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Seed Development in Self-pollination of 4X Hyuganatsu and Reciprocal Crosses between 2X and 4X Hyuganatsu, and Overcoming the Self-incompatibility of 2X Hyuganatsu Using Pollen of 4X Hyuganatsu

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Summary

In the present experiment, seed development was investigated in self-pollination of 4X Hyuganatsu and several cross combinations between 2X and 4X Hyuganatsu. In addition, possible methods to overcome self-incompatibility of 2X Hyuganatsu were examined, using pollen of 4X Hyuganatsu as a mentor or pioneer pollen. The results are summarized as follows.

1. Pollen of the 4X was not uniform in size and form as observed by scanning electron microscopy. Its *in vitro* germination was inferior to that of the 2X by 5%. The band patterns of glycoproteins from pollen and pistils of the 4X analyzed by isoelectric focusing electrophoresis were largely different from those of the 2X.

2. In almost all cases one well-developed seed was contained together with many large imperfectly developed seeds in the fruit of the 2X pollinated with the 4X and in the fruit of the self-pollinated 4X, although the fruit set was somewhat different between two pollinations. When the 4X was pollinated with pollen of the 2X, only one fruit was harvested, lacking a well-developed seed. The fruit set of the open-pollinated 4X tree was very low. A few fruits were harvested, containing about 3 well-developed seeds per fruit.

3. Three pollination methods were tried for overcoming the self-incompatibility of the 2X Hyuganatsu. When pollens of the 2X and 4X were pollinated on the left and right sides respectively, of stigmas of 2X Hyuganatsu (differential pollination), the most effective result was obtained on overcoming the self-incompatibility. One hour time-lag double pollination, where pollen of the 2X was used one hour after pollination by the 4X, showed the next best effectiveness. Pollination with mixed pollens of both ploidies was not effective at all.

Introduction

A seedless mutant of Hyuganatsu (*Citrus tamurana* Hort. ex Tanaka) was found as a limb sport at a production area of Kiyotake-cho, Miyazaki Prefecture. It was shown to be a 4X sport on a 2X tree, through a chromosome count in the meiosis of pollen mother cells (7, 14). Since its fruit quality is as good as that of the seeded Hyuganatsu, it is expected to replace the seeded Hyuganatsu in some ten years. In the meantime, the seeded 2X and seedless 4X Hyuganatsu will be mix-planted and several kinds of pollinations between them will be possible in an orchard. In the present experiment, seed development and fruit set in self-pollination of the 4X Hyuganatsu and reciprocal crosses between the 2X and 4X Hyuganatsu were investigated to obtain fundamental information for the production of seedless fruits (8). In addition, a few pollination trials using pollen of the 4X Hyuganatsu were undertaken to attempt to overcome the self-incompatibility of 2X Hyuganatsu.

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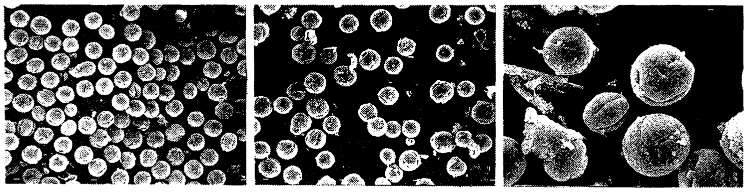
Materials and Methods

The 4X sport and several adult trees of 2X Hyuganatsu grown in a production area of Kiyotake-cho, Miyazaki Prefecture were used as experimental materials. In addition, some adult trees of the 2X in another orchard in Takaoka-cho, Miyazaki Prefecture were used for the experiment on overcoming the self-incompatibility of 2X Hyuganatsu.

In the flowering time in May, pollen of the 2X and 4X Hyuganatsu was collected from the flowers one day before anthesis, put into a plastic con-



A B C

Fig. 1. Scanning electron micrographes of 2X and 4X Hyuganatsu pollen.
A: The 2X Hyuganatsu pollen (×350).
B: The 4X Hyuganatsu pollen (×350).
C: Ibid (×1000).

tainer with some silica gel as a desiccant, and stored in a freezer at −18°C. The flowers used for the pollen collection were frozen in liquid nitrogen at −196°C immediately after picking and stored in a freezer at −18°C for protein analysis of pistils.

1. Morphological and physiological observation of pollens and pistils

The pollen viability of the 4X was compared with that of the 2X through an *in vitro* germination test on an agar medium. Then, the pollen grains of the 2X and 4X were observed by a scanning electron microscope. The sample was prepared with a routine procedure (9).

Protein analysis of pollen and pistils was undertaken as follows. From 100 mg of pollen and 400 mg pistils collected from each of 2X and 4X trees, a crude extract of buffer-soluble proteins was prepared and brought into isoelectric focusing electrophoresis (13). Glycoproteins among the proteins distributed on a cellulose acetate film were detected by color development with the conkanavalin-peroxidase dyeing system (10).

2. Pollination experiment

The pollination scheme is shown in Table 3. In every pollination, 30 flowers one day before anthesis were emasculated and pollinated by hand. The pollinated flowers were bagged for a week to prevent contamination by foreign pollen. At harvest, fruits were weighed and seeds were counted.

3. Trials to overcome the selfincompatibility of 2X Hyuganatsu

Three methods of artificial pollination were carried out on 2X trees as pistillate parent. The first was time-lag double pollination (12). Thirty mature flowers one day before anthesis were emasculated and pollinated with pollen of the 4X followed by self-pollination of the 2X one hour later. The second method is what we call differential pollination (4). A half side of stigma surface of 30 emasculated mature flowers of the 2X Hyuganatsu was pollinated with pollen of the 4X, and another half side was self-pollinated with pollen of the 2X itself. The third method is the typical mentor pollen method (5). Pollen of the 2X was mixed with pollen of the 4X in a volume ratio of 1 : 1 and pollinated onto the stigma of 30 emasculated mature flowers. All the pollinated flowers were bagged for a week to be free from foreign pollen. At harvest season, the fruits were picked and weighed, and seeds were counted.

Table 1. Germination (%) of pollen of 2X and 4X Hyuganatsu.

Sucrose concentration of the medium (%)	2X	4X
10	27.9±7.82	23.9±5.71
20	33.1±5.52	27.6±3.17
30	25.9±3.77	20.4±4.66
Average	29.0±6.68	24.0±5.48

Results and Discussion

1. Morphological and physiological observation of pollen and pistils

Fig. 1 shows the pollen of the 2X and 4X observed under scanning electron microscopy. The pollen of the 4X was very irregular in size and form as compared with the uniform pollen of the 2X. The germination of pollen of the 4X was inferior to that of 2X by nearly 5% (Table 1).

Table 2. Distribution of pollen and pistil glycoproteins of 2X and 4X Hyuganatsu analyzed by isoelectric focusing electrophoresis.

Material			pH gradient 2–3	3–4	4–5	5–6	6–7	7–8	Total
Pistil	2X	T[z]	2	4	3	7	6	0	22
		S	0	0	0	1	0	0	1
	4X	T	4	8	6	9	6	0	33
		S	2	4	3	3	0	0	12
Pollen	2X	T	1	7	11	9	6	0	34
		S	0	1	0	0	0	0	1
	4X	T	4	6	11	10	7	0	38
		S	3	0	0	1	1	0	5

[z] T: Total band number, S: Specific band number.

Table 3. Fruit set, fruit weight and seed numbers obtained from crosses of 2X and 4X Hyuganatsu.

Cross combination	Fruit set (%)	Fruit weight (g)	Well-developed seed [z]			Imperfectly developed seed [y]		
			L	M	S	L	M	S
2X×4X	40.0	187.25±26.02	1.08± 1.19	0.17±0.37	0.17±0.37	16.0±7.33	9.07±6.38	14.92±9.83
4X×4X	23.3	183.79±37.13	1.14± 1.00	0.29±0.70	0	21.7±7.34	2.43±2.19	15.29±6.27
4X×2X	3.3	139.0	0	0	0	3.0	0	37.0
4X Open	6.7	171.81±36.15	3.25± 7.65	0.06±0.24	0.13±0.33	14.38±9.29	4.25±6.11	18.81±2.93
2X Open		186.58±34.33	18.23±11.16	0.85±1.41	0.31±0.61	1.46±3.10	8.15±6.17	11.92±9.29

[z] M: 1/2～3/4 of the large seed in length, S: Less than 1/2 of the large seed in length.
[y] L: More than 7 mm in length, M: 3～7 mm in length, S: Less than 3 mm in length.

Yamashita and Katsuki (11) observed the same type of irregularity and poor germination in pollen of 4X Natsudaidai.

Table 2 summarizes the band patterns of glycoproteins on both pollen and pistils of the 2X and 4X analyzed by isoelectric focusing electrophoresis. The band patterns of the 2X and 4X were remarkably different from each other. The numbers of total and specific bands of pollen of the 4X were 38 and 5, respectively. These were greater than those of the 2X. This tendency was also found in pistils; the numbers of total and specific bands of the 4X were 33 and 12, respectively, both of which were far beyond those of the 2X. It has been generally believed that glycoproteins in pistil are closely connected with the function of gametophytic incompatibility (5). Therefore, further information of glycoprotein sequences in pistil from crosses using 2X and 4X Hyuganatsu possibly provides a further understanding of the self-incompatibility.

2. Pollination experiments

Table 3 shows the fruit set and seed formation in the crosses of the 2X and 4X. In the self-pollination of the 4X, fruit set was 23.3% and the fruit contained one well-developed seed in almost all cases. When crossed with the 4X, the 2X showed 40% fruit set, and almost all of the fruit contained one well-developed seed just like in the self-pollination of the 4X. In the above cases, imperfectly developed seeds of more than 7 mm length appeared in all of the harvested fruits. This suggests that the presumably fertilized ovules were aborted because of chromosomal and physiological abberations during their development (2, 8).

Gametophytic incompatibility has been explained so far by the function of S-genes. Self-incompatibility at the diploid level can be reversed at the autotetraploid level. Brewbaker (1) explained this fact as a competitive action of S-genes. No incompatible reactions developed certainly in the two crosses above mentioned.

Abortion of seed from the 2X crossed with the 4X is supposed to be due to disproportion in the number of chromosomes between embryo and endosperm (3). In this connection, Nishiyama (6) proposed the polar nucleus activation theory based upon a pollination experiment with oats, in which he explained reasonably the mechanism of seed abortion. The seed abortion that appeared in the self-pollination of the 4X Hyuganatsu is rather difficult to explain because of the normal seeds obtained from the 4X Hyuganatsu crossed with Hassaku or Natsudaidai (7). In this case it is assumed that the seed abortion in the self-pollination of the 4X Hyuganatsu is probably due to its abnormal pollen.

When the 4X Hyuganatsu was pollinated with the 2X Hyuganatsu, only one fruit was harvested, having no well-developed seed. This is possibly related to gametophytic incompatible reactions caused by S-genes. In such reactions, no eggs could be fertilized because of no pollen tubes in styles or ovules (15).

In the open pollination of the 4X Hyuganatsu, the fruit set was 6.7%, and an average of 3 well-developed seeds were contained per fruit. In this production area where we carried out the experiment, many Natsudaidai trees were planted rather closely to the 4X tree. Therefore, it is reasonable that the 4X contained several well-developed seeds in the fruit.

3. *Trials to overcome the self-incompatibility of the 2X Hyuganatsu*

Table 4 shows the effect of pollen of the 4X used in three different pollination methods to overcome the self-incompatibility. Differential pollination was the most effective among the three treatments tried in the present experiment. In the harvested fruits, a half side of the fruit was filled with well-developed seeds, while the other half had imperfectly developed seeds (Fig. 2). Since the cross of the 2X with the 4X did not produce any well-developed seeds, 190 well-developed seeds obtained from the 16 harvested fruits of this differential pollination were considered to be self-fertilized with pollen of the 2X, not with pollen of the 4X. It is quite difficult to explain this phenomenon of seed development. It will be studied throughly in our further research.

With the one hour time-lag double pollination, when crossed first with the 4X and then with the 2X, 92 well-developed seeds were obtained from the 7 harvested fruits. In this double pollination, the pollen tubes of the 4X behaved just like a pioneer in the pistils. They might have organized an

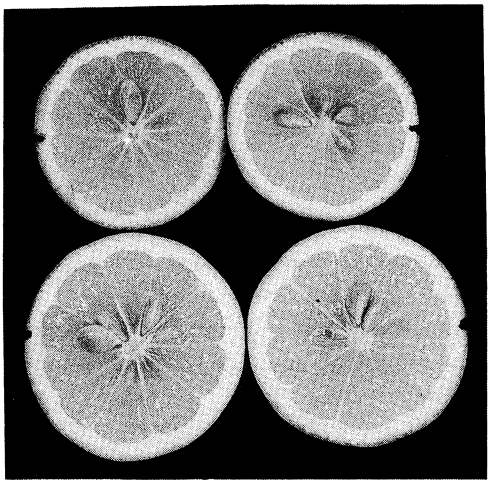


Fig. 2. Transverse sections of fruits obtained in the differential pollination. The upper half of the fruits contained well-developed seeds, while the lower half had imperfectly developed seeds.

Table 4. Several trials using pollen of 4X Hyuganatsu to overcome self-incompatibility of the 2X Hyuganatsu.

Treatment	No. of flowers pollinated	No. of fruits harvested	Fruit weight (g)	Well-developed seed[z]				Imperfectly developed seed[y]		
				L	M	S	T	L	M	S
Time lag double pollination	30	7	145.1	58	33	1	92	54	18	178
Differential pollination	30	16	160.7	140	48	2	190	91	73	415
Pollination with pollen mixture	30	5	128.8	3	0	0	3	30	20	145

[z] M: 1/2 ~ 3/4 of the large seed in length, S: Less than 1/2 of the large seed in length.

[y] L : More than 7 mm in length, M: 3 ~ 7 mm in length, S: Less than 3 mm in length.

accepting system in the pistils of the 2X Hyuganatsu, through which the incompatible pollen of the 2X could reach the ovules for fertilization.

Pollination of mixed pollen of the 2X and 4X Hyuganatsu was not effective at all. The fruits harvested had no well-developed seeds and averaged 6 large imperfectly developed seeds more than 7 mm in length per fruit.

From these data, there is one important problem to consider. Planting of the 2X Hyuganatsu trees mixed with 4X Hyuganatsu trees as pollinizer is a good idea to produce seedless or few-seeded fruit. When the differential or the time-lag double pollination occurs, however, comparatively high-seeded fruits might be produced.

Literature Cited

1. BREWBAKER, J. L. 1954. Incompatibility in autotetraploid *Trifolium repens.* 1. Competition and self-incompatibility. Genetics 39 : 307–316.
2. ESEN, A. and R. K. SOOST. 1971. Unexpected triploids in Citrus: their origin, identification, and possible use. J. Hered. 62 : 329–333.
3. ESEN, A. and R. K. SOOST, 1973. Relation of unexpected polyploids to diploid megegametophytes and embryo : endosperm ploidy ratios. Proc. Int. Citriculture. 1973 : 53–63.
4. LAZUKOV, M. I. 1964. Overcoming incompatibility in pear species by the differential pollination method. Plant Breeding Abstract 35 : 2572.
5. NETTACOURT, D. de. 1977. Incompatibility in Angiosperms. p. 70–71, 105–106. Springer-Verlag, Berlin.
6. NISHIYAMA, I. 1984. Interspecific cross-incompatibility system in the genus *Avena.* Bot. Mag. Tokyo. 97 : 219–231.
7. SHIMOGORI, Y., S. MUTAGAMI and T. YAMAMOTO. 1987. Characteristics of a seedless bud-sport of Hyuganatsu. Abstract of 1987 Fall Meeting of Japan. Soc. Hort. Sci. : 10–11. (In Japanese).
8. YAMASHITA, K. 1976. Production of seedless fruits in Hyuganatsu, *Citrus tamurana* Hort. ex Tanaka, and Hassaku, *Citrus hassaku* Hort. ex Tanaka, through pollination with pollen grains from the 4X Natsudaidai, *Citrus natsudaidai* Hayata. J. Japan. Soc. Hort. Sci. 45 : 225–230.
9. YAMASHITA, K. 1977. A few physiological and morphological differences of floral and foliar organs observed by zymography and scanning electron microscopy. Bull. Fac. Agr. Miyazaki Univ. 24 : 89–95.
10. YAMASHITA, K. 1981. Stigma exudate application for overcoming self-incompatibility of Hyuganatsu and Hassaku. Proc. Int. Soc. Citriculture. 1981 : 48–51.
11. YAMASHITA, K. and H. KATSUKI. 1981. Comparison of the pollen behavior of the 2X and 4X Natsudaidai in the pistils of Hyuganatsu observed by scanning electron microscopy. Bull. Fac. Agr. Miyazaki Univ. 27 : 419–424.
12. YAMASHITA, K. and S. TANIMOTO. 1985. Studies on self-incompatibility of Hassaku (*Citrus hassaku* Hort. ex Tanaka) II. Glycoprotein composition in pistils soon after self-pollination, and several trials to overcome self-incompatibility. J. Japan.. Soc. Hort. Sci. 54 : 178–183.
13. YAMASHITA, K., T. GAUDE, C. DUMAS, Ch. GRASSELLY and P. CROSSA-RAYNAUD. 1987. Protein analysis on pistils and pollens of almonds with special reference to Sf, a self-fertile gene. J. Japan. Soc. Hort. Sci. 56 : 268–272.
14. YAMASHITA, K. and K. YAMAGUCHI. 1988. Development of the 4X Hyuganatsu pollen and the behavior of its nuclei in pollen tubes. Abstr. Japan. Soc. Hort. Autumn Meet. 2–3. (In Japanese).
15. YAMASHITA, K. and M. TAMAI. 1990. Pollen tube elongation in pistils on the reciprocal crosses of the 2X and 4X Hyuganatsu, and acquisition of their 3X hybrids through *in vitro* culture of the embryos. Abst. Japan. Soc. Hort. Spring Meet. : 740. (In Japanese).

4Xヒュウガナツの自家受粉ならびに2Xヒュウガナツとの正逆交雑における種子形成とその花粉を利用した2Xヒュウガナツの自家不和合性打破

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摘　要

2Xならびに4Xヒュウガナツを材料として受粉試験を行い，種子形成について調査した．又，4Xヒュウガナツの花粉をメントールポーレンとして使用し，2Xヒュウガナツの自家不和合性打破の可能性について調査した．

走査型電子顕微鏡で4Xヒュウガナツの花粉を観察したところ，大きさや形は斉一ではなく，*in vitro* の花粉発芽率は2Xヒュウガナツよりも5％程度劣った．又，4Xヒュウガナツの花粉ならびに雌ずいに含まれる糖タンパク質を等電点電気泳動によって分析したところ，そのパターンは2Xヒュウガナツのパターンとは全く異なっていた．

2Xヒュウガナツ×4Xヒュウガナツあるいは4Xヒュウガナツの自家受粉では，着果率に多少の差が見られたものの，両組合せとも1果当たりに1粒の完全種子と多数の不完全種子が含まれていた．ところが，4Xヒュウガナツ×2Xヒュウガナツでは，収穫果はわずかに1果で，完全種子は含まれていなかった．又，4Xヒュウガナツの自然受粉では，着果率はやや低く，収穫果には2，3粒の完全種子が含まれていた．

2Xヒュウガナツの自家不和合性打破を目的として試みたいくつかの処理の中で，柱頭の片側に4Xヒュウガナツの花粉を受粉し，反対側に2Xヒュウガナツの花粉を受粉するいわゆる対峙受粉がもっとも有効であった．又，4Xヒュウガナツの花粉を受粉し，1時間後に2Xヒュウガナツの花粉を受粉する二重受粉も，対峙受粉に次いで有効であったが，両者の花粉を混合して受粉した場合には全く効果が見られなかった．