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Application of Plant Biotechnology for Breeding of Flower Crops in the Genus *Dianthus*

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Introduction

Flower crops may have some advantages in the application of biotechnology, such as somatic hybridization and genetic transformation, for their genetic improvement. Since flower crops are usually evaluated for their ornamental value, somatic hybrids or transgenic plants with some ornamental value can be used commercially without any critical breeding especially in vegetatively propagated species. In addition, sterile plants occasionally obtained from somatic hybridization between distantly related species can also be used by clonal propagation using tissue culture methods. However, in contrast with food crops, only a few studies had previously been reported on the application of biotechnology in flower crops except for several Solanaceous species[1,2].

The genus *Dianthus* belongs to the family Caryophyllaceae and consists of more than 300 species. This genus contains some important flower crops, such as *D. caryophyllus* (carnation), *D. chinensis, D. barbatus* and *D. plumarius,* among which carnation in particular is known to be one of the world's most important flower crops and several cell and tissue culture methods including meristem, callus and cell suspension cultures have already been developed[3]. Although somatic hybridization and genetic transformation techniques are expected to be applied to the genetic improvement of floral and marketable qualities in *Dianthus* species, as with many other flower crops only a few studies have been reported on these techniques. In this review, we report the establishment of plant regeneration systems from several sources of explants, production of somatic hybrids by protoplast fusion, and genetic transformation in the genus *Dianthus.* The possibility of the practical application of biotechnology in *Dianthus* and the related genera are also discussed.

Plant regeneration in *Dianthus*

Establishment of efficient systems of plant regeneration from cultured explants is indispensable for realizing the application of biotechnology, such as somatic hybridization and *Agrobacterium*-mediated genetic transformation, for plant breeding. We examined the requirements for plant regeneration from protoplasts, and leaf and petal explants.

1. *Plant regeneration from protoplasts*

Although several papers have already appeared on protoplast culture in *Dianthus*[4-6], protoplast-derived plants were obtained only in an interspecific hybrid cultivar between *D. caryophyllus* and

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Table 1. Difference in shoot regeneration from leaf mesophyll protoplast-derived calli among the several species and cultivars in *Dianthus*[7].

Dianthus species	Shoot regeneration (%)
D. caryophyllus	
cv. Lena	0
cv. Scania	1.1
cv. Coral	0
cv. White Sim	0
cv. Chabaud	0
D. chinensis	
cv. Gosun-sekichiku	31.2
cv. Seiyou-sekichiku	23.3
cv. Snow Fire	35.3
D. barbatus	1.1
D. plumarius	2.6
D. superbus	0
D. japonicus	0
D. caryophyllus × *D. chinensis*	
cv. Eolo	4.2
cv. Mikado-nadeshiko	8.3
D. chinensis × *D. barbatus*	
cv. Telstar Scarlet	43.3

Protoplast-derived calli were placed on MS medium containing 1 mg/*l* NAA and 5 mg/*l* zeatin, and data were recorded after 4 months.

D. chinensis[5]. Therefore, we screened various genotypes and explants for establishing an efficient sysetm for plant regeneration from protoplasts in *Dianthus* species.

First, leaf mesophyll protoplasts were isolated from 17 genotypes listed in **Table 1** and cultured under the same conditions[7]. In all cultivars tested, viable protoplasts were isolated at high yields and some of these protoplasts divided and formed colonies. However, shoot regeneration was markedly different among the species as shown in **Table 1.** High frequency regeneration was obtained from *D. chinensis* and its interspecific hybrid cultivars, while only low frequency or no shoot regeneration was obtained from the other species. These results suggest that *D. chinensis* may have a high shoot regeneration ability, which is genetically controlled and can be transferred to the interspecific cultivars. Thus, *D. chinensis* appear to be a suitable material as a target for applying biotechnology and also be used as a genetic source for improving plant regeneration ability of other recalcitrant species.

High frequency shoot regeneration from leaf mesophyll protoplasts was not achieved in most *Dianthus* species except for *D. chinensis* despite of the attempts for modifying culture media and conditions. Therefore, we then screened various protoplast sources such as hypocotyls and young petals for obtaining efficient plant regeneration using two recalcitrant species, *D. caryophyllus* cv. Chabaud (a seed-propagated cultivar) and *D. barbatus*[8]. Protoplasts isolated from young petals of two species showed low yield and low division frequency. On the other hand. division frequency of hypocotyl-derived protoplasts was higher than that of leaf-derived protoplasts, although protoplast yield in hypocotyls was slightly lower than in leaves. Shoot regeneration from protoplasts was markedly different among the protoplast sources in *Dianthus* species. Among the sources examined, relatively high frequency shoot regeneration was obtained in hypocotyl- and petal-derived proto-

Teble 2. Difference in shoot regeneration from protoplast-derived calli among different sources for protoplast isolation in two *Dianthus* species[8].

Dianthus species	Protoplast sources	Shoot regeneration (%)
D. caryophyllus		
cv. Chabaud	leaves	0
	hypocotyls	4.5
	petals	1.2
D. barbatus	leaves	0.8
	hypocotyls	7.6
	petals	5.5

Protoplast-derived calli were placed on MS medium containing 1 mg/*l* NAA and 5 mg/*l* zeatin, and data were recorded after 4 months.

plasts in both species, while only low frequency or no shoot regeneration occurred in leaf-derived protoplasts (**Table 2**). These results suggest that hypocotyl is a suitable source for protoplasts with high yield and high regeneration ability in these recalcitrant species. However, since many *Dianthus* cultivars (particularly those of *D. caryophyllus*) are vegetatively propagated, petals may be suitable for protoplast isolation in those cultivars. Further experimentation should be directed to increase the yield and division frequncy of petal-derived protoplsts.

Generally, protopast-derived shoots regenerated roots and were successfully transferred to the greenhouse. In *D. barbatus* and some interspecific cultivars, however, most of the shoots continuously produced flowers in *vitro* and often failed to regenerate normal roots. In all cultivars tested in **Table 1,** at least some morphological differences including reduced growth and early flowering were observed in protoplast-derived plants.

2. *Adventitious shoot regeneration from cultured petal explants of D. caryophyllus*

To date, adventitious shoot regeneration in *D. caryophyllus* has been reported from hypocotyls[9], shoot apices[10], axillary buds[11], stems[12] and petals[12-14]. We selected leaves, stems and petals and used for inducing adventitious shoot regeneration mainly using cv. Scania[15]. Among the explants examined, petal explants regenerated shoots most effectively, while the others regenerated only few shoots (**Table 3**). Furthermore, regeneration frequency was also highly dependent on the petal stage, and a significant decrease in the regeneration ability was observed in petals from fully-opened flowers (**Table 3**). High frequency shoot regeneration of up to 80% was also obtained from all other *D. caryophyllus* cultivars listed in **Table 1** by using petal explants harvested from flower buds, despite minor differences in frequency among the cultivars. Thus, it was shown that the petal explants were suitable for inducing high frequency adventitious shoot regeneration in *D. caryophyl-*

Table 3. Comparison in adventitious shoot regeneration frequency from various explants of *D. caryophyllus* cv. Scania[15].

Explant	Regeneration frequency (%)
Leaf	7
Stem	12
Petal (from flower buds)	93
Petal (from fully-opened flowers)	43

Explants were placed on MS medium containing 5 μM NAA and 5 μM thidiazuron, and data were recorded after 2 months.

lus. Precocious flowering *in vitro* including the development of petal-like leaves in petal-derived shoots, which have been reported previously[13], was also occasionally observed in our study. This precocious flowering was, however, effectively prevented by adding 10 to 100 μM GA_3 to the shoot induction and rooting media.

As mentioned above, successes in protoplast culture and plant regeneration in our study were largely dependent on genetic and physiological factors of donor materials. The importance of the donor plant factors in regeneration was also demonstrated in the study of antibiotic-induced somatic embryogenesis of several *Dianthus* cultivars[16]. The plant factors, thus, were the most critial ones for successful establishment of plant regeneration systems in the genus *Dianthus.* Although systems for protoplast culture and plant regeneration in many flower crops are not fully established and we are still far from understanding the cardinal processes of these systems, analyses of various factors including genetic and physiological factors of donor plant materials may lead to the development of such systems and may help to increase knowledge of the affecting factors.

Somatic hybridization and genetic transformation in *Dianthus*

Although the application of biotechnology to flower crops is behind in development, somatic hybridization and genetic transformation have recently been demonstrated in some flower crops other than Solanaceous species[17-21]. In addition, in the last ten years some progress has been shown in the cloning and characterization of genes controlling floriculturally desirable traits[22-24]. These studies indicate that, although late in coming, biotechnology is gradually becoming applicable to flower crops. We examined, therefore, the somatic hybridization of various interspecific or intergeneric combinations, and *Agrobacterium*-mediated genetic transformation in *Dianthus.*

1. *Somatic hybridization*

We initially chose two species, *D. chinensis* and *D. barbatus,* and fused protoplasts isolated from leaves of both species by PEG[25]. *D. chinensis* has shown to have a high shoot regeneration ability from leaf-derived protoplasts, while those of *D. barbatus* has regenerated shoots at a very low frequency under the same cultural conditions. In addition, protoplast-derived shoots of *D. barbatus* have always shown precocious flowering *in vitro.* Therefore, in this study, we expected that a somatic hybrid could be detected by shoot regeneration with precocious flowering and expression of novel flower color.

Somatic hybrid shoos produced flowers immediately after shoot regeneration, which were

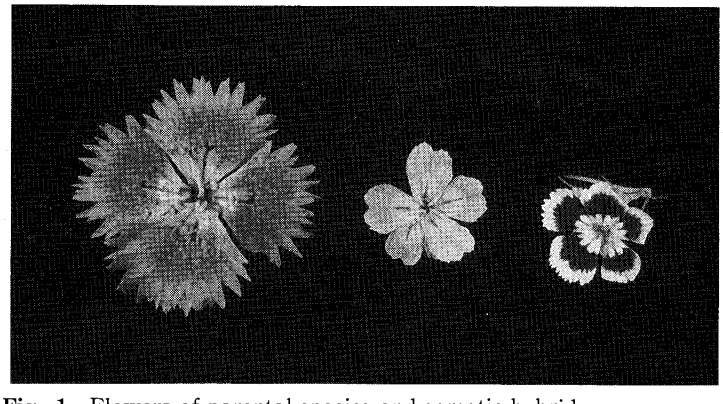

Fig. 1 Flowers of parental species and somatic hybrid.
From left to right ; *D. chinensis,* somatic hybrid, *D. barbatus*[25].

distinctly different from the parental ones (**Fig. 1**). The hybridity of the shoot was confirmed by esterase isozyme and nuclear rDNA analyses. This somatic hybrid was aneuploid, but it is still unclear whether specific elimination of parental chromosomes occurred. Some flowers of this somatic hybrid developed stamens with mature pollen grains and pollen fertilities of up to 60%. Like protoplast-derived plants of *D. barbatus,* this somatic hybrid was severely dwarfed and continuously produced flowers before and after transferring to the greenhouse. However, during the prolonged culture of these abnormal plants both *in vitro* and in the greenhouse, normal often developed from them. Therefore, further characterization of both normal and abnormal shoots derived from the same somatic hybrid plant is now in progress. Trials on seed production from these plants are also being performed.

In the somatic hybridization experiment mentioned above, no special method was employed to increase the efficiency for the selection of somatic hybrids. We, next, employed the selection method using IOA inactivation and the regeneration ability of protoplasts[26], which required neither special plant materials nor special equipment or techniques. Leaf mesophyll protoplasts of *D. caryophyllus,* and those of *D. chinensis* inactivated by IOA treatment were fused by PEG. Since protoplasts of *D. caryophyllus* divide to form callus but not regenerate shoots, fusion-derived calli which regenerated shoots could be tentatively selected as somatic hybrids. From five independent fusion experiments, plants were regenerated from four colonies, and for three of them their hybridity was confirmed by esterase isozyme and RAPD analyses. These plants exhibited intermediate characteristics of both parents in flower morphology (**Fig. 2**). In contrast with the somatic hybridization experiments between *D. chinensis* and *D. barbatus,* all three somatic hybrid plants looked normal and had amphidiploid chromosome number. However, all of them were completely male sterile with undeveloped stamen.

Thus, these two experiments of somatic hybridization suggest that this somatic hybridization technique could be successfully applied to obtain interspecific hybrids in the genus *Dianthus.* Although the combinations of the species used in these somatic hybridization were sexually compatible ones, direct production of amphidiploid or aneuploid plants will have some advantages in the breeding of these flower crops. Further evaluation of the characterization of these somatic hybrid plants is needed. In these studies, it was also confirmed that the regeneration ability is dominantly transmitted to somatic hybrids in this genus. Therefore, it is expected that somatic

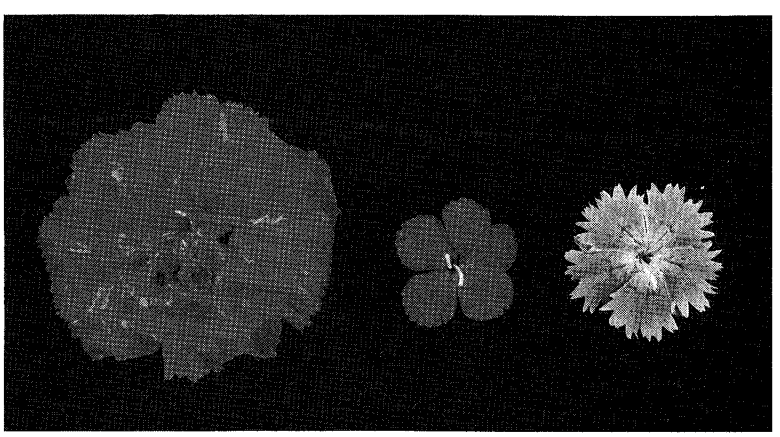

Fig. 2 Flowers of parental species and somatic hybrid.
From left to right ; *D. caryophyllus,* somatic hybrid, *D. chinensis*[26].

hybrids could be easily obtained by utilizing the species combination with high regeneration ability in at least one parent.

On the other hand, we also examined the production of somatic hybrids between sexually incompatible intergeneric combinations, *Dianthus* and *Gypsophila*. In the intergeneric hybridization experiment using protoplasts isolated from cell suspension cultures of *D. caryophyllus* and *G. paniculata*, a hybrid cell line was obtained[27]. Although hybrid plants did not regenerate from an intergeneric somatic hybrid callus, this callus still had some morphogenetic potential probably derived from *G. paniculata* as revealed by root organogenesis. In addition, conservation in this callus of the greater part of nuclear genomes from both parents was revealed by isozyme and rDNA analyses. Thus, notable genomic incompatibility probably do not exist between *Dianthus* and *Gypsophila*, and failure in plant regeneration may be due to the lack of plant regeneration ability in the original cell cultures of both parents. Therefore, intergeneric somatic hybrid plants between these two genera will possibly be obtained by using protoplasts with high shoot regeneration ability as a fusion parent. In fact, several shoots possessing both RAPD markers specifically amplified in the parents were obtained from protoplast fusion between IOA-treated hypocotyl protoplasts of *D. barbatus* and suspension cultured-cell protoplasts of *G. paniculata*[28].

2. *Agrobacterium*-mediated genetic transformation of *D. caryophyllus*

Recently, Lu *et al.* provided a possibility of genetic transformation of *D. caryophyllus*, which has been demonstrated by using the *Agrobacterium*-mediated method[19]. They screened *Agrobacterium* strains for efficient transformation, and transgenic shoots were selected on kanamycin-containing medium after inoculation of stem explants. Over 200 transgenic plants expressing both NPT II and GUS genes were established in soil and flowered in their experiment. We also made an attempt to transform several cultivars of *D. caryophyllus* by *Agrobacterium*-based vectors using petal explants[29], with which an efficient adventitious shoot regeneration system has been developed[15]. Petal explants harvested from flower buds of *D. caryophyllus* cultivars listed in **Table 1** were co-cultivated with either a disarmed *A. tumefaciens* strain LBA4404 or a virulent strain of *A. rhizogenes* A13. Both bacterial strains harboured the binary vector pBI121 which contained NPT II and GUS genes. Co-cultivated explants were cultured on regeneration medium containing kanamycin, and several shoots were selected for kanamycin resistance. However, among the over 100 shoots selected, only two derived from the co-cultivation of cv. White Sim petals with *A. tumefaciens* LBA4404 showed GUS activity measured by histochemical staining. Thus, transformation frequency was still low and further study should be directed to screen suitable selection markers as well as to screen "virulent" *Agrogacterium* strains and "susceptible" *Dianthus* genotypes, in order to increase transformation frequency. On the other hand, transformation of *Dianthus* species mediated by direct DNA uptake also should be examined using the protoplast culture system developed in our study[7,8].

Conclusions and further prospects

Some flower crops are now considered as attractive targets for applying biotechnology for breeding, although a few papers have previously been reported on somatic hybridization and genetic transformation as well as protoplast culture and plant regeneration in flower crops. Here, we made a choice of the genus *Dianthus*, which contains many important flower crops, as a target and esntablished systems for protoplast culture and plant regeneration. In addition, we provided the possibility for the application of somatic hybridization and genetic transformation to genetic improvement of *Dianthus* species. However, many problems still remain unsolved, among which

occasional regeneration of shoots or plants with abnormal morphology is the most serious problem. In the case of somatic hybridization and genetic transformation, the development of systems for true to-type plant regeneration is essential. Generally, abnormal shoots or plants regenerated in *Dianthus* were severely dwarf and showed precocious flowering, such as protoplast-derived shoots of *D. barbatus*[7], petal-derived shoots of *D. caryophyllus*[15] and somatic hybrid plants between *D. chinensis* and *D. barbatus*[25]. Similar observations have already been reported in *D. caryophyllus*[13]. These abnormal characters appear to be induced by some physiological disorders rather than to arise as a result of genetic variations, as plants with normal morphology sometimes developed from abnormal ones during prolonged culture both *in vitro* and in the greenhouse. The mechanism involved in the appearance of these abnormal characteristics, however, has not been identified at present. Breeding of flower crops in the genus *Dianthus* by biotechnology will start to develop rapidly when this problem can be overcome.

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