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Somatic hybridization between *Dianthus chinensis* and *D. barbatus* through protoplast fusion

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Summary. Protoplasts isolated from leaf mesophyll cells of *Dianthus chinensis* and *D. barbatus* were fused by polyethylene glycol (PEG). Calli exhibiting vigorous growth were selected from the PEG-treated protoplasts and shoots were regenerated from one of these calli after 5 months of culture. These shoots readily rooted and continuously produced flowers in the in-vitro condition. The data on flower color, chromosome number, and esterase isozyme patterns indicated that this plantlet was an interspecific somatic hybrid. The hybridity of the plantlet was also confirmed by nuclear rDNA analysis. This report provides the possibility of applying the somatic hybridization technique for the genetic improvement of the genus *Dianthus*.

Key words: *Dianthus* – Protoplast fusion – Somatic hybridization – Ornamental plants – *D. chinensis* – *D. barbatus*

Introduction

Recent developments in the somatic hybridization technique have enabled us to utilize it for the genetic improvement of ornamental, as well as crop, species. To-date, successful results of somatic hybridization have been reported in several ornamental species including *Petunia* (Power et al. 1976) and *Rudbeckia* (Al-Atabee et al. 1990).

The genus *Dianthus* contains many important ornamental species such as the carnation (*D. caryophyllus*), as well as *D. chinensis*, *D. barbatus* and *D. plumarius*. Recently, it has been possible to regenerate plants from the protoplasts of certain *Dianthus* species and cultivars (Nakano and Mii 1992). Nevertheless, the successful production of somatic hybrids in this genus has yet to be achieved. In this paper, we report the production of an interspecific somatic hybrid plant between *D. chinensis* and *D. barbatus*.

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Materials and methods

Plant materials

D. chinensis cv Gosun-sekichiku and *D. barbatus* were used as plant material. The seeds of these two species were obtained from Sakata Seed Corporation, Yokohama, Japan. They were maintained as axenic shoot cultures on half-strength MS medium (Murashige and Skoog 1962), lacking growth regulators but with 2% sucrose and 0.8% agar, at 27 °C under continuous illumination (35 μ mol photons $m^{-2}s^{-1}$) with fluorescent lamps.

Protoplast isolation and fusion

Protoplasts were isolated from fully expanded leaves of both parental species according to the methods of Nakano and Mii (1992). The purified protoplasts were suspended in an 0.5 M mannitol solution at a density of 1×10^6/ml. Protoplast suspensions of both species were mixed in equal volumes and 1 ml of the mixture was further mixed with an equal amount of polyethylene glycol (PEG) solution [40% (w/v) PEG (MW 4000) and 50 mM $CaCl_2 \cdot H_2O$ in 50 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) buffer, pH 6.5] in 6 cm Petri dishes. After incubation at 25 °C for 30 min, 10 ml of washing solution (50 mM $CaCl_2 \cdot 2H_2O$ in 0.5 M mannitol solution, pH 10.5) was added gradually. The PEG-treated protoplasts were collected by centrifugation (120 g for 3 min) and washed twice with an 0.5 M mannitol solution.

Protoplast culture and plant regeneration

PEG-treated protoplasts were suspended in MS medium containing 5 mg/l naphthaleneacetic acid (NAA), 1 mg/l zeatin, 2% sucrose and 0.5 M mannitol at a density of 1×10^5/ml.

Three-millilitre aliquots of protoplast suspensions were dispensed into 6-cm diameter Petri dishes, which were then maintained at 27 °C in the dark. After 2 months of culture, visible colonies (approximately 1 mm diameter) were transferred to the callus proliferation medium and vigorously growing calli were further transferred to the shoot regeneration medium (Nakano and Mii 1992). Regenerated shoots were detached from the calli and subcultured into half-strength MS medium containing 2% sucrose and 0.8% agar for rooting. All cultures during and after callus proliferation were kept at 27 °C under continuous illumination (35 μ mol photons $m^{-1} s^{-1}$) with fluorescent lamps. Finally, rooted plantlets were transferred to sterilized soil and grown in the green house.

Isozyme analysis

Leaf tissues (1 g) of in-vitro grown plantlets were homogenized in a mortar with 1 ml of extraction buffer (1 M sucrose and 56 mM 2-mercaptoethanol in 0.2 M tris-HCl buffer, pH 8.5). The homogenate was centrifuged and the supernatant was used as an enzyme extract. Electrophoresis and isozyme staining for esterase were performed according to the method of Wetter and Dyck (1983).

rDNA analysis

Total DNAs were extracted from in-vitro growing plantlets according to the method of Rogers and Bendich (1985). DNAs digested with restriction enzymes were separated on agarose gels and blotted onto nylon membrane filters (Hybond-N, Amersham). Analyses of rDNA were performed using a non-radioactive DNA labeling and detection (Boehringer Mannheim). The DNA fragment containing the entire rDNA sequences of rice (Takaiwa et al. 1985) was prepared from plasmid pRR217 and used as a probe.

Chromosome counts and pollen viability

Root tips were excised from in-vitro growing plantlets and treated with 8-hydroxyquinoline (2 mM) for 3 h at 27 °C. Root tips were then rinsed with distilled water and hydrolyzed for 3 min at 60 °C with 1 N HCl, and stained with aceto-orcein.

Mature pollen grains taken from in-vitro flowering plantlets were stained with 2% acetocarmine for assessing pollen fertility.

Results and discussion

The present study was carried out to examine the possibility of somatic hybridization in the genus *Dianthus*. We have chosen two species, *D. chinensis* and *D. barbatus*, for the following reasons. *D. chinensis* has been shown to have a high shoot regeneration ability from leaf mesophyll protoplasts, while the protoplast-derived callus of *D. barbatus* has regenerated shoots at a very low frequency under the same culture conditions (Nakano and Mii 1992). In addition, protoplast- or cotyledon-derived shoots of *D. barbatus* produced flowers immediately after shoot regeneration in an in-vitro condition (unpublished results). Therefore, we expected that somatic hybrids could be detected by shoot regeneration with precocious flowering and the expression of novel flower color and morphology.

Fusion frequency for the mixed parental mesophyll protoplasts after PEG treatment was approximately 2%, but the frequency of heterokaryon formation could not be determined because of the similarity of both parental protoplasts. PEG-treated protoplasts started to divide after 10 days of culture and formed visible colonies (approximately 1 mm in diameter) after 2 months. On transferring these colonies to the callus proliferation medium, some calli showed vigorous growth compared to the other calli and those of control parental cultures. These vigorously growing calli were then picked up and transferred to the regeneration medium. Among 30 calli transferred, two formed shoot buds and one of them regenerated many shoots 2 months after transfer. In the control parental cultures, approximately 30% of protoplast-derived calli of *D. chinensis* regenerated shoots, whereas no shoot regeneration was observed from protoplast-derived calli of *D. barbatus*.

Shoots obtained from PEG-treated protoplasts produced flowers immediately after shoot regeneration (Fig. 1). The flower color of these shoots (light pink with red stripes) was distinctly different from those of both parental plants (*D. chinensis*: deep pink, *D. barbatus*: red with white rim) (Fig. 2). Therefore, it

Fig. 1. Shoots derived from PEG-treated protoplasts which produced flowers immediately after shoot regeneration

Fig. 2. Flowers of the parental species and the somatic hybrid. *DC*, *D. chinensis*; *SH*, somatic hybrid; *DB*, *D. barbatus*

Fig. 3. Somatic hybrid plantlet flowering in vitro

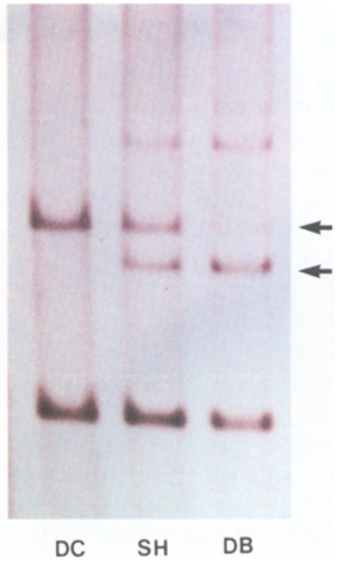

Fig. 5. Blot-hybridization of digoxigenin-labeled rDNA fragments to *Eco*RV-digested total DNAs of the parental species and the somatic hybrid. *Arrows*, from top to bottom, indicate the 6.5-kbp and 6.0-kbp fragments respectively. *DC*, *D. chinensis*; *SH*, somatic hybrid; *DB*, *D barbatus*

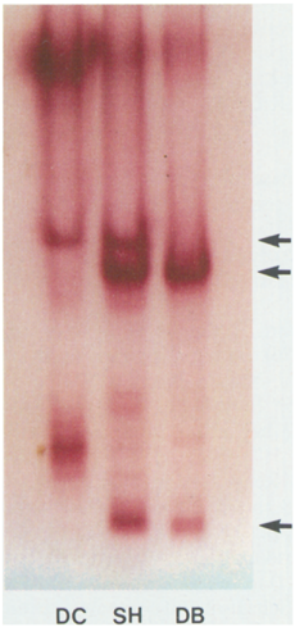

Fig. 4. Esterase isozyme patterns for the parental species and the somatic hybrid. *Arrows* indicate bands specific to the parental species. *DC*, *D. chinensis*; *SH*, somatic hybrid; *DB*, *D. barbatus*

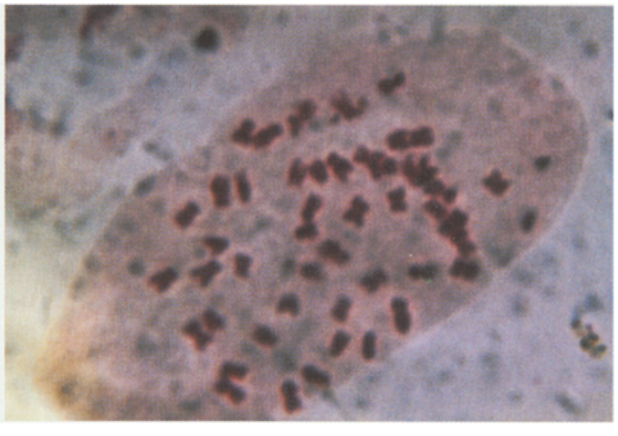

Fig. 6. Chromosomes in a root-tip cell of the somatic hybrid

was concluded that these shoots were derived from a fusion product between *D. chinensis* and *D. barbatus*. The shoots which grew up to 1–2 cm in height were detached from the callus and transferred to half-strength MS medium lacking growth regulators but

with 2% sucrose and 0.8% agar. Roots were formed within 2 weeks and the plantlets thus obtained continuously produced flowers in an in-vitro condition (Fig. 3). These plantlets were severely dwarf with short internodes and dark green leaves as compared with parental plantlets growing in-vitro. The addition of 10 mg/l of gibberellic acid (GA_3) to the rooting medium induced elongation of shoots but did not stop flowering. Transfer of the cultures to a short day condition (8 h photoperiod) did not affect flowering. Continuous flower production was still observed after acclimatization though most plantlets ultimately died after transfer to soil.

To confirm the hybridity of the regenerated plantlet, esterase isozyme analysis and restriction endonuclease analysis of rDNA were employed. Figure 4 shows the esterase isozyme patterns obtained. Zymograms were distinctly different between *D. chinensis* and *D. barbatus* and the regenerated plantlet had common bands of both parents. Restriction endonuclease analysis of rDNA provided additional evidence for hybridization. Among the restriction enzymes tested, *Eco*RV was shown to be the best one for discriminating between rDNA fragments of *D. chinensis* and *D. barbatus* (Fig. 5). A clear and specific rDNA fragment originating from the nuclear DNA of *D. chinensis* was 6.5 kbp in length, while that of *D. barbatus* was 6.0 kbp. *Eco*RV-digested nuclear DNA of the regenerated plantlet contained both 6.5- and 6.0-kbp fragments. These results confirmed that the regenerated plantlet is indeed an interspecific somatic hybrid between *D. chinensis* and *D. barbatus*.

The results from chromosome counts of the somatic hybrid revealed that it had $2n = 52$ (Fig. 6), which was less than the sum of *D. chinensis* ($2n = 30$) and *D. barbatus* ($2n = 30$). Thus the somatic hybrid obtained in this experiment was aneuploid. It would appear that a certain number of chromosomes were eliminated during protoplast culture and/or shoot regeneration. However, because of the difficulty in identifying the individual chromosomes of each species, it is still unclear whether specific elimination of parental chromosomes occurred. Although most flowers of the somtic hybrid plantlets were male sterile with undeveloped stamens, some flowers developed stamens with mature pollen grains. Pollen fertilities of these flowers were up to 60% as determined by acetocarmine staining.

In this study, we have obtained an interspecific somatic hybrid without any artificial selection. The somatic hybrid was regenerated from a callus, which showed vigorous growth on the callus proliferation medium. These results suggest that at least some hybrid vigor was expressed during the callus proliferation stage. Similar observations have been reported in an intraspecific combination involving *Solanum tuberosum* (Austin et al. 1985; Waara et al. 1989), in interspecific combinations in *Datura* species (Schieder 1978) and *Brassica* species (Taguchi and Kameya 1986), as well as an intergeneric combination between *Lycopersicon esculentum* and *Solanum muricatum* (Sakamoto and Taguchi 1991).

The somatic hybrid obtained in the present study was severely dwarf and continuously produced flowers. These abnormal characters appear to be induced by some physiological disorders rather than as a consequence of somaclonal variation, aneuploidy or genomic incompatibility, because comparable morphologies have often been observed in the regenerants from protoplasts of several *Dianthus* cultivars (Nakano and Mii 1992). Further experimentation should be directed to offset the production of these abnormal characters.

In this study, we confirm the possibility of applying the somatic hybridization technique for the genetic improvement of ornamental plants belonging to the genus *Dianthus*. Some of the hybrid plants are now growing in the greenhouse and further characterization and seed production of this somatic hybrid are now in progress.

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