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A novel interspecific hybrid plant between *Hydrangea scandens* ssp. *chinensis* and *H. macrophylla* via ovule culture

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Abstract In the genus *Hydrangea*, *H. macrophylla* is the most popular species. For this species, numerous cultivars with showy colorful flowers have been bred since the early 1900s through selection of natural mutants and intraspecific crosses among a limited number of early ancestral varieties. Although breeding of *H. macrophylla* has been successful, further improvements in flower shape, flower color, and growth habit are desirable. *H. scandens* ssp. *chinensis* is a small shrub that is native to South and Southeast Asia and valued for its evergreen foliage, winter flowering and broad adaptability in mild climates. Cross-pollination between *H. scandens* ssp. *chinensis* and *H. macrophylla*, and subsequent ovule culture, resulted in the production of an interspecific hybrid plant. The hybridity of this plant was confirmed by RAPD analysis. The hybrid plant had flower and leaf morphologies intermediate between the two parental species. Since the hybrid showed more vigorous growth than both parents, had evergreen foliage, and flowered in winter, it has sufficient horticultural merit for commercialization and may be suitable for greenhouse culture.

Key words: Distant hybridization, interspecific hybrid, *Hydrangea macrophylla*, *Hydrangea scandens*, ovule culture.

Distant hybridization has been used in ornamental crop breeding to produce novel and often unpredictable characters and to introgress desirable genes from one taxon to another (Langton 1987). Distant hybridization usually involves crossing of different species within the same genus (Kato et al. 2001), but crosses are sometimes attempted between different genera (Amano et al. 2007). Barriers to distant hybridization may be prezygotic, postzygotic, or both, and are likely to be more severe in crosses between more distantly related taxa than in those between closely related taxa (Ladizinsky 1992). Embryo rescue through embryo or ovule culture has been a valuable *in vitro* tool for circumventing the postzygotic barrier and to produce new cultivars in various ornamental crops, e.g., *Gypsophila* (Kishi et al. 1994), *Lilium* (Van Tuyl et al. 1991), *Primula* (Amano et al. 2006) and *Sandersonia* (Morgan et al. 2001).

The genus *Hydrangea* consists of at least 23 species of erect or climbing, and deciduous or evergreen shrubs (McClintock 1957) which are distributed in East Asia, the eastern part of North America, and towards the western seaboards of Central and South America. The genus is divided into the sections *Hydrangea*, which contains mainly temperate climate species, and *Cornidia*, which consists of climbing species from tropical and subtropical areas. Species of the section *Hydrangea* have a number of ornamentally attractive characteristics, and almost all of the cultivated species in this genus are members of the section *Hydrangea*.

H. macrophylla is native to East Asia and is one of the most popular species in the section *Hydrangea*. The popularity of this species is due in part to its versatility as both a florist and a landscape plant. *H. macrophylla* and its cultivars are mainly cultivated in Europe, North America, and East Asia. Since the introduction of *H. macrophylla* into England by Joseph Banks in 1789, numerous cultivars with a wide spectrum of flower color have been bred through selection of natural mutants and intraspecific crosses among a limited number of early varieties and cultivars (Lawson-Hall and Rothea 1995; Van Gelderen and Van Gelderen 2004). Although breeding of *H. macrophylla* has been successful to some extent, the present cultivars generally have a narrow genetic base. Distant hybridization is an important tool for broadening genetic variability in commercial cultivars of *H. macrophylla*. Several projects to apply distant hybridization for improvement of *H. macrophylla* were initiated recently in Japan and USA. However, no satisfactory results were obtained from these projects, with produced hybrids being weak (Kudo and Niimi 1999a, 1999b; Kudo 2000; Reed et al. 2001; Kudo et al. 2002; Reed 2004; Jones and Reed 2006).

Abbreviations: CTAB, cetyl trimethyl ammonium bromide; MS, Murashige and Skoog; PCR, polymerase chain reaction; RAPD, randomly amplified polymorphic DNA.

This article can be found at http://www.jspcmb.jp/

H. scandens ssp. *chinensis*, which belongs to the section *Hydrangea*, is a small attractive evergreen shrub (up to 1–2 m) native to warm temperate and subtropical regions of South and Southeast Asia (McClintock 1957; Van Gelderen and Van Gelderen 2004). This species has several attractive characteristics, such as winter flowering and small corymbs of about 15–20 cm in diameter with white flowers, but is not commonly used as an ornamental plant. Although hybridization between *H. scandens* ssp. *chinensis* and *H. macrophylla* could potentially combine the evergreen foliage and winter flowering of *H. scandens* ssp. *chinensis* with ornamental characteristics of *H. macrophylla*, such as its wide spectrum of flower color, hybridization between these two species has not yet been reported. The present study describes the production and characterization of an interspecific hybrid between *H. scandens* ssp. *chinensis* and *H. macrophylla* via ovule culture.

Materials and methods

Plant materials, pollination and ovule culture

One genotype of *H. scandens* ssp. *chinensis* and *H. macrophylla* 'Blue Ring' were used in the present study. Both parents were maintained in a greenhouse whose temperature was kept at 10°C in winter. Flowering of both parents was synchronized by controlling temperature and photoperiod. Cross-pollination using fresh pollen was made between *H. scandens* ssp. *chinensis* (female parent) and *H. macrophylla* (male parent). Each flower of *H. scandens* ssp. *chinensis* was emasculated one day before anthesis.

Capsules were collected 90 days after pollination and surface-disinfected for 15 min with sodium hypochlorite solution (1% available chlorine) containing a drop of Tween 20 per 200 ml, followed by three rinses with sterile distilled water. After disinfection, enlarged ovules were excised aseptically from each capsule and placed on 2 g l^{-1} gellan gum-solidified half-strength MS medium (Murashige and Skoog 1962), containing 20 g l^{-1} sucrose without any plant growth regulators. Cultures were maintained as previously described (Kudo and Niimi 1999a; Kudo 2000). Seedlings that germinated normally were subcultured every 3–4 weeks onto fresh medium of the same composition until they were large enough for rooting. After rooting, the plants were transplanted into plastic pots containing sterile moist vermiculite. The pots were covered with plastic bags, and the plants were acclimatized for three weeks and then cultivated under the same conditions as the parental plants.

RAPD analysis

Total DNA was extracted from leaf tissues by a modified CTAB method (Rogers and Bendich 1985). The DNA polymerase chain reaction system (Promega Co. USA) was used for RAPD analysis. Two kinds of random 10-mer primers (Operon Technologies Inc. USA), OPA03 (5′-AGTCAGCCAC-3′) and OPA04 (5′-AATCGGGCTG-3′), were used. The PCR amplification reaction contained 50 ng template DNA in a final volume of 10 μl. DNA fragments were amplified by an initial denaturation step at 95°C for 3 min, followed by 40 cycles of 93°C for 1 min, 40°C for 2 min and 72°C for 2 min, and a final elongation step at 72°C for 3 min using a Perkin-Elmer 2400 thermal cycler (Perkin-Elmer Co. USA). Electrophoresis of amplified products was conducted on a 2% (w/v) agarose gel, with a 100-bp DNA ladder as a size marker. All analyses were replicated at least three times.

Hybrid characterization

Ten plants, propagated vegetatively by cutting from each of the hybrid, *H. scandens* ssp. *chinensis* and *H. macrophylla*, were cultivated in the greenhouse as described above. Morphological characterization was carried out at the flowering stage. Flower color was determined visually with the aid of the JHS Color Chart (Japan Horticultural Plant Standard Color Chart 1984).

Results and discussion

Ovule culture

From 60 cross-pollinated flowers, 3,363 enlarged ovules were produced. By culturing these ovules *in vitro*, a total of 1,120 putative hybrid seedlings were obtained. About 60% of the seedlings were successfully transferred to greenhouse conditions; most of the others showed arrested growth with chlorotic foliage and died within several weeks after the transfer. Forty-two putative hybrids showing evergreen foliage and winter flowering were preliminarily selected and their characteristics evaluated under greenhouse conditions. Finally, one single putative hybrid plant (Cm1) with the highest ornamental value was selected.

Characterization of the hybrid Cm1

The hybrid nature of Cm1 was confirmed clearly with reproducible results obtained by RAPD analysis (Figure 1). When the primer OPA03 was used (Figure 1A), 950-bp and 550-bp bands common to *H. macrophylla* and Cm1 were obtained, whereas these bands were not detected in *H. scandens* ssp. *chinensis*. When the primer OPA04 was used (Figure 1B), a 750-bp band was detected in both *H. macrophylla* and Cm1, but not in *H. scandens* ssp. *chinensis*; in contrast, 700-bp and 400-bp bands were detected in *H. scandens* ssp. *chinensis* and Cm1, but not in *H. macrophylla*. Cm1 thus possessed both male and female parent-specific bands.

Further characterization of Cm1 also confirmed its hybridity (Table 1, Figure 2, 3, 4). In general, Cm1 has flower and leaf morphologies that are intermediate between the two parental species. Cm1 plants showed more vigorous growth than both parents. The flower color (sepal color of decorative flowers) of Cm1 was pink, and the intensity of the pink color was dependent on growing temperature: more intense in spring with warm day temperatures than in winter with low day temperatures. Fragrance was noted in flowers of *H. scandens* ssp. *chinensis*, but not in Cm1. Unexpectedly,

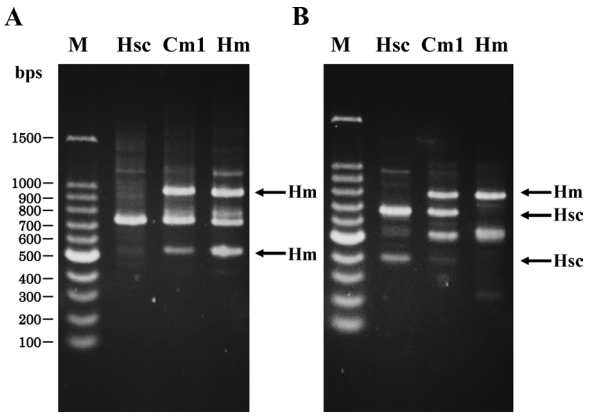

Figure 1. RAPD profiles of *Hydrangea scandens* ssp. *chinensis* (Hsc), an ovule culture-derived plant (Cm1), and *H. macrophylla* (Hm) using the primer OPA03 (A) or OPA04 (B). Arrows (Hm) and (Hsc) indicate bands specific to *H. scandens* ssp. *chinensis* and *H. macrophylla*, respectively. Lane M represents a molecular size marker (100-bp DNA ladder); bps, base pairs.

Figure 2. Flowering plant of the hybrid Cm1. Bar=10 cm.

Figure 3. Inflorescences of *Hydrangea scandens* ssp. *chinensis* (left), the hybrid Cm1 (center) and *H. macrophylla* (right). Bars=3 cm.

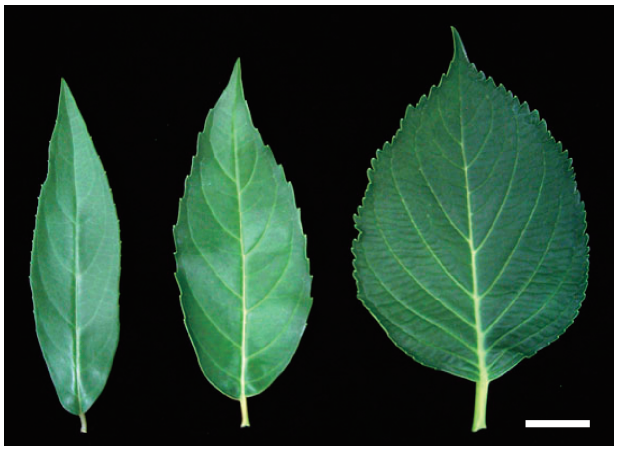

Figure 4. Leaves of *Hydrangea scandens* ssp. *chinensis* (left), the hybrid Cm1 (center) and *H. macrophylla* (right). Bar=3 cm.

Cm1 produced double flowers (Table 1, Figure 3) in spite of utilization of single-flowered plants as parents. It is thought that accumulation of several double-flowered genes results in the double-flowered phenotype, and that the single-flowered phenotype is dominant to the double-flowered one in *Hydrangea* breeding (Shoji Sakamoto, personal communication). There are several possible explanations for the formation of double-flowered progeny in crosses between *H. scandens* ssp. *chinensis* and *H. macrophylla*: (1) interactions between diverged sequences of the parental genomes; (2) global genomic rearrangements of the hybrid; and (3) widespread epigenetic reprogramming during development of floral organs (McClintock 1984; Comai et al. 2000; Grant-Downton and Dickinson 2006). At present, it is unclear which explanation is correct, although the generation of a double-flowered phenotype by interspecific hybridization suggests the possibility of broadening the spectrum of flower shape in *Hydrangea* breeding. The unexpected occurrence of a double-flowered phenotype by distant hybridization has previously been reported in interspecific hybridization between *Primula sieboldii* and *P. kisoana* (Mii 1989).

Cm1 had fully developed anthers, which contained numerous pollen grains with abnormal shapes. Self-pollination of Cm1 and backcross-pollination to *H. macrophylla* resulted in no seed production. Cm1 would therefore be difficult to use as a male parent for further breeding. To restore pollen fertility, it may be necessary to produce amphidiploids by artificial chromosome doubling (Nimura et al. 2003).

Table 1. Comparison of the hybrid Cm1 with the parental species[a]

Characteristics	*H. scandens* ssp. *chinensis*	Hybrid Cm1	*H. macrophylla*
Growth habit			
Leaf duration	Evergreen	Evergreen	Deciduous
Winter dormancy	Non-dormant	Non-dormant	Dormant
Flowering time	Mid-winter	Mid-winter	Spring to early summer
Growth vigor	Medium	Vigorous	Medium
Inflorescences			
Size of inflorescence	Small	Large	Medium
Inflorescence diameter (cm)	16 ± 6	26 ± 4	21 ± 5
Decorative sterile flowers (ray flowers)			
Sepal shape (single or double)	Single	Double	Single
Flower color (sepal color)	Creamy white	Pink	Blue to purple
JHS Color Chart No.[b]	2902	9702	7604
Diameter of decorative sterile flowers	Small to medium	Large	Large
Sepal margin	Entire	Serrate	Serrate
Non-decorative fertile flowers (true flowers)			
Petal shape (single or double)	Single	Single	Single
Flower color (petal color)	Creamy white to light yellow	Pink	Blue to purple
Fragrance	Scented	Unscented	Unscented
Leaves			
Shape (overall shape)	Lanceolate	Elliptic	Orbicular
Leaf tip	Acute	Acuminate	Acuminate
Leaf base	Cuneate	Attenuate	Obtuse
Leaf margin	Entire	Serrate	Serrate
Leaf surface	Glossy	Glossy	Glossy
Number of lateral veins	13 ± 2	16 ± 3	17 ± 2
Ratio of length/width	3.5	2.2	1.5

[a] Values for the inflorescence diameter and number of lateral vein represent the mean ± SE of 10 plants.

[b] Sepal color was checked visually with the aid of the JHS Color Chart [Japan Horticultural Plant Standard Color Chart (Japan Color Research Institute 1984)].

Conclusion

In the present study, interspecific hybridization between *H. scandens* ssp. *chinensis* and *H. macrophylla* has been achieved and a desirable hybrid selected. The hybrid had evergreen foliage and flowered in winter. It has sufficient horticultural merit for commercialization and may be suitable for greenhouse culture as a potted plant. The hybrid was easy to propagate by cutting in spring, following the procedure for *H. macrophylla* (Bailey 1992). Due to the subtropical habitat of *H. scandens* ssp. *chinensis*, it is unlikely that the hybrid would survive winter in the northern part of Japan. However, it may be suitable for cultivation in warmer regions. Finally, it would be worthwhile to investigate the suitability of this hybrid for outdoor culture, since the hybrid exhibits a remontant flowering potential (Adkins and Dirr 2003) and attractive foliage.

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