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Intergeneric somatic hybrid plantlets between *Dianthus barbatus* and *Gypsophila paniculata* obtained by electrofusion

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Abstract Hypocotyl-derived protoplasts of *Dianthus barbatus* that had been pretreated with iodoacetamide were fused electrically with cell suspension culture-derived protoplasts of *Gypsophila paniculata* that could divide to form callus but could not regenerate shoots under the culture conditions used in this study. Electrofusion-derived calli which produced shoots were selected as putative somatic hybrids, and plantlets were subsequently regenerated from 2 of these selected calli. These plantlets, which in vitro produced flowers precociously, were identified as intergeneric somatic hybrids by nuclear ribosomal DNA analysis. Normal plants have not been established up to the present.

Key words *Dianthus barbatus* · Electrofusion · *Gypsophila paniculata* · Intergeneric somatic hybridization · Plantlet regeneration

Introduction

Several species in the family Caryophyllaceae, such as *Dianthus* spp. and *Gypsophila* spp., are widely cultivated as ornamental crops. The breeding of these species has been based on intra- and interspecific hybridization and sport selection, and no intergeneric hybridization has been adopted due to the sexual incompatibility barrier. Somatic hybridization by protoplast fusion offers an alternative method to conventional sexual hybridization for obtaining hybrids between distantly related species (Sink 1991), and intergeneric transfer of desirable traits with respect to floral and marketable qualities can be expected in both *Dianthus* and *Gypsophila* by applying this somatic hybridization technique.

In *Dianthus* species, protoplast culture systems have been established (Nakano and Mii 1992, 1995), and interspecific somatic hybrid plants have also recently been produced (Nakano and Mii 1993a,b). However, intergeneric somatic hybrid plants between the two genera mentioned above have not yet been produced, although callus and root formation have been demonstrated from an intergeneric somatic hybrid between *D. caryophyllus* and *G. paniculata* (Nakano and Mii 1993c). The present study describes the production of intergeneric somatic hybrid plantlets between *D. barbatus* and *G. paniculata* by combining electrofusion and a selection method based on iodoacetamide (IOA) inactivation and regeneration ability (Nakano and Mii 1993b).

Materials and methods

D. barbatus ($2n = 2x = 30$) and *G. paniculata* cv 'Bristol Fairy' ($2n = 2x = 34$) were used as plant materials. Cell suspension cultures of *G. paniculata* were induced and maintained for about 3 months prior to use in protoplast isolation according to the method described previously (Nakano and Mii 1993c).

Protoplasts were isolated from hypocotyls harvested from 7-day-old in vitro seedlings of *D. barbatus* (Nakano and Mii 1995) and from suspension cultures of *G. paniculata* 4 days after subculture (Nakano and Mii 1993c). Prior to electrofusion, protoplasts of *D. barbatus* were treated with 10 m*M* IOA as described previously (Nakano and Mii 1993b). Electrofusion was carried out using a Somatic Hybridizer SSH-1 (Shimadzu Corp, Japan). Protoplasts of each species were suspended in an electrofusion buffer containing 0.5 *M* mannitol, 1 m*M* $CaCl_2 \cdot 2H_2O$, and 5 m*M* 2-*N*-morpholinoethane sulfonic acid (MES) at a density of $0.5–1 \times 10^6$ ml^{-1}. Protoplast suspensions of *D. barbatus* and *G. paniculata* were mixed in an equal volume, and a 1 ml aliquot of the mixture was introduced into a fusion chamber. The protoplasts were then subjected to an alternating current (AC) field of 2 MHz, 50 V cm^{-1} for 20 s, followed by a 30-ms direct current (DC) pulse of 1 kV cm^{-1} that was applied twice. Protoplast culture and subsequent shoot induction from protoplast-derived calli were carried out according to the method of Nakano and Mii (1995). Regenerated shoots were detached from the callus and subcultured onto half-strength MS media (Murashige and Skoog 1962) with or without 1 mg l^{-1} gibberellic acid (GA_3).

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Ribosomal DNA (rDNA) analysis and chromosome counts were performed as previously described (Nakano and Mii 1993a).

Results and discussion

The mean frequency of heterokaryon formation after electrofusion was estimated to be 5.2%, which was higher than that previously obtained from polyethylene glycol (PEG)-mediated fusion between *D. caryophyllus* and *G. paniculata* (2–3%) (Nakano and Mii 1993c). In addition, the electrofusion was less complicated and less time-consuming than PEG-mediated fusion. Therefore, it can be concluded that the electrofusion technique can be effectively used as a routine fusion procedure for somatic hybridization in Caryophyllaceae.

Electrofusion-treated protoplasts started to divide within 7 days in culture and formed a number of visible colonies (about 1 mm in diameter) after 2 months. Under the culture conditions used in this study, cell suspension-derived protoplasts of *G. paniculata* also formed many visible colonies. On the other hand, cell division was never observed in IOA-treated hypocotyl protoplasts of *D. barbatus*, even when they were co-cultured with *G. paniculata* protoplasts. Therefore, visible colonies formed after electrofusion seemed to be derived from either *G. paniculata* protoplasts or heterokaryons. From five independent fusion experiments, over 300 calli were obtained, and these were then transferred to a shoot induction medium. Of these calli, 8 formed green shoot primordia, and of these 8, 2 developed many shoots; these 2 calli were derived from independent fusion experiments. The remaining 6 calli turned brown without shoot development and ultimately died. In the control cultures, calli derived from *G. paniculata* protoplasts never regenerated shoots under the culture conditions used in this study, while a shoot regeneration frequency of about 8% was obtained from those derived from non-IOA-treated protoplasts of *D. barbatus*.

Regenerated shoots from two putative hybrid calli (SH-1 and SH-2) were detached from the calli and transferred to half-strength MS medium with or without GA_3. Shoots from both SH-1 and SH-2 developed a few roots within 2 weeks on both media. All plantlets on plant growth regulator-free medium were severely stunted (dwarf) and flowered precociously in vitro (Fig. 1). The addition of GA_3 to the medium induced shoot elongation but did not affect the flowering of these plantlets. The flowers of both the SH-1 and SH-2 plantlets were red with white centers, while the parental *D. barbatus* and *G. paniculata* used in this study had red and doubled-white flowers, respectively. All of these flowers were male-sterile with undeveloped stamens. SH-1 and SH-2 plantlets also continued to produce flowers during acclimatization but ultimately died after transfer to the greenhouse. Although they have been subcultured for over 2 years on both plant growth regulator-free and GA_3-containing media, plants with a normal growth pattern were not induced from these abnormal plantlets.

Figure 2 shows blot-hybridization patterns of digoxigenin-labeled rDNA fragments to *Bam*HI-diges-

Fig. 1 Intergeneric somatic hybrid plantlets between *D. barbatus* and *G. paniculata* (SH-1). *Bar*: 2 cm

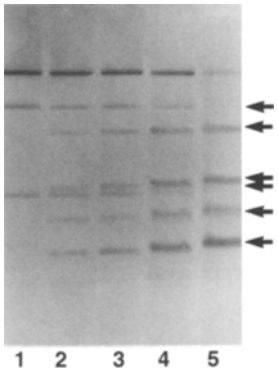

Fig. 2 Blot-hybridization patterns of digoxigenin-labeled rDNA fragments to *Bam*HI-digested total DNAs of parental species and somatic hybrid plantlets. *Arrows*, from *top to bottom*, indicate the 7.6-, 6.7-, 5.6-, 5.5-, 5.1-, and 4.6-kbp fragments, respectively. *Lane 1* *G. paniculata*, *lanes 2* and 3 somatic hybrid SH-1, *lane 4* somatic hybrid SH-2, *lane 5* *D. barbatus*

ted total DNAs of the parental species and putative somatic hybrid plantlets (SH-1 and SH-2). *Bam*HI digestion gave fragments of 6.7, 5.6, 5.1, and 4.6 kbp that were specific to *D. barbatus*, and of 7.6 and 5.5 kbp that were specific to *G. paniculata*. Both SH-1 and SH-2 had fragments specific to both parents. This indicates that both SH-1 and SH-2 are intergeneric somatic hybrids between *D. barbatus* and *G. paniculata*. Chromosome counts of 2 hybrid plantlets revealed that they had about $2n = 55$, which was less than the sum of *D. barbatus* ($2n = 30$) and *G. paniculata* ($2n = 34$), indicating that both SH-1 and SH-2 were asymmetric hybrids.

All of the intergeneric somatic hybrid plantlets obtained in the present study were severely dwarf and flowered precociously. In the genus *Dianthus*, it has already been reported that plantlets showing abnormal characters similar to those observed in the present study were occasionally regenerated from protoplasts (Nakano and Mii 1992, 1995). Although the mechanism involved in the production of such abnormal plants has not yet been identified, these abnormal characters appeared to be induced by some transient physiological disorders rather than to arise as a result of genetic variations since plants not exhibiting these characters sometimes developed from abnormal ones during prolonged culture both in vitro and in the greenhouse (Nakano and Mii 1993d). Therefore, it is possible that the abnormality of the intergeneric somatic hybrid plantlets obtained in the present study was also induced by some physiological disorders and that hybrid plants with a more normal morphology can be obtained from these plantlets even though they remain abnormal for more than 2 years. However, it is also possible that the abnormality observed in this study is caused by some genetic incompatibilities, as has been reported for somatic hybridization studies between distantly related species (Melchers et al. 1978; Gleba and Hoffmann 1980; Harms 1983; Terada et al. 1987; Fahleson et al. 1994). In this case, fertile hybrid plants with a normal morphology may be obtained through an asymmetric hybridization by irradiating protoplasts of one of the parental species prior to fusion, as has been suggested by Hinnisdaels et al. (1988). To elucidate the causal factors of the abnormal growth of the intergeneric hybrids obtained in the present study, further studies are needed, such as an examination of the cultural conditions required for inducing plants with a normal morphology from abnormal hybrid plantlets and/or the production of asymmetric somatic hybrids by the irradiation of one of the parental protoplasts prior to fusion.

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