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Genetic Transformation and Protoplast Culture as the means for Plant Breeding of Some Horticultural Crops

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Genetic transformation and protoplast culture as the means for plant breeding of some horticultural crops

（数種の園芸作物における育種手法としての形質転換とプロトプラスト培養）

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Chapter 1

General Introduction

General Introduction

1. Background of this study

Recently, biotechnological procedures have been employed to proceed plant breeding in addition to traditional breeding by sexual crossing. Especially, somatic hybridization and genetic transformation procedures mediated by *Agrobacterium*, electroporation and particle bombardment are considered to be useful tools for crop improvements.

1.1 Strategies utilizing protoplasts for plant breeding

Establishment of plant regeneration system from protoplasts is one of the essential factors to apply biotechnological procedures to plant breeding. Plant protoplasts have been isolated mainly by enzymatic maceration since Cocking (1960) succeeded in isolating protoplasts from root tip cells by enzyme treatment, and Takebe *et al.* (1968) showed mass isolation of protoplasts from leaves of *Nicotiana tabacum* by using commercially available enzymes. Subsequently, whole plant regeneration from protoplasts of *N. tabacum* was reported by Takebe *et al.* (1971), proving that protoplasts in the state of single cells retained their ability for plant regeneration, which is known as totipotency. Since then, successful results in plant regeneration from protoplasts have been reported in a number of commercially important crops such as rice (*Oryza sativa*), maize (*Zea mays*), potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*), alfalfa (*Medicago* sp.), cucumber (*Cucumis sativus*), eggplant (*Solanum melongena*), lettuce (*Lactuca sativa*) and *Brassica* species, etc. (for review, Bajaj 1989). Using these plant regeneration systems from protoplasts, production of somatic hybrid plants and transgenic plants through polyethylene glycol (PEG)- or electroporation-mediated direct gene transfer has been realized.

Although plant regeneration from protoplasts has been reported in a number of crops, the success is still limited in particular plant species and genotypes. The successful culture of protoplasts and their subsequent regeneration into complete plants depend on a number of factors, which involve genotypes, kind of tissues from which protoplasts are isolated, the physiological conditions under which the plants have been cultured, suitable enzyme solutions, osmotic conditions, incubation period in enzyme solution, culture media and growth regulators, environmental condition of protoplast culture, plating density, and method of culture. Among these factors, genotypes and source tissues of the plants from which protoplasts are isolated are considered to be the most important ones for cell division of the protoplasts, colony formation, induction of adventitious shoots from protoplast-derived calli and whole plant regeneration. At present, no universal procedures which can be adopted to all plant species have been exploited for protoplast culture. Therefore, it is still necessary to establish individual plant regeneration systems from protoplasts corresponding to each species and genotype.

On the other hand, attempts were made to isolate protoplasts from various parts of plant, such as root, leaf, root nodule, coleoptile, fruit tissue, pericarp, flower petal, potato tuber, endosperm, aleurone layer, crown gall tissue, pollen mother cell, pollen tetrad and pollen grain, and callus culture (for review, Bajaj 1977). Furthermore, protoplasts have also been isolated successfully from female gametes in several plant species (for review, Theunis *et al.* 1991). Isolation of female gametes or gametophytes, namely, egg cells or embryo sacs is expected to be utilized for the studies on the mechanism of recognition and fusion between male and female gametes, observation of the process of fertilization in living condition by using isolated embryo sacs, biochemical and genetic analysis of gamete-specific elements, and genetic engineering in combination with *in vitro* fertilization. For these biotechnological uses of isolated female gametes, it is necessary to establish a method for isolating intact ones. However, manipulation

of female gamete and analysis of the gametogenesis are usually very difficult, because female gamete locates deep inside the ovule and is enclosed within sporophytic tissues. Since Bradley (1948) first developed a technique for isolation of embryo sacs from fixed ovules in *Nicotiana* and *Petunia* by HCl hydrolysis, a number of studies have been reported for isolating female gametes by several methods. So far, enzymatic procedures have been described for isolation of intact female gametes or embryo sacs in several plant species, such as *Torenia fournieri* (Mòl 1986), *Lilium longiflorum* (Wagner *et al.* 1989a), *Zea mays* (Wagner *et al.* 1989b), *Petunia* (Van Went and Kwee 1990) and *Crinum asiatum* (Ohshika and Ikeda 1994). In addition to enzymatic maceration, microdissection procedures of ovules after enzyme treatments have successfully been used for isolating embryo sacs or egg cells in *Plumbago zeylanica* (Huang and Russell 1989), *Zea mays* (Kranz *et al.* 1991), *Nicotiana* species (Huang *et al.* 1992), wheat (Kovács *et al.* 1995) and *Nicotiana tabacum* (Tian and Russell 1997). Based on these studies, plant regeneration from *in vitro*-fertilized egg cells of maize (Kranz and Lörz 1993) and from zygote protoplasts of barley (Holm *et al.* 1994) were achieved. At present, a few *in vitro* fertilization models with isolated gametes are available for maize (Faure *et al.* 1994; Kranz and Dresselhaus 1996). For applying these technologies to crop improvements, procedures for isolation, culture and plant regeneration of female gametes are now expected to be established in individual plant species or genotypes, and the techniques are also expected to be applied for direct gene transfer and *in vitro* fertilization between the incompatible cross combinations of species.

1.2 *Agrobacterium*-mediated gene transfer for crop improvements

So far, most of the transgenic plants have been obtained by using two methods, namely, those of *Agrobacterium*-mediated gene transfer and direct gene transfers by means of electroporation, particle bombardment and PEG-mediated ones. The *Agrobacterium*-mediated

procedure has been employed for production of a great number of transgenic plants and considered as the most efficient method for transformation in plants (for review, Dons *et al.* 1991; Deroles *et al.* 1997).

The *Agrobacterium*-mediated technique involves a natural gene transfer system. In nature, *A. tumefaciens* and *A. rhizogenes* have abilities to induce crown gall and hairy root diseases, respectively (for review, Zambryski 1988; Gelvin 1990). Both *Agrobacterium* species carry a large plasmid called Ti in *A. tumefaciens* and Ri in *A. rhizogenes*. A region on these plasmids, called as transferred DNA (T-DNA), is transmitted from *Agrobacterium* to individual plant cells (Zambryski *et al.* 1980, 1989). The most important and widely used vectors are the nononcogenic which allow the regeneration of transformed phenotypically normal plants. These xvectors are available in two types, the cointegrated vector and the binary vector. Cointegrated vectors, such as pGV3805 (Zambryski *et al.* 1983), were firstly constructed by replacing all the oncogenic functions encoded by the T-DNA to pBR322 sequences. Binary vector system involves a host *Agrobacterium* strain containing Ti or Ri plasmid and another plasmid inserted border sequences of T-DNA (Bevan 1984). The T-DNA is randomly integrated into plant genome (Zambryski *et al.* 1982), and stably inherited as a dominant gene in Mendelian fashion (De Block *et al.* 1984; for review, Hooykaas and Schilperoort 1992).

Genetic transformation using *Agrobacterium rhizogenes*

Tepfer (1984) showed that T-DNA on Ri plasmid in *A. rhizogenes* could induce transformed roots known as hairy roots which exhibits vigorous growth and extensive branching with plagiotropism. These two characteristics provide useful markers for selecting the transgenic roots. Therefore, *A. rhizogenes*-mediated transformation has the advantage in the use of these morphological markers compared to other transformation procedures.

It has been recognized that hairy roots induced by *A. rhizogenes* are capable of being regenerated into whole plants in various species. In some species such as *Convolvulus arvensis* (morning glory), shoots were regenerated from hairy roots spontaneously (Tepfer 1984), whereas shoot regeneration was induced by treatments with plant growth regulators in other species. Tepfer (1990) summarized the results on hairy root formation and transgenic plant regeneration from the root cultures in various species and showed that 116 dicotyledonous species successfully produced stable root cultures. However, in 76 out of the 116 species, it was failed to regenerate any shoots. As plant regeneration from hairy roots is still difficult in wide range of species, it is now expected to establish novel shoot regeneration systems for each plant species.

The plants transformed by Ri plasmid vector are also known to exhibit Ri syndrome (or hairy root syndrome) which is usually considered to be unfavorable traits for plant improvements (Tepfer 1984; Ooms *et al.* 1985; Spena *et al*. 1987). These include wrinkled leaves, short internodes, altered flower morphology, etc.. In case of ornamental plant species, however, some of the Ri syndrome such as dwarfness and altered flower morphology are regarded as the favorable traits for increasing additional values. In fact, Pellegrineschi *et al.* (1994), Scorza *et al.* (1994) and Godo *et al.* (1997) evaluated the morphological alterations from the view point of ornamental quality of horticultural plants.

Birot *et al.* (1987) first used *A. rhizogenes* as a binary vector, where the foreign gene was carried by an artificial T-DNA on a second plasmid. In binary vector system, foreign gene can be incorporated at a different site from that of the Ri T-DNA by the co-transformation. In co-transformation, Shahin *et al.* (1986) found that hairy root formation was advantageously used as the markers provided by the Ri T-DNA, and the T-DNA and the second foreign gene(s) segregated independently after meiosis to dispose them in the progeny.

Thus, the transformation systems using *A. rhizogenes* are useful for improving plant morphology and introducing foreign genes, and would be incorporated into traditional breeding programs.

Recent advances in genetic transformation using *Agrobacterium tumefaciens*

Genetic transformation of many dicotyledonous plants has been performed routinely using *A. tumefaciens* (for review, Fisk and Dandekar 1993; Smith and Hood 1995; Deroles *et al.* 1997). This method is effective for integrating only one or a few copies of the disarmed bacterial T-DNA carrying the transgenes into the plant genome. However, it was found that the host range of *A. tumefaciens* was limited to the species mostly belonging to dicotyledonous plants, as a result of the interaction of genetic informations between host plants and the bacteria (De Cleene and De Ley 1976). Recently, in order to extend the host range, novel vectors in *A. tumefaciens* were developed by Hiei *et al.* (1994). They produced an efficient *A. tumefaciens* strain LBA4404 (pTOK233) which had *virB*, *virC* and *virG* genes from the *Kpn*1 fragment of pTiBo542 in a hypervirulent strain of *A. tumefaciens* A281 characterized by Hood *et al.* (1986), Jin *et al.* (1987) and Komari (1990), and succeeded to transform rice (*Oryza sativa* L.), which is a monocotyledonous plant.

Highly efficient plant regeneration systems from various explants, callus and cell cultures are now needed to apply *A. tumefaciens* method for genetic transformation. Development of plant regeneration systems in combination with those for constructing vectors and infection procedures will extend the host range of *A. tumefaciens* to the species and genotypes which are known to be recalcitrant.

2. Aim of the study and outline of the thesis

As described above, establishment of the reproducible culture systems for plant regeneration from protoplasts and procedures for genetic transformation is indispensable to apply biotechnologies for plant breeding. These techniques are needed to develop for each plant species and genotypes according to their property in plant regeneration ability and applicability of the transformation method. Therefore, in this study, I examined the applicability of biotechnologies for several horticultural crops such as saintpaulia (*Saintpaulia ionantha* Wendl.), *Dianthus* cultivars, *Silene armeria* L., snapdragon (*Antirrhinum majus* L.) and grapevine (*Vitis vinifera* L.) which are known to be recalcitrant for application of these biotechnologies.

In chapter 2, successful result on establishment of plant regeneration system from protoplasts in saintpaulia is obtained by inducing and utilizing cell suspension culture with plant regeneration ability as a source of protoplasts.

In chapter 3, protoplast culture of several *Dianthus* cultivars and *Silene armeria* L. is studied in respect to shoot regeneration ability from protoplast-derived calli among *Dianthus* cultivars, and culture requirements for suspension cell-derived protoplasts in *S. armeria* L. as a fundamental study for somatic hybridization and electroporation-mediated transformation.

In chapter 4, histological observation on embryo sacs in ovules and isolation of the cells from embryo sacs in *Dianthus* are examined as a basic study for development of novel regeneration system and its application to plant breeding through in vitro fertilization and direct gene transfer.

In chapter 5, bialaphos-stimulated shoot regeneration from hairy roots of snapdragon (*Antirrhinum majus* L.) transformed by *Agrobacterium rhizogenes* is described. This study shows a novel physiological function of bialaphos, which is known to act as a herbicide.

In chapter 6, production of transgenic herbicide-resistant snapdragon by introducing *bar*

gene through Ri binary vector system is described. In this chapter, I tried to evaluate the traits induced by Ri T-DNA and *bar* gene.

In chapter 7, production of transgenic grapevine (*Vitis vinifera* L. cv. Koshusanjaku) plants by co-cultivation of embryogenic calli with *Agrobacterium tumefaciens* and by selecting secondary embryos is described. In this study, *A. tumefaciens* strain LBA4404 (pTOK233), which was reported to have high infection ability in monocotyledonous plants, was used for inoculation.

Finally, in chapter 8, the general significance of the results obtained in this thesis is summarized and discussed. Furthermore, prospects for expanding these studies are also discussed.

Chapter 2

Plant regeneration from cell suspension-derived protoplasts of *Saintpaulia ionantha* Wendl.

Introduction

The Gesneriaceae contains many important ornamental genera such as *Saintpaulia*, *Sinningia*, *Achimenes* and *Streptocarpus*. Among them, *Saintpaulia ionantha* Wendl., commonly called saintpaulia or African violet, involves numerous cultivars with varied flower color, leaf color and shape, and is one of the commercially most popular ornamental species. Breeding of this species has only been achieved by intraspecific hybridization and sport selection, and neither interspecific nor intergeneric hybridization has been incorporated (Grout 1990).

Recent developments of biotechnology such as somatic hybridization and genetic transformation provide additional means for further improvement of saintpaulia with respect to floral and marketable qualities. For utilizing these techniques, it is indispensable to establish an efficient system for protoplast culture to plant regeneration in this species. To date, several micropropagation systems have been developed for saintpaulia (Geier 1983; Smith and Norris 1983; Cassells and Plunkett 1984; Mølgaard *et al.* 1991). However, plant regeneration from saintpaulia protoplasts has not been demonstrated, although several reports on protoplast isolation and culture have appeared (Hughes 1977; Bilkey and Cocking 1982). In the present study, we describe successful regeneration of plants from cell suspension culture-derived protoplasts of saintpaulia.

Materials and methods

Plant materials

Expanded young leaves, 4.0 to 5.0 cm long, of *Saintpaulia ionantha* Wendl. cv. Pink Veil (purchased from local market) were harvested from stock plants grown in a greenhouse and surface-sterilized with 1% sodium hypochlorite solution for 10 min followed by 3 rinses with

sterilized distilled water. Leaf laminae were cut into 5 mm square pieces and placed with the abaxial side down on MS medium (Murashige and Skoog 1962) supplemented with 1 mg l^{-1} each of NAA and BA, 2% (w/v) sucrose and 0.2% (w/v) gellan gum (Gelrite; Kelco, Division of Merck and Co. Inc., San Diego, CA) in 90 x 20 mm plastic Petri dishes containing 40 ml of the medium. The pH of the culture media used for adventitious shoot induction from leaves and plantlet subculture was adjusted to 5.8 prior to autoclaving at 121 °C for 15 min. After 2 months of culture, adventitious shoots that developed from the leaf explants were excised and transferred to half-strength MS medium containing 2% (w/v) sucrose and 0.2% (w/v) gellan gum for rooting. Plantlets thus obtained were subcultured monthly on the same medium. Stock cultures were kept at 25 °C under 24 h illumination (35 μmol m^{-2} s^{-1}) with fluorescent lamps (National FL30SN).

Cell suspension cultures

Leaf laminae, taken from *in vitro*-grown plantlets 3 weeks after subculture, were cut into 5 x 5 mm pieces and placed on 0.2% (w/v) gellan gum-solidified B5 medium (Gamborg *et al.* 1968) supplemented with 2% (w/v) sucrose and 0, 1, 5 or 10 mg l^{-1} of auxins (NAA or 2,4-D) in combination with 0, 1, 5 or 10 mg l^{-1} BA, with or without 2 g l^{-1} casein hydrolysate (NZ Amine, Type A; Wako Pure Chemical Industries, Ltd.). For culture, 15 explants were placed in 90 x 20 mm plastic Petri dishes containing 40 ml of the medium. Two replicates of dishes were made for each treatment. The pH of the culture media was adjusted to 5.8 and the dishes were maintained at 25 °C in the dark.

For establishing cell suspension cultures, proliferating friable calli (ca. 1 g f. wt.) on the initiated explants were transferred to 100 ml Erlenmeyer flasks, each of which contained 40 ml

of B5 liquid medium supplemented with 1 mg l^{-1} 2,4-D and 2% (w/v) sucrose, at pH 5.8. Cell suspension cultures were maintained at 25 °C under 24 h illumination (35 μmol m^{-2} s^{-1}) on a gyratory shaker at 100 cycles min^{-1}. Subcultures were performed every 10 days by transferring ca. 0.8 g f. wt. of cells to 40 ml of fresh medium/flask.

Protoplast isolation and culture

Protoplasts were isolated from cell suspension cultures 4 days after subculture. One g f. wt. of suspension cells were incubated in 10 ml of filter-sterilized (Millipore, 0.45 μm pore size) enzyme solution containing 2% (w/v) Cellulase Onozuka RS and 1% (w/v) Macerozyme R-10 (both Yakult Pharmaceutical Co. Ltd., Japan), 0.05% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Japan), 1% (w/v) Driselase (Kyowa Hakko Kogyo Co. Ltd., Japan), 5 mM $CaCl_2 \cdot 2H_2O$, 5 mM MES and 0.2 M mannitol. The pH of the enzyme solution was adjusted to 5.8. After 4 h of incubation at 25 °C with gentle gyratory shaking (30 cycles min^{-1}), the mixture was passed successively through nylon sieves (60 and 30 μm) and the protoplasts were collected by centrifugation (120 x g, 3 min). Protoplasts were washed twice with half-strength MS medium containing 0.2 M mannitol by resuspension and centrifugation (120 x g, 3 min). Viability of protoplasts was assessed with FDA staining (Widholm 1972).

Protoplasts were cultured at 1 x 10^5/ml in 35 x 10 mm plastic Petri dishes containing 2 ml of 0.1% (w/v) gellan gum-solidified B5 medium. Twice the concentration of protoplasts, 2 x 10^5/ml, in double-strength liquid medium was mixed with an equal volume of 0.2% (w/v) gellan gum solution at 40 °C just before culture (Mii *et al.* 1991). Various combinations of auxins (NAA, 2,4-D or picloram), and sugars (sucrose, glucose or fructose) or sugar alcohol (mannitol) were evaluated in the culture medium. The pH of culture media was adjusted to 5.8. All dishes

were sealed with Parafilm® and maintained at 25 °C in the dark. Plating efficiency (PE), defined as the percentage of dividing protoplasts, was obtained after 14 days of culture.

Plant regeneration

After 2 months of culture, protoplast-derived macro-colonies, ca. 1 mm diameter, were transferred to B5 medium containing 1 mg l^{-1} 2,4-D in order to proliferate callus for a month. Protoplast-derived calli were then transferred onto B5 medium containing 1 mg l^{-1} each of NAA and BA for callus preconditioning. After 2 months, these preconditioned calli were then transferred for shoot induction onto B5 media containing various concentrations of NAA (0, 0.01, 0.1 or 1 mg l^{-1}) and BA (0, 1 or 5 mg l^{-1}).

Regenerated shoots were detached from the callus and transferred for rooting to half-strength MS medium lacking plant growth regulators. All media used during and after callus proliferation were supplemented with 2% (w/v) sucrose and solidified with 0.2% (w/v) gellan gum, adjusted to pH 5.8. Cultures were maintained at 25 °C under 24 h illumination (35 μmol m^{-2} s^{-1}) with fluorescent lamps (National FL30SN).

Regenerated plantlets with a well-established root system were washed carefully to remove the gellan gum and transferred to pots (9 x 9 cm) containing vermiculite. Potted plants were acclimatized in a transparent plastic cabinet covered with polyethylene bags at 20 °C under 24 h illumination (45 μmol m^{-2} s^{-1}) with fluorescent lamps. After 2 to 3 weeks, acclimatized plants were transferred to the greenhouse.

Results and Discussion

Protoplasts have previously been isolated mechanically or enzymatically from leaves,

petioles or cultured petiole cross-sections of *in vitro*-grown saintpaulia plantlets (Hughes 1977; Bilkey and Cocking 1982). However, protoplast division leading to plant regeneration was not demonstrated in these studies. In our preliminary experiments, only a few protoplasts could be isolated from leaves of stock cultures by the enzyme solution and incubation environment used in this study (data not shown). On the other hand, it has been reported that cell suspension cultures were suitable as a donor source for protoplasts of several species including *Limonium perezii*, in which protoplast isolation from leaves was likewise very difficult or impossible (Kunitake and Mii 1990). Therefore, we initially attempted to induce friable calli from leaf disks of saintpaulia on B5-based medium containing 2,4-D or NAA in combination with BA, with or without casein hydrolysate (Table 1). On media containing NAA with or without casein hydrolysate, compact calli and/or adventitious shoots were preferentially produced, respectively. The compact calli produced on the media containing casein hydrolysate only proliferated as compact calli during the subculture on the same media. Friable calli were only induced on medium containing 1 mg l^{-1} 2,4-D and 2 g l^{-1} casein hydrolysate (Table 1), on which 70% of leaf disks produced friable calli. However, these friable calli also proliferated, when transferred to B5 medium without casein hydrolysate. These results indicate that casein hydrolysate may play an important role in induction of friable calli, but not be necessary for maintaining the calli obtained in this study. Casein hydrolysate has already been demonstrated to promote cell division, proliferation or plant regeneration in several tissue culture systems probably by providing amino acids (Ochatt and Power 1988; Kunitake and Mii 1990).

By transferring the leaf-derived friable calli into liquid B5 medium containing 1 mg l^{-1} 2,4-D, fine cell suspension cultures were readily established, from which about a 5-fold increase in f. wt. was obtained within 10 days after subculture. Protoplasts were readily isolated from

suspension cells 4 days after subculture and the successive size filtrations (Fig. 1A). Protoplast yields of 1-3 x 10^7/g f. wt. suspension cells were routinely obtained and their viability was more than 90% as assessed with FDA.

Protoplasts started to divide after 3 to 6 days in culture (Fig. 1B). The highest percentage of dividing protoplasts (12.7%) was obtained in B5 medium with 0.2 M sucrose and 2,4-D (Table 2). The other two auxins also induced cell division, but with frequencies below 6%.

In B5 medium containing 0.1 M sucrose, protoplast bursting was frequently observed immediately after the cultures were initiated and no cell division occurred. Protoplasts most efficiently divided in B5 medium containing 0.2 M sucrose at 12.9% (Table 3). Division frequency decreased as sucrose concentration increased over 0.2 M. Therefore, various sugars, sucrose, glucose or fructose, and sugar alcohol (mannitol) were also tested at 0.2 M singly or in combination (Table 4). Protoplast division was observed only in B5 media containing sucrose. The highest percentage of dividing protoplasts (18.6%) was obtained from the medium containing 0.1 M sucrose in combination with 0.1 M mannitol. The other sugars, glucose and fructose, appeared inhibitory to cell division.

In several plant species, growth of protoplast-derived colonies has shown to be promoted by lowering the osmoticum concentration of the culture medium (Berry *et al.* 1982; Kunitake and Mii 1990). In saintpaulia, however, protoplasts cultured in gellan gum-solidified B5 medium containing 1 mg l^{-1} of 2,4-D and 0.1 M each of sucrose and mannitol continued to divide and more than 200 macro-colonies per dish were formed after 2 months of culture without adding or changing the culture medium (Fig. 1C and D).

Macro-colonies developed into vigorously growing friable calli one month after transfer to the callus proliferation medium. These friable calli were transferred for preconditioning onto B5

medium containing 0.1 mg l^{-1} each of NAA and BA, on which they became compact and occasionally developed adventitious roots after 2 months. Four months after transfer to shoot induction media, green shoot primordia appeared on the surface of the calli and adventitious shoots arose (Fig. 1E). Among the media tested, shoot regeneration occurred only on B5 medium supplemented with 0.01 mg l^{-1} NAA and 5 mg l^{-1} BA at a frequency of 27%. On all other media, the calli continued to proliferate or eventually turned brown after 8 months of culture.

In this study, the preconditioning of protoplast-derived calli on B5 medium containing both NAA and BA seem to be essential for subsequent shoot induction. Neither calli without the preconditioning treatment nor those preconditioned with both 2,4-D and BA or with only NAA regenerated shoots. The importance of a preconditioning treatment prior to shoot regeneration has also been demonstrated for cultured cell-derived protoplasts of *Actinidia chinensis* (Mii and Ohashi 1988).

More than 100 shoots have so far regenerated from protoplast-derived calli of saintpaulia. Protoplast-derived shoots with 2 to 3 leaves were detached from the callus and transferred to half-strength MS medium, on which they readily developed roots. Regenerated plantlets were successfully established in the greenhouse 9 to 10 months after the initiation of protoplast cultures (Fig. 1F). Acclimatized plants exhibited a normal phenotype with respect to leaf shape and color, and flowered with normal color and 6 months after transfer to the greenhouse (Fig. 1G). Production of both somatic hybrid plants and PEG- or electroporation-mediated transgenic saintpaulia plants using the protoplast culture system developed in this study are now in progress.

Chapter 3

Protoplast culture of several *Dianthus* cultivars and *Silene armeria* L.: comparison of shoot regeneration ability from protoplast-derived calli among *Dianthus* cultivars, and culture of suspension cell-derived protoplasts in *S. armeria* L. as fundamental study for somatic hybridization

Introduction

The genus *Dianthus* includes a lot of important ornamental species, such as carnation (*D. caryophyllus*), *D. chinensis* and *D. barbatus*. In these species, biotechnological techniques such as somatic hybridization and genetic transformation are expected to be applied for further improvements in addition to traditional breeding methods especially in carnation since its economic importance. For application of these techniques, it has been considered that plant regeneration system from protoplasts is indispensable, because protoplasts offer many potential uses such as for somatic hybridization between incompatible species (for review, Hamill and Cocking 1988) and direct gene transfer (Paszkowski *et al.* 1984). So far, successful plant regeneration from protoplasts of several species in *Dianthus* (Nakano and Mii 1992; Kim and Lee 1996), and somatic hybridization among *Dianthus* species (Nakano and Mii 1993a, 1993b) and between *Dianthus* and *Gypsophila* (Nakano *et al.* 1996) have been reported. For further use of protoplast technologies to somatic hybridization and direct gene transfer, it is necessary to establish plant regeneration systems in a broad range of genotypes in genus *Dianthus* as well as in the species of other genus belonging to Caryophyllaceae. In the present study, protoplast culture of several *Dianthus* cultivars and *Silene armeria* is studied in respect to shoot regeneration ability from protoplast-derived calli among *Dianthus* cultivars, and culture requirements for suspension cell-derived protoplasts in *S. armeria* as a fundamental study for somatic hybridization and electroporation-mediated transformation.

Materials and Methods

Plant materials

Three cultivars of *Dianthus chinensis* L., Fire Carpet, Snow Fire and Rose Carpet, and two interspecific cultivars (*D. chinensis* L. x *D. barbatus* L.), Telster White and Telster Orchid, were

used as plant materials. The seeds of these cultivars, which were obtained from Takii & Company Ltd., Kyoto, Japan, were surface-sterilized with sodium hypochlorite solution (1% active chlorine) for 10 min and rinsed 3 times with sterilized distilled water. After sterilization, they were cultured on half-strength MS medium (Murashige and Skoog 1962) lacking plant growth regulators but with 20 g l^{-1} sucrose (Kanto chemical Co. Inc., Tokyo, Japan) and 8 g l^{-1} agar (Wako pure chemical industries, ltd., Osaka, Japan). Plantlets derived from seedlings were routinely subcultured at 26 ± 1 °C under continuous illumination (35 μmol m^{-2} s^{-1}) with fluorescent lamps (National FL30SN) and used for protoplast isolation. Subculture was performed once a month by transferring terminal and lateral cuttings onto the same medium.

Seeds of *Silene armeria* L. were harvested from the plants growing wild around Ohi river at Kakegawa city, Shizuoka prefecture in Japan, at the end of June 1993. These seeds were sterilized and cultured as same as described above.

Protoplast isolation from leaves of several cultivars of Dianthus

Leaves of *in vitro*-grown plantlets 3 weeks after subculture were taken and chopped by using razor blade in CPW solution (Frearson *et al.* 1973) containing 0.5 M mannitol. They were incubated with gentle shaking for 1 h at 26 ± 1 °C in the dark. Then, one gram fresh weight of chopped leaves were transferred to 10 ml of filter-sterilized (Millipore, 0.45 μm pore size) enzyme solution containing 2% (w/v) Cellulase Onozuka RS (Yakult Pharmaceutical Co. Ltd., Japan), 0.1% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Japan), 1% (w/v) Driselase (Kyowa Hakko Kogyo Co. Ltd., Japan), 5 mM MES and 0.5 M mannitol dissolved in CPW solution. The pH of the enzyme solution was adjusted to 5.8. After 5 h incubation at 26 ± 1 °C in the dark with gentle shaking (30 cycles min^{-1}), the mixture was passed through a nylon

sieve (108 μm) and the solution containing protoplasts was laid on density gradient of percoll (Sigma Chemical Co., MO, USA) for purification of protoplasts. Protoplasts were collected by centrifugation (120 x g, 3 min) and washed with CPW solution containing 0.5 M mannitol by resuspension and centrifugation (120 x g, 3 min). Viability of protoplasts was assessed with FDA staining (Widholm 1972).

Protoplast culture and plant regeneration in Dianthus culivars

The protoplasts were cultured at a density of 1 x 10^5/ml in 60 x 15 mm plastic Petri dishes (Falcon 1007, Becton Dickinson & Co., New Jersey, USA) containing 1 g l^{-1} gellan gum (Gelrite; Kelco, Division of Merck and Co. Inc., San Diego, CA) -solidified MS medium containing 5 mg l^{-1} NAA, 1 mg l^{-1} zeatin and 0.5 M glucose as reported by Nakano and Mii (1992) with several modification. The pH of culture media was adjusted to 5.8. All dishes were sealed with Parafilm® and maintained at 26 ± 1 °C in the dark. Division frequency, defined as the percentage of dividing protoplasts, was obtained after 14 days of culture.

After 2 months of culture, visible colonies (ca. 1 mm diameter) were transferred for proliferation to 2 g l^{-1} gellan gum-solidified MS medium containing 5 mg l^{-1} NAA, 1 mg l^{-1} zeatin and 20 g l^{-1} sucrose. For shoot regeneration from protoplast-derived calli, they were further transferred to 2 g l^{-1} gellan gum-solidified MS medium containing 1 mg l^{-1} NAA, 5 mg l^{-1} zeatin and 20 g l^{-1} sucrose one month after subculture to callus proliferation medium. Regenerated shoots were detached from the callus and rooted by transfer to 8 g l^{-1} agar-solidified half-strength MS medium containing 20 g l^{-1} sucrose. Percentage of calli showing shoot regeneration was evaluated 3 months after transfer to the shoot regeneration medium.

Induction of callus and cell suspension cultures from leaves of S. armeria

Leaves taken from *in vitro*-grown plantlets of *S. armeria* were placed on 2 g l^{-1} gellan gum-solidified MS medium supplemented with 1 mg l^{-1} 2,4-D and 30 g l^{-1} sucrose. After one month of culture on the medium, calli (ca. 1 g fresh weight) proliferating on the explants were transferred for establishing cell suspension cultures to 100 ml Erlenmeyer flasks, each of which contained 40 ml of MS liquid medium supplemented with 1 mg l^{-1} 2,4-D and 30 g l^{-1} sucrose. Cell suspension cultures were maintained at 26 ± 1 °C under 24 h illumination (35 μmol m^{-2} s^{-1}) on a gyratory shaker at 100 cycles min^{-1}. Subcultures were performed every 10 days by transferring ca. 0.8 gram fresh weight of cells to 40 ml of fresh medium / flask.

Protoplast isolation and culture in S. armeria

Protoplasts were isolated from cell suspension cultures 4 days after subculture. One gram fresh weight of suspension cells were incubated in 10 ml of filter-sterilized (Millipore, 0.45 μm pore size) enzyme solution which was the same as that used for *Dianthus* as described above, but in concentration of mannitol in the enzyme solution was varied from to 0.2 to 0.8 M mannitol for investigating the effect of the mannitol concentration on protoplast viability and division frequency. After 7 h of incubation at 26 ± 1 °C with gentle gyratory shaking (30 cycles min^{-1}), the mixture was passed successively through nylon sieves (108 and 60 μm) and the protoplasts were collected by centrifugation (120 x g, 3 min). Protoplasts were washed twice by resuspension and centrifugation (120 x g, 3 min) with half-strength MS medium containing mannitol of the same concentration as used for enzyme solution. Viability of protoplasts was assessed with FDA staining (Widholm 1972).

Protoplasts were cultured at 1 x 10^5 / ml in 35 x 10 mm plastic Petri dishes (Falcon 1008, Becton Dickinson & Co., New Jersey, USA) containing 2 ml of 1 g l^{-1} gellan gum-solidified MS medium supplemented with 1 mg l^{-1} 2,4-D.

Double-strength liquid medium containing twice the concentration of protoplasts, 2 x 10^5 / ml, was mixed with an equal volume of 2 g l^{-1} gellan gum solution at 40 °C just before culture (Hoshino *et al.* 1995). Various combinations of sugars (sucrose, glucose or fructose) and sugar alcohol (mannitol) in culture medium were evaluated as carbon sources and/or osmoticums. The pH of culture media was adjusted to 5.8. All dishes were sealed with Parafilm® and maintained at 25 °C in the dark. Division frequency, defined as the percentage of dividing protoplasts, was obtained after 10 days of culture.

Visible colonies were transferred for further callus proliferation onto 2 g l^{-1} gellan gum-solidified MS medium supplemented with 1 mg l^{-1} 2,4-D and 30 g l^{-1} sucrose. One month after transfer, protoplast-derived calli were examined for shoot regeneration on 2 g l^{-1} gellan gum-solidified MS media containing 30 g l^{-1} sucrose, and NAA in combination with BA, TDZ, zeatin or KT-30 at different concentrations.

Results and Discussion

Protoplast culture and comparison of shoot regeneration ability from protoplast-derived calli in Dianthus cultivars

In preliminary experiments, protoplasts isolated from leaves according to the procedure previously reported (Nakano and Mii 1992) were sometimes contaminated with a lot of cell debris after passing successively through nylon sieves (100, 80, 60 and 40 μm). For eliminating

these debris, purification procedures were examined to increase the purity by using density gradient of percoll with centrifugation. The density gradient of percoll has been utilized for purification of protoplasts (Wernicke *et al.* 1979; Griesbach *et al.* 1983) and selection of hybrid cells after fusion treatments (Harms *et al.* 1978; Naton *et al.* 1986, 1992). In the present procedure, protoplasts suspended in washing solution were laid on a layer of washing solution containing 20, 15, 10 and 5% (v/v) percoll from bottom to top in a centrifugation tube. By centrifugation (120 x g) for 3 min, purified protoplasts were collected on 5% (w/v) percoll-containing solution.

The protoplasts as purified above could begin to divide within 5 days and formed visible colonies in all cultivars after 2 moths of culture by using the culture methods as reported by Nakano and Mii (1992). While cell division was observed in all cultivars, the frequency was different among the cultivars. The highest frequency 15.4% was obtained in cv. Telster White after 14 days of culture (Table 5).

After transfer to callus proliferation medium, vigorously growing colonies turned green and adventitious shoots were sometimes observed in some cultivars. One month after transfer to the callus proliferation medium, protoplast-derived calli were transferred to the shoot regeneration medium. Three months after the transfer, adventitious shoots were regenerated from the calli in all cultivars but the frequency were considerably varied among the cultivars varying from 7.8 to 35.3% (Table 5). Among all cultivars, cv. Telster White could regenerate shoots at the highest frequency in addition to high ability of protoplast division.

These results suggested that all the cultivars examined in the present study, especially cv. Telster White, will be utilized for production of both somatic hybrid plants and transgenic plants through PEG- or electroporation-mediated direct gene transfer, because of their plant regeneration ability from protoplasts.

Protoplast isolation and culture in Silene armeria

Effect of mannitol concentration in enzyme solution on protoplast yield and viability were examined in *S. armeria*. The suspension cells bursted or swelled in enzyme solutions containing 0.2 to 0.3 M mannitol. On the other hand, they became small by exclusion of water from cytoplasm at higher concentrations of mannitol (0.7 and 0.8 M). The highest protoplast yield and viability assessed by FDA staining were obtained in enzyme solution containing 0.5 M mannitol (Table 6).

Subsequently, effect of sugars or sugar alcohol was evaluated in protoplast culture medium (Table 7). Protoplasts could divide in media containing sucrose or glucose and the highest division frequency was obtained at 17.3% in 0.5 M sucrose-containing medium.
In this experiment, no protoplast division was observed in media supplemented with fructose, suggesting that it has inhibitory effect on division of *Silene* protoplasts.

Protoplasts were sustained to divide in media supplemented with 2,4-D and sucrose. Visible colonies formed after one month of culture were picked up and transferred to callus proliferation medium. The protoplast-derived calli on the medium continued to grow and occasionally regenerated adventitious roots. However, no shoots were obtained on the same medium. The calli were examined for shoot regeneration on media containing several combinations of plant growth regulators. Although calli turned green on media containing NAA in combination with BA, TDZ or zeatin, adventitious shoots were not regenerated (data not shown). Although adventitious roots were obtained on media supplemented with NAA, and NAA in combination with zeatin one month after transfer, the calli forming roots never regenerated shoots during prolonged culture period for 6 months. Consequently, no any shoots were obtained among all media tested.

In this study, procedures were developed for isolation and culture of protoplasts in *S. armeria*. Using the procedures, intergeneric somatic hybridization between *S. armeria* and *Dianthus* cultivars will be tried.

Chapter 4

Isolation of embryo sacs from ovules by enzymatic treatments and microdissection in *Dianthus*

Introduction

The genus *Dianthus* includes a lot of important ornamental species, such as carnation (*D. caryophyllus*), *D. chinensis* and *D. barbatus*. In these species, biotchnologies are expected to be applied for further improvements in addition to traditional breeding methods especially in carnation since its economic importance.

Recently, techniques have been developed for the isolation and culture of female gametes (reviewed in Theunis *et al.* 1991), and plant regeneration from *in vitro*-fertilized egg cells of maize (Kranz and Lörz 1993) and zygote protoplasts of barley (Holm *et al.* 1994). However, it is still difficult to manipulate female gametophytes in most angiosperms, because female gametogenesis generally takes place deep inside of the sporophytic ovule tissues. So far, enzymatic procedures for isolation of female gametes or embryo sacs have been described in several plant species, such as *Torenia fournieri* (Mòl 1986), *Lilium longiflorum* (Wagner *et al.* 1989a), *Zea mays* (Wagner *et al.* 1989b), *Petunia* (Van Went and Kwee 1990) and *Crinum asiatum* (Ohshika and Ikeda 1994). In addition to enzymatic maceration, microdissection procedures of ovules after enzyme treatments have successfully been adopted for isolating embryo sacs or female gametes in *Plumbago zeylanica* (Huang and Russell 1989), *Zea mays* (Kranz *et al.* 1991), *Nicotiana* spp. (Huang *et al.* 1992), wheat (Kovács *et al.* 1995) and *Nicotiana tabacum* (Tian and Russell 1997). In *Dianthus*, establishment of novel regeneration systems from isolated egg cells, *in vitro* fertilized egg cells and zygotes is now expected to be applied for further breeding of the species involved in this species through direct gene transfer and *in vitro* fertilization between incompatible cross combinations of species. In the present study, isolation of embryo sacs in *Dianthus* is demonstrated based on the results of histological observations on ovules and by utilizing enzymatic procedures in combination with microdissection.

Materials and methods

Plant materials and collection of ovaries

Flowers of interspecific hybrid (*Dianthus chinensis* x *D. barbatus*) cv. Telster White (Takii & Company Ltd.) were collected 1 or 2 days after anthesis from stock plants growing in a greenhouse. They were used both for histological observations to identify localization of embryo sac and especially egg apparatus in an ovule and for isolation of cells from embryo sacs.

Histological observations

For the histological studies on embryo sac in an ovule, both petals and ovary walls were removed from harvested flowers and then ovules with placenta were put into FAA solution (formalin, acetic acid, 50% ethanol, 5:5:90, by vol.) (Sass 1958) for fixation during 1-3 days. The fixed ovules were washed by distilled water (D.W.) and stained in modified Mayer's acid haemalaum (Lillie *et al.* 1965). After 2 hours of incubation at room temperature, ovules were destained in D.W. for a period varying from 2 to 6 hours depending on individual staining intensity. Subsequently, ovules were gradually dehydrated in a graded ethanol series, 50, 75 and 95% for 2, 2 and 24 h, respectively, and then cleared by successive transfer to 95% ethanol : benzyl benzoate (2:1, by vol.), 95% ethanol : benzyl benzoate (1:2, by vol.), and benzyl benzoate : dibutyl phthalate (BBD) (1:1, by vol.) at intervals more than 1 h according to the method of Crane and Carman (1987) with several modifications. Treated ovules were mounted in BBD on a glass well (4 mm in depth) slide with a coverslip and observed under inverted microscope (IMT-2, Olympus) with Nomarski differential interference equipment.

Fixed ovules with placenta were also transferred through a graded ethanol series into absolute n-butyl alcohol and embedded in paraffin (54-56 °C melting point, Wako Pure

Chemical Industries, Ltd., Japan). Longitudinal sections of 10 μm thick were made by using microtome and stained with Mayer's acid haemalaum followed by mounting with Canada balsam on glass slides.

Isolation of cells from ovules

After removing petals from receptacle, flowers of *Dianthus* just before anthesis were surface-sterilized with sodium hypochlorite solution (1% active chlorine) for 10 min and rinsed 3 times with sterilized distilled water. Ovaries were longitudinally cut into 2 pieces by a razor blade and ovary walls were peeled off. Dissected ovaries were put into 2 ml of filter-sterilized (Millipore, 0.45 μm pore size) enzyme solutions, which consisted of different concentrations and combinations of Cellulase Onozuka RS and Cellulase Onozuka R-10 (both Yakult Pharmaceutical Co. Ltd., Japan), Macerozyme R-10, Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Japan), Hemicellulase, Driselase (Kyowa Hakko Kogyo Co. Ltd., Japan), $CaCl_2 \cdot 2H_2O$, 2-(N-morpholino)-ethanesulfonic acid (MES) and MS inorganic elements, in glass Petri dishes (35 mm x 15 mm). Ovules were then detached from placenta in the enzyme solution by using forceps and a razor blade under a dissecting microscope. The enzyme solutions used for the present study were detailed in Table 9. They were incubated on a gyratory shaker (30 cycles min^{-1}) at 25 °C. The pH of the enzyme solutions was adjusted to 5.8 before filter sterilization. After enzyme treatments, ovules were transferred into CPW solution (Frearson *et al.* 1973) containing 0.5 M mannitol, and dissected with a hand-made glass needle for isolating female gametes. The nuclei of the embryo sacs were stained with 4,6-diamidino-2-phenylindole (DAPI; Wako Pure Chemical Industries, Ltd., Japan) according to the method of Kranz *et al.* (1991) and observed under a fluorescence microscope using the UV excitation system (BH2-RFC,

Olympus) (excitation wave length was 365 nm).

Results and Discussion

Histological observations of the embryo sac in the ovule

From microscopic observations, it was shown that ovules were raised on free central placenta (Fig. 2) and the type of ovule was the hemitropous (Fig. 3) in *Dianthus* cv. Telster White. Average number of ovules in an ovary was 74.7 (Table 8).

Nucellar tissue at micropylar region was thick and occupied almost half part of mature ovule (Fig. 3). As a result, the mature embryo sac was located deep in ovule at chalazal portion. All cells of the embryo sac showed a specific morphology and structural organization. One of the main characteristics of these cells was to possess polarity and specific position of the nuclei. Both synergid cells had a large vacuole, locating at the side facing to the central cell (Fig. 4 and 5A-C) and filiform apparatus (Fig. 5C), which was generally observed in many angiosperms (Willemse and van Went 1984) and located at the side of micropylar region. The egg cell was also vacuolated at side of micropylar region, resulted in egg nucleus with cytoplasm at side of the central cell. The average diameters of embryo sac, synergid and egg cell were 208, 33 and 35 μm, respectively (Table 8).

The central cell occupied the largest portion of the embryo sac and was highly vacuolated (Fig. 4). The two polar nuclei which were located near egg apparatus and sometimes protruded into egg apparatus were fused to form one nucleus prior to fertilization. The fused nucleus was the largest among the nuclei in the embryo sac, and contained a large nucleolus which was obviously shown by hematoxylin staining (Fig. 4, and 5B). The antipodal cells were not observed in the mature embryo, suggesting that these cells had already degenerated before maturation of the embryo sac.

Isolation of embryo sacs

Based on the histological observations, we tried to establish a technique for isolating female gamete from ovules by using enzyme treatment and microdissection. In all enzyme mixtures used in this study (Table 9), cells of funiculus initiated to be digested after 30 min of incubation and completely dispersed until 8 h of incubation. In contrast, it was difficult to digest outer integument irrespective of the difference in components of enzyme solutions as shown in Table 9 and ovules kept its shape after prolonged incubation until 48 h. As a result, presence of outer integument inhibited to release an embryo sac.

To isolate embryo sacs, ovules were dissected using glass needles after enzymatic treatments for 12 h (Table 9). By the dissection, unique large cells were released from ovules in all the enzyme treatment except for no. 4 (Table 9). The unique cells (Fig. 6) were estimated as the embryo sacs, because they maintained their original pear-like shape and showed a similar size to embryo sac which was approximately 190 - 210 μm in longitudinal diameter and was extraordinary large compared to other sporophytic cells. They were also released from deep inside of ovule where embryo sacs were located. Although these cells may include female gametes, they are not easily identified from the more numerous nucellar cells. High yields of embryo sacs at approximately 13% were obtained in no. 1 and no. 7 of enzyme solutions, both of which contained 1% (w/v) Driselase. Therefore, Driselase may have a beneficial effect to digest ovule tissues and to release embryo sacs by its enzymatic activity.

Isolated embryo sacs were stained with DAPI to confirm the number and position of nuclei (Fig. 7). The embryo sac contained four nuclei, which were estimated as the nuclei of egg, synergid and central cells from their specific position. The antipodal cells were not observed because these cells might have already degenerated before maturation of the embryo sac.

In this study, isolation procedure of embryo sacs has been developed in *Dianthus*, based on histological observations of ovules by clearing method and serial sectioning. In *Dianthus*, embryo sacs locate deep inside of ovules, compared to other plant species such as *Torenia fournieri* (Mòl 1986), *Zea mays* (Kranz *et al.* 1991) and wheat (Kovács *et al.* 1995) which have embryo sacs near micropylar region. Because of this reason, it was necessary to treat ovules with enzyme solution followed by dissection for isolating embryo sacs. The procedure developed in the present study will offer a new approach to further *in vitro* fertilization studies in *Dianthus*.

Chapter 5

Bialaphos stimulates shoot regeneration from hairy roots of snapdragon (*Antirrhinum majus* L.) transformed by *Agrobacterium rhizogenes*

Introduction

Bialaphos is a tripeptide antibiotic produced by *Streptomyces hygroscopicus* and used as a herbicide. It consists of phosphinothricin and two L-alanine residues (Murakami *et al.* 1986). Recently, a gene which confers resistance to the herbicide bialaphos (*bar*) has been characterized (Thompson *et al.* 1987) and utilized not only for producing the crops with herbicide tolerance under field conditions (De Greef *et al.* 1989) but also as a selectable marker for transgenic plants with agriculturally important genes in some crops such as tobacco (*Nicotiana tabacum* L.) (De Block *et al.* 1987), *Brassica* (De Block *et al.* 1989), potato (*Solanum tuberosum* L.) (De Block 1988) and sugarbeet (*Beta vulgaris* L.) (D'Halluin *et al.* 1992).

Antirrhinum majus L., commonly called snapdragon, has been used as an ornamental plant and also studied for molecular analyses on transposon (Sommer *et al.* 1988), homeotic genes for flower development (Sommer *et al.* 1990, Coen 1991) and flower pigmentation (Sommer and Saedler 1986; Moyano *et al.* 1996). Although transformation system for snapdragon has already been reported by using *Agrobacterium rhizogenes* (Handa 1992; Senior *et al.* 1995), it is still needed to establish a highly efficient system of transgenic plant production for proceeding genetic improvement as well as molecular analysis.

In a preliminary experiment to transform snapdragon for the improvement of herbicide resistance using *Agrobacterium rhizogenes*, an increase in shoot regeneration from hairy roots transformed by a wild type strain of *A. rhizogenes* was noted on medium containing bialaphos. In this paper, we report the detailed results on the stimulation of shoot regeneration from hairy roots of snapdragon by bialaphos treatment and discuss possible physiological mechanisms involved in this stimulative effect.

Materials and methods

Plant materials and bacterial strain

Seeds of snapdragon (*A. majus* cv. Floral Carpet, Sakata Seed Corp.; Yokohama Japan) were germinated on soil and grown in a growth chamber at 20 °C under 16:8 L:D photoperiod (45 μmol $m^{-2}s^{-1}$) by fluorescent lamps (National FL30SN).

A. rhizogenes strain A13 (MAFF02-10266) (Daimon *et al.* 1990) was used in this study. Prior to co-cultivation, 30 ml liquid YEB medium (Vervliet *et al.* 1974) prepared in a 100 ml Erlenmeyer flask was inoculated with strain A13. The bacterial cultures were incubated overnight at 27°C on a rotary shaker (200 cycles min^{-1}).

Transformation by A. rhizogenes

Leaves were taken from growth chamber-grown plants 4 weeks after germination, surface-sterilized with a sodium hypochlorite solution (1% active chlorine) for 10 min and rinsed 3 times with sterile distilled water. Leaf laminae were cut into 5 mm square pieces and inoculated with freshly grown *A. rhizogenes* suspensions (about 1 x 10^8 cells ml^{-1}) using a needle. Inoculated leaf segments were co-cultivated on 2 g l^{-1} gellan gum (Gelrite; Kelco, Division of Merck and Co. Inc., San Diego, CA) -solidified half-strength MS medium (Murashige and Skoog 1962), which included half-strength salts and full-strength organic elements, containing 30 g l^{-1} sucrose. A total of 76 leaf explants were treated with *Agrobacterium* and cultured in 90 mm x 20 mm plastic Petri dishes (Terumo SH-20S, Japan) which contained 40 ml of medium. About 10 explants were placed on each plates. After 5 days of co-cultivation, the leaf segments were transferred to the same medium with the addition of 500 mg l^{-1} cefotaxime (Claforan; Hoechst AG) which was supplemented after autoclaving in order to eliminate *Agrobacterium*. Leaf segments were subcultured every 2 weeks on the same medium. Root tips (about 10 mm) from

adventitious roots produced at the inoculated site of the explants 3 to 8 weeks after inoculation were excised and transferred to the same medium to obtain *Agrobacterium*-free cultures. The pH of the culture media was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. All dishes were sealed with Parafilm and maintained at 25 °C in the dark throughout this experiment.

Opine analysis

Opine detection was performed as described by Isogai *et al.* (1990). About 100 mg fresh weight of cultured root tissue was ground in a microtube containing a bit of quartz sand and two drops of 1N HCl by Pasteur pipette. After centrifugation at 6000 x g for 15 min, 20 μl of supernatant was spotted on a Whatman 3MM paper and subjected to high-voltage paper electrophoresis at 450 V for 2 h (Petit *et al.* 1983). The electrode buffer consisted of formic acid, acetic acid and distilled water (5:15:80, by vol.). The detection of mikimopine, a specific opine to *A. rhizogenes* strain A13, was performed with Pauly reagent (Isogai *et al.* 1990).

PCR analysis

PCR analysis was done as described by Kiyokawa *et al.* (1992). Total DNA was isolated from leaves of putative transformants following the method of Dellaporta *et al.* (1983). The sequence including core T-DNA genes, *rol* genes, was identified by PCR (Saiki *et al.* 1988). Primers 1724C and 1724D, designed by Kiyokawa *et al.* (1992) were used to amplify a 1105 bp DNA fragment which was expected to be the objective sequence. Fifty nanogram of template DNA and 1 μl of 5 μM each primer were mixed with 5 μl of 10 x *Taq* DNA polymerase reaction buffer (Toyobo; Tokyo, Japan), 100 μM (final concentration) of a dNTP mixture (equimolar dATP, dCTP, dGTP and dTTP) and 1 unit of *Taq* DNA polymerase (Toyobo) in a total volume of 50 μl. Forty cycles of PCR were performed in a programmed temperature control system

(PC-7000, Astec, Tokyo) with denaturation of DNA at 93 °C for 1 min, annealing at 55 °C for 1 min 30 sec, and DNA synthesis at 73 °C for 2 min. Amplified DNAs were analyzed by ethidium bromide staining after 1% (w/v) agarose gel electrophoresis at 100 V for 1 h.

Subculture of hairy roots

Hairy roots obtained from leaf segments were subcultured every 5 weeks by transferring 10 mm long root tips to 40 ml of 2 g l^{-1} gellan gum-solidified half-strength MS medium containing 30 g l^{-1} sucrose but lacking PGRs. The cultures were grown at 25 °C in the dark.

Application of PGRs and bialaphos to hairy roots

Root tips 10 mm long were excised from stock cultures of both non-transformed and a hairy roots 4 weeks after subculture, and transferred to 2 g l^{-1} gellan gum-solidified half-strength MS medium supplemented with 30 g l^{-1} sucrose and PGRs or bialaphos. As PGRs, 0, 0.1, 0.5 or 1 mg l^{-1} NAA was added in combination with 0, 1 or 5 mg l^{-1} cytokinin (BA, TDZ or zeatin). Bialaphos was applied at 0, 0.1, 0.5, 0.7, 0.9, 1.2, 3.0 or 5.0 mg l^{-1}. The experiments were carried out with the completely randomized design and repeated 5 times. The data were obtained after 3 months of culture and analyzed by Student-Newman-Keuls test (cited from Snedecor and Cochran 1980). In some experiments, the cultures with bialaphos treatments at 0.1 or 0.5 mg l^{-1} for 1, 2, 3, 4 or 5 weeks were transferred to bialaphos-free medium. Only a single line of both non-transformed and hairy root was used throughout the experiments. The cultures were kept at 25 °C under 24 h illumination (35 μmol $m^{-2}s^{-1}$) with fluorescent lamps (National FL30SN).

Plant regeneration

Regenerated shoots were detached from host roots and transferred to 2.5 g l^{-1} gellan gum-solidified half-strength MS medium containing 20 g l^{-1} sucrose without PGRs, in which only strength of mineral salts was reduced to the half. Regenerated plantlets with well-established root systems were washed carefully to remove the gellan gum and transferred to pots (9 x 9 cm) containing vermiculite. Potted plants were acclimated in a transparent plastic cabinet covered with polyethylene bags at 20 °C under 24 h illumination (45 μmol $m^{-2}s^{-1}$) with fluorescent lamps. After 2 to 3 weeks, acclimatized plants were transferred to the greenhouse.

Results

Establishment of hairy root cultures

A total of 19 axenic adventitious root cultures were established from 76 leaf explants about 3 to 8 weeks after inoculation with *A. rhizogenes* A13 (Fig. 8A). Twelve of the root cultures grew vigorously on medium lacking PGRs and showed a typical hairy root phenotype characterized by fast growth, high lateral branching and lack of geotropism (Fig. 8B). These roots were maintained in the dark at 25 °C.

The transgenic nature of these roots was confirmed by the presence of mikimopine (Fig. 9) and by PCR detection of a 1.1 kbp band which included *rol* genes (Fig. 10). As a result, we confirmed that these adventitious roots which showed vigorous growth on PGR-free medium were hairy roots transformed by *A. rhizogenes.*

Effect of NAA and cytokinins

Initially, NAA and cytokinins were examined for their ability to induce shoot regeneration by using one of the hairy root lines which showed positive responses to mikimopine and PCR detections (Table 10). On media containing only NAA at 0.1, 0.5 or 1 mg l^{-1}, hairy roots grew vigorously without shoot regeneration after 4 months of culture. Although the addition of BA, TDZ or zeatin to these NAA-containing media induced green calli from several hairy roots after one month of culture, no shoots were regenerated. Non-transformed roots also failed to regenerate shoots on the same media (data not shown).

Effect of bialaphos on shoot regeneration

When bialaphos was added to the culture medium at more than 0.5 mg l^{-1}, it inhibited the increase in fresh weight of hairy roots (Table 11). However, addition of bialaphos at 0.5 mg l^{-1} or less had less harmful effect and actually enhanced shoot regeneration at 0.5 mg l^{-1} (Fig. 8C). Adventitious shoots were induced from 10, 14 and 56% of the hairy roots after 3 months of culture on half-strength MS medium supplemented with bialaphos at 0, 0.1 and 0.5 mg l^{-1}, respectively (Table 11). However, transfer of the explants from bialaphos-containing medium to bialaphos-free medium after 7, 14, 21 or 28 days of culture had no stimulatory effect on shoot regeneration (data not shown). On the other hand, non-transformed roots did not show any shoots on the same media.

Plant regeneration

More than 30 shoots were regenerated from hairy roots on media supplemented with bialaphos. Regenerated shoots with 2 to 3 leaves were detached from the hairy roots and transferred to 2.5 g l^{-1} gellan gum-solidified half-strength MS medium containing 20 g l^{-1}

sucrose, on which they readily developed roots. Regenerated plantlets were successfully established in the greenhouse. Acclimatized plants exhibited altered phenotypes such as reduced apical dominance with highly branched stems and short internodes (Fig. 8D). The transgenic nature of the plants was reconfirmed by opine and PCR analyses. Leaves and flowers of the transformed plants were smaller than those of non-transformed plants (Fig. 8E).

Discussion

Bialaphos is a tripeptide antibiotic produced by *Streptomyces hygroscopicus* consisting of two L-alanine molecules and one L-glutamic acid analog called phosphinothricin (Kondo *et al.* 1973; Ogawa *et al.* 1973). When bialaphos is applied to plant tissue, the phosphinothricin released by degradation in plant cells functions as an inhibitor of glutamine synthetase (Bayer *et al.* 1972). The assimilation of ammonium and regulation of nitrogen metabolism in plants are controlled by the function of glutamine synthetase (Miflin and Lea 1977; Skokut *et al.* 1978), which can detoxify ammonium produced during photorespiration, inorganic nitrogen assimilation, and both catabolic and anabolic processes. Therefore, inhibition of glutamine synthetase by phosphinothricin results in the rapid accumulation of ammonium which interrupts the electron-transport systems of both chloroplasts and mitochondria, resulting in the production of free radicals (Krogmann *et al.* 1959; Puritch and Barker 1967) and ultimately the death of the plant. In the present study, high levels of bialaphos were toxic to normal roots and hairy roots, as expected. However, low concentration of bialaphos had a stimulatory effect on shoot regeneration from hairy roots of snapdragon without severe damage to the roots. Hébert-Soulé *et al.* (1995) also reported that somatic embryogenesis in grape (*Vitis* sp. L.) was stimulated by phosphinothricin at 0.5 mg l^{-1} which was the same concentration as used in the present study.

These results suggest that accumulation of the appropriate amount of ammonia caused by bialaphos treatment might act as a stress for stimulating shoot regeneration from hairy roots without leading to death.

Recently, it has been reported that somatic embryogenesis in cell culture of *Daucus carota* could be induced by stress treatments such as high concentration of sodium chloride (Kiyosue *et al.* 1989b) and heavy metal ions (Kiyosue *et al.* 1990) without any application of auxins. Prolonged treatment of carrot seeds with high concentrations of sodium hypochlorite prior to culture also had the same effect (Kiyosue *et al.* 1989a). In these studies, somatic embryos were formed after the removal of the stress. In the present study, however, shoot regeneration from hairy roots was induced on medium containing bialaphos but not observed following treatment for 28 days or less. However, short term treatments with bialaphos at higher concentrations must be examined, because the concentration used in our study might be suboptimal as a stress.

The effect of bialaphos on shoot regeneration was only observed on hairy roots but not on non-transformed roots (Table 11). Therefore, it can be considered that the effect of bialaphos for shoot regeneration observed in this study may be associated with integrated T-DNA into plant cells. T-DNA contains various open reading frames (ORFs), such as an auxin synthesis gene which regulates growth and differentiation. Bialaphos in combination with some products derived from these ORFs may synergistically affect shoot regeneration from transgenic hairy roots. The effect of other related compounds such as phosphinothricin is now being studied to clarify the mechanism involved in the bialaphos-induced shoot regeneration from hairy roots.

Regenerated plants were easily acclimatized and the plants established in soil in pots showed typical Ri syndrome, such as reduced apical dominance with highly branched stems. There was no morphological difference between regenerated plants formed on 0 and 0.5 mg l^{-1}

bialaphos. However, both of them did not set seeds, in contrast to the previous report by Handa (1992).

Although plant regeneration from hairy root cultures of snapdragon has already been reported (Handa 1992; Senior *et al.* 1995), the frequency of shoot regeneration was relatively low. In the present study, shoot regeneration from hairy roots was remarkably enhanced by an herbicide, bialaphos. The effectiveness of bialaphos on the shoot regeneration was also confirmed for the other hairy root strains in our subsequent experiments (unpublished results). As plant regeneration from hairy roots is still difficult in some species, it is possible that the difficulty in some of these recalcitrant species will be overcome by application of bialaphos. The effectiveness of bialaphos on shoot regeneration from hairy roots are now being studied for other recalcitrant plant species.

Chapter 6

Transgenic herbicide-resistant snapdragon (*Antirrhinum majus* L.) produced by bialaphos-stimulated shoot regeneration from hairy roots

Introduction

Antirrhinum majus L., commonly called snapdragon, has been used as an ornamental plant and also studied for molecular analyses on transposon (Sommer *et al.* 1988), homeotic genes for flower development (Sommer *et al.* 1990, Coen 1991) and flower pigmentation (Sommer and Saedler 1986, Moyano *et al.* 1996). Recently, transformation systems for snapdragon have been reported using *Agrobacterium rhizogenes* (Handa 1992, Senior *et al.* 1995), and it is now expected to introduce foreign genes to this species for conferring agronomically useful traits.

Genetically engineered plants with agronomically important traits have already been produced in various crops since the early successful report by Gasser and Fraley (1989). One of the most successful results of the transformation is the production of herbicide-resistant crops, some of which are now widely cultivated and the products are commercially available. The herbicide-resistant trait using *bar* gene encoding phosphinothricin acetyltransferase (PAT) from *Streptomyces hygroscopicus* (Thompson *et al.* 1987) has been produced in several crops and medicinal plants such as tobacco (*Nicotiana tabacum* L.) (De Block *et al.* 1987), potato (*Solanum tuberosum* L.) (De Block 1988), *Brassica napus* and *B. oleracea* (De Block *et al.* 1989), sugarbeet (*Beta vulgaris* L.) (D'Halluin *et al.* 1992), *Atropa belladonna* (Saito *et al.* 1992), phalaenopsis (Anzai *et al.* 1996) and *Scoparia dulcis* L. (Yamazaki *et al.* 1996). PAT inactivates bialaphos, which is a tripeptide antibiotic produced by *Streptomyces hygroscopicus* consisting of two L-alanine molecules and one L-glutamic acid analog called phosphinothricin (Kondo *et al.* 1973, Ogawa *et al.* 1973) that inhibits activity of glutamine synthetase and causes death of plant cells by ammonium accumulation (Bayer *et al.* 1972).

In this paper, we report transfer of *bar* gene into snapdragon and production of fertile transgenic herbicide-resistant plants by using an Ri binary vector system.

Materials and Methods

Plant materials

Seeds of snapdragon (*A. majus* L. cv. Floral Carpet Deep Rose (FCRose), Floral Carpet Orchid (FCO), Floral Carpet Pink (FCP), Floral Carpet Red (FCRed), Floral Carpet White (FCW) and Floral Carpet Yellow (FCY); Sakata Seed Corp.; Yokohama, Japan) were germinated on soil and grown in a growth chamber at 20±1 °C under 16:8 L:D photoperiod (45 μmol $m^{-2}s^{-1}$) by fluorescent lamps (National FL30SN).

Bacterial strains and preparation for inoculation

The plasmid pARK5 (Saito *et al.* 1992) which contains *bar* gene encoding phosphinothricin acetyltransferase under the control of the CaMV 35S promoter, and *nptII* gene encoding neomycin phosphotransferase II under the control of nopaline synthase promoter was transferred from *Escherichia coli* XL1-Blue (Stratagene) to *Agrobacterium rhizogenes* strain A13 (MAFF02-10266) (Daimon *et al.* 1990) harboring a wild mikimopine-type Ri plasmid by the method of triparental mating (Deblaere *et al.* 1987) using pRK2013 in *E. coli* HB101 as a helper plasmid (Figurski and Helinski 1979).

Prior to co-cultivation, 30 ml of 50 mg l^{-1} kanamycin-containing liquid YEB medium (Vervliet *et al.* 1974) (consisting of 5 g l^{-1} beef extract, 1 g l^{-1} yeast extract, 5 g l^{-1} peptone, 5 g l^{-1} sucrose and 2 mM $MgSO_4$, pH 7.2) prepared in a 100 ml Erlenmeyer flask was inoculated with *A. rhizogenes* strain A13 (pARK5). The bacterial cultures were incubated overnight at 28 °C on a rotary shaker (200 cycles min^{-1}). *A. rhizogenes* wild type strain A13 was also used in this study for the comparison.

Transformation by A. rhizogenes

Leaves were taken from growth chamber-grown plants 4 weeks after germination, surface-sterilized with a sodium hypochlorite solution (1% active chlorine) for 10 min and rinsed 3 times with sterile distilled water. Leaf laminae were cut into 5 mm square pieces and inoculated with freshly grown *A. rhizogenes* suspensions (about 1 x 10^8 cells ml^{-1}) using a needle. Inoculated leaf segments were co-cultivated on 2 g l^{-1} gellan gum (Gelrite; Kelco, Division of Merck and Co. Inc., San Diego, CA) -solidified half-strength MS medium (Murashige and Skoog 1962) which included half-strength salts and full-strength organic elements, containing 30 g l^{-1} sucrose. The leaf explants treated with *A. rhizogenes* were cultured in 90 mm x 20 mm plastic Petri dishes (Terumo SH-20S, Japan) which contained 40 ml of medium. About 10 explants were placed on each plate. After 5 days of co-cultivation, the leaf segments were transferred to the same medium with the addition of 500 mg l^{-1} cefotaxime (Claforan; Hoechst AG) which was supplemented after autoclaving in order to eliminate *A. rhizogenes*. Leaf segments were subcultured every 2 weeks on the same medium. Root tips (about 10 mm) from adventitious roots produced at the inoculated site of the explants 3 to 8 weeks after inoculation were excised and transferred to the same medium to obtain *A. rhizogenes*-free cultures. The pH of the culture media was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. All dishes were sealed with Parafilm and maintained at 25 ± 1 °C in the dark throughout this experiment.

Subculture of hairy roots

Hairy root cultures established after elimination of *A. rhizogenes* were subcultured every 5 weeks by transferring 10 mm long root tips to 40 ml of 2 g l^{-1} gellan gum-solidified half-strength MS medium containing 30 g l^{-1} sucrose but lacking PGRs. The cultures were grown at 25 ± 1

°C in the dark.

Opine analysis

Opine detection was performed as described by Isogai *et al.* (1990). About 100 mg fresh weight of cultured root tissue was ground in a microtube containing a bit of quartz sand and two drops of 1N HCl by Pasteur pipette. After centrifugation at 6000 x g for 15 min, 20 μl of supernatant was spotted on a Whatman 3MM paper and subjected to high-voltage paper electrophoresis at 450 V for 2 h (Petit *et al.* 1983). The electrode buffer consisted of formic acid, acetic acid and distilled water (5:15:80, by vol.). The detection of mikimopine, a specific opine to *A. rhizogenes* strain A13, was performed with Pauly reagent Isogai *et al.* (1990).

Selection by bialaphos

It has been reported that growth of hairy roots of snapdragon was completely inhibited by 0.9 mg l^{-1} bialaphos treatment (Hoshino and Mii 1997). According to this result, the hairy root cultures obtained in the present study were placed on medium containing 1 mg l^{-1} bialaphos to select the hairy root clones integrating *bar* gene. The medium used was 2 g l^{-1} gellan gum-solidified half-strength MS medium containing 30 g l^{-1} sucrose which was supplemented with bialaphos prior to autoclaving. Twenty seven hairy root clones were cultured in each 90 mm x 20 mm plastic Petri dish (Terumo SH-20S, Japan) which contained 40 ml of medium. The cultures were grown at 25 ± 1 °C in the dark.

Resistant level of bar-integrated hairy root clones towards bialaphos

To investigate resistant level of *bar*-integrated hairy root clones towards bialaphos, root

tips of 10 mm long were excised from stock cultures of bialaphos-resistant hairy roots or hairy roots transformed by wild type strain of *A. rhizogenes* 4 weeks after subculture and transferred to 2 g l^{-1} gellan gum-solidified half-strength MS medium supplemented with 30 g l^{-1} sucrose and different concentrations of bialaphos. Bialaphos was applied at 0, 0.5, 1, 2, 3, 5, 10, 20, 30, 50 or 100 mg l^{-1}. The media were prepared in 90 mm x 20 mm plastic Petri dishes (Terumo SH-20S, Japan), each of which contained 40 ml of medium. Ten root tips were placed on medium in each dish. Fresh weight of hairy roots on each dish was measured after 5 weeks of culture. The experiments were carried out with the completely randomized design and repeated 3 times. All dishes were maintained at 25 ± 1 °C in the dark.

Plant regeneration

Adventitious shoots were regenerated from hairy root cultures on 1 mg l^{-1} bialaphos-containing medium. Regenerated shoots were detached from host roots and transferred to 2.5 g l^{-1} gellan gum-solidified half-strength MS medium containing 20 g l^{-1} sucrose without PGRs, in which only strength of mineral salts was reduced to the half. Regenerated plantlets with well-established root systems were washed carefully to remove the gellan gum and transferred to pots (9 x 9 cm) containing vermiculite. Potted plantlets were acclimated in a transparent plastic cabinet covered with polyethylene bags at 20 ± 1 °C under 24 h illumination (45 μmol m^{-2} s^{-1}) with fluorescent lamps in the growth chambers.

PCR analysis

Polymerase chain reaction (PCR) was done to detect *rol*, *nptII* and *bar* genes. Total DNA was isolated from leaves of putative transformants following the method of Dellaporta *et al.*

(1983).

Primers 1724C and 1724D, designed by Kiyokawa *et al.* (1992) were used to amplify a 1105 bp DNA fragment which was expected to be the objective sequence including *rol* genes on Ri T-DNA. Fifty nanogram of template DNA and 1 μl of 5 μM each primer were mixed with 5 μl of 10 x *Taq* DNA polymerase reaction buffer (Toyobo; Tokyo, Japan), 100 μM (final concentration) of a dNTP mixture (equimolar dATP, dCTP, dGTP and dTTP) and 1 unit of *Taq* DNA polymerase (Toyobo) in a total volume of 50 μl. Forty cycles of PCR were performed in a programmed temperature control system (PC-7000, Astec, Tokyo) with denaturation of DNA at 93 °C for 1 min, annealing at 55 °C for 1 min 30 sec, and DNA synthesis at 73 °C for 2 min.

Primers used for detecting *nptII* and *bar* genes were designed by Hamill *et al.* (1991) and Anzai *et al.* (1996) to amplify 700 and 412 bp DNA fragments, respectively, which were expected to include the objective sequences. Composition of reaction mixtures and cycles of amplification were the same as those used by Hamill *et al.* (1991) for *nptII* gene and Anzai *et al.* (1996) for *bar* gene, respectively.

Amplified DNAs were analyzed by ethidium bromide staining after 1.5% (w/v) agarose gel (SEA KEM® GTG®, FMC BioProducts, USA) electrophoresis at 100 V for 1 h.

Southern blot analysis

The PCR products after amplification using primers for detecting *bar* gene were electrophoresed in a 1.5% (w/v) agarose gel, transferred to nylon Hybond-N$^+$ filter (Amersham), and hybridized with ECL™ (Amersham)-labelled *bar* gene sequence from plasmid pARK5.

Measurements of morphological characteristics

Several morphological characteristics were investigated in non-transformed and transformed plants on 5-9 July 1997. The morphological characteristics of the flowers, such as length of the corolla, width of lower lip, and length between upper and lower lips were measured.

Pollen fertility

Pollen fertility of the non-transformed and transformed plants was evaluated by staining more than 300 pollen grains per flower with aceto-carmine on 10-15 June 1997. The pollen grains were obtained from ten flowers per plant and observed using microscope (BX-50, Olympus).

Herbicide applications

Two commercially available herbicides, HERBIE™ (Meiji Seika Kaisha, LTD., Japan) and Basta® (Hoechst AG, FRG) containing 18% bialaphos and 20% D,L-glufosinate (phosphinothricin), respectively, were used for evaluation of herbicide resistance. Both herbicides at 0.5% solution diluted by distilled water were sprayed to the leaves of non-transformed and transformed plants.

Results

Selection of hairy roots by bialaphos

Within 3 to 8 weeks after inoculation with *A. rhizogenes* A13, adventitious roots appeared on leaf explants at the sites inoculated by needles irrespective of the presence of pARK5 (Fig. 11). Totally 24 and 45 adventitious root cultures were obtained from all cultivars examined after

inoculation with wild type and pARK5-containing strains of *A. rhizogenes*, respectively (Table 12). Adventitious roots were excised from host explants and placed on PGR-free medium. Vigorously growing roots on this medium were selected and subjected to opine assay (Fig. 12) to confirm integration of Ri T-DNA. As a result, 27 root clones obtained from all cultivars examined except for cv. FCW showed a presence of mikimopine following opine assay. Therefore, we concluded that these adventitious roots which showed vigorous growth on PGR-free medium were hairy roots transformed by *A. rhizogenes*.

For selecting resistant hairy root clones to bialaphos, root tips (10 mm long) were excised from these hairy roots and placed on 1 mg l^{-1} bialaphos-containing medium, where hairy roots transformed by a wild type strain of *A. rhizogenes* A13 could not survive as reported by Hoshino and Mii (1997). As a result, 12 out of 22 hairy root strains induced by inoculation with *A. rhizogenes* A13 (pARK5) revealed resistance to bialaphos (Table 12), indicating that frequency of co-transformation with both Ri T-DNA and T-DNA of pARK5 was 54.5%.

Resistant level of bar-inserted hairy root clones to bialaphos

It has been reported that growth of hairy roots transformed by wild type *A. rhizogenes* was inhibited by bialaphos at more than 0.9 mg l^{-1} (Hoshino and Mii 1997). In the present study, growth of hairy root clone FCY.A13.2 transformed by wild type strain of *A. rhizogenes* was completely inhibited by addition of bialaphos at 1 mg l^{-1} or more, whereas *bar*-integrated hairy root clones, both FCY5.3.1 and FCY5.2.3 showed resistance up to 100 mg l^{-1} bialaphos (Table 13 and Fig. 13). Furthermore, the growth of *bar*-integrated hairy root clone FCY5.3.1 was enhanced by addition of bialaphos at 30 to 50 mg l^{-1} and that of FCY5.2.3 at 2 to 5 mg l^{-1}, respectively (Table 13).

Regeneration of transgenic plants from bar-integrated hairy roots

It has been shown that low concentration of bialaphos at 0.5 mg l^{-1} stimulated shoot regeneration from hairy roots of snapdragon transformed by wild type strain of *A. rhizogenes* (Hoshino and Mii 1997). The similar effect of bialaphos on shoot regeneration was also observed in bialaphos-resistant hairy roots at a higher concentration, i.e. seven out of 12 *bar*-integrated hairy root clones regenerated shoots on medium containing 1 mg l^{-1} bialaphos (Fig. 14). All of the *bar*-integrated hairy root clones from cv. FCO, FCP, FCRose and FCY expected for one clone of cv. FCRose showed shoot regeneration on 1 mg l^{-1} bialaphos-containing medium.

Regenerated shoots with 2 to 3 leaves were detached from the hairy roots and transferred to 2.5 g l^{-1} gellan gum-solidified half-strength MS medium containing 20 g l^{-1} sucrose, on which they readily developed roots. Regenerated plantlets were successfully established in the growth chamber after acclimatization. The plants thus established exhibited altered phenotypes known as Ri syndrome such as reduced apical dominance with highly branched stems and short internodes.

The transgenic nature of these regenerated plants was confirmed by PCR and Southern blot analyses. The integration of the Ri T-DNA was shown by detecting a 1.1 kbp band which includes *rol* genes (Fig. 15). The presence of T-DNA of pARK5 was indicated in all bialaphos-resistant regenerants examined by using PCR amplification of 700 bp fragment for *nptII* and 412 bp fragment for *bar* gene, respectively (Figs. 16 and 17). The integration of *bar* gene was also confirmed by Southern hybridization with PCR products. A DNA fragment containing the coding sequence of *bar* gene from plasmid pARK5 was used as a probe and hybridized with

PCR products as shown in Fig. 17. As a result, it was proved that PCR products after amplification included *bar* gene sequence (Fig. 18). These results indicate that T-DNAs of both Ri plasmid and pARK5 were integrated in the plant genome.

Flower characteristics and pollen fertility

The flower shape of the transformed plants greatly altered from that of non-transformed plants (Table 14). The length of corolla of clone FCY5.3.1 was shorter than that of the control plants. In contrast, the width of lower lip was almost the same between the transgenic and control plants. Characteristic feature of transformed plants was shown in length between upper and lower lips, which was shorter than that of control plants (Table 14 and Fig. 19A and B).

The pollen fertility was different among transformed plants (Table 15). Clone FCY5.3.1 had almost the same pollen fertility as the control plants. On the other hand, the pollen fertility of other transformed plants was lower than that of the control plants. No difference in pollen fertility was observed between *bar*-integrated and only wild type Ri T-DNA-integrated plants. Also the size of pollen grain did not differ between non-transformed (clone FCY.A13.4) and transformed (clone FCY5.3.1) plants (Fig. 20).

Resistance to herbicides

To confirm resistance of the transformants towards the commercial formulation of the herbicides, we applied the HERBIE™ and Basta® separately to regenerated plants of clone number FCY5.3.1 and FCO5.1.1. Non-transformed plants of cv. FCY and FCO and regenerated plants from hairy roots of these cultivars transformed by wild type strain of *A. rhizogenes* A13 were also used as controls.

When HERBIE™ or Basta® was applied to single leaves of control plants, they turned brown from the tip within 2 days (Fig. 21A) and died completely within 7 days. Whereas, leaves of clones FCY5.3.1 and FCO5.1.1 continued to survive and showed resistance to both herbicides in the same treatment (Fig. 21B). Application of herbicides to whole plants also showed resistance in *bar*-integrated plants, whereas both cv. FCP and clone FCY.A13.2 died within 7 days after herbicide application (Fig. 22).

Discussion

It has been known that transferred Ri T-DNA in plant cells could induce hairy roots. Ability of hairy root induction is utilized for selection of transformed cells and has an advantage over other transformation methods such as *A. tumefaciens*-mediated one because of the unnecessity of any marker genes. Recently, the binary vector systems using *A. rhizogenes* have been applied in several plant species, such as potato (*Solanum tuberosum* L.) (Visser *et al.* 1989), *Atropa belladonna* (Saito *et al.* 1992), sweet potato (Otani *et al.* 1993) and *Scoparia dulis* L. (Yamazaki *et al.* 1996). The frequencies of co-transformation in these studies ranged from 20 to 50%. In the present study, frequency of co-transformation evaluated from the transgenic plants totally obtained (22 plants) irrespective of the difference in cultivars was relatively high (54.5%).

The plants transformed by Ri plasmid vector are also known to exhibit Ri syndrome which is usually considered to be unfavorable traits for plant breeding. In case of ornamental plant species, however, some of the Ri syndromes such as dwarfness are expected to be used as novel traits to increase ornamental quality. In fact, Pellegrineschi *et al.* (1994), Scorza *et al.* (1994) and Godo *et al.* (1997) tried to evaluate the morphological alterations as useful traits in

horticultural plants. In the present study, we obtained several clones which exhibited varying degree of morphological alterations such as varied flower morphology, increased flower number and dwarfness (Table 14). These traits are now expected to be utilized to improve snapdragon cultivars.

In the present study, it was shown that *bar*-integrated hairy root clones revealed resistance to high concentrations of bialaphos at up to 100 mg l^{-1} in *in vitro* culture, whereas hairy roots transformed by wild type *A. rhizogenes* were sensitive at 1 mg l^{-1}. This indicates that *bar*-integrated hairy roots of snapdragon were conferred with high level of resistance to bialaphos and that *bar* gene is useful not only to confer with agronomically important trait but also to select transformed clones exclusively. All *bar*-integrated plants after acclimatization also revealed resistance to standard level of herbicide application. The herbicide resistant trait will be useful as weed control in field and garden, and decrease labor for eliminating weeds.

The herbicide resistant plants obtained in this study were fertile and set seeds. Therefore, it can be expected that usefulness of the integrated foreign genes, both *bar* gene as conferring herbicide resistance and Ri T-DNA as inducing phenotypic alterations for snapdragon breeding will be evaluated through the genetical analyses of the progenies such as on copy number and stabilities of the gene expression. As segregation of *bar* gene and Ri T-DNA is expected in the progeny population, it is possible to produce fertile herbicide resistant snapdragon without Ri syndromes via *A. rhizogenes*-mediated transformation system established in the present study.

Chapter 7

Production of transgenic grapevine (*Vitis vinifera* L. cv. Koshusanjaku) plants by co-cultivation of embryogenic calli with *Agrobacterium tumefaciens* and selecting secondary embryos

Introduction

Grapevines (*Vitis* spp.) are one of the major fruit crops throughout the world. For the genetic transformation of grapevines, it is necessary to establish highly efficient system of plant regeneration from cell and tissue cultures. Recently, induction of somatic embryogenesis from leaves (Stamp and Meredith 1988; Matsuta and Hirabayashi 1989), ovaries (Gray and Mortensen 1987), ovules (Rajasekaran and Mullins 1985), immature zygotic embryos (Emershad and Ramming 1994) and anthers (Stamp and Meredith 1988; Gray and Mortensen 1987; Mozsár and Süle 1994) has been reported in several species of *Vitis*. These embryogenic cultures have been shown to be useful materials for *Agrobacterium*-mediated transformation in *Vitis* species (Martinelli and Mandolino 1994; Nakano *et al.* 1994). However, transformation efficiency of embryogenic callus in co-cultivation with *Agrobacterium* has not yet been studied in detail. In the present study, therefore, we tried to establish a protocol for genetic transformation of *Vitis* by using embryogenic calli and showed the recovery of transgenic plants with GUS activity among secondary embryos formed on somatic embryos in the presence of kanamycin.

Materials and methods

Plant materials and induction of embryogenic calli

Embryogenic calli were initially induced from leaf segments excised from *in vitro*-subcultured plantlets of *V. vinifera* cv. Koshusanjaku according to the method of Matsuta and Hirabayashi (Matsuta and Hirabayashi 1989) with several modifications (Nakano *et al.* 1994). The embryogenic calli were maintained by subculturing monthly on Nitsch's medium (1969) (Nitsch and Nitsch 1969) lacking vitamins, inositol and glycine but supplemented with 1 μM 2,4-D and 30 g l^{-1} sucrose, and solidified with 2 g l^{-1} gellan gum (Wako Pure Chemical

Industries, Ltd.). The friable callus cultures containing no appreciable embryos established after several subcultures were used as the material for co-cultivation with *Agrobacterium tumefaciens*.

Agrobacterium tumefaciens strains

The *A. tumefaciens* strain LBA4404 (pTOK233) which was provided by Japan Tobacco Inc. (Hiei *et al.* 1994) was used for the transformation studies. The plasmid pTOK233 possessed the inserted *virB*, *virC* and *virG* genes derived from supervirulent Ti-plasmid pTiBo542 (Watson *et al.* 1975), which is known to retain an ability to enhance the virulence of *A. tumefaciens* against plant cells (Jin *et al.* 1987). The T-DNA region of pTOK233 contained neomycin phosphotransferase II (NPTII) under the control of nopaline synthase (NOS) promoter, hygromycin phosphotransferase (HPT) under the control of cauliflower mosaic virus (CaMV) 35S promoter, and β-glucuronidase (GUS) with an intron fused to CaMV 35S promoter.

Another *A. tumefaciens* strain used was EHA101 (Hood *et al.* 1986) which was harboring plasmid pIG121Hm (Ohta *et al.* 1990.) containing NPTII, HPT and GUS in the T-DNA region as pTOK233.

Co-cultivation of embryogenic calli with Agrobacterium

Prior to co-cultivation, 30 ml liquid AB medium (Chilton *et al.* 1974) containing 50 mg l^{-1} hygromycin was prepared in 100 ml Erlenmeyer flask and inoculated with *A. tumefaciens* strains LBA4404 (pTOK233) or EHA101 (pIG121Hm). The bacterial cultures were incubated for more than 30 hrs at 28°C with reciprocal shaking (130 min^{-1}).

Two gram fresh weight of embryogenic calli 3 weeks after subculture were put on nylon membrane (40 μm pore size) and then exposed with 5 ml of freshly grown suspension culture of

bacteria. Inoculated embryogenic calli were washed several times with sterilized distilled water and placed for co-cultivation on 2 g l^{-1} gellan gum-solidified Nitsch's medium (1969) containing 30 g l^{-1} sucrose and 1 μM 2,4-D with or without 100 μM acetosyringone. After 5 days of co-cultivation in the dark, embryogenic calli were transferred for eliminating *Agrobacterium* to the same medium lacking acetosyringone but containing 500 mg l^{-1} cefotaxime (Claforan; Hoechst AG), which was supplemented after autoclaving. After 2 weeks, the cultures were transferred for inducing somatic embryogenesis to 2 g l^{-1} gellan gum-solidified Nitsch's medium (1969) lacking plant growth regulators but containing 20 g l^{-1} sucrose and 500 mg l^{-1} cefotaxime, and kept under 24 h illumination (35 μmol $m^{-2}s^{-1}$) with fluorescent lamps (National FL30SN). Four weeks after the bacterial inoculation, the cultures were placed on the same medium but supplemented with 50 mg l^{-1} kanamycin which was filter-sterilized (Millipore, 0.45 μm pore size) and added after autoclaving the medium for selecting transformed cells. The embryogenic calli with somatic embryos were subcultured every 2 weeks on the same medium with kanamycin. The pH of the culture media used throughout this experiment was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. All the calli and somatic embryos were cultured in 90 mm x 20 mm plastic Petri dishes (Terumo SH-20S, Japan), each containing 40 ml of medium. All the dishes were sealed with Parafilm® and maintained at 26 ± 1 °C.

GUS histochemical assay

Histochemical localization of GUS gene expression was detected in kanamycin-resistant calli, somatic embryos and leaves of regenerated plants according the method of Jefferson *et al.* (1987) with several modifications. For staining, tissues were incubated at 37°C for 3-5 hours in

Na-phosphate buffer (50 mM, pH 7.0) which contained 1 mM X-Gluc (5-bromo-4-chloro-3-indolylglucuronide : Wako Pure Chemical Industries, Ltd.). To remove chlorophyll, leaf tissues were washed several times with 70% ethanol after the incubation. The tissues developing indigogenic dye were evaluated as those expressing GUS gene.

Plant regeneration

Heart- to torpedo-shaped embryos derived from embryogenic calli were picked by forceps and placed on 2 g l^{-1} gellan gum-solidified Nitsch's medium (1969) containing 20 g l^{-1} sucrose and 50 mg l^{-1} kanamycin. The cultures were transferred monthly onto the same medium. Regenerated plantlets with well-expanded 5 to 6 leaves were washed carefully to remove the gellan gum and transferred to pots (9 x 9 cm) containing vermiculite. Potted plants were acclimatized in a transparent plastic cabinet covered with polyethylene bags at 20 ± 1 °C under 24 h illumination (45 μmol $m^{-2} s^{-1}$) with fluorescent lamps in the growth chambers.

DNA extraction and PCR analysis

Total genomic DNAs were isolated from leaves of putative transgenic plants following the method of Harding and Roubelakis-Angelakis (1994). Specific oligonucleotide primers for detecting GUS and NPTII gene sequences (Hamill *et al.* 1991) were used to identify the presence of these genes in the genomic DNAs. Fifty nanogram of template DNA and 5 μM primers each at 1 μl were mixed with 2.5 μl of 10 x Taq DNA polymerase reaction buffer, 100 μM (final concentration) of a dNTP mixture (equimolar dATP, dCTP, dGTP and dTTP) and 1 unit of Taq DNA polymerase (Toyobo, Japan) in a total volume of 25 μl. Forty cycles of PCR were performed in a programmed temperature control system (PC-7000, Astec, Tokyo). A

single cycle consisted of the following steps: denaturation of DNA at 92 °C for 1 min, annealing at 55 °C for 1 min, and DNA synthesis at 73 °C for 1.5 min. Amplified DNAs were detected after running on 1.5% (w/v) agarose gel electrophoresis using TAE as a running buffer at 100 V for 1 h.

Results and discussion

Prior to co-cultivation experiments, sensitivity of embryogenic calli to kanamycin was investigated to select transformed cells. In our preliminary experiment, kanamycin at 50 mg l^{-1} showed toxicity to embryogenic calli of *V. vinifera* cv. Koshusanjaku and completely inhibited the growth of calli with browning 4 weeks after transfer (Nakano *et al.* 1994). As a result, we employed 50 mg l^{-1} kanamycin to carry out selection of transformed cells after co-cultivation with *A. tumefaciens*.

The calli used in this study (Fig. 23) were friable and brownish white, and had retained embryogenic potential with high plant regeneration ability for more than 4 years. These embryogenic calli were subjected to GUS assay 7 days after inoculation. GUS-positive cells were observed as shown in Fig. 24, whereas no endogenous GUS activity was detected in non-co-cultivated tissues. Frequency of blue spots obtained by GUS histochemical assay was different between the 2 bacterial strains examined and LBA4404 (pTOK233) always showed higher number of GUS spots than EHA101 (pIG121Hm) (Table 16). It has been reported that addition of acetosyringone to co-culture media or bacterial suspensions was effective to increase transformation efficiency in several plant species such as *Brassica* (Charest *et al.* 1989), apple (James *et al.* 1993) and tomato (Davis *et al.* 1991; Lipp João and Brown 1993). In the present study, effectiveness of acetosyringone was also confirmed in grapevine. The number of GUS

spots was drastically increased by adding acetosyringone to co-culture medium in both bacterial strains although the data were fluctuated among the 2 repeated experiments (Table 16). The highest frequency of GUS spots was obtained when acetosyringone was used with *A. tumefaciens* stain LBA4404 (pTOK233).

In the present study, it was revealed that *A. tumefaciens* strain LBA4404 (pTOK233) could transform *Vitis* at high transformation frequency compared to EHA101 (pIG121Hm) (Table 16). It has previously been reported that the former bacterial strain had an ability to introduce foreign genes to rice (Hiei *et al.* 1994; Aldemita and Hodges 1996), which was a recalcitrant plant for transformation by *Agrobacterium*. Predominance of LBA4404 (pTOK233) for the transformation of *Vitis* may be due to the plasmid pTOK233 which possessed *virB*, *virC* and *virG* derived from supervirulent Ti-plasmid pTiBo542 (Hiei *et al.* 1994).

Embryogenic calli could produce embryos within 1 month after co-cultivation treatment. However, these embryos turned brown at some part of the tissues due to the damage caused by transferring to 50 mg l^{-1} kanamycin-containing medium. During the subsequent culture on kanamycin-containing medium, they initiated to produce secondary embryos on survived tissues which were expected to originate from transformed cells (Fig. 25). Four months after bacterial inoculation, approximately 20-30% of the secondary embryos showed GUS expression as shown in Fig. 26 (Table 17). It is possible that the GUS-negative secondary embryos were transgenic ones with NPTII gene but lost or silenced GUS gene, or escaped ones which did not integrate any foreign genes. Therefore, we tried to recover transgenic plants from secondary embryos by using selection of kanamycin.

Thus, secondary embryo-derived plants were successfully regenerated on kanamycin-containing medium after co-cultivation with LBA4404 (pTOK233) or EHA101 (pIG121Hm) of

A. tumefaciens. GUS-positive plants were selected among regenerated plants showing GUS activity in excised leaves (Fig. 27). They were readily acclimatized after transfer to pots in soil when they grew into the plants with fully expanded 4-5 leaves, and successfully transferred to the growth chambers (Fig. 28).

GUS-positive plants were subjected to DNA analysis using PCR for detecting GUS and NPTII sequences. The reaction using total genomic DNAs extracted from leaves allowed to detect 1.2 and 0.7 kbp bands which coincided with GUS and NPTII genes, respectively (Fig. 29A and B). As a result, we could confirm that they were transgenic plants with both GUS and NPTII genes.

In the previous study, we produced transgenic plants of grapevine via *A. rhizogenes*-mediated transformation of embryogenic calli (Nakano *et al.* 1994). However, it was needed to select transgenic plants which had no introduced undesirable Ri T-DNA. In the present study, we succeeded in introducing only the marker genes by using disarmed *A. tumefaciens*. Transformation of embryogenic calli with *A. tumefaciens* has previously been reported by Martinelli and Mandolino (1994). However, they did not show the transformation efficiency. In the present study, we developed the method for producing transgenic plants of *V. vinifera* L. by selecting the secondary embryos which were resistant to kanamycin after co-cultivation of embryogenic calli with *A. tumefaciens*. After selection with kanamycin, approximately 40% of the regenerated plants were transgenic with NPTII and GUS genes (Table 17). Relatively high transformation efficiency shown in the present study will encourage the introduction of some useful genes for grapevine breeding. Further studies will be concentrated to produce transgenic *Vitis* plants with useful genes such as those encoding disease resistance by using the method established in the present study.

Chapter 8

Conclusion

Conclusion

Although successful application of biotechnologies has been reported in a number of crop plants such as rice (Kyozuka and Shimamoto 1995), maize (Wilson *et al.* 1995), soybean (Widholm 1995), barley and wheat (Mendel and Teeri 1995), it is still limited in restricted plant species and genotypes. Therefore, it is still necessary to establish the systems for plant regeneration and transformation from protoplasts, cells and tissues in individual plant species and genotypes. In the first half of this thesis, protoplast culture systems established in saintpaulia, *Dianthus* cultivars and *Silene armeria* were described. Also, isolation of female gametes of *Dianthus* was attempted as the application of the protoplast technology. In the latter part of this thesis, genetic transformation was demonstrated in snapdragon and grapevine.

In saintpaulia (*Saintpaulia ionantha* Wendl.), protoplasts were previously isolated mechanically or enzymatically from leaves, petioles and cultured petiole cross-sections of *in vitro*-grown saintpaulia plantlets (Hughes 1977; Bilkey and Cocking 1982). However, protoplast division leading to plant regeneration was not demonstrated in these studies. Also in the preliminary experiments of the present thesis, no plant regeneration was obtained, although only a few protoplasts could be isolated from leaves of stock cultures (Hoshino, unpublished data). Therefore, at first, induction of suspension culture was attempted from friable calli as a source of protoplast isolation. Cell suspension culture was induced from friable calli obtained from leaf explants on B5 medium containing 1 mg l^{-1} 2,4-D and 2 g l^{-1} casein hydrolysate. Protoplasts isolated from the suspension cells could start to divide and form colonies. Shoots were regenerated from protoplast-derived calli on shoot induction media. Regenerated plantlets were successfully established in the greenhouse. By using the protoplast culture system developed in this study, production of both somatic hybrid plants and PEG- or electroporation-

mediated transgenic plants would be possible.

In chapter 3, shoot regeneration abilities of the protoplast-derived calli were compared among *Dianthus* cultivars, and culture requirements of suspension cell-derived protoplasts were examined in *Silene armeria* L.. Among the *Dianthus* cultivars examined, cv. Telster White showed the highest frequencies of both cell division and shoot regeneration. In contrast, no shoots were regenerated in *S. armeria* L., although cell suspension-derived protoplasts could divide and form colonies. Using the culture conditions obtained in this study, intergeneric somatic hybrids will be produced between *S. armeria* L. and *Dianthus* species through protoplast fusion using the *Dianthus* cultivars which retain plant regeneration ability.

Chapter 4 demonstrated that isolation of embryo sacs was possible in *Dianthus* by enzymatic maceration followed by dissection with glass needles based on histological observations. The result would offer a new approach to *in vitro* fertilization studies in *Dianthus*.

In chapter 5, it was found that bialaphos was effective for shoot regeneration from hairy roots of snapdragon (*Antirrhinum majus* L.) transformed by *Agrobacterium rhizogenes*. Some possible mechanisms involved in this phenomenon were considered according to the results of the several experiments. It is also suggested that bialaphos might be utilized for shoot regeneration from hairy roots in other recalcitrant plant species.

In chapter 6, transgenic herbicide-resistant plants of snapdragon were produced by introducing *bar* gene through Ri binary vector system based on the results in chapter 5. The transgenic plants exhibited altered phenotypes known as Ri syndrome such as reduced apical dominance with highly branched stems and short internodes, and also showed resistance to a standard level of commercial herbicides applied.

Grapevine (*Vitis* spp.) is one of the recalcitrant plants to introduce a foreign gene. In chapter 7, it was found that embryogenic calli as a source of inoculation and strains of

Agrobacterium were the important factors for transformation of grapevine. Transgenic grapevine (*V. vinifera* L. cv. Koshusanjaku) plants were successfully produced by co-cultivation of embryogenic calli with *Agrobacterium tumefaciens* and selection of the secondary embryos.

The methods thus established in the present study for protoplast culture, isolation of embryo sacs and *Agrobacterium*-mediated transformation will provide useful tools for further production of novel plants with desirable traits not only in the crops studied in the present study but also in other horticultural crops through somatic or gamete-somatic hybridization and genetic transformation.

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Figures and Tables

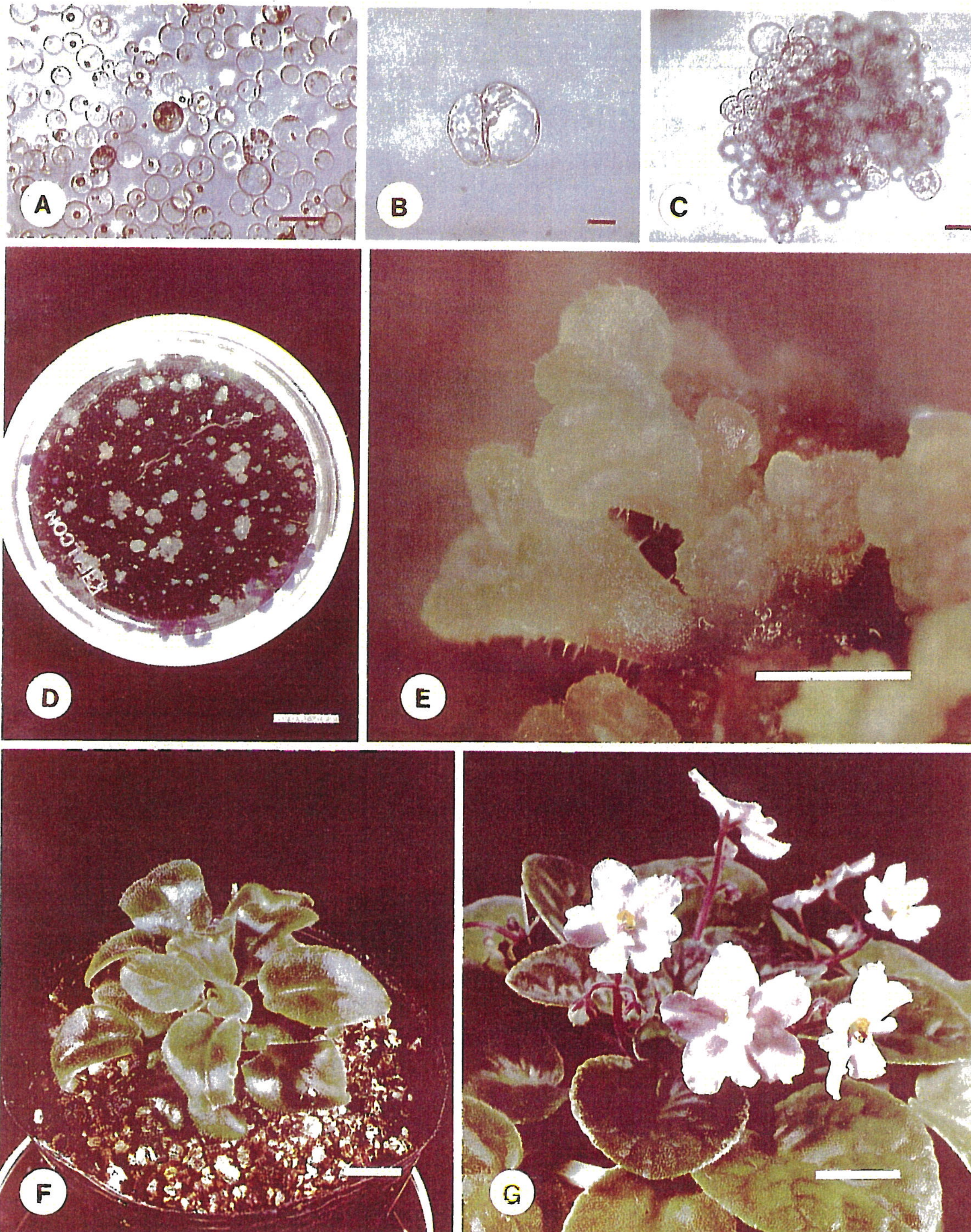

Fig. 1. Plant regeneration from protoplasts of saintpaulia. **A.** Protoplasts isolated from suspension cultured-cells. Bar = 40 μm. **B.** First division of protoplasts after 5 days of culture. Bar = 15 μm. **C.** Protoplast-derived colony after 2 months of culture. Bar = 90 μm. **D.** Protoplast-derived visible colonies after 2 months of culture. Bar = 0.5 cm. **E.** Adventitious shoot regeneration from a protoplast-derived callus on B5 medium containing 0.01 mg l^{-1} NAA and 5 mg l^{-1} BA 9 months after the initiation of protoplast culture. Bar = 1 cm. **F.** A protoplast-derived plant established in a pot. Bar = 1 cm. **G.** A regenerated plant in the flowering stage. Bar = 1 cm.

Fig. 2. Longitudinally dissected ovary of *Dianthus* one day after anthesis. Ovules were raised on free central placenta Bar = 2.5 mm.

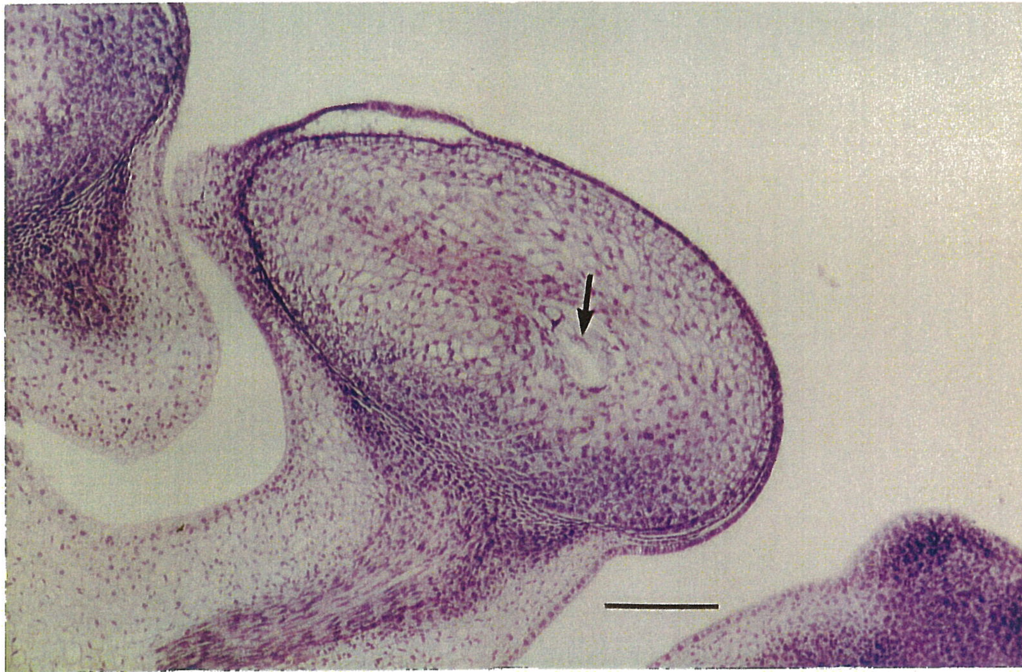

Fig. 3. Sectioned ovule of *Dianthus*, showing hemitropous type. Nucellar tissue at micropylar region was thick and occupied almost half part of mature ovule. Note the embryo sac (arrow) which locates deep in chalazal portion. Bar = 200 μm.

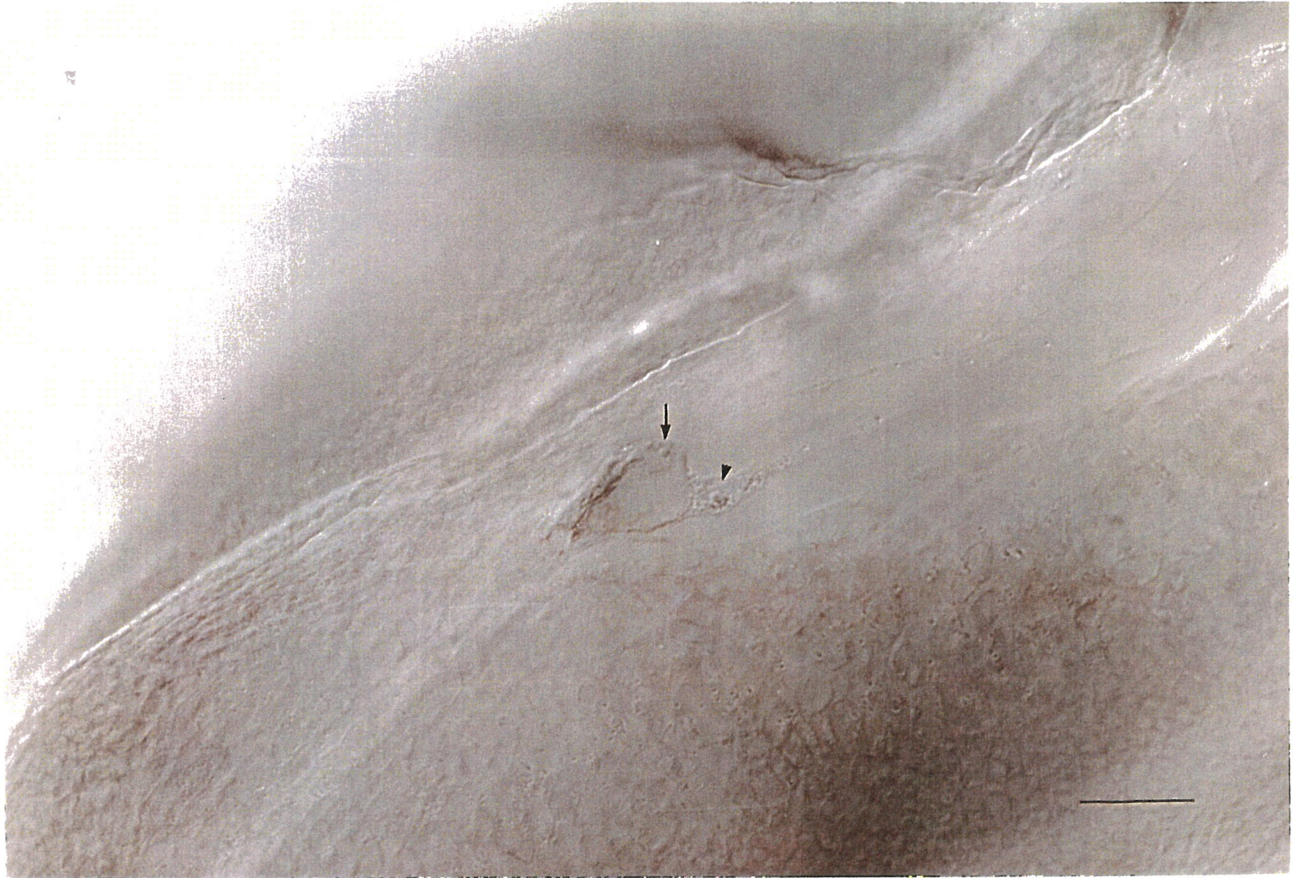

Fig. 4. Microscopic observation of embryo sac under differential interference-contrast optics after staining with acid-haemalaum and clearing with BBD. Nucleus of egg cell (*arrow*) and polar nucleus (*arrowhead*) are in focus. Bar = 40 μm.

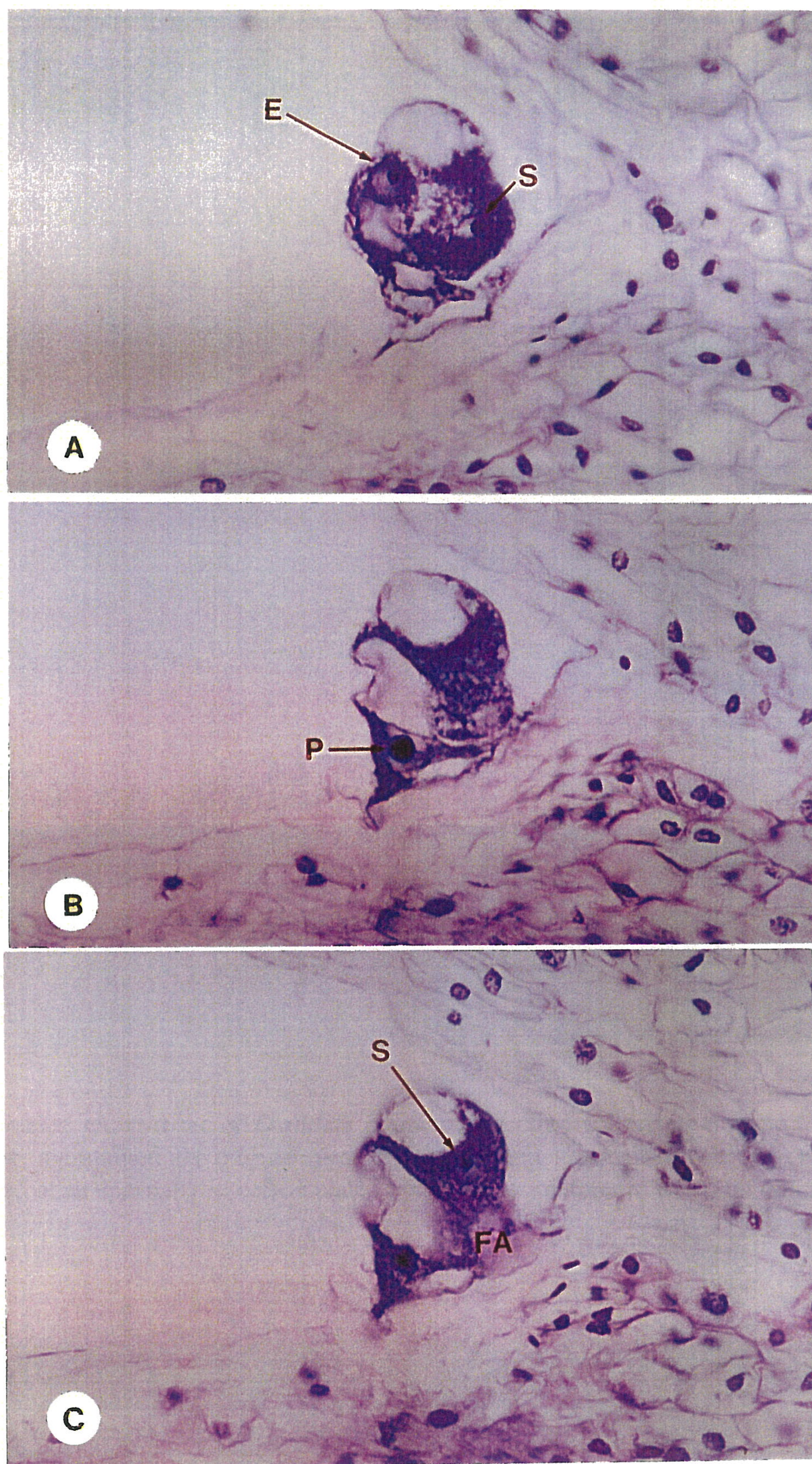

Fig. 5. Serial sections (10 μm thick) of an ovule of *Dianthus* showing egg apparatus one day after anthesis. The cells of egg apparatus possess polarity and specific position of the nuclei and vacuoles. Nucleus of egg cell locates at the side facing to central cell (A), whereas nuclei of synergid cells, which are characterized with filiform apparatus, locate at the opposite side (A and C). One polar nucleus derived from fusion of 2 nuclei in central cell was located near egg apparatus at this developmental stage (B). Each bar = 40 μm.

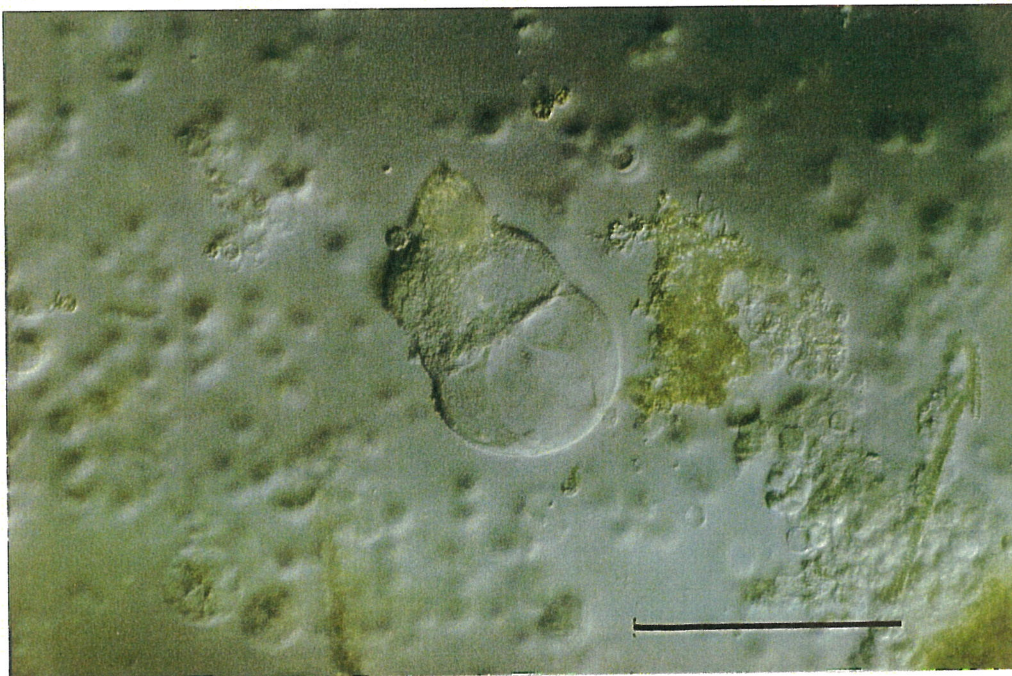

Fig. 6. Isolated embryo sac of *Dianthus* by microdissection following enzyme treatment. The embryo sac maintained its original pear-like shape and size which was extraordinary large compared to other sporophytic cells isolated by the same treatment. Bar=200 μm.

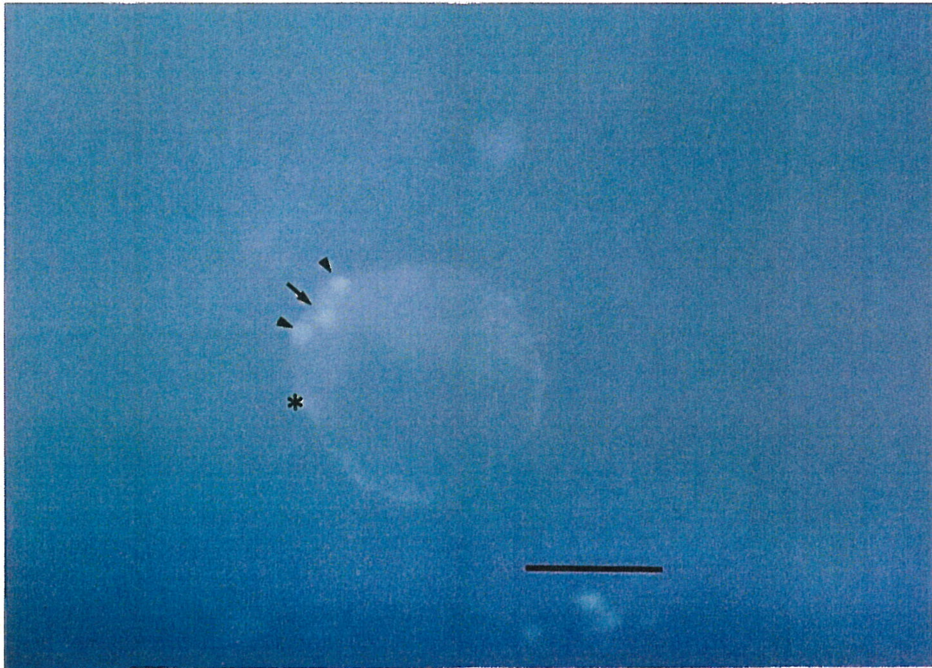

Fig. 7. Isolated embryo sac after DAPI staining. The embryo sac consisted of nuclei of egg cell (*arrow*), synergid cells(*arrowhead*) and central cell (position of asterisk, out of focus in this figure). Bar=200 μm.

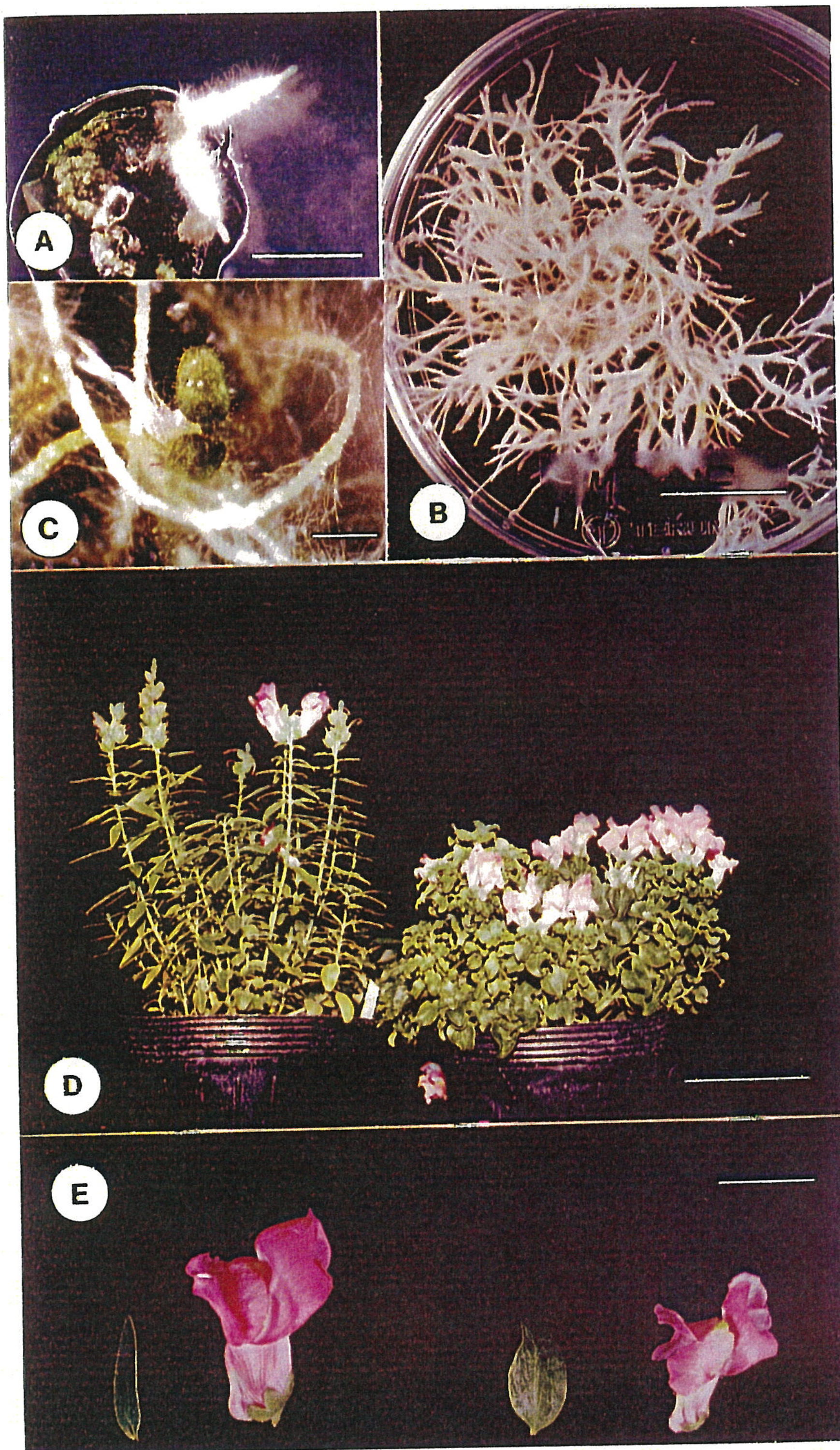

Fig. 8. Bialaphos-stimulated shoot regeneration and subsequent plant regeneration from hairy roots of snapdragon. **A.** Adventitious roots produced from leaf segment on 2 g l^{-1} gellan gum-solidified half-strength MS medium containing 30 g l^{-1} sucrose 6 weeks after inoculation with *A. rhizogenes* A13. Bar = 5 mm. **B.** Root culture of snapdragon induced by inoculation with *A. rhizogenes* A13. The roots showed a typical hairy root phenotype characterized by fast growth, high lateral branching and lack of geotropism at one month of culture of the root segment in the dark after elimination of bacteria. Bar = 2 cm. **C.** Shoot regeneration from hairy root culture on 0.5 mg l^{-1} bialaphos-containing medium. Bar = 5 mm. **D.** Growth habit of non-transformed plant (left) and transformed plant (right). Bar = 5 cm. **E.** Flowers and leaves of non-transformed plant (left) and transformed plant (right). Bar = 1.5 cm.

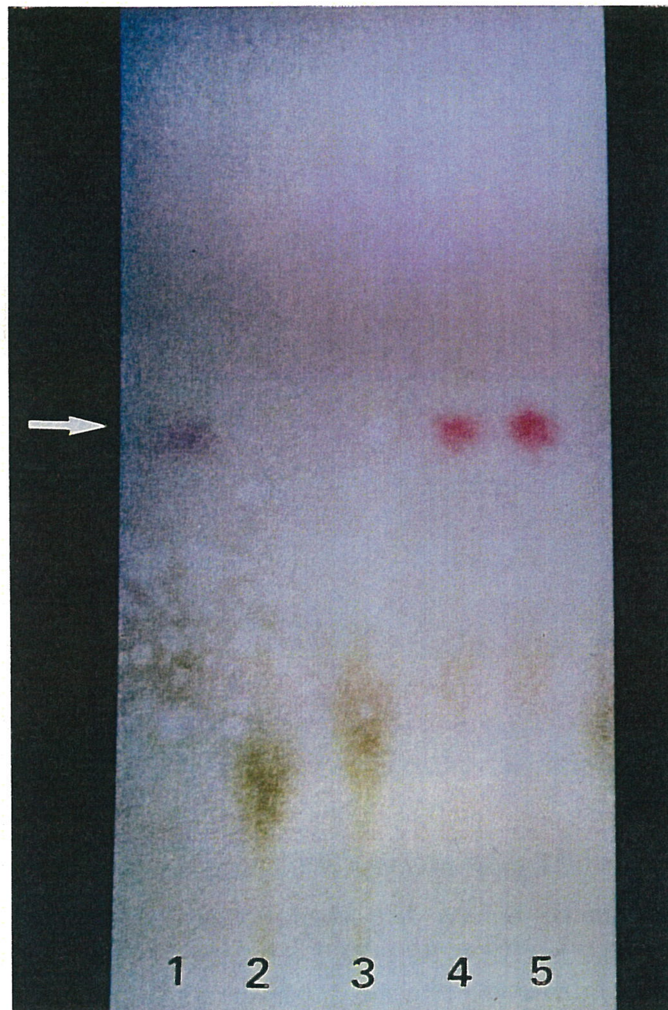

Fig. 9. Mikimopine detection in leaves and roots of regenerated plants derived from hairy roots of snapdragon. Lane 1, hairy roots of tobacco transformed with *A. rhizogenes* A13; lane 2, leaf of a non-transformed plant; lane 3, root of a non-transformed plant; lane 4, leaf of a transformed plant; lane 5; root of a transformed plant. Arrow indicates the position of mikimopine spots.

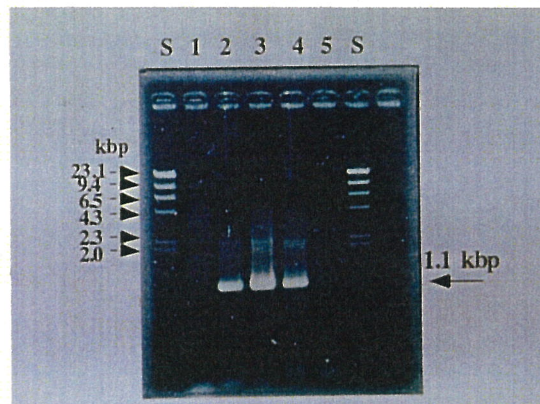

Fig. 10. PCR amplification of the core T-DNA region from DNAs isolated from *A. rhizogenes* A13, non-transformed and transformed plants. M, λ/*Hind* III and øX174/*Hae* III molecular marker (23.13, 9.42, 6.56, 4.36, 2.32, 2.03, 1.35, 1.08, 0.87 and 0.60 kbp fragments from top to bottom); lane 1, non-transformed plant; lane 2, plasmid DNA extracted from *A. rhizogenes* A13, as the positive control; lane 3 and 4, transformed plants; lane 5, non-transformed plant. Arrow indicates the position of the expected 1.1 kbp fragment including *rol* genes. Lane 3 and 4 show that these plants contained *rol* genes from Ri plasmid.

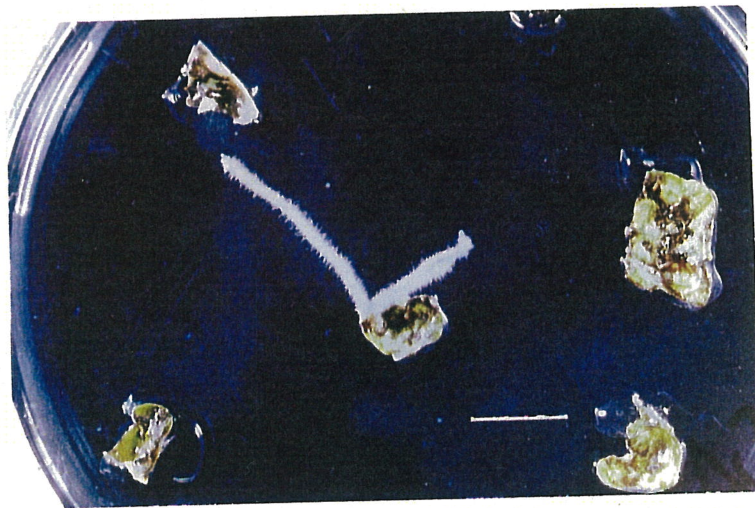

Fig. 11. Adventitious roots produced from leaf segment on 2 g l^{-1} gellan gum-solidified half-strength MS medium containing 30 g l^{-1} sucrose 4 weeks after inoculation with *A. rhizogenes* A13 (pARK5). Bar = 10 mm.

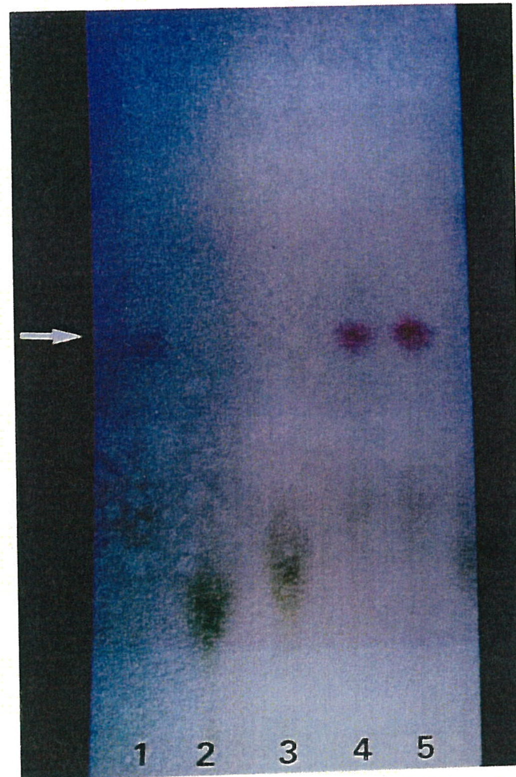

Fig. 12. Mikimopine detection in adventitious roots of snapdragon induced by inoculation with *A. rhizogenes* A13 (pARK5). Lane 1, hairy roots of tobacco transformed with *A. rhizogenes* A13 as a positive control; lane 2 and 3, roots of non-transformed plants; lane 4 and 5, adventitious roots produced after inoculation with *A. rhizogenes* A13 (pARK5). Arrow indicates the position of mikimopine spots.

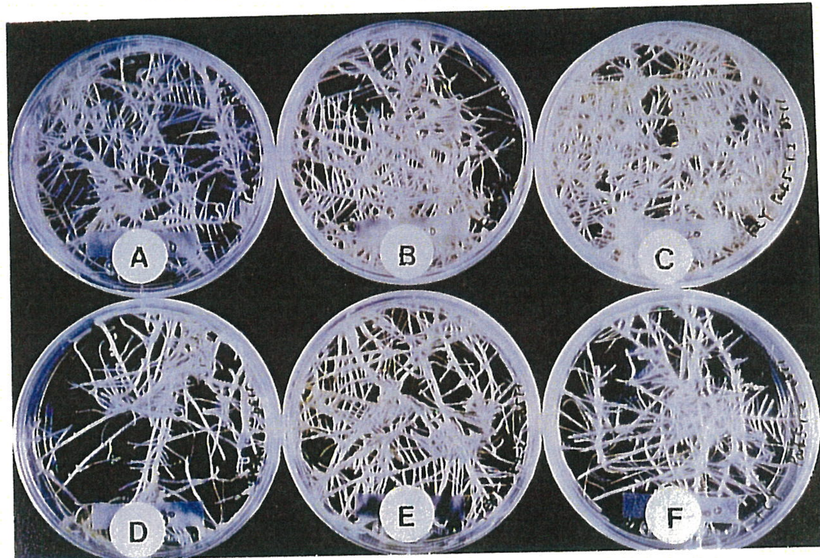

Fig. 13. Growth of *bar*-integrated hairy roots (clone FCY5.3.1) on media supplemented with (A) 0, (B) 10, (C) 20, (D) 30, (E) 50 and (F) 100 mg l^{-1} bialaphos, respectively.

Fig. 14. Shoot regeneration from hairy root culture on 1 mg l^{-1} bialaphos-containing medium. Arrows indicate adventitious shoots formed on hairy root culture.

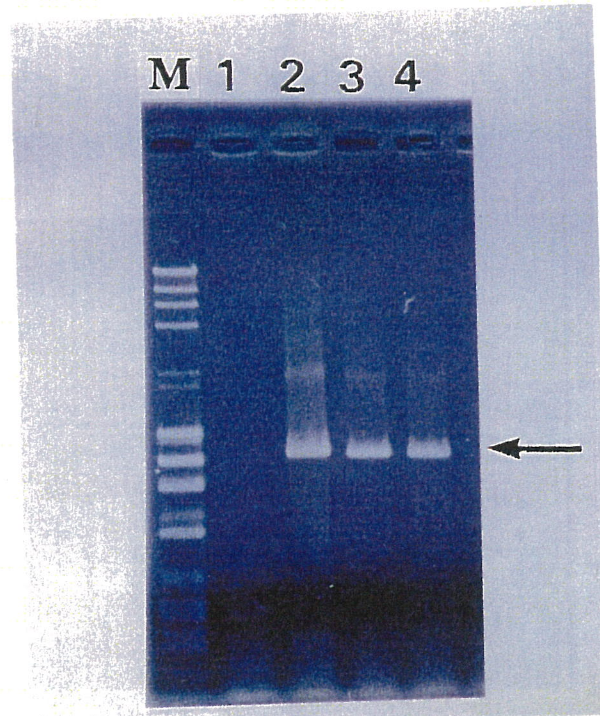

Fig. 15. PCR amplification of the core T-DNA region from DNAs isolated from *A. rhizogenes* A13, and non-transformed and transformed plants. M, λ/*Hin*d III and øX174/*Hae* III molecular marker (23.13, 9.42, 6.56, 4.36, 2.32, 2.03, 1.35, 1.08, 0.87 and 0.60 kbp fragments from top to bottom); lane 1, non-transformed plant; lane 2, plasmid DNA extracted from *A. rhizogenes* A13, as the positive control; lane 3 and 4, transformed plants. Arrow indicates the position of the expected 1.1 kbp fragment including *rol* genes. Lanes 3 and 4 show that these plants contained *rol* genes from Ri plasmid.

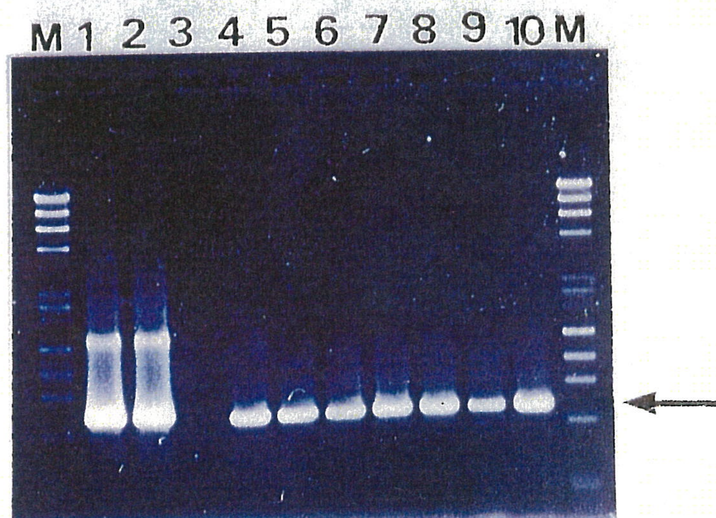

Fig. 16. PCR amplification of *nptII* gene from DNAs isolated from *A. rhizogenes* A13 (pARK5), and non-transformed and transformed plants. M, λ/*Hin*d III and øX174/*Hae* III molecular marker (23.13, 9.42, 6.56, 4.36, 2.32, 2.03, 1.35, 1.08, 0.87 and 0.60 kbp fragments from top to bottom); lane 1 and 2, plasmid DNA extracted from *A. rhizogenes* A13 (pARK5) as the positive control; lane 3, non-transformed plant; lane 4 to 10, transformed plants derived from different hairy root clones. Arrow indicates the position of the expected 700 bp fragment including *nptII* gene. Lanes 4 to 10 show that these plants contained *nptII* gene from pARK5.

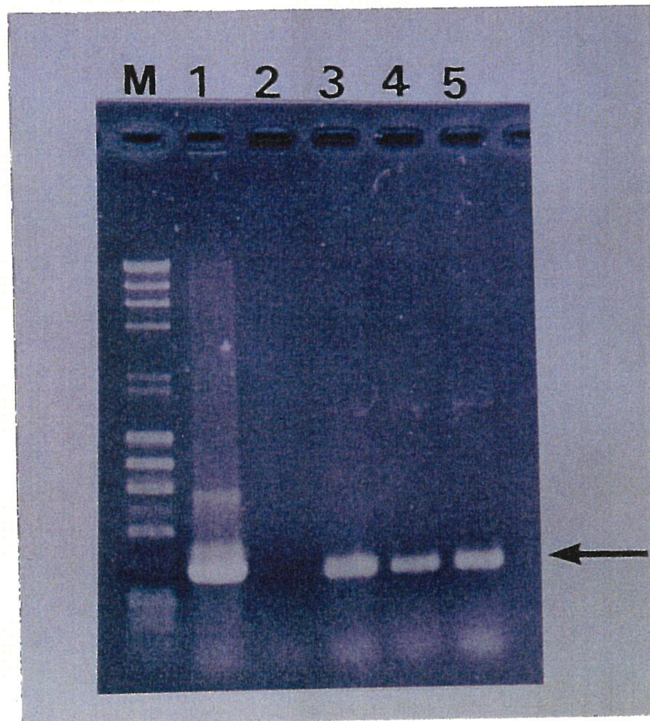

Fig. 17. PCR amplification of *bar* gene from DNAs isolated from *A. rhizogenes* A13 (pARK5), and non-transformed and transformed plants. M, λ/*Hin*d III and øX174/*Hae* III molecular marker (23.13, 9.42, 6.56, 4.36, 2.32, 2.03, 1.35, 1.08, 0.87 and 0.60 kbp fragments from top to bottom); lane 1, plasmid DNA extracted from *A. rhizogenes* A13, as the positive control; lane 2, non-transformed plant; lane 3 to 5, transformed plants derived from different hairy root clones. Arrow indicates the position of the expected 412 bp fragment including *bar* gene.

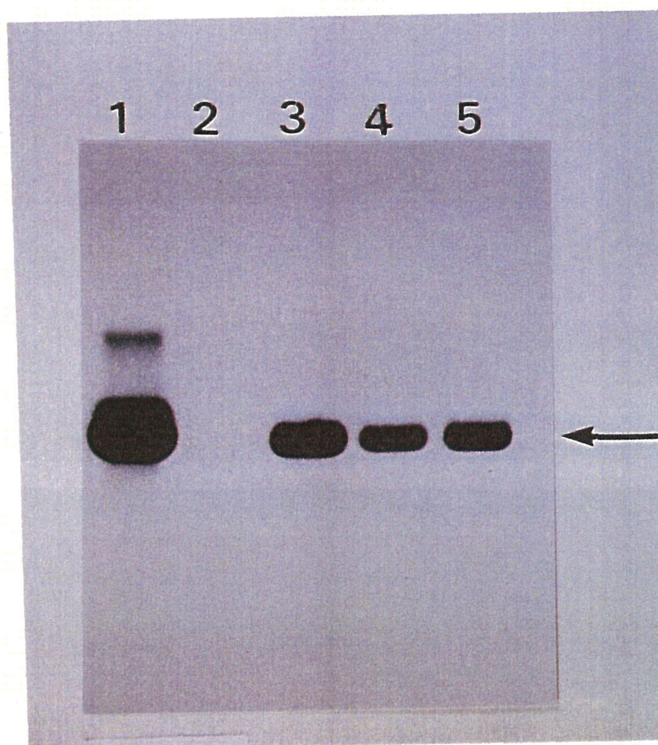

Fig. 18. Southern analysis of bialaphos-resistant plants following PCR amplification using primers for detecting *bar* gene. The PCR products on the agarose gel shown in Fig. 7 were used for blotting to nylon filter. The filter was hybridized with plasmid pARK5 as a probe. Lane 1, plasmid DNA extracted from *A. rhizogenes* A13, as the positive control; lane 2, non-transformed plant; lane 3 to 5, transformed plants derived from different hairy root clones. Arrow indicates 412 bp fragment which includes *bar* gene sequence. Lanes 3 to 5 show that these plants contained *bar* gene sequence from pARK5.

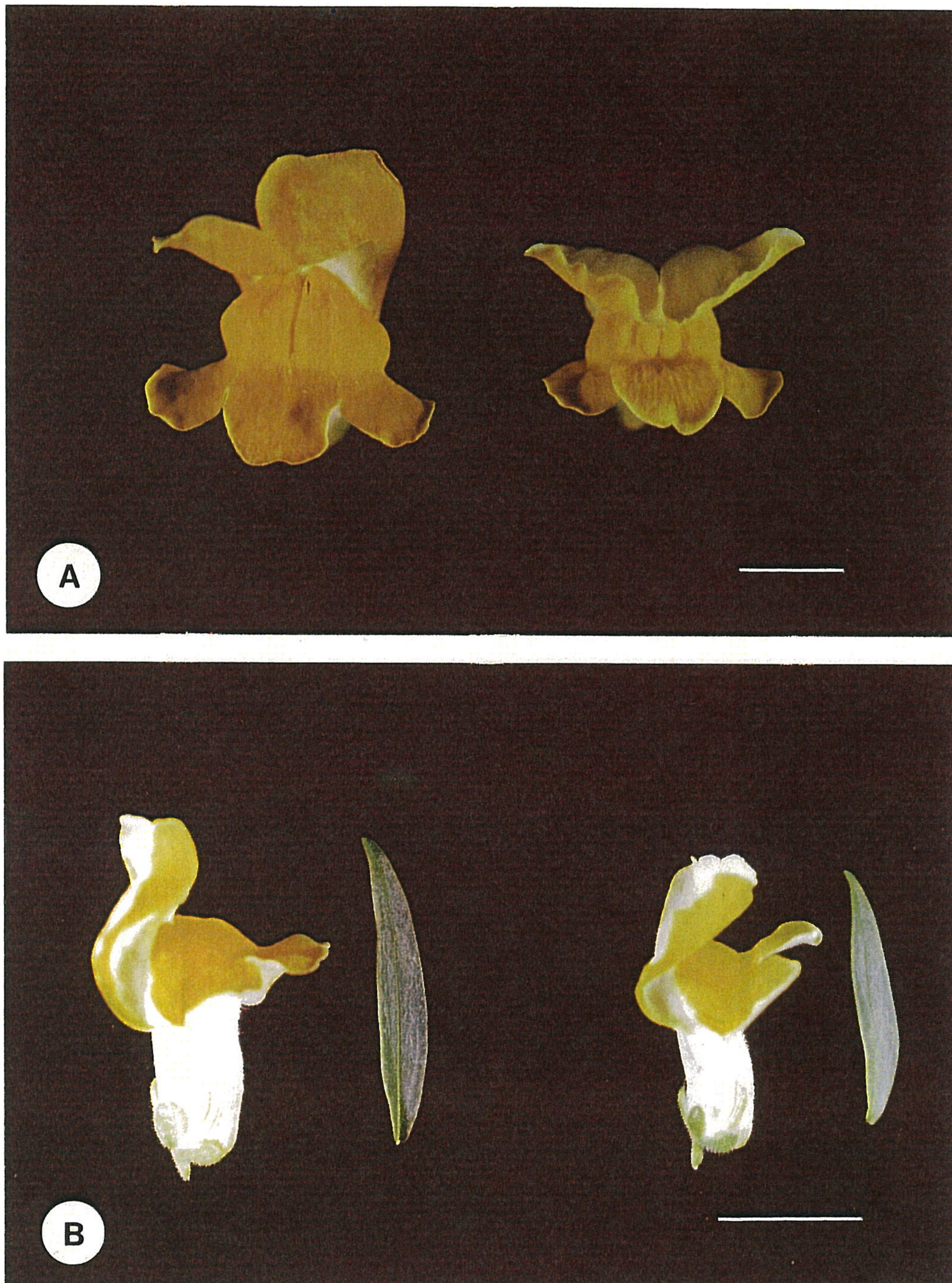

Fig. 19. Comparison of flower morphology between non-transformed and transformed plants.
(A) Flowers of non-transformed plant (cv. FCY) (left) and transformed plant (clone FCY5.3.1) (right), showing flatting of flower shape without changing in size of lower lips. Bar = 1 cm.
(B) Flowers and leaves of non-transformed plant (cv. FCY) (left) and transformed plant (clone FCY5.3.1) (right), showing difference in length between upper and lower lips. Bar = 1.5 cm.

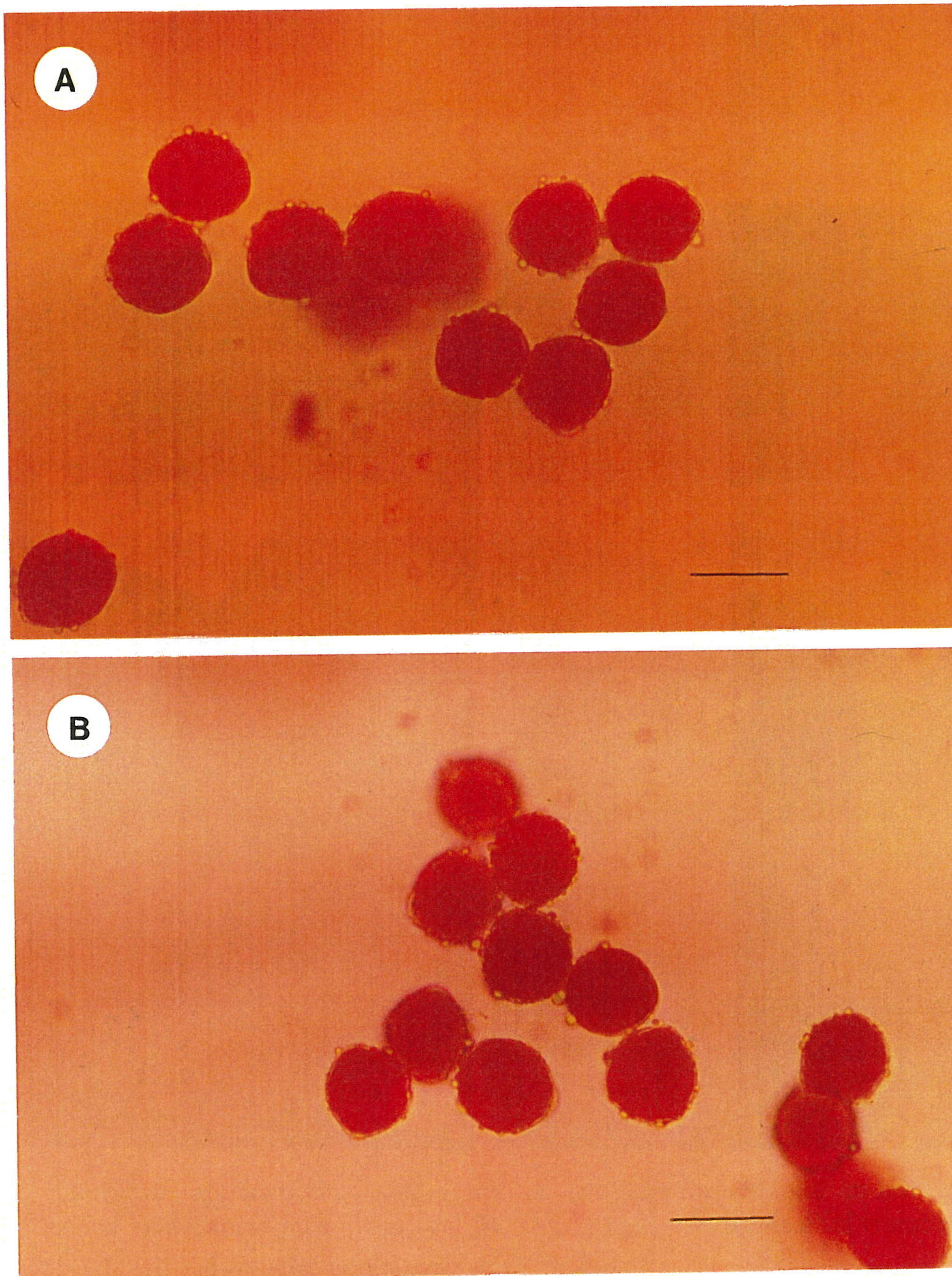

Fig. 20. Comparison of pollen fertility between non-transformed and transformed plants. Mature pollen grains of non-transformed plant (cv. FCY) (A) and transformed plant (clone FCY5.3.1) (B) were stained with aceto-carmine. Bar = 40 μm.

Fig. 21. Herbicide application to single leaf of non-transformed and transformed plants. Damaged leaf of non-transformed plant (cv. FCY) (A) and resistant leaf of transformed plant (clone FCY5.3.1) (B) 2 days after application of 0.5% Basta®.

Fig. 22. Difference in the response to herbicide application between non-transformed plant (cv. FCY) (left) and transformed plant expressing the *bar* gene (clone FCY5.3.1) (right) 7 days after application of 0.5% HERBIE™. Note the wilting of the non-transformed plant.

Fig. 23. Embryogenic callus used for co-cultivation with *A. tumefaciens*. Bar= 10 mm

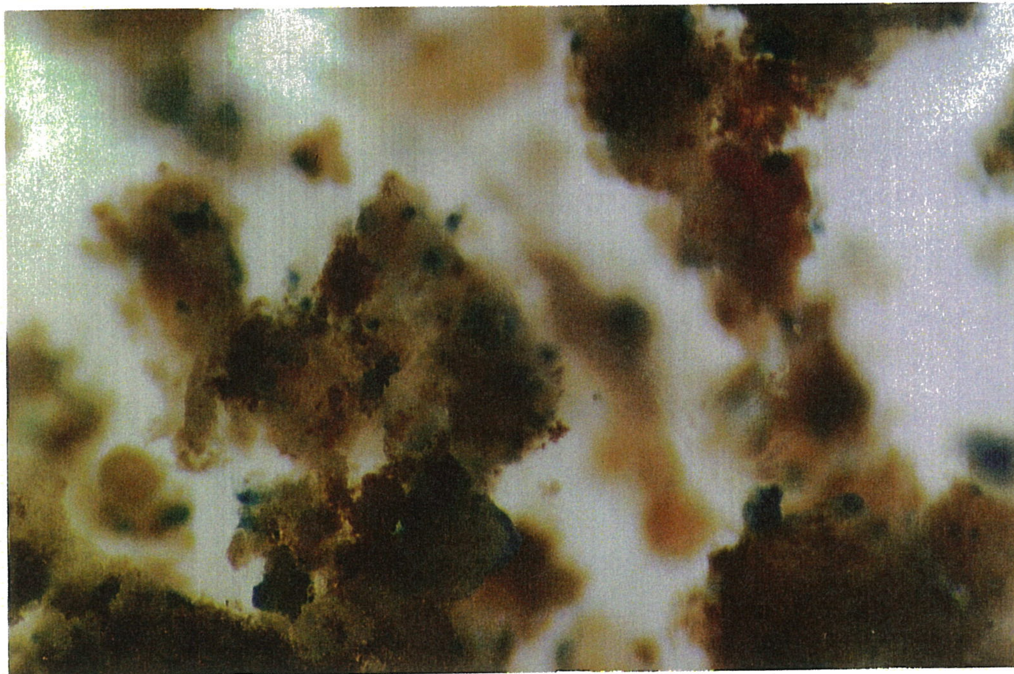

Fig. 24. Embryogenic calli showing GUS activities 7 days after co-cultivation.

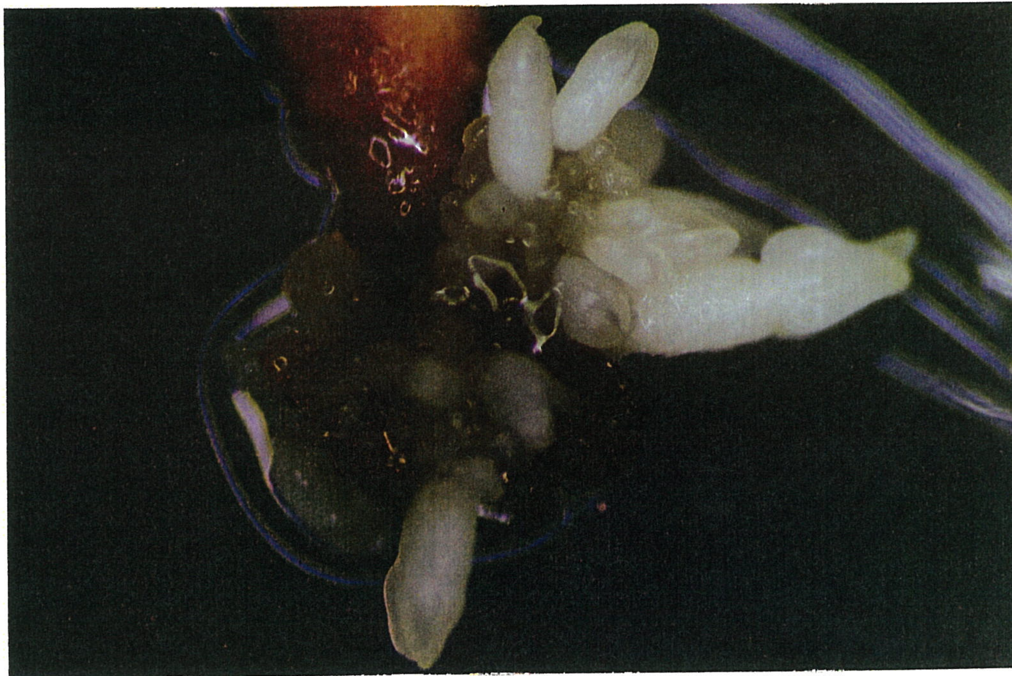

Fig. 25. Secondary embryos formed at the base of a somatic embryo derived from embryogenic callus after 3 moths of culture. The original somatic embryo turned dark brown on 50 mg l^{-1} kanamycin-containing medium while secondary embryos could grow on the same medium.

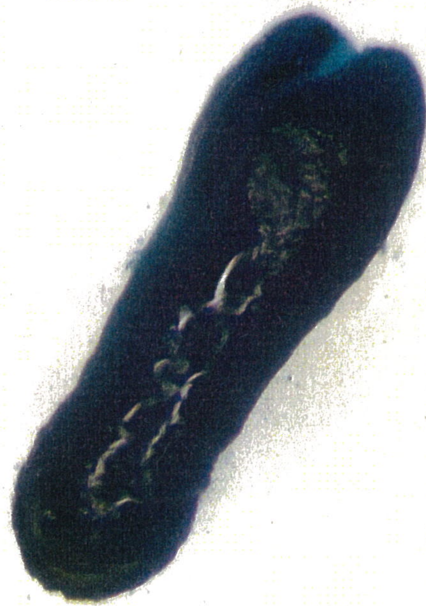

Fig. 26. Secondary embryo developed 4 months after bacterial inoculation showing GUS activity.

Fig. 27. A Leaf showing GUS activity excised from a regenerated plant derived from secondary embryo.

Fig. 28. Regenerated plant having GUS activity established in a pot after transfer to the growth chamber.

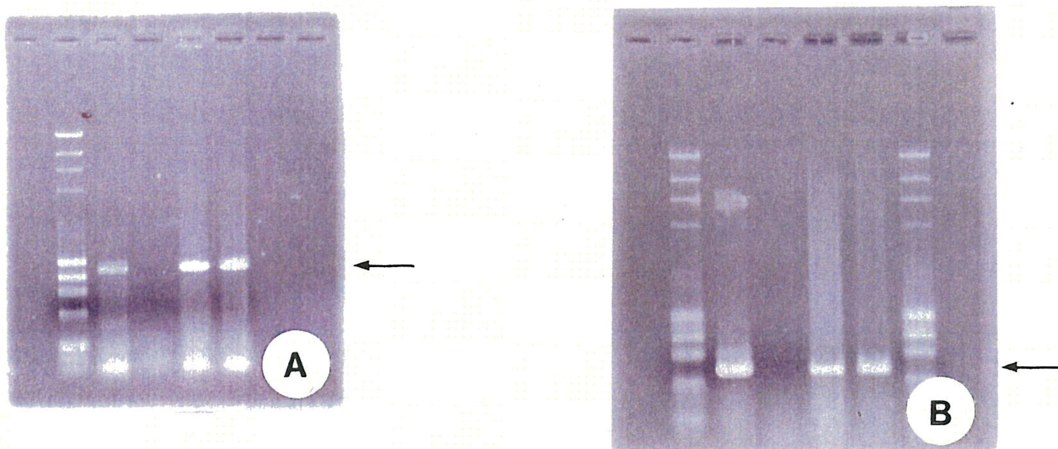

Fig. 29. PCR analysis of transgenic *Vitis* plants.
(A) Detection of GUS gene.
(B) Detection of NPTII gene.

Lane M, λ/*Hin*d III and øX174/*Hae* III as molecular markers (23.13, 9.42, 6.56, 4.36, 2.32, 2.03, 1.35, 1.08, 0.87 and 0.60 kbp fragments from top to bottom); lane 1, plasmid pTOK233 as a positive control; lane 2, non-transformed plant; lane 3 and 4, transgenic plants. Arrows indicate the position of the expected 1.2 and 0.7 kbp fragments including GUS and NPTII genes, respectively.

Table 1. Effect of plant growth regulators and casein hydrolysate on adventitious shoot, root, friable calli and compact calli formation from saintpaulia leaf disks.

Plant growth regulators (mgl^{-1})	casein hydrolysate (0)							casein hydrolysate (2 g l^{-1})						
		NAA			2,4-D				NAA			2,4-D		
	0	1	5	10	1	5	10	0	1	5	10	1	5	10
BA 0	S,R	S,R	R	R	C	–	–	S,R	–	C	C	F	–	–
1	S	S	S,R	–	–	–	–	–	–	–	C	C	C	C
5	S	S	S	–	C	–	–	C	S	S,R	C	C	C	C
10	–	–	–	C	–	–	–	–	–	C	C	C	C	C

All responses scored 2 months after culture.

S, adventitious shoot formation; R, adventitious root formation; F, friable callus formation; compact callus formation; –; No responses.

Table 2. Effect of auxins on plating efficiency of saintpaulia protoplasts.

Auxin (1 mg l^{-1})	Plating efficiency (%)
NAA	6.0 ± 1.3
2,4-D	12.7 ± 1.4
Picloram	4.6 ± 0.5

Each value represents the mean ± SE of at least 3 independent experiments.
Protoplasts were cultured in 0.1 % gellan gum-solidified B5 medium containing 0.2 M sucrose and 1 mg l^{-1} auxins.

Table 3. Effect of sucrose concentration on plating efficiency of saintpaulia protoplasts.

Sucrose (M)	Plating efficiency (%)
0.1	0
0.2	12.9 ± 1.2
0.3	8.9 ± 0.8
0.4	7.3 ± 1.2
0.5	6.0 ± 1.0

Each value represents the mean ± SE of at least 3 independent experiments. Protoplasts were cultured in 0.1 % gellan gum-solidified B5 medium containing 1 mg l^{-1} 2,4-D and various concentrations of sucrose.

Table 4. Effect of sugars and sugar alcohol on plating efficiency of saintpaulia protoplasts.

Carbon source	Plating efficiency (%)
0.2M fructose	0
0.2M glucose	0
0.2M sucrose	13.0 ± 1.1
0.1M sucrose + 0.1M mannitol	18.6 ± 0.8
0.1M sucrose + 0.1M glucose	4.5 ± 2.7
0.1M glucose + 0.1M mannitol	0

Each value represents the mean ± SE of at least 3 independent experiments.
All media contained 1 mg l^{-1} 2,4-D and solidified with 0.1 % gellan gum.

Table 5. Differences in frequency of protoplast division and shoot regeneration from protoplast-derived calli among several cultivars in *Dianthus.*

Cultivars	Division frequency (%)[1]	Shoot regeneration (%)[2]
D. chinensis		
cv. Fire Carpet	13.5 ± 5.1	14.3 ± 8.7
cv. Snow Fire	10.9 ± 3.1	18.7 ± 5.9
cv. Rose Carpet	7.2 ± 4.7	7.8 ± 7.6
D. chinensis x *D. barbatus*		
cv. Telster White	15.4 ± 2.1	35.3 ± 5.5
cv. Telster Orchid	8.3 ± 3.8	12.3 ± 3.3

The values represent the mean ± S.E. of 3 independent experiments.
1) Percentage of protoplasts showing at least one cell division after 14 days of culture.
2) Percentage of calli showing shoot regeneration 3 months after transfer to the shoot regeneration medium.

Table 6. Effect of mannitol concentration in enzyme solution on protoplast yield and viability assessed by FDA staining in *Silene armeria* L..

Mannitol concentration (M)	Yield (x 10^5 / g f.w.)	Viability (%)[1)]
0.2	2.2 ± 0.42	62.1 ± 3.5
0.3	6.3 ± 0.36	69.3 ± 2.2
0.4	5.9 ± 0.32	77.5 ± 4.5
0.5	11.2 ± 0.25	76.2 ± 3.3
0.6	10.2 ± 0.43	74.9 ± 2.9
0.7	7.7 ± 0.66	71.8 ± 5.5
0.8	7.4 ± 0.22	62.3 ± 2.8

The values represent the mean ± S.E. of 3 independent experiments.
1) The data were assessed by FDA staining.

Table 7. Effect of sugars and sugar alcohol on cell division frequency of *Silene armeria* L..

Sugars and sugar alcohol	Division frequency (%)[1)]
0.5 M fructose	0
0.5 M glucose	10.9 ± 3.0
0.5 M sucrose	17.3 ± 4.5
0.25 M sucrose + 0.25 M mannitol	13.8 ± 3.0
0.25 M sucrose + 0.25 M glucose	12.4 ± 3.6
0.25 M glucose + 0.25 M mannitol	12.9 ± 2.6
0.25 M glucose + 0.25 M fructose	0

1) Percentage of protoplasts showing at least one cell division after 10 days of culture.
Each value represents the mean ± S.E. of at least 3 independent experiments.
All media contained 1 mg l^{-1} 2,4-D and solidified with 1 g l^{-1} gellan gum.

Table 8. Number of ovules in an ovary, and diameters of egg cells, synergid cells and embryo sacs.

cultivar	number of ovules / ovary[1)]	diameter of embryo sac (μm)[2)]	diameter of egg cell (μm)[2)]	diameter of synergid cell (μm)[2)]
Telster White	74.7 ± 4.9	208 ± 11	35.2 ± 2.1	32.9 ± 1.8

1) The data were expressed as the average of 10 ovaries.
2) The data were obtained by measuring 30 ovule samples treated by clearing method under inverted microscope.

Table 12. Induction of hairy roots conferring bialaphos-resistance in *Antirrhinum majus* L. after inoculation with *Agrobacterium rhizogenes*.

cultivar	bacterial strain	number of explants	number of adventitious roots	frequency of root formation (%)[1]	number of opine-positive roots	frequency of opine-positive roots (%)[2]	bialaphos ^{r} PCR*bar* +[3]	frequency of *bar*-positive roots (%)[4]
Floral Carpet Orchid	A13	52	5	9.6	1	1.9	0	0
	A13 (pARK5)	50	11	22.0	5	10.0	2	40
Floral Carpet Pink	A13	48	14	29.2	3	6.3	0	0
	A13 (pARK5)	47	8	17.0	4	8.5	4	100
Floral Carpet Red	A13	38	0	0	0	0	0	0
	A13 (pARK5)	54	11	20.4	3	5.6	1	33.3
Floral Carpet Deep Rose	A13	23	1	4.3	1	4.3	0	0
	A13 (pARK5)	15	4	26.7	2	13.3	1	50
Floral Carpet White	A13	41	2	4.9	0	0	0	0
	A13 (pARK5)	46	3	6.5	0	0	0	0
Floral Carpet Yellow	A13	41	2	4.9	0	0	0	0
	A13 (pARK5)	42	8	19.0	8	19.0	4	50

1) (number of adventitious roots / number of explants) x 100
2) (number of opine-positive roots / number of adventitious roots) x100
3) number of hairy roots showing both resistance to bialaphos and *bar*-integration confirmed by PCR.
4) (number of *bar*-integrated roots / number of opine-positive roots) x100

Table 10. Effect of plant growth regulators on root growth and callus formation in hairy root cultures of snapdragon.

PGR (mg l^{-1})		No. of explants with root growth	No. of callus-forming explants
none		10	0
NAA			
0.1		7	3
0.5		8	2
1		10	0
NAA	BA		
0	1	1	9
0	5	0	10
0.1	1	2	8
0.1	5	3	7
0.5	1	5	5
0.5	5	0	10
1	1	2	8
1	5	0	10
NAA	TDZ		
0	1	1	9
0	5	2	8
0.1	1	0	10
0.1	5	3	7
0.5	1	1	9
0.5	5	1	9
1	1	0	10
1	5	0	10
NAA	Zeatin		
0	1	0	10
0	5	0	10
0.1	1	7	3
0.1	5	0	10
0.5	1	2	8
0.5	5	0	10
1	1	0	10
1	5	0	10

A single line of hairy root was used for the experiment. Each treatment consisted of 10 hairy root segments (10 mm each). The data were recorded after 4 months of culture.

Table 11. Effect of bialaphos treatment on fresh weight, shoot regeneration frequency and number of shoots per explant in root cultures of non-transformed and transformed snapdragon.

bialaphos concentration ($mg\ l^{-1}$)	fresh weight (g)[1)]	shoot regeneration (%)[2)]	No. of shoots / explant[3)]
non-transformed roots			
0.0	0.85[b]	0[a]	0[a]
0.1	0.79[b]	0[a]	0[a]
0.5	0.60[b]	0[a]	0[a]
0.7	0.31[a]	0[a]	0[a]
0.9	0.13[a]	0[a]	0[a]
1.2	0.11[a]	0[a]	0[a]
3.0	0.08[a]	0[a]	0[a]
5.0	0.08[a]	0[a]	0[a]
transformed roots			
0.0	3.35[b]	10[a]	0.12[a]
0.1	3.17[b]	14[a]	0.16[a]
0.5	2.63[b]	56[b]	0.61[b]
0.7	0.86[a]	0[a]	0[a]
0.9	0.08[a]	0[a]	0[a]
1.2	0.10[a]	0[a]	0[a]
3.0	0.08[a]	0[a]	0[a]
5.0	0.08[a]	0[a]	0[a]

Each treatment consisted of 10 hairy root segments (10 mm long, 8 mg each) and the experiment was repeated 5 times.

1) Total fresh weight of 10 root segments after 3 months of culture.

2) Percentage of the roots producing adventitious shoots after 3 months of culture.

3) Average number of shoots per explant after 3 months of culture.

Data on transformed roots were separately analyzed from those on non-transformed roots by Student-Newman-Keuls Test ($P<0.05$). Means in the same column followed by the same letter are not significantly different.

Table 9. Efficiency of embryo sacs isolation from ovules after treatment with different composition of enzyme solution.

enzyme composition[1]	Cellulase Onozuka RS	Cellulase Onozuka R-10	Macerozyme R-10	Pectolyase Y-23	Hemicellulase	Driselase	$CaCl_2 \cdot 2H_2O$	MES[2]	MS inorganic elements	embryo sacs / ovules[3] (%)
1	2%	-	-	0.1%	-	1%	-	5mM	-	17 /122 (13.9)
2	-	1%	0.5%	-	-	-	5mM	5mM	-	1 / 62 (1.6)
3	2%	-	0.5%	0.1%	-	-	-	-	1/2 conc.	3 / 71 (4.2)
4	2%	-	0.5%	-	0.5%	-	-	-	1/2 conc.	0 / 44 (0)
5	2%	-	-	0.05%	-	-	10mM	5mM	-	3 / 59 (5.1)
6	-	2%	0.5%	-	-	-	10mM	5mM	-	2 / 67 (3.0)
7	2%	-	1%	0.05%	-	1%	5mM	5mM	-	18 / 133 (13.5)

All enzyme compositions were supplemented with 0.5 M mannitol.

1) Dissolved in CPW solution (Frearson et al. 1973).

2) MES: 2-(N-morpholino)-ethanesulfonic acid

3) Number of embryo sacs released after dissection / number of ovules dissected

Table 13. Effect of different concentrations of bialaphos on growth of hairy roots of *Antirrhinum majus* L. transformed by wild type strain of *A. rhizogenes* A13 and those induced by *bar*-introduced A13.

bialaphos conc.		fresh weight (g)	
(mg l^{-1})	cont (FCY.A13.2)	FCY5.3.1	FCY5.2.3
0	2.32±0.55	1.28±0.11	2.08±0.14
0.5	1.92±0.23	1.49±0.44	1.53±0.70
1	0.06±0.02	1.14±0.09	2.35±0.75
2	0.07±0.02	1.29±0.06	2.75±0.41
3	0.05±0.02	1.28±0.12	4.89±2.80
5	0.06±0.02	1.44±0.32	2.93±0.11
10	0.08±0.01	1.18±0.28	1.71±0.45
20	0.06±0.02	1.21±0.39	1.57±0.05
30	0.06±0.02	1.63±0.12	2.46±0.31
50	0.06±0.02	1.76±0.41	1.32±0.20
100	0.05±0.01	0.90±0.13	1.32±0.08

Table 14. Flower characteristics of transformed and non-transformed plants of *Antirrhinum majus* L..

Clone number	length of corolla (cm)	width of lower lip (cm)	length between upper and lower lips (cm)
FCY (control)	3.58 ± 0.14	2.12 ± 0.16	2.48 ± 0.12
FCY5.3.1	3.09 ± 0.14	2.04 ± 0.10	1.51 ± 0.32

Twenty flowers were measured.
Each value represents the mean ± S.D.

Table 15. Comparison of pollen fertilities between non-transformed and transformed plants of *Antirrhinum majus* L..

Clone number	Pollen fertility (%)
FCY (control)	98.4 ± 0.8
FCY5.3.1 (pARK5)	96.3 ± 1.2
FCO5.4 (pARK5)	75.3 ± 5.3
FCY.A13.5.4 (A13 wild)	90.2 ± 2.5
FCY.A13.4 (A13 wild)	92.3 ± 1.9

Pollen fertilities of non-transformed and transformed plants were checked by staining more than 300 pollen grains per flower with aceto-carmine.
Each value represents the mean ± S.D.

Table 16. Effect of acetosyringone on GUS activity in embryogenic callus of *Vitis vinifera* cv. Koshusanjaku following co-cultivation with *Agrobacterium tumefaciens.*

Bacterial strains	Acetosyringone (μM)[1]	Number of GUS spots /100 mg callus[2] exp.1	exp.2
EHA101(pIG121Hm)	0	5	2
	100	31	165
LBA4404(pTOK233)	0	15	13
	100	56	695

1) Acetosyringone was added to co-culture medium.
2) Data were obtained 7 days after inoculation.

Table 17. Effect of bacterial strains on transformation efficiency of embryogenic calli of *Vitis vinifera* cv. Koshusanjaku evaluated by GUS activity after co-cultivation with *Agrobacterium tumefaciens*.

Bacterial strain	No. of GUS-positive embryos / no. of embryos tested (%)[2)]	No. of GUS-positive plants / no. of regenerated plants examined (%)[3)]
EHA101(pIG121Hm)	17/80 (21.3)	3/8 (37.5)
LBA4404(pTOK233)	26/84 (31.0)	4/9 (44.4)

Acetosyringone was added to co-culture medium.

1) Data were obtained one month after bacterial inoculation.

2) Number of GUS-positive embryos was counted 4 months after bacterial inoculation.

3) Number of GUS-positive plants was determined by examining GUS activity in leaves 6 months after bacterial inoculation.

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Summary

In this study, I tried to establish necessary protocols for the application of plant biotechnologies to breeding of several horticultural crops. The results obtained are summarized as follows.

1. Plant regeneration from cell suspension-derived protoplasts of *Saintpaulia ionantha* Wendl.

Friable calli were induced on leaf segments of *Saintpaulia ionantha* Wendl. on B5 medium containing 1 mg l^{-1} 2,4-D and 2 g l^{-1} casein hydrolysate. Cell suspension cultures were readily established from these friable calli and protoplasts could be isolated from the cells with yields 1-3 x 10^7 / g f. wt.. By culturing in 0.1% gellan gum-solidified B5 medium supplemented with 1 mg l^{-1} 2,4-D and 0.1 M each of sucrose and mannitol at a density of 1 x 10^5 / ml, the protoplasts divided within 6 days and formed macro-colonies after 2 months of culture. Shoot regeneration from protoplast-derived calli was obtained by sequential treatment of the calli with plant growth regulators: initially with 1 mg l^{-1} each of NAA and BA for 2 months followed by 0.01 mg l^{-1} NAA and 5 mg l^{-1} BA for 4 months. Regenerated plants were established after rooting of the shoots on half-strength MS medium, and successfully transferred to the greenhouse. The regenerated plants grew into flowering stage and showed the same phenotype as the parent plant.

2. Protoplast culture of several *Dianthus* cultivars and *Silene armeria* L.: comparison of shoot regeneration ability from protoplast-derived calli among *Dianthus* cultivars, and culture of suspension cell-derived protoplasts in *S. armeria* L. as fundamental study for somatic hybridization

Shoot regeneration ability from protoplast-derived calli was compared among several

Dianthus cultivars, and culture conditions for suspension cell-derived protoplasts in *S. armeria* L. were studied as a basis for somatic hybridization among these species. The highest frequency of shoot regeneration from protoplast-derived calli was obtained in cv. Telster White among five cultivars of *Dianthus* examined. In *S. armeria*, protoplasts which were isolated from cell suspension cultures maintained in liquid MS medium supplemented with 1 mg l^{-1} 2,4-D showed the highest division frequency in MS medium containing 1 mg l^{-1} 2,4-D and 0.5 M sucrose. Although protoplasts were sustained to divide and could form visible colonies, no adventitious shoots were regenerated on media containing NAA in combination with BA, TDZ, zeatin or KT-30.

3. Isolation of embryo sacs from ovules by enzymatic treatments and microdissection in *Dianthus*

Mature ovules of *Dianthus* (Caryophyllaceae) were histologically observed by using clearing method and serial sectioning to characterize the cells in embryo sac. The results show that mature embryo sac was located deep in chalazal portion of the hemitropous ovule due to thick nucellus tissue at micropylar region. For the isolation of the embryo sacs, ovules collected from ovaries of flowers 1 or 2 days after anthesis were treated with enzyme solution, which was prepared for digesting cell walls, on a gyratory shaker. After 12 hours of enzyme treatment, the ovules were dissected by using a glass needle under inverted microscope to release embryo sacs. The embryo sacs characterized by their specific size were successfully released by these successive treatments. Fluorescent staining with 4,6-diamidino-2-phenylindole (DAPI) revealed the nuclei of egg apparatus in the isolated embryo sacs. The procedure for isolating embryo sacs established in this study will offer a new approach to further *in vitro* study on fertilization in *Dianthus*.

4. Bialaphos stimulates shoot regeneration from hairy roots of snapdragon (*Antirrhinum majus* L.) transformed by *Agrobacterium rhizogenes*

Hairy roots of snapdragon (*Antirrhinum majus* L. : Scrophulariaceae) induced by a wild-type strain of *Agrobacterium rhizogenes* were cultured on media containing various concentrations of a phosphinothricin-based herbicide, bialaphos or plant growth regulators (PGRs). Adventitious shoot regeneration from hairy roots was observed with a low frequency (10%) on half-strength Murashige and Skoog medium. Addition of PGRs, NAA in combination with BA, TDZ or zeatin to the medium had no effect on shoot regeneration from hairy roots. Although bialaphos at 0.9 mg l^{-1} or more was toxic to hairy roots, it significantly increased the shoot regeneration frequency up to 56% at 0.5 mg l^{-1}. In contrast, non-transformed roots and leaves regenerated no shoots on media with or without bialaphos. Regenerated shoots detached from host roots readily developed roots on gellan gum-solidified medium. Regenerated plants were successfully transferred to the greenhouse.

5. Transgenic herbicide-resistant snapdragon (*Antirrhinum majus* L.) produced by bialaphos-stimulated shoot regeneration from hairy roots

Transgenic herbicide-resistant snapdragon (*Antirrhinum majus* L.) plants were obtained by co-cultivation of leaf explants with *Agrobacterium rhizogenes* harboring Ri plasmid and pARK5. The T-DNA region of pARK5 contains *bar* gene encoding phosphinothricin acetyltransferase under the control of the cauliflower mosaic virus 35S promoter, and *nptII* gene encoding neomycin phosphotransferase II under the control of nopaline synthase promoter. Transformed hairy roots induced from leaf explants inoculated with *A. rhizogenes* strain A13 (pARK5) could grow on 1 mg l^{-1} bialaphos-containing half-strength MS medium, although hairy roots transformed by wild type strain of *A. rhizogenes* A13 could not survive at the same concentration of bialaphos. Shoot regeneration from the hairy roots integrating T-DNA of

pARK5 was also stimulated at 1 mg l^{-1} bialaphos. Regenerated shoots readily developed roots on 2 g l^{-1} gellan gum-solidified half-strength MS medium. Transgenic nature of the plants was confirmed by opine, PCR and Southern analyses. Regenerated plantlets were successfully established in the growth chamber. Acclimatized plants exhibited altered phenotypes known as Ri syndrome such as reduced apical dominance with highly branched stems and short internodes, and also showed resistance to an applied standard level of commercial herbicides.

6. Production of transgenic grapevine (*Vitis vinifera* L. cv. Koshusanjaku) plants by co-cultivation of embryogenic calli with *Agrobacterium tumefaciens* and selecting secondary embryos

Embryogenic calli induced from leaf segments of grapevine (*Vitis vinifera* L. cv. Koshusanjaku) were co-cultivated for 5 days with *Agrobacterium tumefaciens* strains EHA101 (pIG121Hm) or LBA4404 (pTOK233), both of which contained the plasmid carrying the neomycin phosphotransferase II (NPTII), hygromycin phosphotransferase (HPT) and the β-glucuronidase (GUS) genes. Putative transgenic calli were selected on 2 g l^{-1} gellan gum-solidified Nitsch's medium (1969) containing 50 mg l^{-1} kanamycin and 20 g l^{-1} sucrose after co-cultivation with *A. tumefaciens*. Transformation frequency of the embryogenic calli evaluated by GUS histochemical assay was increased by the addition of acetosyringone to co-culture medium. Complete transgenic plants were selected among secondary embryos formed on the surface of embryos in the presence of kanamycin. Finally, kanamycin-resistant plants expressing GUS gene were obtained. PCR analysis confirmed their transgenic nature by detecting GUS and NPTII genes.

摘要

近年、細胞融合や遺伝子導入などの細胞工学的手法が様々な作物の育種に応用され、個々の植物について多くの事例が報告されている。これらの手法を応用するには、プロトプラストからの植物体再生系やカルスや外植片からの不定芽再生系の確立が必須である。しかしながら、培養技術について、全体を統括した知識体系として整えられていないのが現況であり、細胞工学的手法を適用するには、個々の植物に対応した新しい培養系や遺伝子導入系を確立していくことが重要であると考えられる。本論文では、数種の園芸作物を材料に、細胞工学的手法による育種を進めるための基礎的な知見を得るために、個々の植物に応じた培養技術の開発を試みた。

本論文は、第 1 章の序論から第 8 章の結論まで全 8 章から構成されている。第 1 章では序論として、プロトプラスト培養とその応用および *Agrobacterium* による形質転換におけるこれまでの知見を概観し、本論文の基幹となる知識体系を整理した上で、材料に用いた各園芸植物に細胞工学的手法を適用する際の問題点について論じた。

第 2 章では、セントポーリア (*Saintpaulia ionantha* Wendl.) のプロトプラストからの植物体再生系の開発を試みた。セントポーリアは、葉から容易に不定芽を再生するため、組織培養のモデル植物として用いられているが、現在利用できる範囲の酵素では葉肉からプロトプラストを単離することができず、また、プロトプラストの単離に適し、かつ不定芽再生能を有するカルスの誘導も困難であった。本研究において、2,4-D とカゼイン加水分解物を組み合わせて培地に添加することにより、不定芽再生能を有するカルスが誘導されることを見出した。このカルスより誘導した懸濁培養細胞からプロトプラストを単離し、培養条件について様々な検討を行うことにより、プロトプラストからの植物体再生系を確立することができた。本研究で得られた培養系は、遺伝子導入や細胞融合に利用できるものと期待される。

第 3 章では、ナデシコ科に属するナデシコ属 (*Dianthus*) 植物の 5 品種とムシトリナデシコ (*Silene armeria* L.) についてプロトプラストの培養条件について検討した。本研究は、ムシトリナデシコと園芸的に重要なナデシコ属の間で細胞融合により属間雑種を作出することを目的としている。ナデシコ属の 5 品種では、テルスターホワイトがプロトプラストの分裂率、プロトプラスト由来カルスからの不定芽

再生率がともに高いことが示された。ムシトリナデシコについては、葉片から 2,4-D を用いて懸濁培養細胞を誘導し、プロトプラストの単離を行った。培養条件の検討を行うことにより、プロトプラストからカルスまで形成させることができた。しかし、このカルスは不定根を分化するものの、植物体再生には至らなかった。

第 4 章では、ナデシコ属植物の雌性配偶子の単離を試みた。本研究は、試験管内受精や遺伝子導入の材料に雌性配偶子を利用するために、その単離および培養系の確立を目的とした。はじめに、連続切片と透明法による胚珠内部の観察を行い、胚嚢の位置および胚嚢を構成する細胞の識別を行った。得られた観察結果に基づいて酵素処理とガラス針により胚珠の切開を行い、胚嚢の単離を行った。今後、卵細胞の単離方法に関する研究を発展させ、試験管内受精や遺伝子導入に応用されることが期待される。

第 5 章では、*Agrobacterium rhizogenes* によって誘導したキンギョソウ (*Antirrhinum majus* L.) 毛状根からの不定芽分化が、除草剤である bialaphos によって高められることを示した。その作用機作として、bialaphos によるグルタミン合成酵素の阻害によって生じるアンモニアの蓄積が、間接的に不定芽分化に作用していることを推察した。

第 6 章では、遺伝子導入による除草剤耐性のキンギョソウの作出を試みた。Ri プラスミドと除草剤耐性遺伝子である *bar* を持つプラスミド pARK5 を保持する *Agrobacterium rhizogenes* を用いて接種を行い、除草剤 bialaphos に抵抗性を示す毛状根を選抜した。毛状根からの不定芽再生は、1 mg l^{-1} bialaphos によって促進され、順化した植物体は、頂芽優性の低下、分枝の増加、節間が詰まる等の Ri シンドロームとして知られる形態的変異を呈し、また、同時に除草剤に対する抵抗性も示した。

第 7 章では、ブドウ (*Vitis vinifera* L.) 品種甲州三尺の embryogenic callus に *Agrobacterium tumefaciens* を接種することにより、遺伝子導入系の確立を試みた。抗生物質耐性とレポーター遺伝子を利用して形質転換カルスを選抜し、分化の過程で二次胚を選抜することにより、キメラでない形質転換体の作出方法を確立した。得られた植物体について、PCR による導入遺伝子の確認を行った。

第８章の結論では、得られた結果を総括し、園芸作物への細胞工学的手法の応用について論述した。

Abbreviations

BA: benzyladenine

CaMV: cauliflower mosaic virus

2,4-D: 2,4-dichlorophenoxyacetic acid

FDA: fluorescein diacetate

f. wt.: fresh weight

MES: 2-(N-morpholino)-ethanesulfonic acid

MS: Murashige and Skoog (1962)

NAA: α-naphthaleneacetic acid

PAT: phosphinothricin acetyltransferase

PCR: polymerase chain reaction

PE: plating efficiency

PEG: polyethylene glycol

PGR: plant growth regulator

TDZ: N-(1,2,3-thiadiazol-5-yl)-N'-phenylurea or thidiazuron