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Production of Interspecific Hybrid Plants through Cotyledonary Segment Culture of Embryos Derived from Crosses between *Hydrangea macrophylla* f. *hortensia* (Lam.) Rehd. and *H. arborescens* L.

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Summary

A sexual incompatibility mechanism prevents reciprocal crosses between *Hydrangea macrophylla* f. *hortensia* (Lam.) Rehd. and *H. arborescens* L. No viable hybrid plants have been produced by conventional hybridization because the progenies exhibit hybrid lethality at a young seedling stage. For circumventing hybrid lethality, we cultured cotyledonary segments of embryos derived from crosses of *H. macrophylla* and *H. arborescens*. The cotyledonary segments formed callus. Fifteen callus lines, which regenerated plantlets, were established from crossings of *H. macrophylla* 'Blue sky' × *H. arborescens* 'Annabelle'. However, only a single callus line among them regenerated plantlets which could be transplanted to soil. Hybridity of the regenerated plants was examined by morphological, cytological characteristics, and RAPD markers: morphologically, the regenerated plants appeared intermediate to the parents; the regenerated plants were aneuploids having a chromosome number, 2n = 42; whereas those of the parents *H. macrophylla* 'Blue sky' and *H. arborescens* 'Annabelle' were 2n = 52 and 2n = 38, respectively; the RAPD banding patterns of the regenerated plants exhibited the same hybrid bands as the parents. The hybridity of the regenerated plants was probably confirmed by the above methods.

Key Words: *Hydrangea macrophylla*, *H. arborescens*, cotyledonary segment culture, interspecific hybrid.

Introduction

A sexual incompatibility mechanism prevents reciprocal crosses between *H. macrophylla* and *H. arborescens* (Kudo and Niimi, 1999). Seeds were produced only when *H. macrophylla* was used as the female parent, but none germinated *in vivo*. No viable hybrid plants have been produced by the conventional ovule culture because the hybrid progenies exhibited hybrid lethality at a young seedling stage (Kudo and Niimi, 1999).

Hybrid lethality is an important barrier which prevent the production of hybrids between distantly related species (Gerstel, 1954; Oka, 1962; Siddiqui and Jones, 1969; Zeven, 1981). Some investigators have attempted to overcome the lethality to produce viable hybrids (Inoue et al., 1997; Reed and Collins, 1978). Lloyd (1975) obtained viable hybrid plants between *Nicotiana suavelens* Lehm. and *N. tabacum* L. through callus culture of cotyledonary segments of hybrid seedlings, whereas Marubashi et al. (1988) accomplished the same by growing them at 36 ℃.

In this paper, we circumvented hybrid lethality between *H. macrophylla* and *H. arborescens* by culturing cotyledonary segments of embryos; the hybridity of the regenerated plants was confirmed by morphological, cytological characteristics, and RAPD markers.

Materials and Methods

Plant material

Parental plants of *H. macrophylla*, 'Blue sky' and 'Blue diamond', and *H. arborescens* 'Annabelle' were grown in a greenhouse under natural day length. Flowers of *H. macrophylla* were emasculated and pollinated with pollen of *H. arborescens*. From 90 to 120 days after pollination, capsules were harvested and surface-disinfected. Fertilized ovules were excised aseptically and cultured as previously described (Kudo and Niimi, 1999). Embryos that emerged from the ovules 30 to 90 days after culture were used.

Callus formation and organogenesis

A modified Murashige and Skoog's medium (Murashige and Skoog, 1962) consisiting of half strength macronutrients and full strength micronutrients, ethylenediaminetetraacetic acid iron salt (Fe-EDTA),

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vitamins, 2 % (w/v), and 0.25 % (w/v) gelrite was adjusted to pH 5.7 before autoclaving at 121 ℃ for 20 min. The basal medium supplemented with or without 8.87 μM 6-benzyladenine (BA) was used for callus and shoot formation. Two cotyledonary segments (2 × 3 mm long) excised from the ovule-culture derived embryos were plated on the medium in a Petri dish (60 mm diam.). Forty segments were cultured on the medium with BA and 10 segments were cultured on the same medium without BA. Cultures were maintained at 25 ℃ under 16-photoperiod with a light flux of 3,000 lx. Calli formed on the segments were disected into small pieces (about 5mm diam.), and transferred onto the fresh medium. The calli were subcultured every three weeks on the same but fresh medium. When adventitious buds formed on the callus, a bud cluster (5 mm diam.) was cut and transferred onto a hormone-free basal medium or a medium supplemented with 2.22 μM BA to elongate the regenerated shoots.

Preparation for establishment of plants in soil

For *in vitro* rooting, 2 to 3 cm long shoots were excised from the bud cluster and transferred to 30 × 90 mm culture tubes containing 15 ml basal medium, 1 % (w/v) sucrose, and 0.25 % (w/v) gelrite. *In vitro* rooted plants were removed from the medium, washed gently to remove the gelrite, and transplanted into 6 cm plastic pots containing sterile moist mixture of peat moss, vermiculite, perlite (1:3:1, v/v). The pots with plantlets were covered with plastic bags to impart some tolerance to moisture stress for 2 weeks; the plantlets were gradually acclimatized to ambient greenhouse conditions.

Confirmation of hybridity of regenerated plants

1. Chromosome number of regenerated plantlets

Young roots, 10 to 14 days old, were excised from 5 individual parental plantlets grown *in vitro* ; they were pretreated with a solution of 0.1 % (w/v) colchicine in distilled water at 8 ℃ for 20 h, and fixed with an aceto-alcohol solution at 4 ℃ for 2 to 4 h. The cell walls were digested with an enzyme solution [4 % Cellulase Onozuka RS (Yakult Co., Ltd), 1 % Macerozyme RS (Yakult Co., Ltd), 1 % Pectolyase Y-23 (Seisin Co., Ltd.), 0.75 mM ethylenediaminetetraacetic acid disodium salt (Na_2-EDTA) and 0.75 mM KCl, pH. 4.0] at 37 ℃ for 60 to 90 min. The remanents were stained with 1 % (w/v) aceto-orsein solution. Chromosomes of four to 10 metaphase plates per plant (average 7 cells) were counted.

2. RAPD analysis

DNA from the leaves of greenhouse-grown regenerated plants and parental plants was extracted according to Wagner et al. (1987). To verify the stability of parent-specific RAPD markers, DNA of 10 individuals of a parental cultivar was extracted and amplified with each primer.

Polymerase chain reactions (PCR) were performed in a volume of 20 μl containing 2.5 μl 10 × PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2 mM $MgCl_2$, 300 μM each of dNTP, 5 picomoles of a single primer, 100 ng of template DNA, and 2.5 units of *Taq* DNA polymerase (Takara Shuzo Co., Japan). Forty random oligonucleotide primers (Kit A and Kit B, Operon Technologies, USA) were used to screen for RAPD markers. Amplification of the DNA template was performed on a Perkin-Elmer 2400 thermal cycler. PCR conditions were as follows: after an initial denaturation step at 94 ℃ for 1 min, the reaction mixture was subjected to amplification for 45 cycles of 1 min denaturation step at 94 ℃, 2 min annealing step at 40 ℃, 3 min elongation step at 72 ℃ ; after the final cycle, the amplified product was extended at 72 ℃ for 10 min. The amplification products were separated by electrophoresis on 1 % (w/v) NuSieve ME agarose (FMC bioproducts, USA) gel in 0.5 × TBE (0.045 M Tris-borate, 0.001 M EDTA, pH 8.0) buffer, stained with 2 $\mu g \cdot ml^{-1}$ ethidium bromide solution and the gels were photographed under UV light. Each test was replicated at least twice. Only distinct and polymorphic amplified DNA bands were scored.

Results

Callus formation and shoot regeneration

Cotyledon segments from ovule-culture derived embryos increased in size within a week after culturing on basal medium with BA. A white friable callus and a green nodular callus formed at the cut end and/or the entire surface of segments 30 to 60 days after culture. The nodular calli were transferred to fresh medium with 8.87 μM BA and subcultured every 3 weeks. No calli proliferated on the basal medium without BA, whereas with BA, clusters consisting of adventitious buds, growing leaves and bud-like structure were formed on the callus surface (Fig. 1 A); plantlets rarely formed elongated shoots in the cultures. Shoot elongation occurred only after transferring the adventitious bud cluster to fresh medium with 2.22 μM BA. When the pale green shoots elongated (gradually became vitreous) to more than 10 mm; they were excised and transferred to 200 ml glass vessels; then they became compact and dark green. Some shoots rooted in 2 weeks after transfer, whereas others rooted only after they were cultured on hormone free basal medium for 2 weeks. The plantlets with well-developed roots were transferred to soil in a greenhouse and grew well. Results from two cross combinations are summarized in Table 1. In the *H. macrophylla* 'Blue sky' × *H. arborescens* 'Annabelle' cross, twenty one callus lines were obtained from the cotyledonary segments. Eleven of 21 callus lines formed nodular leaves, but only 4 formed elongated normal

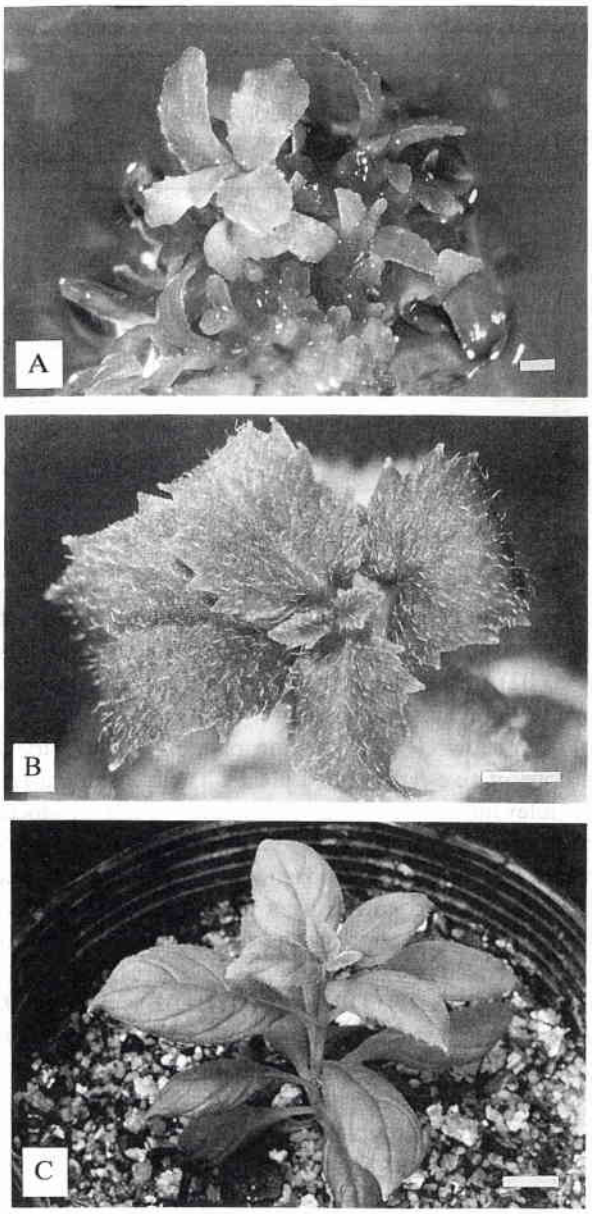

Fig. 1. Plant regeneration via cotyledonay segment culture. (A) Cluster of adventitous buds formed on callus from cotyledonary segments in *Hydrangea macrophylla* 'Blue sky' × *H. arborescens* 'Annabelle' crosses. The photograph was taken 90 days after culture. Scale = 5 mm. (B) A normal shoot regenerated from BA-1 callus line. Scale = 5 mm. (C) A regenerated plant from BA-1 callus line grown in greenhouse conditions. Scale = 10 mm.

shoots (Fig. 1 B). Eventually, a single callus (BA-1) line produced plantlets which were transferred to a greenhouse (Fig. 1 C). Nine shoots were regenerated from the BA-1 line, but only one shoot grew vigorously and developed roots. Other shoots became chlorotic and browning and died shortly after being transplanted to a rooting medium. The surviving plantlet was re-propagated *in vitro* on the basal medium without any hormones. Seven plantlets which were recovered and acclimatized to greenhouse conditions took 12 to 15 months from the beginning of ovule culture to be transferred in soil. Five plants, regenerated from the BA-1 line, grew normally in a greenhouse but they did not reach the flowering stage until 6 months after being transplanting in soil.

Callus from embryos of *H. macrophylla* 'Blue diamond' × *H. arborescens* 'Annabelle' generally grew faster and more abundantly than did those of *H. macrophylla* 'Blue sky' × *H. arborescens* 'Annabelle'. In embryos of *H. macrophylla* 'Blue diamond' × *H. arborescens* 'Annabelle' crosses, 34 callus lines were obtained from the cotyledonary segments, of which 11 developed shoots. The shoot tip became brown and its growth was arrested. These plantlets did not developed sufficiently to be transplanted to soil.

Confirmation of hybridity of regenerated plants

1. Morphological characteristic

The morphology of regenerated plants grown in a

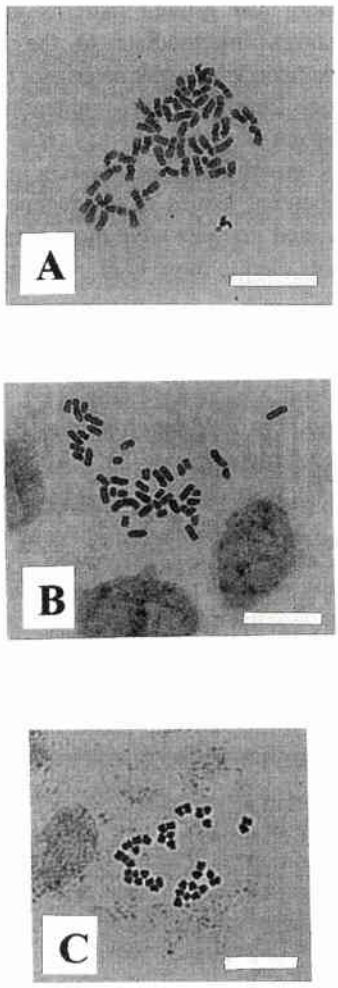

Fig. 2. Metaphase plate of chromosomes in the root tips of *Hydrangea macrophylla* 'Blue sky', 2n = 52 (A), a regenerated plants from BA-1 callus line, 2n = 42 (B) and *H. arborescens* 'Annabelle', 2n = 38 (C). Scale = 10 μm.

Table 1. Production of putative hybrid plants between *Hydrangea macrophylla* and *H. arborescens* by cotyledonary segment culture.

Cross combination	Medium[w]	No. of cotyledonary segments cultured	No. of callus[x] lines obtained	No. of callus[y] lines forming normal shoots *in vitro*	No. of callus lines[z] from which plants were transferred in soil	No. of plantlets grown in a greenhouse
H. macrophylla 'Blue sky'						
× *H. arborescens* 'Annabelle'	+	40	21	4	1	5
	−	10	0	—	—	—
H. macrophylla 'Blue diamond'						
× *H. arborescens* 'Annabelle'	+	40	34	11	—	—
	−	10	0	—	—	—

[z] No. of callus lines from which putative hybrid plants were transferred in soil 8 months after culture of cotyledonary segments. The putative hybrid plants were recovered from a single callus line (BA-1).
[y] No. of callus lines formed normal shoots 120 days after culture of cotyledonary segments.
[x] No. of callus lines obtained 60 days after culture of cotyledonary segments.
[w] + and − indicate basal medium with and without 8.87 μM BA, respectively, used for callus induction.

greenhouse from BA-1 line was investigated. The regenerated plants had growth patterns and morphological appearances intermediate to the parents. *H. arborescens* 'Annabelle' is characterized by extended woody stems with long internodes, subtending by thinly textured, pubescent leaves; whereas *H. macrophylla* 'Blue sky' has green stems subtending glossy, thick leaves. The pubescent leaves of the regenerated plants with deeply serrated margins were more similar to those of *H. arborescens* than to those of *H. macrophylla*.

2. Chromosome number

The chromosome numbers of 5 regenerated plants transplanted to soil are 2n = 42 (Fig. 2 B), whereas the chromosome numbers of *H. macrophylla* 'Blue sky' and *H. arborescens* 'Annabelle' are 2n = 52 (Fig. 2 A) and 2n = 38 (Fig. 2 C), respectively.

3. RAPD analysis

RAPD banding patterns produced with DNA from 10 individuals of a parental cultivar were identical (data not shown). Reproducible parent-specific makers were selected for the identification of hybridity. Of the 40 primers examined, 4 (OPA-09, OPA-20, OPB-01 and OPB-02) turned out to be informative and to give at least one polymorphic band.

Fig. 3 shows RAPD banding patterns of the parents and one of five regenerated plants. Table 2 summarises the relevant results with 4 primers which produced parent-specific polymorphic bands. The present (+) or absent (−) symbols demonstrate that the regenerated plants can be recognized by a unique banding pattern. Bands from the parents are present in the fingerprint of the regenerated plants.

Primers OPA-09, OPA-20, OPB-01 and OPB-02 yielded reproducible patterns and gave polymorphic markers. The primer OPB-01 yielded banding patterns of regenerated plant resembling that of the male parent as well as producing two male-specific and a female-specific RAPD markers. The 0.94-kb and 0.47-kb bands are present in the male parent and the regenerated plants, but not in the female parent. In contrast, the 2.14-kb band was exclusively present in the female parent (Fig. 3). The primer OPA-20 also produced a

Table 2. Results of RAPD analysis to confirm the hybridity of regenerated plants.

Primer	No. of polymorphic bands	Size of polymorphic band (kb)	Presence or absence of the band in the parents and regenerated plants[z]		
			Hydrangea macrophylla 'Blue sky'	Regenerated plants	*H. arborescens* 'Annabelle'
OPA-09	2	1.98	+	+	−
		0.90	+	+	−
OPA-20	1	1.12	−	−	+
OPB-01	3	2.14	+	−	−
		0.94	−	+	+
		0.47	−	+	+
OPB-02	1	2.71	+	+	−

[z] +and - indicate presence and absence of the bands, respectively.

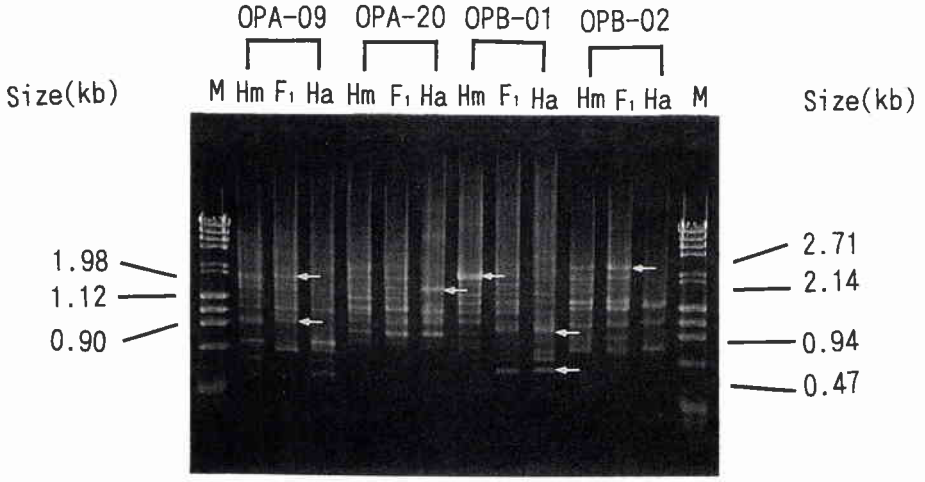

Fig. 3. RAPD profiles of *Hydrangea macrophylla* 'Blue sky' (Hm), regenerated plant (F_1) and *H. arborescens* 'Annabelle' (Ha). The profiles were generated by primer OPA-9, OPA-20, OPB-1 and OPB-2. Arrows indicate parent-specific polymorphic bands. Lane M represents molecular size marker (λ /*Hind* III digest- ϕ X174/*Hae* III digest).

male-specific RAPD marker. Although the 1.12-kb band is present in the male parent, the regenerated plants and the female parent are devoid of the band. The primer OPA-09 and OPB-02 produced female-specific RAPD markers in the regenerated plants. The 1.98-kb and 0.90-kb bands from the OPA-09 primer and 2.71-kb band from the OPB-02 primer are present in the female parent and the regenerated plants, but absent in the male parent. Identical RAPD patterns were detected in all five regenerated plants.

Discussion

In this study, we developed a cotyledonary segment culture for circumventing the hybrid lethality between *H. macrophylla* and *H. arborescens*. The derived plants were demonstrated to be hybrids by morphological traits, cytological characteristics and RAPD banding pattern.

The cotyledonary segment culture method is useful only for crosses of *H. macrophylla* 'Blue sky' × *H. arborescens* 'Annabelle'; no plants were recovered from crosses of *H. macrophylla* 'Blue diamond' × *H. arborescens* 'Annabelle'. Differences in the recovery of plants from the two crosses indicate that the genotype of female parent could significantly affect hybrid recovery. Kudo and Niimi (1999) obtained fewer embryos of *H. macrophylla* 'Blue sky' × *H. arborescens* 'Annabelle' than from those of *H. macrophylla* 'Blue diamond' × *H. arborescens* 'Annabelle' through the ovule culture method, indicating that embryo recovery and plantlet regeneration are independent events with different responses during *in vitro* culture. The responses may be attributed to differences in genetic compatibilities and regeneration capacity between parental germplasm. Consequently, plantlets were regenerated from only a single callus line in *H. macrophylla* 'Blue sky' × *H. arborescens* 'Annabelle'.

Several sexual barriers are known to limit the production of hybrids between distantly related species. In several crosses between relatives of barley, wheat, and cotton, seedling lethality is common, and the weak, chlorotic hybrids die before reaching maturity (Brar and Khush, 1986). Hybrid lethality or weakness may be due to the action of specific genes, disharmony between the nucleous of one species and the cytoplasm of another. In this study the production of hybrid plants from only a single callus line appears to result from the elimination of specific genes that cause lethality of hybrids during callus and/or shoot formation. Callus may be associated with overcoming hybrid lethality in that its growth activates transposable elements and stimulate the appearance of stress-induced enzyme (McClintock, 1984). Although the effect of culture conditons on *in vitro* mutagenesis remains unclear, Hirochika et al. (1996) reported that retrotransposons of rice were activated under tissue culture conditions; he suggested that the retrotransposons are involved in mutations induced by tissue culture.

Overcoming the hybrid lethality between interspecific crosses has been reported in *Nicotiana* (Inoue et al., 1997; Lloyd, 1975; Reed and Collins, 1978). Lloyd (1975) cultured cotyledonary segments of hybrids between *N. suaneolens* and *N. tabacum* on a modified MS medium containing plant growth regulators before the hybrids exhibited lethal symptoms; he succeeded in obtaining viable hybrid plants via callus. We consider that the callus phase is necessary to ovecome hybrid lethality; whereas plant growth regulators enhance the screening of cells with the mutant gene responsible for the lethality.

In this study, the regenerated hybrid plants showed aneuploidy. Chromosome numbers of the regenerated

plants were 2n = 42, whereas the chromosome numbers of *H. macrophylla* 'Blue sky' and *H. arborescens* 'Annabelle' were 2n = 52 and 2n = 38, respectively; therefore, the regenerated plants seemed to have incomplete chromosome complements of the parents. Chromosome numbers of *H. macrophylla* and *H. arborescens* have been reported to be 2n = 36 (McClintock, 1957); however, no detailed study has been published. This contradiction is the subject for a future study. The mechanism whereby hybrid plants with aneuploidy are obtained is not clear. However, genetic distance between parental plants or cultural conditions may play a role in the occurrence of aneuploid. DeVerna et al. (1990) produced the hybrid plants of *Lycopersicon esculentum* × *Solanum rickii* by means of a sesquidiploid bridging hybrid. Of four hybrid progeny obtained, one was diploid and three were aneuploid. Aneuploids from cell and callus cultures have also been obtained in several plant species (Sacristan and Melchers, 1969). Heinz and Mee (1971) obtained callus lines of sugarcane. Each line has a different number of chromosomes. Similar differences were noted among regenerated plants. In callus culture of tetraploid alfalfa, aneuploidy, and chromosome doubling were common among plants regenerated from callus (Bingham and McCoy, 1986).

RAPD profile has recently been used for the identification of hybriditiy in several interspecific hybrid plants (Rieseberg, 1993; McCoy, 1993; Magdalita, 1997). In this study, RAPD analysis provided further evidence that plants regenerated from line BA-1 of *H. macrophylla* × *H. arborescens* are hybrids from their RAPD analyses. However, the use of RAPD markers have some limitations in their application as it can not detect parental alleles. This can be overcome by RFLP markers, although this requires more time and large amount of DNA. Alternatively, RAPD markers can be converted into sequence-tagged sites to allow parental alleles to be identified (Monna et al., 1994).

Although the cotyledonary segment culture method is useful, further work will be required to improve its efficiency. The composition of the medium and the genotype of the parents should be optimized. We are currently testing this method with other *H. macrophylla* cultivars as female parents.

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胚珠培養由来胚の子葉片培養法によるセイヨウアジサイとアメリカノリノキとの種間雑種の作出

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摘　要

前報でセイヨウアジサイ [*Hydrangea macrophylla* f. *hortensia* (Lam.) Rehd.] とアメリカノリノキ (*H. arborescens* L.) の種間交雑において，交配後の胚珠培養で得られた実生が発芽直後あるいは幼苗期に枯死することを報告した．本研究では，雑種致死を回避し健全な雑種植物を得るため胚珠培養由来胚の子葉片を培養する方法を検討した．

セイヨウアジサイ2品種を種子親，アメリカノリノキ1品種を花粉親にした種間交雑で胚珠培養により得た実生の子葉片を植物ホルモン添加培地で培養し，カルスを誘導した．'ブルースカイ' を種子親にした場合，シュートを分化する15のカルス系統を選抜したが，鉢上げ後ガラス温室で順調に生育したのは1系統のみであった．再分化幼植物の雑種性を検定した結果，形態は両親のほぼ中間を示し，染色体数は，種子親の 'ブルースカイ' は 2n =52本，花粉親の 'アナベル' は 2n=38本であったのに対し，再分化植物のそれは 2n=42本で異数性を示した．さらに RAPD のバンドパターンは両親のバンドを併せもった．以上から再分化植物の雑種性は高いと判断された．

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