confirmation of the *S* genotypes of cultivars, which were predicted by us (Wakana *et al.*, 1998; Ngo, 2001) on the basis of the data for the segregation distortion of the GOT isozymes in their progenies. Namely, the high successful rates for pollen tube growth are correspond with the full cross compatibility in the crosses with cultivars whose *S* genotypes were prospected by the segregation distortion in their progenies, and the low successful rates for pollen tube growth are correspond with the semi cross compatibility in the crosses with cultivars whose *S* genotypes were estimated similarly. In this case, the existence of small variation in the rates among semi-compatible crosses and among fully compatible crosses is seemed to attribute to the difference in viability of embryo sac, size of pistils and fertility of pollen. For example, pummelo cultivars such as ‘Banpeiyu’ have large gynoecia containing many viable ovules and large transmitting tissue for pollen tube growth in comparison to those of other *Citrus* cultivars. Yamamoto *et al.* (2000) reported that in self-pollination of self-compatible cultivars number of pollen tubes reaching the style base varied greatly depending upon plants or cultivars, i.e., the number is 6.2 per style in tachibana, 10.3 in ‘Allen Eureka’ lemon, 7.7 and 10.4 in two sour orange cultivars, ranged from 22.2 to 38.8 for

pummelo relative cultivars and from 11.3 to more than 100 for mandarin cultivars. Thus, we must pay attention to the parental characteristics for pollen tube growth, when controlled and restricted pollination is carried out to determine whether the pollination is either semi- or full-compatible or not. The rates of pollen grains germinating and reaching the style base was very lower than the expected rate of 50% for semi compatible crosses and 100% for full compatible crosses. This is due to low fertility and/or low viability of pollen grains or pollen tubes due to physiological problems other than cross-incompatibility (Sedgley, 1976 and 1977).

The pollen tube behaviors in the present controlled and restricted pollinations in 53 cross combinations support the cultivar *S* genotypes predicted on the base of the results of GOT isozyme analyses and, moreover, suggest that several cultivars have an *S* allele in common. When the genotype of 'Banpeiyu' is defined as  $S_1S_2$ , the cultivar *S* genotypes supported in this study are 'Honda Buntan' ( $S_1S_2$ ), 'Kawano Natsudaidai' ( $S_2S_3$ ), 'Zadaidai' ( $S_1S_3$ ), 'Tosa Buntan' ( $S_1S_3$ ), 'Shishiyuzu' ( $S_1S_3$ ) and 'Hyokan' ( $S_1S_3$ ). The cultivars or plants that have either  $S_1$  or  $S_2$  allele are 'Taiwan Buntan' ( $S_1S_2$  or  $S_2S_3$ ), Nagashima No. 12 ( $S_1S_2$  or  $S_2S_3$ ), Ipoh No. 1 ( $S_2S_3$ ; unpublished), Nagashima No. 8 ( $S_1S_2$  or  $S_2S_3$ ), 'Bansei Siam Buntan' ( $S_1S_2$  or  $S_2S_3$ ), Nagashima No. 7 ( $S_1S_2$  or  $S_2S_3$ ), 'Hino Buntan' ( $S_1S_2$  or  $S_2S_3$ ), 'Kaophuang' ( $S_1S_2$  or  $S_2S_3$ ), Nagashima No. 4 ( $S_1S_2$  or  $S_2S_3$ ), Ipoh No. 5 ( $S_2S_3$ ; unpublished), Vietnam No. 2 ( $S_1S_2$  or  $S_2S_3$ ) and 'Shatienyu' ( $S_1S_2$  or  $S_2S_3$ ). Recently, Mori *et al.* (2009) and Kim *et al.* (2009) carried out pollination with pollen grains of  $S_1$  hybrids homozygous for one of four *S* alleles ( $S_1$ ,  $S_2$ ,  $S_3$  and  $S_4$ ) and demonstrated that the *S* genotype of 'Kawano Natsudaidai' is  $S_2S_3$  and that of 'Tosa Buntan' is  $S_1S_3$ . Furthermore, the present result suggests the possibility that 'Shatienyu' and Vietnam No. 2 have the same  $S_x$  allele that is not  $S_1$ ,  $S_2$  and  $S_3$ , and 'Mato Buntan', Bangladesh No. 2 and 'Tanikawa Buntan' have the same  $S_y$  allele that is not  $S_1$ ,  $S_2$  and  $S_3$ .

The estimation of *S* genotypes of *Citrus* cultivars and plants in the present study is the first step for genotyping many *Citrus* cultivars with an *S* gene. Further research for the *S* genotyping will contribute to the breeding of self-incompatible *Citrus* cultivars that produced seedless fruit.

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