Frequent Occurrence of Precocious Flowering in Zygotic Seedlings Derived from Crosses with a Monoembryonic and Male Sterile Acid Citrus Hybrid between Yuzu (C. junos Sieb. ex Tanaka) and Hanayu (C.hanaju Hort.exShirai)

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We successfully produced a male sterile and monoembryonic acid citrus hybrid plant that generated seedlings showing precocious flowering with high rates. Of 26 crosses with 16 acid citrus cultivars, three ('Sudachi'×'Yuzu', 'Hanayu'×'Kabosu' and 'Hanayu'×'Yuzu') were firstly found to generate precociously flowered seedlings with low rates. Second, 23 hybrid seedlings with 'Hanayu', 'Kabosu' and 'Sudachi' in their parents were crossed with 'Hanayu', 'Kabosu' and 'Yuzu', and then one cross (HY–16×'Yuzu') was found to generate precocious seedlings with a high rate of 6%. When the hybrid plant HY–16 derived from 'Hanayu'×'Yuzu' was crossed with 'Yuzu' in different years, it constantly generated seedlings showing precocious flowering with a rate of about 6%, but it did not generate them when it was crossed with 'Hanayu'. When HY–16 was crossed with grapefruit cultivars, it generated seedlings exhibiting precocious flowering with very high rates of about 15%. The seedlings derived from 'Hanayu'×'Yuzu' and selfing of 'Hanayu' and 'Yuzu' generated seedlings showing precocious flowering with low rates of about 1%. HY–16 was a male sterile and monoembryonic plant with fruitful and seedy characters. The precociously flowered seedlings derived from crosses with HY–16 developed 6–33 leaves until flower bud formation that occurred between February to August on the shoot tip. Three–fifths of the seedlings for HY–16×'Yuzu' and one–second for HY–16×grapefruit were male sterile. These results suggested that HY–16 accumulated genes related to precocious flowering and that this hybrid was useful for rapid genetic analysis of various characters such as male sterility in *Citrus*.

INTRODUCTION

The very long juvenility from seed to flowering is one of major barriers to develop in citrus breeding (Soost and Cameron, 1975; Soost, 1988). *Citrus* seedlings with very short juvenility are of great interest for breeders and geneticists to shorten the generation cycle during breeding and genetic analysis. However, induction of flower bud initiation in juvenile *Citrus* seedlings has so far been very limited (Soost, 1988). The most effective method to shorten the citrus juvenile phase is top working of juvenile seedlings on adult

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trees and training of the grafted seedlings on horizontal trellis established in citrus orchard. However, this method is not able to shorten juvenility of seedlings beyond three to five years.

The juvenile phase of citrus seedlings was different in different species and cultivars, i.e., three years for lime, five for lemon and more than ten for pummelo and its hybrids such as tangelo (Hackett, 1985; Soost, 1988). So far as acid citrus concerned, most of their zygotic seedlings, except for those of lime and lemon, have very long juvenile phases more than ten years. The juvenility of most of these seedlings was shortened to four to eight years when they were top–grafted on the branches of adult satsuma trees. In this case, however, the effect of top–grafting on the shortening of juvenile phase was different for different hybrid plants and a large number of seedlings did not flower up to eight years after seed germination (unpublished). Among these seedlings, it was found that only a few of them showed precocious flowering. Unlike the short juvenile plants found among trifoliate orange (*Poncirus trifoliata* Raf.) seedlings reported by Yadav *et al.*, (1980), they never flowered after second year as reported in seedlings of grapefruit (Furr and Reece, 1947; Iwamasa and Oba, 1973; Davenport, 1990; Hield *et al.*, 1996).

Although most acid citrus cultivars are moderately or highly polyembryonic, some acid citrus cultivars such as 'Yuzu', 'Hanayu' and 'Lisbon' lemon show very low degree of polyembryony and produce monoembryonic seeds and zygotic seedlings with high frequencies (Wakana *et al.*, 2005). Therefore, they are useful as seed parents for the production of zygotic seedlings of *Citrus* cultivars. In addition, we found that almost all polyembryonic acid citrus cultivars were heterozygous for alleles governing polyembryony. Hence, these polyembryonic cultivars are expected to generate monoembryonic hybrid seedlings that generate zygotic seedlings with high ability of precocious flowering.

The aim of this study is (1) to detect acid citrus cultivars generating zygotic seedlings with the ability of precocious flowering, (2) to choose the monoembryonic and zygotic seedlings generating seedlings with high ability of precocious flowering and (3) to ascertain the usefulness of the selected seedlings for genetic analysis of some characters such as male sterility.

MATERIALS AND METHODS

Plant material

Thirteen acid *Citrus* cultivars (2n=2x=18), 'Yuzu' (*C. junos* Sibb. *ex* Tanaka), 'Kizu'(*C. kizu* hort. *ex* Y. Tanaka),'Hanayu' (*C. hanaju* hort. *ex* Shirai), 'Kabosu' (*C. sphaerocarpa* Tanaka)), 'Eureka' and 'Lisbon' lemons (*C. limon* Burm. f.), 'Kabusu' sour orange (*C. aurantium* Linn.), 'Chinotto' (*C. myrtifolia* Rafin.), 'Bouquet des Fleurs' (*C. aurantium* Linn. var. *crispa* Y. Tanaka), 'Kusaie lime' (*C. limonia* Osbeck), 'Etrog' citron (*C. medica* Linn.) 'Balotin' bergamot (*C. balotina* Poit. *et* Turp.), 'Mexican lime (*C. aurantifolia* Swingle) and a tetraploid form of diploid 'Hanayu' (Wakana *et al.*, 2005) were used as plant materials to produce zygotic seedlings. The thirteen cultivars were about 25–year–old trees grown at Sasaguri orchard of the University Farm, Kyushu University, Fukuoka.

Crosses were carried out for 26 cross combinations in 1989 and 1990. The seeds derived from the crosses were collected at November and divided into monoembryonic and polyembryonic ones after the removal of the seed coats, and then embryos of monoembryonic seeds and a large embryo showing different color from the other embryos within each polyembryonic seed were placed on wet filter paper at 25 °C. After germination of the embryos, they were planted and carried to a greenhouse by early April of the next year. The minimum temperature of the greenhouse was controlled at 10 °C during winter conditions. The hybridity of these seedlings was determined either by their different morphology of leaves, stems and spines from that of nucellar or by GOT isozyme analysis (Wakana *et al.*, 1998). The seedlings determined as hybrids in the crosses were top-grafted on the branches of adult 'Miyagawa-wase Unshiu' satsuma mandarin trees grown at the Sasaguri orchard of Kyushu University Farm. Each hybrid plant name was given according to the abbreviation of cross and plant number, e.g., HY-16 means a sixteenth zygotic seedling derived from 'Hanayu'×'Yuzu'.

To produce zygotic seedlings showing precocious flowering with high frequency, further crossings with 23 of these hybrid seedlings were carried out in 2002 according to the procedure mentioned above. In fourteen of the 23 cross combinations, 'Hanayu' was used as a seed parent, while in the remaining nine crosses with monoembryonic hybrid seedlings, 'Hanayu', 'Kabosu' and 'Yuzu' were used as pollen parents. The zygotic seedlings derived were planted and carried to a greenhouse of which the minimum temperature was controlled at 10 °C during winter. Number of seedlings showing precocious flowering in each cross was scored.

To certify the ability of the seedling showing precocious flowering, it was crossed with 'Hanayu', 'Yuzu', 'Marsh' and 'Foster Pink' grapefruit in 2003 and 2004. The zygotic seedlings were grown under the conditions mentioned above. Number of seedlings showing precocious flowering in each cross was scored and time of precocious flowering, morphology of the flowers and number of the leaves in the seedlings were also scored.

RESULT

Segregation of seedlings showing precocious flowering

Of the 26 crosses between acid citrus cultivars, three crosses, 'Sudachi'×'Yuzu', 'Hanayu'×'Kabosu' and 'Hanayu'×'Yuzu', generated each only one hybrid seedling showing precocious flowering in the greenhouse (Table 1). This result suggested that 'Yuzu', 'Hanayu', 'Kabosu' and 'Sudachi' have genes related to precocious flowering of their zygotic seedlings.

In 23 back crosses with the hybrid seedlings of these four cultivars, only one back cross (HxY-16×'Yuzu') generated seven precociously flowered seedlings with a relatively high rate of 6.0% (Table 2). The hybrid seedling HxY-16 was a male sterile and monoembryonic plant.

The HY-16×'Yuzu' cross constantly generated seedlings exhibiting precocious flowering with the rates of about 6% in 2004 and 2005 (Table 3) as observed in 2003. However, back cross of HY-16 with 'Hanayu' did not generate precociously flowered seedlings. When HY-16 was pollinated with 'Marsh' and 'Foster Pink' grapefruits, it generated precociously flowered seedlings with high rates of 15.2% and 15.8% respectively. On the other hand, only one zygotic seedling exhibiting precocious flowering was generated from each of the 'Hanayu'×'Hanayu', 'Hanayu'×'Yuzu' and 'Yuzu'×'Yuzu'

Table 1. The occurrence of precocious flowering among three– to five–year–old zygotic seedlings of acid citrus cultivars, 1989 and 1990.

Cross combination	No. of zygotic seedlings	
	Examined	Precocious (%)
Sudachi×Kabosu	35	0
Sudachi×Yuzu	36	1 (2.8)
Sudachi×Lisbon lemon	15	0
Kabusu×Lisbon lemon	34	0
Hanayu×Kabosu	88	1 (1.1)
Hanayu×Chinotto	30	0
Hanayu×Sudachi	34	0
Hanayu×Bouquet	4	0
Hanayu×Yuzu	80	1 (1.3)
Yuzu×Kabosu	71	0
Yuzu×Kizu	17	0
Yuzu×Kusaie lime	45	0
Yuzu×Etrog citron	10	0
Yuzu×Kabusu	71	0
Yuzu×Balotin bergamot	8	0
Yuzu×Yuzu	24	0
Yuzu×Lisbon lemon	6	0
Eureka lemon×Yuzu	17	0
Mexican lime×Kabosu	6	0
Lisbon lemon×Kabosu	124	0
Lisbon lemon×Chinotto	64	0
Lisbon lemon×Kizu	51	0
Lisbon lemon×Kabusu	54	0
Lisbon lemon×Bouquet	4	0
Lisbon lemon×Yuko	16	0
Lisbon lemon×Yuzu	78	0

crosses, whereas precocious flowering was not observed in the hybrid seedlings generated from 'Hanayu' 4x×'Hanayu' 4x and 'Yuzu'×'Hanayu' crosses.

Morphology of seedlings showing precocious flowering

Flower bud initiation occurred only at the shoot apex of seedlings. The calyxes of flowers of most precocious seedlings were imperfect and abnormal. The petals were also imperfect and abnormal, i.e., most of the flowers consisted of three to five irregular–shaped petals. In the flowers, degenerated pistils were rarely observed, but in almost all flowers pistils appeared to be normal. Flower–bud abscission before anthesis was observed at three precocious seedlings derived from HY–16×'Yuzu' and HY–16× 'Foster Pink' grapefruit (Table 3).

The anthers of flowers of precocious seedlings from 'Hanayu'×'Hanayu', 'Hanayu'× 'Yuzu', 'Yuzu'×'Yuzu', 'Hanayu'×'Kabosu' and 'Sudachi'×'Yuzu' were yellow and produced normal pollen, whereas those from HY–16×'Yuzu', HY–16×'Mash' and HY–16× 'Foster Pink' were either white or yellow (Fig. 1). The yellow–colored anthers produced much pollen but no pollen was produced from the white–colored anthers. Among the

Table 2. Precocious flowering in three– to five–month–old hybrid seedlings derived from back crosses with 'Hanayu', 'Kabosu' and 'Yuzu' 2001and 2002.

Cross[a]	Degree of nucellar embryony in seed parent	No. of zyotic seedlings examined	No. of seedlings showing precocious flowering (%)
Hanayu×HxL–1	low	50	0
Hanayu×HxL–32	low	76	0
Hanayu×HxL–41	low	38	0
Hanayu×HxL–35	low	14	0
Hanayu×HxS–15	low	67	0
Hanayu×HxS–27	low	58	0
Hanayu×HxC–17	low	39	0
Hanayu×HxK–31	low	44	0
Hanayu×HxK–35	low	65	0
Hanayu×HxK–53	low	52	0
Hanayu×HxK–57	low	84	0
Hanayu×HxY–6	low	9	0
Hanayu×HxY–13	low	17	0
Hanayu×HxY–27	low	45	0
HxL–28×Hanayu	monoembryonic	161	0
HxL–12×Hanayu	monoembryonic	67	0
HxL–10×Hanayu	monoembryonic	39	0
HxK–14×Hanayu	monoembryonic	72	0
HxK–14×Kabosu	monoembryonic	88	0
HxY–16×Hanayu	monoembryonic	15	0
HxY–16×Yuzu	monoembryonic	117	7 (6.0)
HxC–11×Hanayu	monoembryonic	132	0
LxK–28×Hanayu	monoembryonic	109	0

[a] H: Hanayu, L: Lisbon lemon, S: Sudachi, C: Chinotto, K: Kabosu, Y: Yuzu.

Table 3. Segregation of zygotic seedlings showing male sterility in three– to eight–year–old zygotic seedlings derived from crosses with acid citrus cultivars.

Cross combination	Year	No. of zygotic seedlings				
			Precociously flowered			
		Examined	Male fertile	Male sterile	Flower–bud abscission	Total (%)
HY–16[a]×Hanayu	2004	338	0	0	0	0 (0)
HY–16×Yuzu	2004	555	23	15	0	38 (6.8)
HY–16×Yuzu	2005	293	12	4	1	17 (5.8)
HY–16×Marsh	2005	79	7	4	1	12 (15.2)
HY–16×Foster Pink	2005	247	18	19	2	39 (15.8)
Hanayu×Hanayu	2005	121	0	1	0	1 (0.8)
Hanayu 4x×Hanayu 4x	2005	97	0	0	0	0 (0)
Hanayu×Yuzu	2005	200	0	1	0	1 (0.5)
Yuzu×Yuzu	2005	85	0	1	0	1 (1.2)
Yuzu×Hanayu	2005	68	0	0	0	0 (0)

[a] HY–16: a 15–year–old zygotic seedling derived from 'Hanayu'×'Yuzu'.

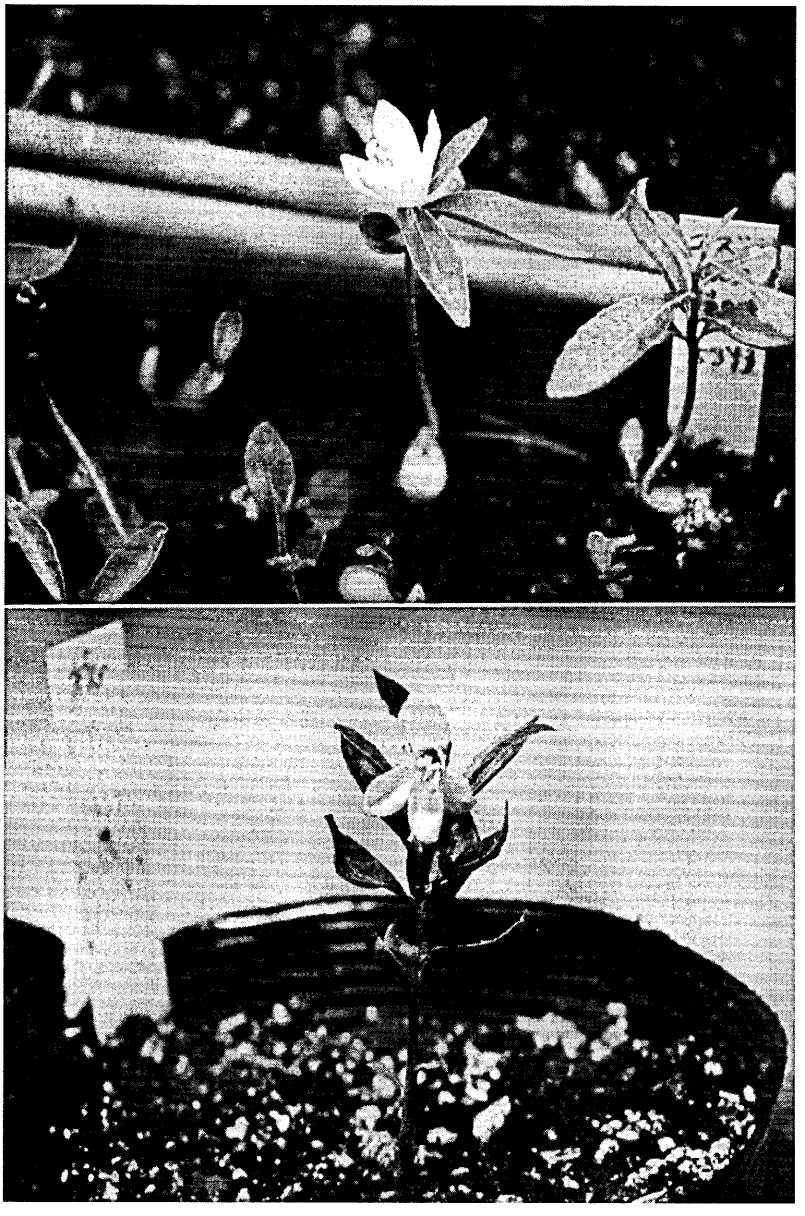

Fig. 1. Precocious flowering in a three–month–old zygotic seedling derived from self–pollination of 'Yuzu' (upper) and a five–month–old zygotic seedling obtained from HY–16×'Foster Pink' grapefruit (lower).

Table 4. Precocious flowering time in three– to seven–month–old seedlings derived from crosses with 'Hanayu', 'Yuzu', 'Marsh' grapefruit' 'Foster Pink' grapefruit and a hybrid between 'Hanayu' and 'Yuzu' (HY16), 2005.

Cross	No. of precocious seedlings flowering in indicated time[a]																			
	February		March			April			May			June			July			August		
	M	L	E	M	L	E	M	L	E	M	L	E	M	L	E	M	L	E	M	L
HY–16×Yuzu	0	0	0	0	4	2	1	5	1	0	1	1	0	0	1	1	0	0	0	0
HY16×Marsh	0	0	0	0	0	0	5	4	2	0	0	0	0	0	0	0	0	0	0	0
HY16×Foster Pink	0	0	0	0	0	7	9	8	4	2	2	0	2	1	0	0	1	0	1	0
Hanayu×Hanayu	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Hanayu×Yuzu	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Yuzu×Yuzu	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	0	1	0	0	4	10	15	18	7	2	3	1	2	1	1	2	1	0	1	0

[a] E: early; M. middle; L: late.

Table 5. Number of leaves in precociously flowered seedlings, 2005.

	No. of precocious seedlings with indicated No. of leaves at anthesis													
	6	7	8	9	10	11	12	13	14	15	23	24	26	33
HY–16×Yuzu	0	0	4	2	3	3	3	0	0	0	1	0	0	0
HY16×Marsh	2	0	0	1	3	0	2	2	1	0	0	0	0	0
HY16×Foster Pink	3	3	1	5	9	3	4	2	4	2	0	1	1	1
Hanayu×Hanayu	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Hanayu×Yuzu	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Yuzu×Yuzu	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	7	3	5	8	15	6	10	4	5	2	1	1	1	1

precocious seedlings, a few with pale–yellow–colored anthers that did not release pollen were also found, but were included in male fertile seedlings in Table 3.

The first precocious flowering was observed in the seedling from Yuzu×Yuzu in late February. The maximum flowering time was April in which 43 seedlings flowered, and then number of seedlings flowering was gradually decreased by late July (Table 4). In a hybrid seedling derived from HY–16×'Foster Pink' grapefruit, first precocious flowering occurred on a primary shoot at 13 April, and second precocious flowering occurred on a secondary shoot at 18 July, 2005.

Number of developed leaves in the precocious seedlings with one primary flower ranged from 6 to 33 (Table 5). In this case, there was an obvious tendency that the later the flowering time, the more the number of leaves increased.

DISCUSSION

Iwamasa and Oba (1975) observed precocious flowering in some seedlings of grapefruit (*C. paradisi* Macf.), 'Tengu' (*C. tengu* Tanaka), 'Banokan' (*C. grandis* var. *banokan* Tanaka), three tangelos (*C. paradisi*×*C. reticulata*) and a hybrid between satsuma mandarin (*C. unshiu* Marc.) and pummelo (*C. grandis* L.), but no precocious flowering was observed in 17 other taxa. Thus, it was believed that precocious flowering appears to occur mainly in grapefruit and grapefruit hybrids or pummelo hybrids (Soost, 1988). However, the present result of crosses with 13 acid citrus cultivars (species) indicates that 'Yuzu', 'Hanayu', 'Sudachi' and 'Kabosu' have genes (alleles) related to precocious flowering in their hybrid seedlings, since each of the six crosses, 'Sudachi'× 'Yuzu', 'Hanayu'×'Kabosu', 'Hanayu'×'Yuzu', 'Hanayu'×'Hanayu', 'Hanayu'×'Yuzu' and 'Yuzu'×'Yuzu', generated one hybrid seedling showing precocious flowering. The four cultivars (species) are the most important acid citrus in Japan. Hence, these findings may help rapid progress in acid citrus breeding and genetics.

The stability of appearance rates of precocious zygotic seedlings in each of the crosses with HY–16 in different years suggests that precocious flowering is a genetic character. The low appearance rates of precocious seedlings in the progenies from the six crosses suggest that the four cultivars do not accumulate so many genes that they might generate a large number of precocious hybrid seedlings. It is also suggested that 'Yuzu' accumulated more genes (alleles) associated with precocious flowering than 'Hanayu', since when HY–16 was pollinated with them the hybrid seedlings with genes of 'Hanayu' did not show precocious flowering. Hence, the production of monoembryonic hybrid plants that generate precocious hybrid seedlings with high frequencies is required for the rapid progress in the acid citrus breeding and genetic analysis. The monoembryonic hybrid plant HY–16 produced in this study is satisfactory to us to carry out the breeding and analysis.

Furr and Reece (1947) firstly reported the appearance of seedlings showing precocious flowering in grapefruit. Holland *et al.* (1995) investigated precocious flowering of grapefruit seedlings grown *in vitro* and reported that about 30–40% of the seedlings showed precocious flowering. The dramatic increase and the stability in the rates of precocious hybrid seedlings from HY–16 crossed with two grapefruit cultivars indicates that they have accumulated multiple alleles controlling precocious flowering in their progenies. Although the instability of morphological and cytological characters indicated by Soost (1988) was often observed in the calyxes and petals, and sometimes in the ovaries and stamens of precocious flowers, the anthers were clearly divided into two categories fertile (yellow) and sterile (white), except for one or two flowers in each HY–16 cross wherein intermediate fertility (anther color: pale–yellow) was observed. This suggests that male sterility in HY–16 inherited and segregated in the progenies of each cross. Since HY–16 is monoembryonic and male sterile, it will be highly useful not only for precocious flowering study but also for genetic analysis of male sterility in *Citrus*.

Holland *et al.* (1995) found with an *in vitro* technique that precocious flowering in grapefruit seedlings occurred irrespective of the presence of their cotyledons, *i.e.*, the leaves of the seedlings are responsible for the precocious flowering. Our study suggests that differentiation of more than six leaves is necessary for the flower bud initiation in precocious *Citrus* seedlings, and that the maximum number of leaves necessary for the precocious flowering was 33. However, further experiments will be necessary for these hybrid seedlings to certify the range of leaf numbers responsible for the flower bud initiation.

Effect of low temperature on flower bud initiation of adult citrus trees in some cultivars had been demonstrated (Garcia–Luis *et al.*, 1922; Poerwanto and Inoue, 1990). Iwamasa and Oba (1975) reported that precocious flowering of grapefruit seedlings only occurred in those grown at temperatures between 20°C and 10°C, from November to March in Japan. Holland *et al.* (1995) investigating with grapefruit seedlings reported that in those grown at constant temperature of 26°C in a greenhouse none or 2% of the plants showed precocious flowering. Based on these results, Holland *et al.* (1995) suggested that the inductive conditions required for mature and precocious flowering are similar. Our results of precocious flowering in the zygotic seedlings from various crosses almost support this suggestion. However, the occurrence of precocious flowering in July and August suggests that this is not a rule, since the minimum outdoor temperatures of June were about 20°C and Maximum ranged from 24 to 34°C in 2005. Under tropical or subtropical temperature conditions, flower bud initiation occurs in mature citrus trees. Even in Japanese temperate conditions, second flowering occurs on summer shoots followed by first flowering on spring shoots in some citrus trees such as 'Kizu', suggesting that cold conditions are not essential for flower bud initiation. The difference in the reaction of flower bud initiation between citrus cultivars and between zygotic seedlings may be due to their genetic constitution. To ascertain this hypothesis, it is necessary to produce monoembryonic hybrid plants that generate zygotic seedlings showing precocious flowering with high rates.

In addition to the ability to generate precocious seedlings with high frequencies, HY–16 established in this study has superior characters such as monoembryony available for genetic analysis and male sterility useful for seedless cultivar breeding. Nakano *et al.* (2000) proposed three genes that control male sterility in the progenies of 'Kiyomi' (*C. unshiu*×*C. sinensis*), but the gene system was not satisfactory. Since male sterile progenies segregated in many crosses with acid citrus cultivars, use of HY–16 as a seed parent for crossings with these cultivars may contribute to the resolution of male sterility inheritance and breeding of seedless acid cultivars, as well as physiological and genetic researches of precocious flowering in *Citrus*.

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