

Progress on studies for seedless breeding of citrus in Japan

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Abstract: Seedlessness is a desirable characteristic for both fresh and processed citrus markets and one of the most important breeding objectives. In this paper, progress on studies for seedless breeding of citrus in Japan is reviewed. Among the several types of male sterility, anther abortion is the strictest male sterility in citrus and was shown to be controlled by both nuclear and cytoplasmic genes. Several seedless cultivars with male sterility have been developed. The mechanism and inheritance of the strictest female sterility derived from 'Mukaku Kishu' (*Citrus kinokuni* hort. ex Tanaka) were clarified and seedless cultivar and parental lines with this female sterility have been released. Some self-incompatible cultivars show seedlessness when coupled with parthenocarpy. *S* (self-incompatibility) genotypes of several cultivars have been estimated. Tetraploid plants, as parents of triploid offspring, were obtained from nucellar seedlings, and by ploidy mutation, colchicine treatment, and protoplast fusion. Triploid plants were produced from the combination of not only tetraploid and diploid crosses but also diploid and diploid crosses. New triploid seedless cultivars were bred by programmed cross-breeding and protoplast fusion.

1. Introduction

Seedlessness is a desirable characteristic for both fresh and processed citrus markets (Vardi *et al.*, 2008). In fact, major citrus cultivars on a global level such as 'Valencia,' navel orange [*Citrus sinensis* (L.) Osbeck] and 'Marsh' grapefruit (*C. paradisi* Macfad.) are seedless (Reuther, 1988). Although a major early-maturing citrus, satsuma mandarin (*C. unshiu* Marcow.), is seedless, almost all mid- and late-maturing citrus, such as natsudaidai (*C. natsudaidai* Hayata), iyo (*C. iyo* hort. ex Tanaka), and hassaku (*C. hassaku* hort. ex Tanaka), are seedy in Japan (Iwamasa, 1988). Thus, breeding new seedless cultivars is an urgent issue to develop the Japanese citrus industry.

To develop seedless citrus at the diploid ($2x=18$) level, utilization of sterility is essential (Iwamasa, 1966). Sterility can be divided into three types: male sterility, female sterility, and self-incompatibility. In addition, selection of triploid ($3x=27$) individuals is useful for breeding seedless cultivars (Ollitrault *et al.*, 2007). Therefore, various kinds of investigations on the mechanism and genetic factors underlying seedlessness in citrus have been conducted and many breeding works have been carried out to develop new seedless cultivars, many of them carried out in Japan over the last few decades. In this review, the progress on studies for seedless breeding of citrus in Japan, with regard

to 1) seedlessness at the diploid level and 2) ploidy manipulation for seedless breeding, is discussed.

2. Seedlessness at the diploid level

Male sterility

The degree of male (pollen) sterility is variable in citrus, and usually pollen sterile accessions produce seedless or low-seedy fruits when cultivated in solid blocks. Male sterility couples with parthenocarpy to produce seedless fruits when cross-pollination is prevented. Even in mixed planting with pollen fertile accessions, male sterility reduces seed production and increases the percentage of seedless fruits because those accessions have a smaller chance of fertilization than male fertile ones (Yamamoto *et al.*, 1993, 1995).

Iwamasa (1966) and Ollitrault *et al.* (2007) summarized the various levels of male sterility at the diploid level in citrus (Table 1). Chromosome aberration was one of the most important phenomena causing pollen sterility. Asynapsis in 'Mukaku Yuzu' (*C. junos* Sirbold ex Tanaka) is genetically controlled, while that in 'Eureka' lemon (*C. limon* (L.) Burm. f.) and 'Mexican' lime (*C. aurantifolia* (Cristm.) Swingle) is induced by low temperature (Nakamura, 1943; Iwamasa and Iwasaki, 1962; Iwamasa, 1966). Reciprocal translocation is found to cause pollen sterility of 'Valencia' orange (*C. sinensis*) (Iwamasa, 1966). Inversion is the cause of partial pollen sterility of 'Mexican' lime (*C. aurantifolia*) (Iwamasa, 1966). Male sterility that is not caused by chromosome aberration is also well known. Anther abor-

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Table 1 - Diagrammatic representation of various kinds of the male-sterility in citrus, according to the sequential order of development (Modified from Iwamasa, 1966)

Developmental stage	Nature of sterility	Cultivar or hybrid	Reference
Initiation of anther development	Anther abortion	Satsuma mandarin × Sweet orange, etc.	Iwamasa, 1966
↓			
Archivesporial stage			
↓			
Resting stage	Degeneration of PMCs	Washington Navel Tahiti lime Lemon × Valencia, etc. Satsuma mandarin × Trifoliate orange	Osawa, 1912 Uphof, 1931 Frost, 1948 Iwamasa, 1966
↓			
Meiosis			
I-division	Asynapsis (genic) Asynapsis (by low temp.)	Mukaku Yuzu Eureka lemon Mexican lime	Iwamasa, 1966 Nakamura, 1943 Iwamasa and Iwasaki., 1962
↓			
	Translocation	<i>C. assamensis</i> Valencia orange	Naithani and Raghuvanshi, 1958 Iwamasa, 1966
↓			
	Inversion	<i>C. assamensis</i> , etc Mexican lime	Raghuvanshi, 1962 a Iwamasa, 1966
↓			
II-division	Failure of spindle	Marsh grapefruit	Raghuvanshi, 1962 b
↓			
Liberation from tetrad	Degeneration	Jaffa orange	Oppenheim and Frankel, 1929
↓			
Mitotic division	Degeneration	Satsuma mandarin	Nakamura, 1943
↓			
Mature pollen grain			

tion in satsuma mandarin (*C. unshiu*) hybrids is the strictest male sterility in citrus (Iwamasa, 1966). The sterile stamen appears only as the filament, and no pollen grains are produced (Fig. 1). Male sterility of ‘Washington’ navel (*C. sinensis*), ‘Tahiti’ lime (*C. latifolia* Tanaka), and some other hybrids is due to early degeneration of pollen mother cells (PMCs) (Osawa, 1912; Uphof, 1931; Frost, 1948; Iwamasa, 1966). Pollen sterility of satsuma mandarin (*C. unshiu*) is caused by plural sterility such as abnormal behavior and degeneration of pollen grains (Nakamura, 1943; Yang and Nakagawa, 1969, 1970).

To develop new seedless cultivars efficiently, genetic analysis of male sterility has been conducted. Among these studies, genetic analysis of anther abortion has progressed remarkably. This male sterility is due to genocyttoplasmic interaction [satsuma mandarin (*C. unshiu*), ‘Encore’ mandarin (*C. nobilis* Lour. × *C. deliciosa* Ten.), yuzu (*C. junos*), and lemon (*C. limon*) possess sterile cytoplasm] and is probably controlled by more than one major gene (Iwamasa, 1966; Yamamoto *et al.*, 1992 a, b, 1997; Nakano *et al.*, 2001; Dewi *et al.*, 2013 a). Dewi *et*

al. (2013 a) postulated that a dominant nuclear fertility-restoring gene system comprising one epistatic gene and two complementary genes controls the restoration of male



Fig. 1 - Flowers of male-sterile (aborted anthers, MS) and male-fertile (normal anthers, MF) citrus.

fertility and male-sterile anther size in citrus plants with sterile cytoplasm. Nakano *et al.* (2000) found DNA markers linked to aborted anther for juvenile screening of male-sterile plants. Male-sterile (aborted anther) progenies were also determined at the early period of seedling growth to exhibit precocious flowering, a phenomenon in which very young seedlings have flowers (Dewi *et al.*, 2013 b). Another type of male sterility, inheritance of pollen fertility/sterility, was also studied (Ueno, 1986). Some pollen-sterile progenies arose from two pollen fertile parents.

In citrus, not only somatic hybrids but also cybrids were produced by symmetric protoplast fusion (Saito *et al.*, 1993; Moriguchi *et al.*, 1996; 1997; Tokunaga *et al.*, 1999). Yamamoto and Kobayashi (1995) produced a cybrid having the sweet orange (*C. sinensis*) nuclear genome and satsuma mandarin (*C. unshiu*) cytoplasmic genome by fusion between satsuma mandarin protoplasts isolated from embryogenic callus and sweet orange mesophyll protoplasts. The cybrid is useful for seedless breeding because it has sterile cytoplasm derived from satsuma mandarin.

Several new seedless cultivars with male sterility were released in Japan (Nishiura *et al.*, 1983; Okudai *et al.*, 1991; Matsumoto *et al.*, 1991, 2003; Yoshida *et al.*, 2005 c) (Fig. 2, Table 2). All cultivars with aborted anther possess cytoplasm derived from satsuma mandarin (*C. unshiu*).

Female sterility

Female sterility is a very important trait which is closely related to seedlessness. Yamamoto *et al.* (1995) reported

that the degree of female fertility/sterility is rated on the basis of the average number of seeds per fruit obtained through hand pollination. A high positive correlation ($r = 0.93^{**}$) was found between the number of seeds of hand-pollinated fruits and that of open-pollinated fruit. This result indicated that female sterility is directly related to seediness. Female sterility estimated by the above-mentioned method was revealed to be a heritable characteristic (Yamamoto *et al.*, 2001).



Fig. 2 - Male-sterile seedless citrus 'Setoka'.

Table 2 - Seedless cultivars and parental lines of citrus released in Japan

	Cultivar or Parental line	Cross combination	Note
Male sterility	Kiyomi	Miyagawa wase (<i>Citrus unshiu</i>) × Trovita (<i>C. sinensis</i>)	
	Seiho	Kiyomi × Minneola (<i>C. paradisi</i> × <i>C. tangerina</i>)	
	Tsunokaori	Kiyomi × Okitsu wase (<i>C. unshiu</i>)	
	Amaka	Kiyomi × Encore (<i>C. nobilis</i> × <i>C. deliciosa</i>)	
	Setoka	(Kiyomi × Encore) No. 2 × Murcott (probably tangor)	
	Harehime	E647 (Kiyomi × Osceola) × Miyagawa wase (<i>C. unshiu</i>)	
	Tsunokagayaki	(Kiyomi × Okitsu wase) No. 14 × Encore (<i>C. nobilis</i> × <i>C. deliciosa</i>)	
Female sterility	Southern Yellow	Tanikawa Buntan (<i>C. maxima</i>) × Mukaku Kishu (<i>C. kinokuni</i>)	
	Citrus parental line Norin No. 5	Lee (Clementine × Orlando) × Mukaku Kishu (<i>C. kinokuni</i>)	
	Citrus parental line Norin No. 6	King mandarin (<i>C. nobilis</i>) × Mukaku Kishu (<i>C. kinokuni</i>)	
Self-incompatibility	Ariake	Seike navel (<i>C. sinensis</i>) × Clementine (<i>C. clementina</i>)	
Triploid	Puchimaru	Oval kumquat (<i>Fortunella margarita</i>) × tetraploid Meiwa kumquat (<i>C. crassifolia</i>)	
	White Love	Cybrid having lemon (<i>C. limon</i>) nuclear and sudachi (<i>C. sudachi</i>) cytoplasmic genome + Haploid clementine (<i>C. clementina</i>)	Somatic hybrid
	Tokushima 3X No. 1	Tetraploid sudachi HS4 (<i>C. sudachi</i>) × sudachi Ryokuko-kei (<i>C. sudachi</i>)	
	Yellow Bell	Open-pollinated seedling of diploid Michitani-line Villafranca (<i>C. limon</i>)	

'Mukaku Kishu', a bud variant of the seedy kinokuni mandarin (*Citrus kinokuni* hort. ex Tanaka), is completely seedless and considered to have the strictest female sterility in citrus. Yamasaki *et al.* (2007, 2009) studied the mechanism of expression of seedlessness derived from 'Mukaku Kishu'. In fruits of 'Mukaku Kishu', specific very small and swollen seeds called "type A seeds" were observed. The expression of 'Mukaku Kishu'-type seedlessness is characterized by formation of "type A seed" with an immature seed coat and an embryo arrested at an early stage. However, arrested embryo development in the "type A seed" is not caused by endosperm abortion. This female sterility is controlled by two major genes: sterility and fertility are dominant and recessive, respectively (Nesumi *et al.*, 2001). New seedless cultivar and parental lines with this sterility were bred in Japan (Yoshida *et al.*, 2005 a, b) (Fig. 3, Table 2).

Navel orange (*C. sinensis*) and satsuma mandarin (*C. unshiu*) have strong female sterility; only a few seeds were developed when they were hand-pollinated (Miki, 1921; Nagai and Tanikawa, 1926; Nishiura and Iwasaki, 1963; Yamamoto *et al.*, 1995). Osawa (1912) observed degeneration of the embryo sac in both navel orange and satsuma mandarin. Nesumi *et al.* (2000) assumed that the female sterility of satsuma mandarin is controlled by two major genes: sterility and fertility are recessive and dominant, respectively, and they were mapped on a linkage map (Omura *et al.*, 2000).

Self-incompatibility

Self-incompatibility is a genetically controlled phenomenon preventing seed set in self-pollinated plants producing functional gametes. Self-incompatibility in citrus is a very important trait for fruit production. Without parthenocarpy, it requires cross pollination to achieve stable fruit production (Nagai and Tanikawa, 1926; Miwa, 1951). However, its coupling with parthenocarpy could produce seedless fruit (Iwamasa and Oba, 1980; Yamamoto *et al.*, 1995; Yamamoto and Tominaga, 2002). Thus, much research has been conducted to determine self-incompatibility of many accessions (Nagai and Tanikawa, 1926; Miwa, 1951; Nishiura and Iwasaki, 1963; Iwamasa and Oba, 1980; Yamamoto and Tominaga, 2002; Yamamoto *et al.*, 2006, 2012).

The incompatibility system of citrus is of the gametophytic type and Soost (1965, 1969) proposed *S* (self-incompatibility) genotypes of some accessions. Since then,

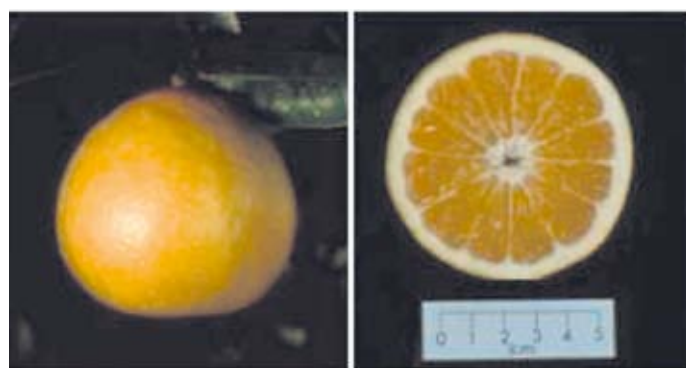


Fig. 3 - Female-sterile seedless citrus 'Southern Yellow'.

the source of *S* genotypes has been less well elucidated. Recently, however, research has progressed remarkably *via* certain methods. Pollen tube growth was strongly inhibited in incompatible pollination; those pollen tubes exhibited abnormal behaviors, namely twisted and heavy and irregular callose deposition. On the other hand, in compatible pollination, many normal pollen tubes penetrated into the style (Ngo *et al.*, 2001). Self-incompatibility *S* genotypes of several citrus cultivars were estimated by the observation of pollen tube behavior in the styles after controlled pollination with a restricted number of pollen grains on their stigmas (Ngo *et al.*, 2010). Cross-incompatible, cross-semi-compatible, and cross-full-compatible relationships were clarified based on the results of the number of pollen tubes that reached the base of the style. From these results, *S* genotypes of several cultivars were estimated. The *S* genotype could be estimated with the aid of allozymes produced by the glutamate oxaloacetate transaminase isozyme gene (*Got-3*), which appeared to be linked to the *S* gene (Ngo *et al.*, 2011). The most efficient way to determine the *S* genotype is considered to be pollination with pollen homozygous for the *S* genotype. Kim *et al.* (2010, 2011) revealed the *S* genotype of some cultivars by pollination of homozygous *S*₁ seedlings of 'Hirado buntan' [*C. maxima* (burm.) Merr.] and 'Banpeiyu' (*C. maxima*). There are no differences in estimated *S* genotypes among the three above-mentioned methods. Table 3 shows the estimated *S* genotypes of several accessions.

Incompatibility *S* alleles are distributed widely, not only in self-incompatible accessions but also self-compatible ones such as satsuma mandarin (*C. unshiu*), grapefruit (*C. paradisi*), and 'Dancy' (*C. tangerina* hort. ex Tanaka) (Soost, 1965, 1969; Vardi *et al.*, 2000). Thus, self-incompatible individuals can be produced from cross combinations between two self-

Table 3 - Estimated *S* genotype of citrus accessions (Kim *et al.*, 2011; Ngo *et al.*, 2010, 2011)

Accession	Latin name	Estimated <i>S</i> genotype ⁽²⁾
Banpeiyu	<i>Citrus maxima</i> (Burm.) Merr.	<i>S</i> ₁ <i>S</i> ₂
Tosa Buntan	<i>C. maxima</i> (Burm.) Merr.	<i>S</i> ₁ <i>S</i> ₃
Iriki Buntan	<i>C. maxima</i> (Burm.) Merr.	<i>S</i> ₁ <i>S</i> ₂
Kaopang	<i>C. maxima</i> (Burm.) Merr.	<i>S</i> ₁ <i>S</i> ₂
Soyu	<i>C. maxima</i> (Burm.) Merr.	<i>S</i> ₁ <i>S</i> ₂
Hassaku	<i>C. hassaku</i> hort. ex Tanaka	<i>S</i> ₄ <i>S</i> ₅
Yuge-hyokan	<i>C. yuge-hyohan</i> hort. ex Yu. Tanaka	<i>S</i> ₆ <i>S</i> ₇
Shishiyuzu	<i>C. pseudogulgul</i> hort. ex Shirai	<i>S</i> ₁ <i>S</i> ₆
Hyuganatsu	<i>C. tamurana</i> hort. ex Tanaka	<i>S</i> ₁ <i>S</i> ₈
Tachibana No. 1	<i>C. tachibana</i> (Makino) Tanaka	<i>SfS</i> ₈
Rough lemon	<i>C. jambhiri</i> Lush.	<i>SfS</i> ₁
Zadaida	<i>C. aurantium</i> L.	<i>SfS</i> ₁
Kinukawa	<i>C. glaberima</i> hort. ex Tanaka	<i>SfS</i> ₂
Kawano		
Natsudaidai	<i>C. natsudaidai</i> hort. ex Tanaka	<i>SfS</i> ₂

⁽²⁾ Sf: self-compatible.

compatible parents, for example, 'Orlando' and 'Minneola' arose from 'Duncan' grapefruit and 'Dancy' combination (Swingle *et al.*, 1931). In Japan as well, self-incompatible seedless 'Ariake' was bred by crossing self-compatible 'Seike' navel orange (*C. sinensis* (L.) Osbeck) and self-incompatible clementine (*C. clementina* hort. Tanaka) (Yamada *et al.*, 1995; Yamamoto *et al.*, 2006) (Fig. 4, Table 2).



Fig. 4 - Self-incompatible seedless citrus 'Ariake'.

3. Ploidy manipulation for seedless cultivar breeding

Although spontaneous triploid ($3x=27$) accessions were very rare in citrus (Krug, 1943; Krug and Bacchi, 1943; Noro and Kajimoto, 1955), many triploid hybrids have been produced by artificial hybridization. Since these triploids are seedless, it could be considered that producing triploids is a useful way to promote seedless breeding in citrus efficiently. Tachikawa *et al.* (1961) conducted one of the earliest programmed triploid breeding projects. First, they produced tetraploid materials ($4x=36$) by colchicine treatment and then triploid ($2x=18$) hybrids were obtained from these tetraploid and diploid cross combinations. As they showed, since triploids arise from tetraploid and diploid crossing, tetraploid plants are important for triploid breeding. Therefore, various tetraploid accessions were obtained by certain methods.

Oiyama *et al.* (1980) selected spontaneous autotetraploids from nucellar seedlings of polyembryonic cultivars. They revealed the leaf morphological characteristics of tetraploids: thick and broad leaves and reduced number of stomata per area. Kawase *et al.* (2005) obtained Meiwa kumquat (*Fortunella crassifolia* Swingle) autotetraploid from 500 seedlings. An autotetraploid also arose as a bud sport (Yamao *et al.*, 1993). Colchicine treatment is useful for the production of autotetraploids in many higher plants. In citrus in particular, various tetraploids were produced by this treatment. Colchicine treatment of seeds was effective in polyembryonic cultivars (Yahata *et al.*, 2004). However, this treatment of monoembryonic seeds is a problem because the tetraploids obtained by this treatment are not true-to-type. Oiyama and Okudai (1986) resolved this problem through a

combination of colchicine treatment of isolated small buds and their micrografting. They successfully produced autotetraploids from three monoembryonic cultivars. Moreover, in another eight autotetraploids, monoembryonic citrus was produced using the same method (Kaneyoshi *et al.*, 2008).

It has been revealed that although tetraploids arise from both diploid \times tetraploid and tetraploid \times diploid crosses, the latter combination is more effective (Cameron and Burnett, 1978; Kaneyoshi *et al.*, 2008) and an unbalanced ploidy ratio between embryo and endosperm is considered to cause this phenomenon (Esen and Soost, 1973). Thus, artificially produced monoembryonic autotetraploids were important as seed parents for triploid breeding. On the other hand, triploid hybrids sometimes appeared from diploid-diploid crosses (Esen and Soost, 1971; Oiyama and Okudai, 1983; Yasuda *et al.*, 2010); the appearance of triploids is due to the unreduced gametophyte of one parent (Esen and Soost, 1971).

Biotechnological methods such as protoplast fusion have contributed to the progress of citrus triploid breeding. Since somatic hybrids are tetraploids in general (Ohgawara *et al.*, 1985), they are important parents for triploid breeding. Somatic hybrids derived from crosses between navel orange (*C. sinensis*) + satsuma mandarin (*C. unshiu*), grapefruit (*C. paradisi*), yuzu (*C. junos*) and 'Murcott' (artificial hybrid) were registered as parental lines (Kobayashi *et al.*, 1995). Triploid hybrids could be produced directly by means of protoplast fusion. Somatic hybrids produced from diploid and haploid fusion became triploids (Kobayashi *et al.*, 1997). The haploid parents were obtained by diploid \times triploid cross (Oiyama and Kobayashi, 1993).

Table 2 shows the triploid cultivars released in Japan. Among the four cultivars, 'Puchimaru' (Yoshida *et al.*, 2003) (Fig. 5), 'White love', Tokushima 3X No. 1' (Toku-

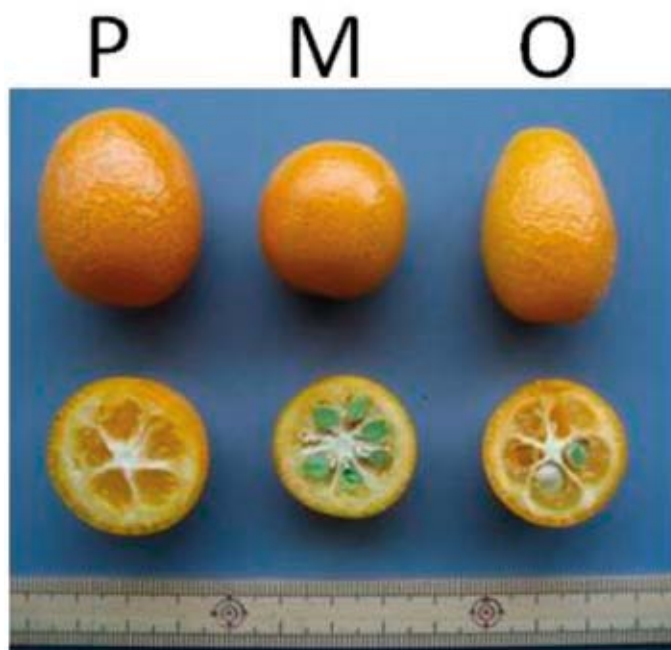


Fig. 5 - Triploid seedless kumquat 'Puchimaru' and diploid seedy Meiwa kumquat and Oval kumquat. P= Puchimaru, M= Meiwa kumquat, and O= Oval kumquat.

naga *et al.*, 2005), and ‘Yellow Bell’ (Kaneyoshi *et al.*, 2014) were derived from diploid × tetraploid cross, protoplast fusion between diploid and haploid, tetraploid × diploid cross, and diploid × diploid cross, respectively.

4. Conclusions

Seedless breeding of citrus has progressed rapidly in Japan over the last few decades. The production of new seedless cultivars is increasing in contrast to the decrease in production of seedy conventional cultivars such as ‘Kawano Natsudaikai’ (*C. natusdaikai*) and ‘Miyauchi Iyokan’ (*C. iyo*). Various kinds of cross combinations using cultivars or parental lines with sterility have been conducted actively and further polyploid breeding is being carried out to produce new triploid plants. In addition, methods to shorten the long juvenile period of citrus have developed in Japan (Okudai *et al.*, 1980; Mitani *et al.*, 2008), making it easier to breed, compared to a few decades ago, various types of new seedless cultivars.

Understanding the mechanism and hereditary mode of each type of sterility will contribute to produce seedless individuals efficiently and effectively. Biotechnological techniques are also very useful for producing seedless materials. The results of genome analysis have provided useful information for breeding new seedless citrus with female sterility (Garcia *et al.*, 2000). Moreover, owing to the progress of recent DNA analysis technologies, the draft whole genome of sweet orange (*C. sinensis*) has already been reported (Xu *et al.*, 2013). The combination of conventional breeding study, biotechnology, and genome analysis is considered to be essential to breed new superior seedless cultivars in citrus.

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