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Isolation and Characterization of Microprotoplasts from Propyzamide-treated Cell Suspension Cultures of *Hemerocallis hybrida*

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To produce intergeneric hybrid plants with one or a few chromosomes via microprotoplast fusion in the Liliaceous ornamentals, we developed a system for isolating microprotoplasts from micronucleated cells in suspension cultures of *Hemerocallis hybrida* cv. Stella d'Oro (2n = 2x = 22). Micronucleation was efficiently induced with a micronucleus index up to 19.1% by the following sequential treatment of suspension cultures: initially with 2 mM hydroxyurea for 24 h and then with 8 μM propyzamide for 60 h with the addition of 20 μM cytochalasin-B to the cultures 20 h after the initiation of the propyzamide treatment. Following the enzyme treatment of the micronucleated cells and ultra-centrifugation with a continuous iso-osmotic gradient of Percoll solution, microprotoplasts were isolated, each of which had a small nucleus surrounded by a thin rim of cytoplasm. Microprotoplasts less than 10 μm in diameter were obtained with yield of 2.9×10^4 cells per 1 ml packed cell volume of suspension cells through sequential filtration with nylon sieves with decreasing pore sizes (50, 20 and 10 μm). The DNA content of the microprotoplasts was less than the 2C level as indicated by flow cytometric analysis, and the relative fluorescence intensity in some of their nuclei corresponded to one or a few chromosomes.

Key Words: ***Hemerocallis hybrida*, cytochalasin-B, micronucleus, microprotoplast, propyzamide.**

Introduction

Like microcells in mammalian (Fournier 1982), microprotoplasts in higher plants (Ramulu *et al.* 1999) contain only one or a few chromosomes. Therefore, the microprotoplast fusion technique, i.e. cell fusion between microprotoplasts and somatic cell protoplasts, can be used for partial genome transfer in higher plants (Verhoeven *et al.* 1991, Ramulu *et al.* 1992, 1995, 1999). Several asymmetric hybrid plants have so far been produced by this technique in the Solanaceous species (Ramulu *et al.* 1996a,b) and in the genus *Helianthus* (Binsfeld *et al.* 2000).

We aim to produce intergeneric hybrid plants with one or a few alien chromosomes via microprotoplast fusion for genetic improvement and chromosome studies in the Liliaceous ornamental plants. For applying this technique, it is essential to develop efficient systems for inducing micronucleated cells as well as for isolating microprotoplasts (Verhoeven and Ramulu 1991, Ramulu *et al.* 1993). To date, microprotoplasts have been successfully prepared from micronucleated suspension cells of *Nicotiana plumbaginifolia*, *Solanum tuberosum*, *S. brevidens*, *Beta vulgaris* (Ramulu *et al.* 1992) and *Helianthus giganteus* (Binsfeld *et al.* 2000). We had previously succeeded in the efficient induction of micronucleation in cell suspension cultures of *Hemerocallis hybrida* by sequentially treating them with a DNA synthesis inhibitor, hydroxyurea (HU), followed by a spindle toxin, propyzamide (PRO) (Saito and Nakano 2001a). Here, we describe the isolation of microprotoplasts from the suspension cultures of *H. hybrida* sequentially treated with HU and PRO.

Materials and Methods

Cell suspension cultures

Cell suspension cultures of *H. hybrida* cv. Stella d'Oro (2n = 2x = 22), which consisted of fine cell clumps and showed a fast growth (Saito and Nakano 2000), were used in the present study. The cultures were maintained by subculturing every 3 days in MS medium (Murashige and Skoog 1962) containing 10 mg l^{-1} picloram and 3% sucrose at 25°C in the dark on a rotary shaker (100 cycle min^{-1}).

Micronucleation

HU as a DNA synthesis inhibitor and cytochalasin-B (CB) as a microfilament-disrupting agent (both from Sigma-Aldrich Chemie Gmbh, Steinheim, Germany), and PRO as a spindle toxin (Wako Pure Chemical Industries Ltd., Osaka, Japan) were used in the present study. All the stock solutions were prepared in water-free dimethylsulfoxide (DMSO) and stored at −20°C. For synchronizing cell division, HU at a final concentration of 2 mM was added to the suspension cultures 12 h after subculture. After treatment with HU for 24 h, the cells were washed three times with HU-free fresh medium and then re-cultured in a medium containing 8 μM PRO for inducing micronucleation (Saito and Nakano 2001a). For further promoting micronucleation, CB at a final concentration of 20 μM was added to the cultures 12 h or 20 h after the initiation of the PRO treatment. All the cultures were maintained at 25°C in the dark on a rotary shaker

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(100 cycle min^{-1}).

During the PRO treatment, random sampling of suspension cells, ca. 0.1 ml packed cell volume (PCV) each, was made at certain intervals, and the metaphase index (MI: percentage of metaphase-arrested cells) and micronucleus index (MNI: percentage of micronucleated cells) were determined, as previously described (Saito and Nakano 2001a).

Protoplast isolation

Suspension cells were incubated in an enzyme solution consisting of MS medium, 0.5% Macerozyme R-10, 2% Cellulase Onozuka RS (both from Yakult, Honsha Co., Tokyo, Japan), 8 μM PRO, 20 μM CB, 10 mg l^{-1} picloram, 5 mM 2-morpholinoethanesulfonic acid (MES) and 0.5 M sorbitol, at 25°C in the dark on a rotary shaker (30 cycle min^{-1}). After 6 h of enzyme treatment, the mixture was passed through a nylon sieve (pore size 50 μm), and the protoplasts were freed from debris by flotation on a 0.8 M sucrose solution with centrifugation (800 cycle min^{-1} for 3 min). The protoplasts were washed twice with a 0.5 M sorbitol solution by re-suspension and centrifugation (800 cycle min^{-1} for 3 min). They were then suspended in a 0.5 M sorbitol solution containing 20 μM CB and maintained on ice until ultra-centrifugation.

Microprotoplast isolation

Continuous iso-osmotic gradients of Percoll were prepared by the addition of 0.5 M sorbitol to the Percoll solution (Sigma-Aldrich) followed by ultra-centrifugation (40,000 cycle min^{-1}, 200,000 *g* in the center of tube) for 30 min at 4°C in a Hitachi ultra-centrifuge 70P-72 (Hitachi Koki Co., Ltd., Tokyo, Japan) using a 6 × 13 ml swing-out rotor RPS40T (Hitachi). The top layer (45 mm from the top) was removed from the preformed gradient, and 5 ml of the protoplast suspension maintained on ice was layered on the top followed by ultra-centrifugation (40,000 cycle min^{-1} for 1.5 h). All the bands obtained by ultra-centrifugation were gathered together and then diluted with a 0.5 M sorbitol solution. Protoplasts and microprotoplasts were washed twice with a 0.5 M sorbitol solution by re-suspension and centrifugation (800 cycle min^{-1} for 3 min). The (micro)protoplast suspensions thus obtained were sequentially filtered using nylon sieves with decreasing pore sizes (50, 20 and 10 μm).

For cytological analysis, (micro)protoplasts obtained after sequential filtration were dropped under a bridge with cover glasses, and observed under either a light microscope (LEICA DMLB, Leica, Wetzlar, Germany) or an UV microscope (UFX-II, Nikon, Tokyo, Japan) after staining with 4,6-diamidino-2-phenylindole (DAPI).

Flow cytometric analysis

Relative nuclear DNA content of the (micro)protoplasts was determined by using a flow cytometer (PA; PERTECH GmbH, Münster, Germany). Samples, 0.5 ml each of the (micro)protoplast suspensions, were stained for 10 min with 2 ml of Solution B (High Resolution DNA kit, Type P) containing DAPI prior to the flow cytometric analysis. As a control, untreated suspension cells were also analyzed. In this case, the cells (ca. 0.1 g fresh weight) were chopped up with a razor blade in 0.5 ml of Solution A (High Resolution DNA kit, Type A) in a plastic Petri dish for isolating nuclei, and stained for 10 min with 2 ml of Solution B (Saito and Nakano 2001b).

Results

Micronucleation

Fig. 1 shows the changes in the MI during the PRO treatment without CB addition and those in the MNI during the PRO treatments with or without CB addition following a 24-h pre-treatment with HU in suspension cultures of *H. hybrida*. In the absence of CB, the MI started to increase 12 h after the initiation of the PRO treatment, and the maximum MI of 7.8% was obtained after 20 h. Micronuclei also started to be observed 12 h after the initiation of the PRO treatment. The number of micronucleated cells (Fig. 2A) gradually increased thereafter, and the maximum MNI of 13.1% was obtained after 60 h.

Addition of 20 μM CB affected considerably the MNI during the PRO treatment, although the maximum MNI was obtained 60 h after the initiation of the PRO treatment in all the treatments examined. The addition of CB 20 h after the initiation of the PRO treatment significantly ($P < 0.01$)

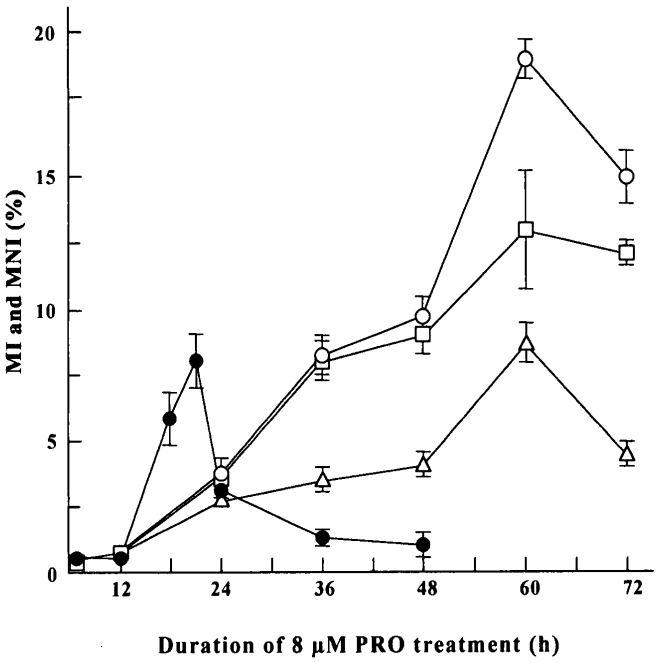

Fig. 1. Changes in the MI and MNI after the initiation of the treatment with 8 μM PRO in cell suspension cultures of *Hemerocallis hybrida* pre-treated with HU. ●, MI in the absence of CB; □, MNI in the absence of CB; △, MNI when 20 μM CB was added to the cultures 12 h after the initiation of the PRO treatment; ○, MNI when 20 μM CB was added 20 h after the initiation of the PRO treatment. Vertical bars represent SE of the mean of 4 independent experiments.

increased the maximum MNI up to 19.1%. On the other hand, the addition of CB after 12 h rather inhibited micronucleation, in which the maximum MNI decreased to 8.6%. Therefore, in the following experiments, CB was added to the cultures 20 h after the initiation of the PRO treatment. Application of CB to the cultures not subjected to the PRO treatment could not induce micronucleation (data not shown).

Isolation and enrichment of microprotoplasts

In our preliminary experiments, enzyme treatment for 6 h was sufficient for isolating large numbers of viable protoplasts from the suspension cultures of *H. hybrida* (data not shown). Therefore, in the present study, suspension cells, which had been treated for 54 h with PRO, were incubated for 6 h in an enzyme solution containing both PRO and CB. After the enzyme treatment, both mononucleated and micronucleated protoplasts (Fig. 2B) were obtained.

Following ultra-centrifugation of the purified protoplasts, one large band and several small bands were obtained in the Percoll gradient at various distances. The large band appeared at around 4 cm from the top of the centrifuge tube, along with small bands just below the large band. All of these bands contained vacuoplasts or cytoplasts, evacuolated protoplasts and microprotoplasts. Upper parts of the large band contained mainly cytoplasts or vacuoplasts (Fig. 2C). Separation of each band was very difficult because these structures were close to each other. Therefore, all of them were gathered together and sequentially filtered using nylon sieves with decreasing pore sizes (50, 20 and 10 μm), which gave a population containing predominantly smaller microprotoplasts (Fig. 2D). Table 1 shows the effect of sequential filtration on the yield of microprotoplasts with different sizes. After filtration using two sieves with pore sizes of 50 and 20 μm (double filtration), microprotoplasts below 10 μm and of 10-20 μm in diameter were obtained with yields of 5.0×10^4 and 2.1×10^4 per 1 ml PCV of suspension cells, respectively. Further filtration using a sieve with a pore size of 10 μm (triple filtration) led to a decrease in the yield of microprotoplasts below 10 μm to 2.9×10^4 per 1 ml PCV of suspension cells. Fig. 2 E and F show the same microprotoplasts stained with DAPI, which were imaged by using either transmitted (Fig. 2E) or UV light (Fig. 2F). Since, in most cases, microprotoplasts and DAPI-stained micronuclei were nearly equal in size, each microprotoplast had a micronucleus surrounded by a thin rim of cytoplasm (Fig. 2E, F). The size of the microprotoplasts appeared to depend upon that of the micronucleus.

DNA contents of the (micro)protoplasts obtained through sequential filtration were analyzed by flow cytometry. Fig. 3 shows histograms of the relative fluorescence

Table 1. Microprotoplast yields obtained per 1 ml PCV of suspension cells of *Hemerocallis hybrida* cv. Stella d'Oro

Size of sieves	50 + 20 μm		50 + 20 + 10 μm
Microprotoplast size	< 10 μm	10-20 μm	< 10 μm
Yield (× 10^4)	5.0 ± 1.0	2.1 ± 0.2	2.9 ± 0.2

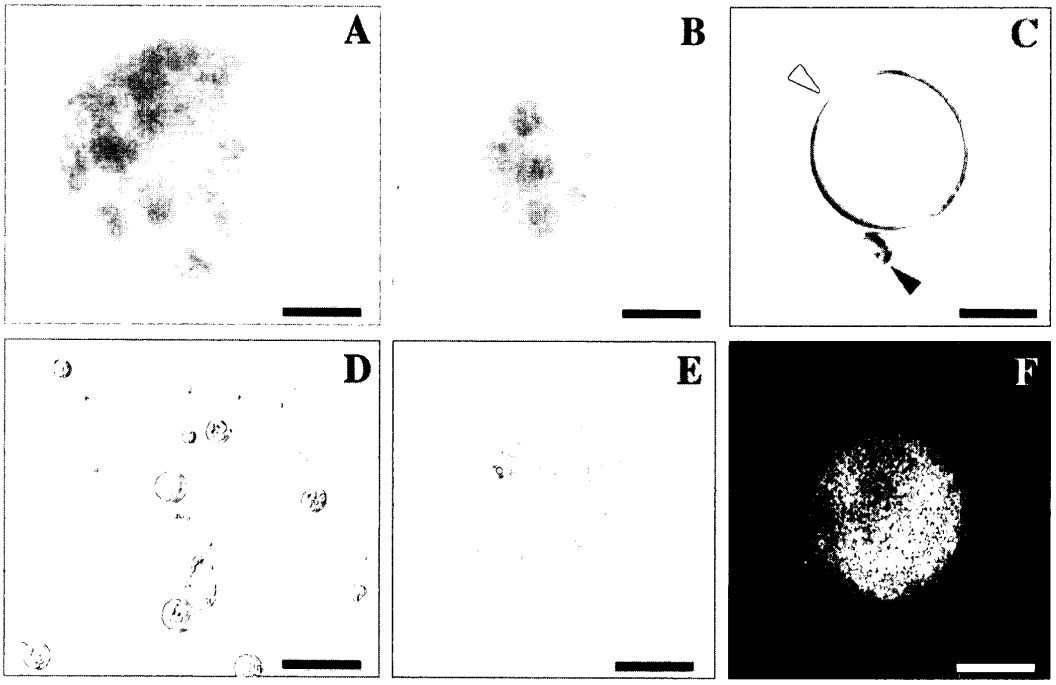

Fig. 2. Induction of micronucleation and isolation of microprotoplasts in cell suspension cultures of *Hemerocallis hybrida*. (A), cell with several micronuclei; (B), protoplast with several micronuclei; (C), vacuoplast (white arrow head) and microprotoplast (black arrow head) obtained after ultra-centrifugation; (D), microprotoplasts purified through sequential triple filtration. (C) and (D) were imaged by using differential interference contrast in transmitted light. (E, F), DAPI-stained microprotoplast under light microscopy and UV microscopy, respectively. Bars in (A) – (D) = 20 μm. Bars in (E) and (F) = 5 μm.

intensity of the nuclei from the control suspension cells (2n = 2x = 22) not subjected to PRO and CB treatments (Fig. 3A), and the populations obtained after double (Fig. 3B) or triple filtration (Fig. 3C). Histogram from the control suspension cells showed two peaks corresponding to 2C and 4C nuclei. After double filtration, the frequency of the nuclei with a DNA content below the 2C level increased markedly, but some of the 2C nuclei still remained. After triple filtration, almost all the 2C as well as 4C nuclei were eliminated and the DNA content of most of the nuclei was below the 2C level, indicating that a population that exclusively contained microprotoplasts could be obtained by triple filtration (Fig. 3C). In addition, the peak of 2C nuclei (22 chromosomes) was located at a relative fluorescence intensity of around 10 (Fig. 3A), while part of these microprotoplasts showed relative fluorescence intensities of ca. 0.4 to 2.0 (Fig. 3C), indicating that the DNA content of the micronucleus was equivalent to 1 to 5 chromosomes.

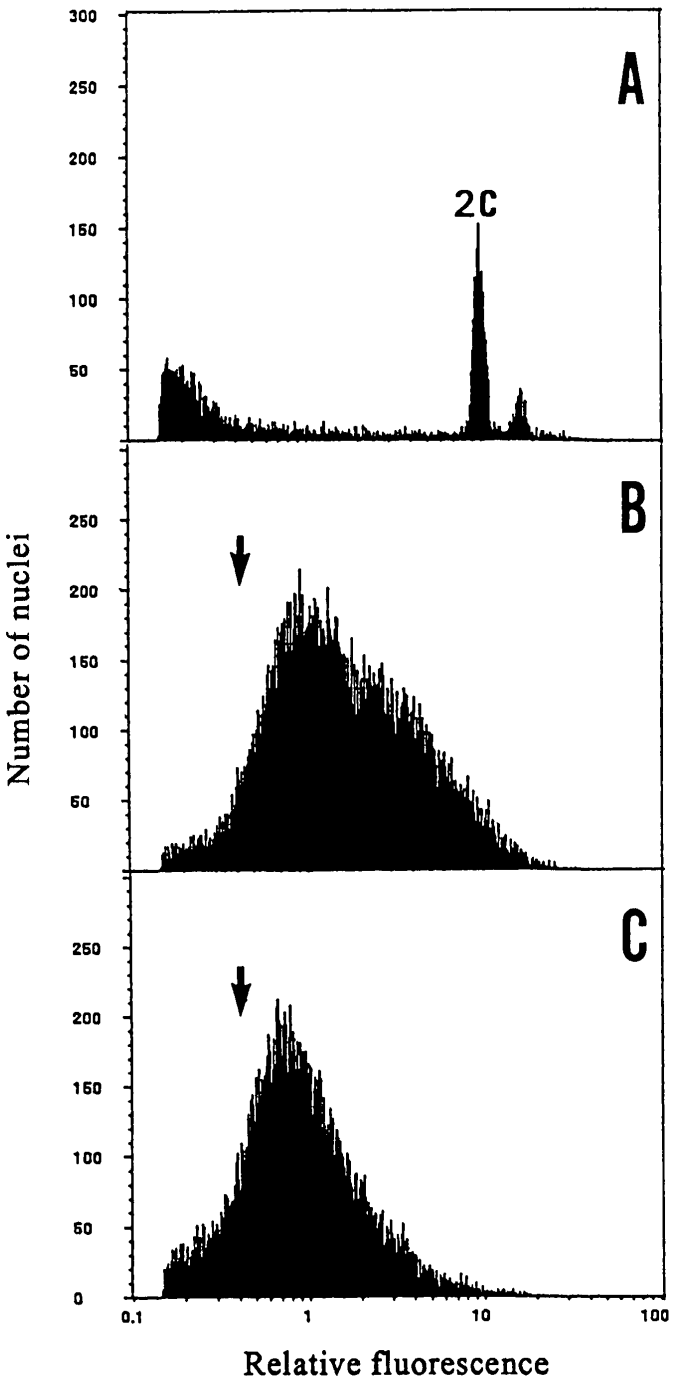

Fig. 3. Histograms from FCM analysis of nuclear DNA contents of suspension cells and microprotoplasts of *Hemerocallis hybrida*. (A), suspension cells with 22 chromosomes; (B), microprotoplasts after double filtration using sieves with 50- and 20-μm pore size; (C), microprotoplasts after triple filtration using sieves with 50-, 20- and 10-μm pore size. Arrows show the relative fluorescence intensity equivalent to one chromosome.

Discussion

In the present study, we demonstrated the possibility of achieving efficient micronucleation as well as successful isolation of microprotoplasts in the Liliaceous ornamental plant, *H. hybrida* cv. Stella d'Oro. The maximum MNI of 19.1% was obtained through the following sequential treatment of suspension cultures: (1) after 12 h of subculture, the cultures were treated with 2 mM HU for 24 h; (2) the cultures were then treated with 8 μM PRO for 20 h; (3) the cultures were finally treated with both 8 μM PRO and 20 μM CB for 40 h (60 h after the initiation of the PRO treatment). Microprotoplasts with yields up to 2.9×10^4 per 1 ml PCV of suspension cells were routinely obtained by protoplasting of the sequentially treated suspension cultures, ultra-centrifugation of the isolated protoplasts, and purification via triple filtration using nylon sieves with decreasing pore sizes (50, 20 and 10 μm).

In the previous study, we examined the effects of HU and several spindle toxins on the induction of micronucleation in suspension cultures of *H. hybrida*, and the highest MNI of 14.7% was obtained by sequential treatment of the cultures with HU and PRO (Saito and Nakano 2001a). In the present study, furthermore, the addition of CB to the cultures during the PRO treatment was also found to affect micronucleation. A significant increase in the MNI was obtained by the addition of CB 20 h after the initiation of the PRO treatment, at the time when the MI reached a peak in the control cultures without CB addition. Stimulation of micronucleation by CB had already been reported for human cells: although it had been considered to be difficult to obtain micronucleated human cells by sole treatment with colcemid, micronucleation could be induced by the addition of CB to the cultures during the colcemid treatment (Fournier 1982). As in the case of human cells, CB also stimulated micronucleation of cultured cells of *Solanum tuberosum* and *Nicotiana plumbaginifolia* (Ramulu *et al.* 1990, 1993), and *Helianthus giganteus* (Binsfeld *et al.* 2000) during spindle toxin treatment. In these previous studies, CB was added to the cultures when the MI reached a peak (Ramulu *et al.* 1990, 1993, Binsfeld *et al.* 2000). Favorable results were obtained in the present study for suspension cultures of *Hemerocallis hybrida*. Ramulu *et al.* (1993) demonstrated that micronuclei produced through spindle toxin treatment could be stably maintained without undergoing fusion by the

addition of CB, resulting in the enhancement of the yield of micronuclei.

In the present study, however, the application of CB 12 h after the initiation of the PRO treatment inhibited micronucleation of cultured cells of *H. hybrida*. Since microfilaments play an important role in positioning the nucleus in the center of the interphase cell, treatment of the cells with cytochalasin, a microfilament-disrupting agent, caused the displacement of the nucleus (Katsuta and Shibaoka 1988). Since CB application 12 h after the initiation of the PRO treatment decreased the MI (data not shown), cell-cycle progression from interphase to metaphase might be inhibited by CB through the displacement of the nucleus, which in tern led to the decrease in the MNI in the present study.

The isolation of a large number of microprotoplasts is important for successful asymmetric somatic hybridization (Verhoeven and Ramulu 1991, Ramulu *et al.* 1993). In the present study, we succeeded for the first time in isolating microprotoplasts from cell suspension cultures in monocotyledonous plants. As in the case of *N. plumbaginifolia* (Ramulu *et al.* 1993) and *Helianthus giganteus* (Binsfeld *et al.* 2000), microprotoplasts of *H. hybrida* were successfully isolated through enzymatic protoplasting and ultracentrifugation in the presence of both spindle toxin and CB. In addition, as suggested by Binsfeld *et al.* (2000), cold treatment at 4°C during ultra-centrifugation also appeared to be important for efficient isolation of microprotoplasts: cold treatment might stabilize the formed micronuclei and their plasma membrane system. Ultra-centrifugation at 100,000 *g* for 2 h and at 27,000 *g* for 1 h was performed for isolating microprotoplasts from cultured dicotyledonous plant cells (Verhoeven and Ramulu 1991, Ramulu *et al.* 1993, Binsfeld *et al.* 2000) and mammalian cells (Landolph and Fournier 1983), respectively. However, in our preliminary experiments, ultra-centrifugation at such speeds was not sufficient for efficient isolation of microprotoplasts of *H. hybrida*, and a much higher speed, ca. 200,000 *g*, was needed (data not shown). These differences may be due to physical and/or physiological differences in the cells or protoplasts used for isolating the microprotoplasts.

In the present study, smaller microprotoplasts of *H. hybrida* could successfully be purified from a miscellaneous population by sequential filtration through 3 nylon sieves with decreasing pore sizes as in the cases of *N. plumbaginifolia* (Ramulu *et al.* 1993, 1999) and *Helianthus giganteus* (Binsfeld *et al.* 2000). Flow cytometric analysis revealed that smaller microprotoplasts with DNA contents equivalent to those of small numbers (ca. 1-5) of chromosomes were enriched after sequential triple filtration.

Although the yield of the microprotoplasts obtained in the present study was relatively low compared with that in the other plant species (Ramulu *et al.* 1993, Binsfeld *et al.* 2000), our system for preparing microprotoplasts from suspension cultures of *H. hybrida* may pave the way for the transfer of one or a few chromosomes via cell fusion from this species to other sexually incompatible species, for example *Lilium* × *formolongi*, in which protoplast-to-plant systems have been developed (Mii *et al.* 1994, Godo *et al.* 1996), leading to the production of intergeneric chromosome addition lines in the Liliaceous ornamentals. Attempts to produce asymmetric intergeneric hybrids via microprotoplast fusion are currently in progress using this system.

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Literature Cited

Binsfeld, P.C., R. Wingender and H. Schnabl (2000) Characterization and molecular analysis of transgenic plants obtained by microprotoplast fusion in sunflower. Theor. Appl. Genet. 101: 1250-1258.

Fournier, R.E.K. (1982) Microcell-mediated chromosome transfer. *In* "Techniques in Somatic Cell Genetics" Shay, J.W. (ed.), Plenum Press, New York. p. 309-327.

Katsuta, J. and H. Shibaoka (1988) The roles of the cytoskeleton and the cell wall in nuclear positioning in tobacco BY-2 cells. Plant Cell Physiol. 29: 403-413.

Landolph, J.R. and R.E.K. Fournier (1983) Microcell-mediated transfer of carcinogen-induced ouabain resistance from C3H/10 T1/2Cl 8 mouse fibroblasts to human cells. Mut. Res. 107: 447-463.

Murashige, T. and F. Skoog (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Ramulu, K.S., H.A. Verhoeven, P. Dijkhuis and L.J.W. Gilissen (1990) A comparison of APM-induced micronucleation and influence of some factors in various genotypes of potato and *Nicotiana*. Plant Sci. 69: 123-133.

Ramulu, K.S., P. Dijkhuis, H.A. Verhoeven, I. Famelaer and J. Blaas (1992) Microprotoplast isolation, enrichment and fusion for partial genome transfer in plants. Physiol. Plant. 85: 315-318.

Ramulu, K.S., P. Dijkhuis, I. Famelaer, T. Cardi and H.A. Verhoeven (1993) Isolation of sub-diploid microprotoplasts for partial genome transfer in plants: enhancement of micronucleation and enrichment of microprotoplasts with one or a few chromosomes. Planta 190: 190-198.

Ramulu, K.S., P. Dijkhuis, E. Rutgers, J. Blaas, W.H.J. Verbeek, H.A. Verhoeven and C.M. Colijn-Hooymans (1995) Microprotoplast fusion technique: a new tool for gene transfer between sexually-incongruent plant species. Euphytica 85: 255-268.

Ramulu, K.S., P. Dijkhuis, E. Rutgers, J. Blaas, F.A. Krens, J.J.M. Dons, C.M. Colijn-Hooymans and H.A. Verhoeven (1996a) Microprotoplast-mediated transfer of single specific chromosomes between sexually incompatible plants. Genome 39: 921-933.

Ramulu, K.S., P. Dijkhuis, E. Rutgers, J. Blaas, F.A. Krens, W.H.J. Verbeek, C.M. Colijn-Hooymans and H.A. Verhoeven (1996b) Intergeneric transfer of partial genome and direct production of monosomic addition plants by microprotoplast fusion. Theor. Appl. Genet. 92: 316-325.

Ramulu, K.S., P. Dijkhuis, J. Blaas, F.A. Krens and H.A. Verhoeven (1999) Microprotoplast-mediated chromosome transfer

(MMCT) for direct production of monosomic addition lines. *In* "Methods in Molecular Biology, Plant Cell Culture Protocols" Hall,R.D. (ed.), Huhana Press Inc., Totowa, NJ. 111: p. 227-242.

Saito,H. and M.Nakano (2000) Establishment and characterization of cell suspension cultures of *Hemerocallis hybrida*. Bull. Fac. Agr. Niigata Univ. 53: 1-8.

Saito,H. and M.Nakano (2001a) Partial synchronization of cell division and micronucleation in suspension-cultured cells of *Hemerocallis hybrida*: the effects of hydroxyurea and various spindle toxins. Breed. Sci. 51: 301-307.

Saito,H. and M.Nakano (2001b) Flow cytometric analysis of partially synchronized suspension cultures of *Hemerocallis hybrida*: Plant Biotech. 18: 229-231.

Verhoeven,H.A. and K.S.Ramulu (1991) Isolation and characterization of microprotoplasts from APM-treated suspension cells of *Nicotiana plumbaginifolia*. Theor. Appl. Genet. 82: 346-352.

Verhoeven,H.A., K.S.Ramulu, L.J.W.Gilissen, I.Famelaer, P.Dijkhuis and J.Blaas (1991) Partial genome transfer through micronuclei in plants. Acta Bot. Neerl. 40: 97-113.