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Intergeneric hybridization of marguerite (*Argyranthemum frutescens*) with annual chrysanthemum (*Glebionis carinatum*) and crown daisy (*G. coronaria*) using ovule culture

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Abstract To diversify flower color and growth habit of marguerite (*Argyranthemum frutescens*), intergeneric crossing was carried out using marguerite as the seed parent and annual chrysanthemum (*Glebionis carinatum*) or crown daisy (*G. coronaria*) as the pollen parent. After cross-pollination, seedlings were successfully obtained by applying ovule culture. Ovule culture-derived plants showed novel characteristics in flower shape and color (orange, reddish brown, or wisteria pink) that are not observed in marguerite. Some also showed novel flowering habits such as perpetual flowering. The results indicate that these ovule culture-derived plants were intergeneric hybrids and that the hybrids obtained in the present study may be useful for further breeding of marguerite, especially for introducing valuable characteristics such as a wide range of flower color.

Key words: *Argyranthemum*, *Glebionis*, intergeneric hybridization, ovule culture.

Marguerite (*Argyranthemum frutescens*) is a perennial plant native to the Canary Islands, Spain (Bramwell et al. 2001) and Madeira, Portugal (Press et al. 1994). However, horticulturally, it has been treated as an annual plant in Japan. Marguerite seems to have been introduced into Japan in the 1860s or 1870s (Kitamura et al. 1988). The introduced strain was 'Zairai Shiro', which is still cultivated as a cut flower in Japan.

Although several cultivars have been introduced since then, the white flower was mostly used in Japan because demand for this color as a cut flower was high. However, a gardening boom occurred in Japan after the Osaka Flower Expo in 1990, influenced by the usage of ornamental plants in European flowerbeds, and a number of marguerite cultivars with various characteristics have since been introduced. With this drastic change in market demand, breeding efforts have been initiated on this crop to produce novel cultivars that suit the Japanese climate.

Wide hybridization, such as interspecific and intergeneric hybridization, has efficiently been used with the aid of embryo rescue techniques such as embryo and ovule culture to breed novel cultivars (Sharma et al. 1996), and should be a useful strategy to produce novel marguerite cultivars. The 23 (Yokoi 2003) or 24 (Bremer et al. 1994) wild species recorded in the genus *Argyranthemum* might be considered as the available germplasm for the breeding of marguerite, but most of them have white flowers and diversity in flower color and other characters is limited. Therefore, it is necessary to consider the possibility of producing intergeneric hybrids using species in closely related genera. Intergeneric hybridization of marguerite with certain species of related genera has in fact been performed at Shizuoka Economic Botanical Garden since the 1960s, and one commercial cultivar, 'Izu Yellow' (yellow flower color, large capitulum diameter and good leaf shape), has successfully been developed by crossing with crown daisy (*Glebionis coronaria*) (Furusato 1977, 1978).

Like marguerite, crown daisy, which is native to the Mediterranean coast, had previously been classified as a species of *Chrysanthemum*. However, crown daisy has now been transferred to *Glebionis* or *Ismeria* (Sutton 2001) by the revision of Compositae classification (Brummitt 1997). In Japan, crown daisy is usually treated as a species of *Glebionis*. This species has pure yellow flowers and shows strong plant vigor. The genus *Glebionis* also contains an annual plant, annual *Chrysanthemum* (*G. carinatum*), which is native to Morocco and has diversified flower colors not seen in marguerite. Although annual chrysanthemum can be crossed with crown daisy (Kitamura et al. 1988), there have been no reports on crossing with marguerite.

Abbreviations: IAA, indole-3-acetic acid; MS, Murashige and Skoog.
This article can be found at http://www.jspcmb.jp/

In the present study, we used ovule culture following cross-pollination to examine cross-compatibility between marguerite and six species from related genera, including *G. carinatum* and *G. coronaria* , and characterized the ovule culture-derived plants in order to introduce diversified flower colors and growth habits of related species into marguerite.

Materials and methods

Cross-pollination

Three marguerite genotypes, one cultivar with white flowers ('Fairy White') and two breeding lines with pink flowers ('97-30-5' and '97-31-1'), were used as seed parents. Six species from related genera, annual chrysanthemum (*Glebionis carinata*), crown daisy (*G. coronaria*), snow daisy (*Chrysanthemum paludosum*), yellow daisy (*C. multicaule*), German chamomile (*Matricaria chamomilla*) and swan river daisy (*Brachycome iberidifolia*), were used as pollen parents. Plants of the seed parents were planted in unglazed pottery pots (16 cm in diameter) and those of the pollen parents were planted in polyethylene pots (10 cm in diameter). All plants were cultivated in a plastic house where the temperature was kept above 5°C from December to March.

Disk florets of the 2nd and 3rd columns from the outside of the capitulum of marguerite were used for cross-pollination. According to the crossing method for daisies (Tanaka 1982), ray florets were removed from capitula, pollen grains of the dehisced anthers were removed by blowing, and capitula were covered with paper bags. Three days later, disk florets were pollinated using a brush and the capitula were again covered with the paper bags. Pollen grains collected on the day of anthesis were used in most pollination experiments, but in some seasons pollen grains were stored in a refrigerator at 5°C for a few days before use.

Ovule culture

About three weeks after cross-pollination, capitula were harvested and surface-disinfected with sodium hypochlorite solution (1% available chlorine) for 10 min. Ovule culture was performed as follows. Disk florets of the 2nd and 3rd columns from the outside of the capitulum were isolated, and about 20 florets were cultured in three culture tubes (24×120 mm) each containing 8 ml of a modified MS medium consisting of half-strength mineral salts supplemented with 0.2 mg l^{-1} IAA, 30 g l^{-1} sucrose and 2 g l^{-1} gellan gum (Murashige and Skoog 1962). Cultures were maintained at 25°C under a 16-h photoperiod (4,000 lx). After ovule culture for two months, the number of germinating plants was recorded.

Ovule culture-derived plantlets showing normal growth were removed separately from the culture tubes and transplanted to plastic trays containing expanded vermiculite for acclimatization. Acclimatized plants were then transplanted to polyethylene pots (10 cm in diameter) and cultivated under the same conditions as the parental plants.

Characterization of ovule culture-derived plants

Plant type (rounded, spreading or upright), degree of branching, leaf shape, flower color and capitulum diameter of the parental and ovule culture-derived plants were examined at the flowering stage according to the Technical Protocol (marguerite) specified by the Ministry of Agriculture, Forestry and Fisheries of Japan.

Results

Cross-pollination

In 2000, cross-compatibility between marguerite and six species from related genera was examined, using three marguerite genotypes as seed parents (Table 1). Two months after pollination, capitula were harvested, and about 80 (without ovule culture) or 60 (with ovule culture) disk florets were isolated from each capitulum and used for germination tests. When ovule culture was not applied, no germination was observed in any of the six cross combinations. On the other hand, a few germinating plants were obtained by ovule culture from crosses involving annual chrysanthemum or crown daisy as the pollen parent. The percentage of pollinated capitula from which germinating plants were obtained by ovule culture was higher in marguerite×crown daisy (26.2%) than in marguerite×annual chrysanthemum (12.9%). No germinating plants were obtained in the other four cross combinations even when ovule culture was applied.

Table 1. Number of germinating plants obtained from intergeneric crosses using marguerite as the seed parebt with or without ovule culture[a]

Pollen parent	Without ovule culture			With ovule culture		
	No. of capitula pollinated	No. of capitula with germinating plants (%)	No. of germinating plants	No of capitula pollinated	No. of capitula with germinating plants (%)	No. of germinating plants
Annual chrysanthermum	22	0 (0)	0	70	9 (12.9)	16
Crown daisy	25	0 (0)	0	61	16 (26.2)	26
Snow daisy	15	0 (0)	0	25	0 (0)	0
Yellow daisy	14	0 (0)	0	15	0 (0)	0
German chamomile	15	0 (0)	0	14	0 (0)	0
Swan river daisy	11	0 (0)	0	14	0 (0)	0

[a] Three marguerite genotypes, 'Fairy White', '97-30-5' and '97-31-1', were used. About 80 (without ovule culture) or 60 (with ovule culture) disk florets were isolaed from each capitulum and subjected to germination tests.

Table 2. Number of plants obtained from intergeneric crosses using marguerite as the seed parent and crown daisy or annual chrysanthemum as the pollen parent by ovule culture[a]

Marguerite genotype used as the seed parent	Pollen parent									
	Annual chrysanthemum					Crown daisy				
	No. of capitula pollinated	No. of capitula with germinating plants (%)	No. of germinating plants	No. of acdirratized plants	No. of plants bloomed	No. of capitula pollinated	No. of capitula with germinating plants (%)	No. of germinating plants	No. of acdirratized plants	No. of plants bloomed
'Fairy White'	22	1 (4.5)	3	1	1	20	2 (10.0)	4	0	0
'97-30-5'	20	2 (10.0)	5	1	1	18	8 (44.4)	10	10	8
'97-31-1'	28	6 (21.4)	8	4	3	23	6 (26.1)	12	8	8
Total	70	9 (12.9)	16	6	5	61	16 (26.2)	26	18	16

[a] About 60 disk florets were isolated from each capitulum and subjected to ovule culture.

Table 3. Morphological characterization of bloomed plants obtained from intergeneric crosses using marguerite as the seed parent and crown daisy or annual chrysanthemum as the pollen parent by ovule culture

Plant number	Cross combination		Morphological characteristics[a]					Notes
	Seed parent	Pollen parent	Plant type	Brenching[b]	Leaf color	Flower color[c]	Capitulum diameter[d]	
00-157-1	'Fairy White'	Annual chrysanthemum	—	—[e]	—	Yellow	Very small	Slow growth, died after blooming
00-139-1	'97-30-5'	Annual chrysanthemum	Rounded	Moderate	Pale green	Wisteria pink/ white	Medium	Good flower shape and color, died after blooming
00-154-1	'97-31-1'	Annual chrysanthemum	Spreading	Many	Pale green	Orange/ dark orange	Large	Good flower shape and color, perpetual flowering
00-235-1			Spreading	Many	Pale green	Reddish brown	Medium	Leafshape similar to annual chrysanthemum
00-235-2			Rounded	Moderate	Pale green	Pale pink/ dark pink	Medium	Many flowers, perpetual flowering
00-138-1	'97-30-5'	Crown daisy	Upright	Moderate	Pale green	White	Medium	Deformed petals
00-141-1			Upright	Few	Pale green	White	Medium	
00-142-1			Upright	Few	Pale green	White/yellow	Medium	Flower shape similar to 'Zairai Shiro'
00-189-1			Upright	Few	Pale green	White/yellow	Medium	
00-190-1			Upright	Few	Pale green	White	Medium	Narrow petals, green stems
00-191-1			Upright	Few	Pale green	White	Medium	Lack of petals
00-198-1			Upright	Few	Pale green	White	Large	Narrow and cup-shaped petals
00-199-1			Upright	Few	Pale green	White	Medium	
00-144-1	'97-21-1'	Crown daisy	Upright	Few	Pale green	White/yellow	Medium	
00-144-2			Upright	Few	Pale green	White/yellow	Medium	Round petals
00-144-3			Upright	Few	Pale green	White	Medium	Flat petals
00-144-4			Upright	Few	Pale green	White	Medium	
00-145-1			Upright	Few	Pale green	White/yellow	Large	Spoon-shaped petals
00-145-2			Upright	Few	Pale green	White/yellow	Large	
00-145-3			Upright	Few	Pale green	White/yellow	Medium	Spoon-shaped petals
00-217-1			Upright	Few	Pale green	White/yellow	Small	Large plant height
Parental plants	'Fairy White'		Upright	Many	Green	White	Small	
	'97-30-5'		Rounded	Many	Dark green	Pale pink	Small	
	'97-31-1'		Rounded	Many	Green	Pale pink	Small	Many pollen grains
	Crow daisy		Upright	Few	Pale green	White	Large	
	Annual chrysanthemum		Upright	Few	Pale green	Red purple or yellow	Large	Various flower colors

[a] Morphological characteristics are expressed according to the Marguerite Varietal Characteristic Systematic Investigation Standard specified by the Ministry of Agriculture.
[b] Few, 0–2 branches per plant, moderate, 3–6 branches per plant; many, 7 or more branches per plant.
[c] Petal main color/color of the near-basal region of the petal.
[d] Very small, below 5 cm; small, 5–6 cm; medium, 6–7 cm; large, over 7 cm.
[e] Not investigated.

Based on these results, intergeneric cross-pollination and subsequent ovule culture were conducted in 2001 using three marguerite genotypes as seed parents and crown daisy and annual chrysanthemum as pollen parents (Table 2). For both marguerite×annual chrysanthemum and marguerite×crown daisy, the percentage of pollinated capitula from which germinating plants were obtained was higher for breeding lines of marguerite than for 'Fairy White' as the seed parent. In total, 16 and 26 germinating plants were obtained by ovule culture for marguerite×annual chrysanthemum and marguerite×crown daisy, respectively. However, many plants from marguerite×annual chrysanthemum lacked roots and did not grow well *in vitro*. Consequently, they could not be acclimatized and transplanted to pots. Finally, 5 and 16 ovule culture-derived plants for marguerite×annual chrysanthemum and marguerite×crown daisy, respectively, reached the flowering stage.

Characterization of ovule culture-derived plants

The five flowering plants from marguerite×annual chrysanthemum and 16 from marguerite×crown daisy were characterized morphologically (Table 3). For marguerite×annual chrysanthemum, flower color (the main color of the petal of ray florets) of the five ovule culture-derived plants was varied: pale pink, orange, reddish brown, wisteria pink, or yellow. For some plants, the near-basal region of the petal showed a deeper or paler color than the main color. For all five plants, the most basal part of the petal was yellow in color, a character specific to the pollen parent annual chrysanthemum (Figure 1). Capitulum diameter was as large as or larger than that of the seed parent marguerite (Figure 1). Among the five plants, pollen production was observed only in one (00-235-1; pollen fertility was not checked). Leaf shape of the five plants was similar to that of annual chrysanthemum. Although marguerite usually produces no flowers in Japan after June, due to high temperatures, all five ovule culture-derived plants showed perpetual flowering: they produced flowers from the beginning of March until the begininng of July, and flowering reached its peak at the end of April.

In marguerite×crown daisy, flower color of the 16 ovule culture-derived plants, which were obtained from crosses using the breeding line '97-30-5' or '97-31-1' as the seed parent, was white. In some plants, the near-basal region of the petal was light yellow in color. Capitulum diameter in some plants was as large as that of crown daisy. Pollen production was observed in four of the 16 plants. Most of the ovule culture-derived plants had upright stems with fewer branches and showed strong plant vigor, and one season flowering in spring, like crown daisy.

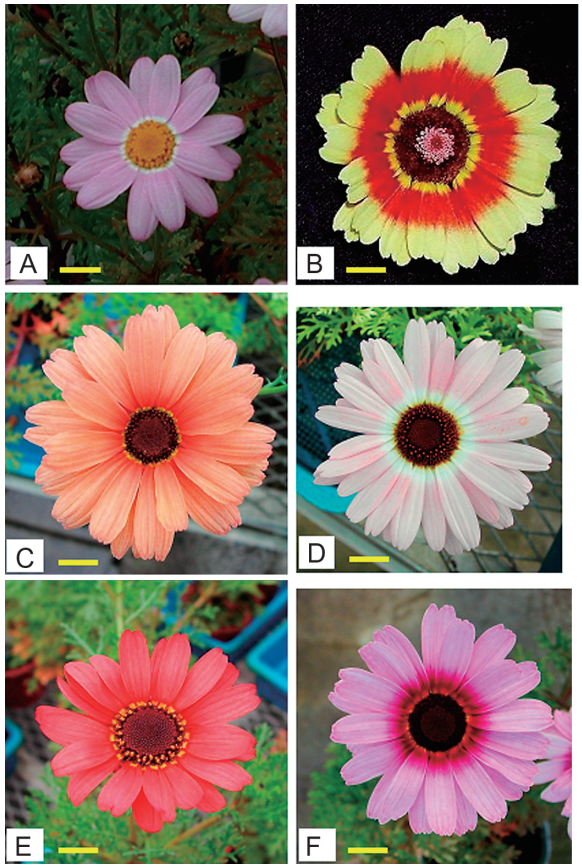

Figure 1. Flowers of intergeneric hybrids and their parents. (A) Marguerite breeding line '97-31-1' used as the seed parent. (B) Annual chrysanthemum used as the pollen parent. (C) Intergeneric hybrid 00-154-1. (D) Intergeneric hybrid 00-139-1. (E) Intergeneric hybrid 00-235-1. (F) Intergeneric hybrid 00-235-2. Bars=1 cm.

Discussion

Ovule culture-derived plants examined in the present study showed novel flower color and shape characteristics that are not observed in marguerite, and some also showed novel flowering habits such as perpetual flowering. These results indicate that the ovule culture-derived plants with novel characteristics were intergeneric hybrids.

Studies on the crossing of *Argyranthemum* with allied genera, including *Glebionis* used in the present study, have not yet been performed except for the combination between marguerite (*A. frutescens*) and crown daisy (*G. coronaria*) (Furusato 1977, 1978). In the present study, intergeneric hybrid plants were successfully obtained in cross combinations of marguerite×annual chrysanthemum and marguerite×crown daisy when ovule culture was applied. The effectiveness of ovule culture for wide hybridization has already been reported in various cross combinations such as *Spiraea thunbergii* and *S. japonica* L. (Iizuka et al. 2001), Hydrangea *macrophylla* f. *hortensia* and *H. quercifolia* (Kudo et al. 2002), and *Cammelia chrysantha* and *C. japonica*

(Nishimoto et al. 2003). According to these reports, interspecific hybrids acquired novel characteristics that had not previously been observed, suggesting the importance of interspecific hybridization for breeding of ornamental plants.

In the present study, the efficiency of hybrid production by ovule culture was higher in marguerite× crown daisy than in marguerite×annual chrysanthemum. Since hybrid plants of marguerite×crown daisy have already been produced (Furusato 1977, 1978), cross-incompatibility may be stronger between marguerite and annual chrysanthemum than between marguerite and crown daisy. In the present study, we have also confirmed that variation in plant vigor and flowering period among different ovule culture-derived plants depends on the marguerite genotype used as the seed parent. Since characteristics of hybrid plants are important for their use in further breeding, it will be necessary to clarify the reason for such variation.

Flower color of all 16 hybrid plants obtained using crown daisy as the pollen parent was white, although flowers of the two marguerite breeding lines used as seed parents were pink. In previous studies, however, only hybrids with yellow flowers were obtained when a marguerite genotype with white flowers as the seed parent was crossed with crown daisy (Furusato 1977, 1978). These results highlight the difficulty of predicting flower color of hybrids, since the mechanism of flower color inheritance in marguerite and related species is unknown. As well as flower color, the hybrids had several characteristics not observed in marguerite, such as upright stems with fewer branches and one season flowering. However, we were unable to find novel characteristics that might be valuable for flowerbed and pot plants in these hybrid plants.

Various flower colors were observed in the five hybrid plants obtained using annual chrysanthemum as the pollen parent. This was considered to be a reflection of the characteristics of the pollen parent annual chrysanthemum. Since these hybrids also had several other characteristics not observed in marguerite, such as strong plant vigor under outdoor conditions and perpetual flowering, they can be usable as flowerbed and pot plants.

The results obtained here indicate that intergeneric hybridization using annual chrysanthemum as the pollen parent may be very effective for introducing valuable characteristics, especially a wide range of flower color, into marguerite. Further studies, such as confirmation of the fertility and production of amphidiploids, will be needed before the hybrids can be used for breeding purposes. Because hybridity was verified only by comparing the morphological characteristics of bloomed plants in the present study, further confirmation of the hybrid nature of these plants, for example using molecular markers, is desirable in the future.

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