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High frequency plant regeneration from anther-derived cell suspension cultures via somatic embryogenesis in *Catharanthus roseus*

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Summary. A system for high frequency plant regeneration from cell suspension cultures in *Catharanthus roseus* is described. Calli were obtained from anthers cultured on Murashige and Skoog's medium supplemented with 1 mgl^{-1} α-naphthaleneacetic acid and 0.1 mgl^{-1} kinetin. After the second subculture on solid medium, embryogenic callus was identified and transferred to liquid medium to initiate suspension cultures. Cells dispersed finely in the medium were subcultured at 14-day intervals. Upon plating onto the basal medium, yellowish compact colonies proliferated from the cells and more than 80% of them gave rise to somatic embryos. Subsequently, plantlets developed from the embryos. Both the plantlets and the source plants showed the normal somatic chromosome number of 2n=2x=16.

Key words: anther culture – *Catharanthus roseus* – plant regeneration – somatic embryogenesis

Abbreviations: MS, Murashige and Skoog; MSNK, MS medium + 1 mgl^{-1} NAA + 0.1 mgl^{-1} kinetin; NAA, α-naphthaleneacetic acid.

Introduction

Catharanthus roseus is a tropical and subtropical plant belonging to the family Apocynaceae. The plant has drawn attention due to the production of useful alkaloid compounds such as vinblastine and vincristine which are used for blood cancer treatment (Lounasmaa and Galambos 1989). Vinblastine and vincristine are produced by coupling two different monomeric indole alkaloids, vindoline and catharanthine (Endo et al. 1988; Misawa et al. 1988; Fujita et al. 1990). In the plant, the former is accumulated at a relatively high level, whereas the latter is at a much lower level. However, in cultured cells or hairy roots, the former is not produced, whereas the latter occurs at a considerable level (Constabel et al. 1982; Lounasmaa and Galambos 1989, Jung et al. 1992a,b). Therefore, it has been considered desirable to produce the dimers by coupling catharanthine obtained from cell or hairy root cultures with vindoline obtained from cultivated plants (Fujita et al. 1990).

On the other hand, Yun et al. (1992) succeeded in enhancing the productivity of scopolamine, a tropane alkaloid, in *Atropa belladonna* by introducing cDNA clone of the gene of hyoscyamine-6β-hydroxylase, a rate-limiting enzyme in the process of catalyzing hyoscyamine to scopolamine, from *Hyoscyamus niger* using Ti-plasmid-mediated transformation. Their results suggest that the productivity of vinblastine or vincristine in *C. roseus* plants may be elevated by manipulating the expression level of the gene for a rate-limiting enzyme, if any, in the biosynthetic process of catharanthine. To do so, a plant regeneration system for this species is prerequisite.

Plant regeneration from leaf segment-derived callus via organogenesis has been reported in *C. roseus* (Constabel et al. 1982). However, the frequency of plant regeneration seems to be too low to be practical for the genetic transformation. Since success was limited to low frequency shoot formation, we decided to develop a regeneration system based on somatic embryogenesis. In preliminary experiments, we failed to obtain somatic embryos from cultured seedling explants. However, when anthers were

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cultured, somatic embryos were produced. This communication describes high frequency plant regeneration in embryogenic cell suspension cultures derived from anthers of *C. roseus*.

Materials and methods

Anther culture. Forty anthers were dissected from surface-sterilized flower buds at the flag leaf stage (3 to 7 days before anthesis) of *Catharanthus roseus* (L.) G. Don (cv. Little Delicata; seeds were purchased from Takii & Company, Kyoto, Japan.) grown in a growth chamber (27℃ day/22℃ night at 16-h photoperiods). Five anthers each were placed in 87x15 mm plastic Petri dishes that contained Murashige and Skoog's (1962) inorganic salts, 100 mgl^{-1} myo-inositol, 0.4 mgl^{-1} thiamine·HCl, 3% sucrose, and 4 gl^{-1} Gelrite [MS basal medium] supplemented with 1 mgl^{-1} α-naphthaleneacetic acid (NAA) and 0.1 mgl^{-1} kinetin at pH 5.8 [MSNK]. The cultures were maintained in the light (about 3 Wm^{-2} from cool-white fluorescent lamps at 16-h photoperiods) at 25℃. Calli formed on anthers were separated and subcultured on MSNK medium every four weeks. To regenerate whole plants, the calli were transferred onto the basal medium and cultured in the light.

Suspension culture. To initiate suspension cultures, callus subcultured on MSNK medium was placed in 50 ml of liquid MSNK medium in 250-ml Erlenmeyer flask on a gyratory shaker set at 100 rpm in the dark. The suspension cultures were subcultured at 14-day intervals.

Assays for embryogenic potential. To test the stability of embryogenic potential, finely dispersed suspension cultures were periodically plated on the solid basal medium after 14 days of subculture and incubated in the dark. After four weeks of culture, they were observed under a dissecting microscope to determine the frequency of somatic embryo formation on colonies proliferated from the plated cells.

Chromosome counts. For chromosome counts, ten root tips from each of the source plant seedlings and randomly selected plantlets from somatic embryos were treated with 0.0025 M 8-hydroxyquinoline for 4 to 5 h at room temperature. After fixation in ethanol: acetic acid (3:1, v:v) solution for 30 min at 4℃, root tips were kept in 80% ethanol at 4℃ for 12 h and then treated with 1 N HCl before staining with aceto-orcein.

Results and Discussion

After two weeks of culture, white friable calli were formed on over 50% of the cultured anthers (Fig. 1A). After four weeks of culture, the anthers turned brown and degenerated. At the time, the calli were separated from the anthers and subcultured on MSNK medium. After the second subculture, organized structures embedded in the calli which were derived from the same anther were observed. One of the calli with structures was transferred onto the basal medium. It gave rise to numerous somatic embryos (Fig. 1B,C), indicating that the calli and the initial structures were embryogenic calli and immature embryos. The somatic embryos were subsequently converted into plantlets (Fig. 1D) and more than 40 plants were transplanted to potting soil and grown until flowering in a growth chamber (Fig. 1E).

The callus maintained mitotic divisions when subcultured on MSNK medium every four weeks. However, when subcultures were conducted at greater than 4-week intervals, numerous embryos arose on the callus. The embryogenic potential of the callus was retained for over one year of subcultures.

After two to three passages of subcultures, yellowish compact cell aggregates began to appear in the suspension cultures. These cultures were plated on solid MSNK medium. After one to two weeks of culture, proliferating yellowish compact microcalli were separated from friable ones under a dissecting microscope (Fig. 2A), and transferred back to liquid MSNK medium for reinitiation of suspension cultures. As subcultures proceeded, the compact cell aggregates became smaller and finely dispersed in the medium. From these cultures two distinct types of cells were observed under a light microscope: small, round, densely aggregated cells with rich cytoplasm (Fig. 2B) and large, elongated, vacuolated, rarely aggregated cells with scarce cytoplasm (Fig. 2C). The appearance of the former was in agreement with that of cereal embryogenic cells and the latter was with that of nonembryogenic cells as described by Vasil and Vasil (1984). The population of the small cells became gradually dominant over that of the large cells and when plated onto the basal medium, these cells gave rise to numerous somatic embryos at a high frequency: after four weeks of culture, greater than 80% of the yellowish compact colonies proliferated from the plated cells formed somatic embryos at early developmental stages (Fig. 2D).

All of the source plant seedlings and regenerants from the initial callus and from suspension cultures showed the normal somatic chromosome number of 2n=2x=16 (Fig. 2E), suggesting that the cells which underwent development into plants had passed through endomitosis or that the initial callus was derived from anther wall.

The frequency of plant regeneration from cultured anthers is usually very low and such was the case with *C. roseus*: one out of 40 anthers produced embryogenic callus, which subsequently developed into plantlets via somatic embryogenesis. A repeated experiment with anthers showed that the frequency was of the same range and that light did not seem to affect the frequency.

Fortunately, the suspension cultures reestablished

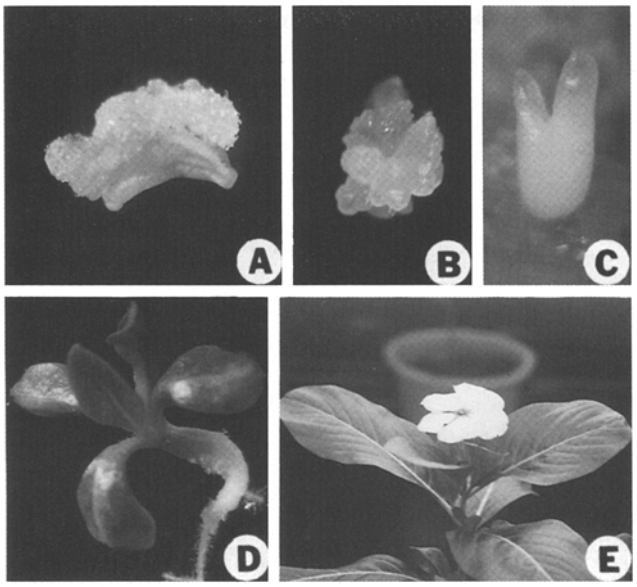

Fig. 1. Plant regeneration from anther-derived callus of *C. roseus*.
A: Callus formed on an anther cultured on MS medium with 1 mgl^{-1} NAA and 0.1 mgl^{-1} kinetin; B: Somatic embryos formed on embryogenic callus after transfer onto MS basal medium; C: Somatic embryo at cotyledonary stage; D: Regenerated plantlet; E: Flowering plant regenerated from somatic embryo.

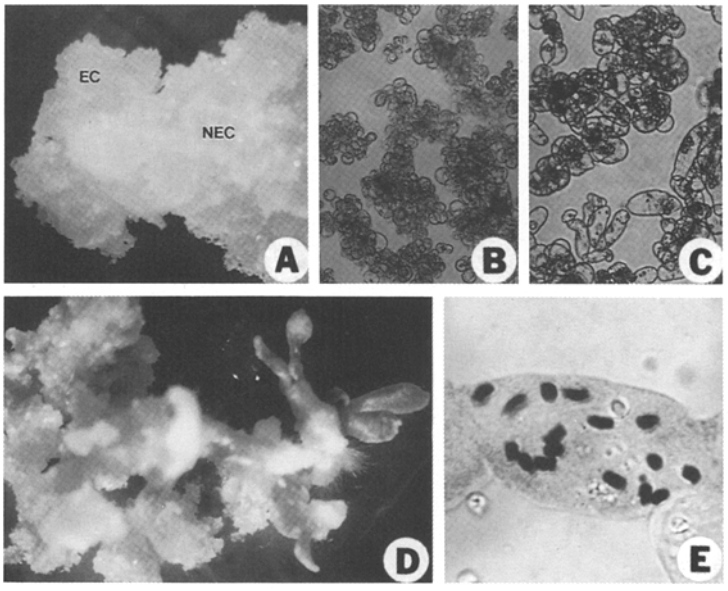

Fig. 2. Cell suspension cultures of *C. roseus*. A: Microcalli proliferated from plated cell suspension cultures (EC: embryogenic callus; NEC: nonembryogenic callus); B: Embryogenic cell suspension culture; C: Nonembryogenic cell suspension culture; D: Somatic embryos and plantlets from plated embryogenic cells. E: Metaphase chromosomes of a regenerant (2n=2x=16).

from the initial callus after one year of subcultures maintained a high embryogenic potential stably. We think that the cell line obtained in this study will be useful in the gene manipulation of *C. roseus* for elevation of the productivity of vinblastine and vincristine. In this way, we may detour the coupling process for the production of dimer alkaloids and cell cultures by

extracting the compounds directly from cultivated plants in the field.

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