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Preparation of Microprotoplasts for Partial Genome Transfer via Microprotoplast Fusion in Liliaceous Ornamental Plants

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Abstract

We aimed to produce intergeneric hybrid plants with only one or a few alien chromosomes via microprotoplast fusion for genetic improvement and chromosome studies in Liliaceous ornamental plants. In order to apply this technique, it is essential to establish an efficient system for mass-preparation of microprotoplasts. We have established 2 different systems for isolating microprotoplasts, one from partially synchronized cell suspension cultures of *Hemerocallis hybrida* and the other from developing microspores of *Lilium longiflorum*. Here, the induction of micronucleated cells, isolation of microprotoplasts, and enrichment of smaller microprotoplasts containing one or a few chromosomes are described for both systems.

Discipline: Biotechnology / Plant breeding
Additional key words: cell suspension culture, *Hemerocallis hybrida*, *Lilium longiflorum*, micronucleation, microsporocyte

Introduction

Somatic hybridization via protoplast fusion can be a useful approach for transferring polygenically controlled traits, unidentified and uncloned genes between sexually incompatible species. However, since most of the hybrids obtained via symmetric protoplast fusion may harbor numerous undesired chromosomes or genes, repeated backcrossing and selection are required for eliminating them. Furthermore, the resulting symmetric hybrids often show weakness and/or sterility, probably due to genomic disharmony, instability and unfavorable combinations[1]. Therefore such hybrids may not be successful immediately as commercial cultivars. To solve these problems, asymmetric fusion, which limits the amount of genetic information introduced from donor cells into hybrids, has been carried out using irradiated protoplasts[26]. However, irradiation treatments often result in chromosome breakage, random deletion and rearrangement[10]. Recently, an alternative asymmetric fusion method using microprotoplasts (microprotoplast fusion) has been developed. Since microprotoplasts contain only one or a few intact chromosomes, a limited number of chromosomes can be transferred via microprotoplast fusion, resulting in the production of chromosome addition lines with even a single and specific, intact chromosome between sexually incompatible species[14,17,25]. To date, chromosome addition lines have successfully been produced in the Solanaceous species[15,16,18] and in the genus *Helianthus*[2].

For applying the microprotoplast fusion method in higher plants, it is essential to establish an efficient system for mass-preparation of microprotoplasts. Microprotoplasts of higher plants have been obtained from 2 types of cell populations partially synchronized in the cell cycle: fast-growing cell suspension cultures[9,23] and microsporocytes[4,6]. For the production of intergeneric asymmetric hybrid plants with one or a few alien chromosomes via microprotoplast fusion for genetic improvement and chromosome studies in Liliaceous ornamental plants, we aimed to develop an efficient and reproducible system for mass-preparation of microprotoplasts. We describe here the preparation of cell suspension culture-

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derived, somatic microprotoplasts in *Hemerocallis hybrida* and of developing microspore-derived, gametic microprotoplasts in *Lilium longiflorum*.

Preparation of somatic microprotoplasts from cell suspension cultures

Scheme for the preparation of somatic microprotoplasts

To date, fast-growing cell suspension cultures have mainly been used as a source of microprotoplasts. In this case, the cultures are generally treated with a DNA synthesis inhibitor and/or a spindle toxin for synchronizing cell division and for inducing micronucleation of suspension cells[17,24], and then microprotoplasts are isolated from the micronucleated cells by enzymatic protoplasting and ultra-centrifugation. A scheme for the preparation of somatic microprotoplasts from cell suspension cultures is shown in Fig. 1.

Preparation of somatic microprotoplasts from cell suspension cultures of *Hemerocallis hybrida*

1. Plant material and establishment of cell suspension cultures

A diploid genotype, *Hemerocallis hybrida* 'Stella d'Oro' (2n = 22), which is a dwarf cultivar (30 to 50 cm in height) with yellow flowers, was used. Cell suspension cultures were initiated from creamy-white calli (Fig. 2B) derived from root segments of *in vitro*-grown plantlets (Fig. 2A)[19]. They consisted of fine clumps with 20 to 50 cells (Fig. 2C), and di-, tetra- and octoploid cells were detected in one-year-old cultures by flow cytometry analysis[20]. Suspension cells were subcultured every 3 days in MS[8] medium containing 10 mg/L picrolam and 30 g/L sucrose at 25°C in the dark on a rotary shaker (100 rpm).

2. Micronucleation

For inducing micronuclei, suspension cells 12 h

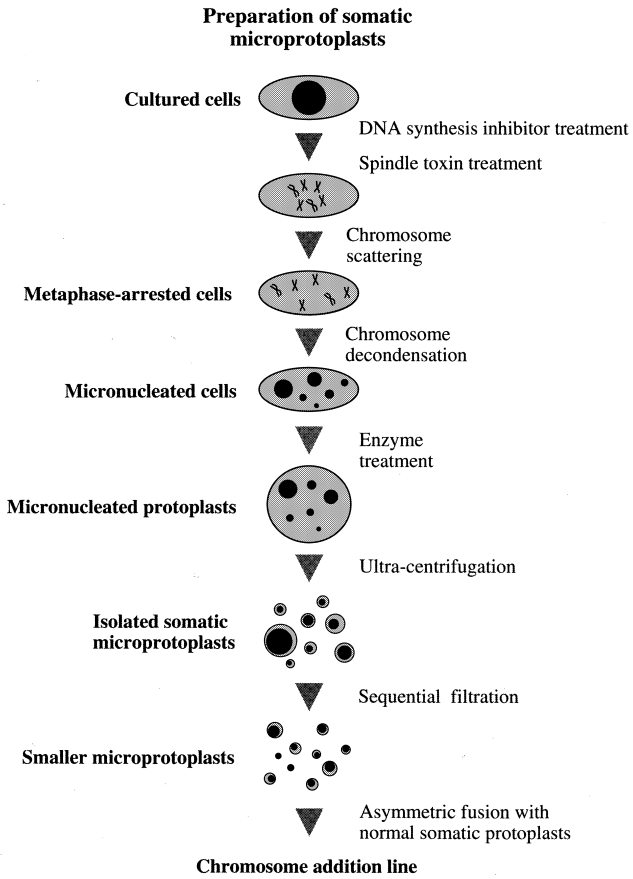

Fig. 1. Scheme for the preparation of somatic microprotoplasts from cell suspension cultures in higher plants

For inducing micronuclei, cell suspensions are treated with a DNA synthesis inhibitor followed by a spindle toxin. Somatic microprotoplasts are isolated from micronucleated protoplasts by ultra-centrifugation and enriched by sequential filtration using nylon sieves with decreasing pore sizes.

after subculture were initially treated with 2 mM of the DNA synthesis inhibitor, hydroxyurea for 24 h, and then with 8 μM of the spindle toxin, propyzamide for 60 h. The percentage of micronucleated cells (micronucleus index; MNI) of 14.7% and micronuclei ranging from 1 to 7 per cell were obtained by the application of the propyzamide treatment[21]. Although other spindle toxins, amiprophos-methyl and butamiphos, had been used for inducing efficient micronucleation in suspension-cultured cells of *Nicotiana plumbaginifolia*[11], *Solanum tuberosum*[12,13,24] and *Helianthus giganteus*[2], their efficiency on the induction of micronucleation in *Hemerocallis hybrida* was limited[21]. To our knowledge, successful application of the propyzamide treatment for inducing micronucleation had not been reported previously in either plant or mammalian cells. Micronucleation efficiency was further enhanced by treating the suspension cultures with the microfilament-disrupting agent, cytochalasin B. The highest MNI of 19.1% was obtained by the following sequential treatments of the 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 at 20 h after the initiation of the propyzamide treatment (Fig. 2D)[22].

3. Isolation of micronucleated protoplasts[22]

At 78 h after the initiation of the sequential treatments described above, suspension cells were incubated for 6 h in a cell wall-digesting enzyme solution consisting of MS medium, 2% Cellulase Onozuka RS, 0.5% Macerozyme R-10, 10 mg/L picrolam, 5 mM 2-morpholinoethanesulfonic acid (MES), 0.5 M sorbitol, 8 μM propyzamide and 20 μM cytochalasin B at 25°C in the dark on a rotary shaker (30 rpm) to isolate micronucleated protoplasts (Fig. 2E). After the enzyme treatment, protoplast suspensions were filtered through a nylon sieve (pore size 50 μm), and the protoplasts were washed twice with a 0.5 M sorbitol solution containing 20 μM cytochalasin B, and maintained on ice until ultracentrifugation.

4. Isolation of somatic microprotoplasts[22]

Continuous iso-osmotic gradients of PERCOLL were prepared by the addition of 0.5 M sorbitol to PERCOLL followed by ultra-centrifugation (40,000 rpm, 200,000 *g* in the center of tube) for 30 min at 4°C in a Hitachi ultra-centrifuge 70P-72 using a 6×13 mL swing-out rotor RPS40T. 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 rpm for 1.5 h). Following ultra-centrifugation, one large band and several small bands were obtained in the 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 mainly vacuoplasts or cytoplasts, evacuolated protoplasts and microprotoplasts (Fig. 2F). Upper parts of the large band contained mainly cytoplasts or vacuoplasts. Separation of each band was very difficult because these structures were close to each other. Therefore, all of them were gathered together.

5. Enrichment and characterization of somatic microprotoplasts[22]

In order to enrich microprotoplasts containing one or a few chromosomes, the gathered suspensions were diluted with a 0.5 M sorbitol solution and then sequentially filtered through nylon sieves with decreasing pore sizes (50, 20 and 10 μm). The sequential filtration resulted in a population containing predominantly smaller microprotoplasts (Fig. 2G), and microprotoplasts below 10 μm in diameter were obtained with a yield of 2.9×10^4 per 1 mL packed cell volume of suspension cells. Since, in most cases, microprotoplasts and DAPI-stained micronuclei were nearly equal in size (Fig. 2H, I), each microprotoplast had a micronucleus surrounded by a thin rim of cytoplasm. The size of the microprotoplasts appeared to depend upon that of the micronucleus. The DNA content of almost all of the populations obtained after the sequential filtration was below the 2C level, and the relative fluorescence intensity in some of the nuclei corresponded to one or a few chromosomes as indicated by flow cytometry analysis.

Preparation of gametic microprotoplasts from developing microspores

Scheme for the preparation of gametic microprotoplasts

This approach does not require additional treatments with a DNA synthesis inhibitor for synchronizing cell division, because the meiotic cycle in higher plants is generally highly synchronous by nature[4,6]. For inducing micronucleation of meiocytes, microsporocytes at the meiotic cycle are treated only with a spindle toxin. In addition, since each micronucleated meiocyte may form microcells during the spindle toxin treatment via cytokinesis, as in the case of normal tetrad formation, microprotoplasts can directly be obtained from the microcell-formed meiocytes by enzymatic protoplasting. Therefore, the ultra-centrifugation process needed to prepare somatic microprotoplasts can be omitted. A scheme for the preparation of gametic microprotoplasts from developing microspores is shown in Fig. 3.

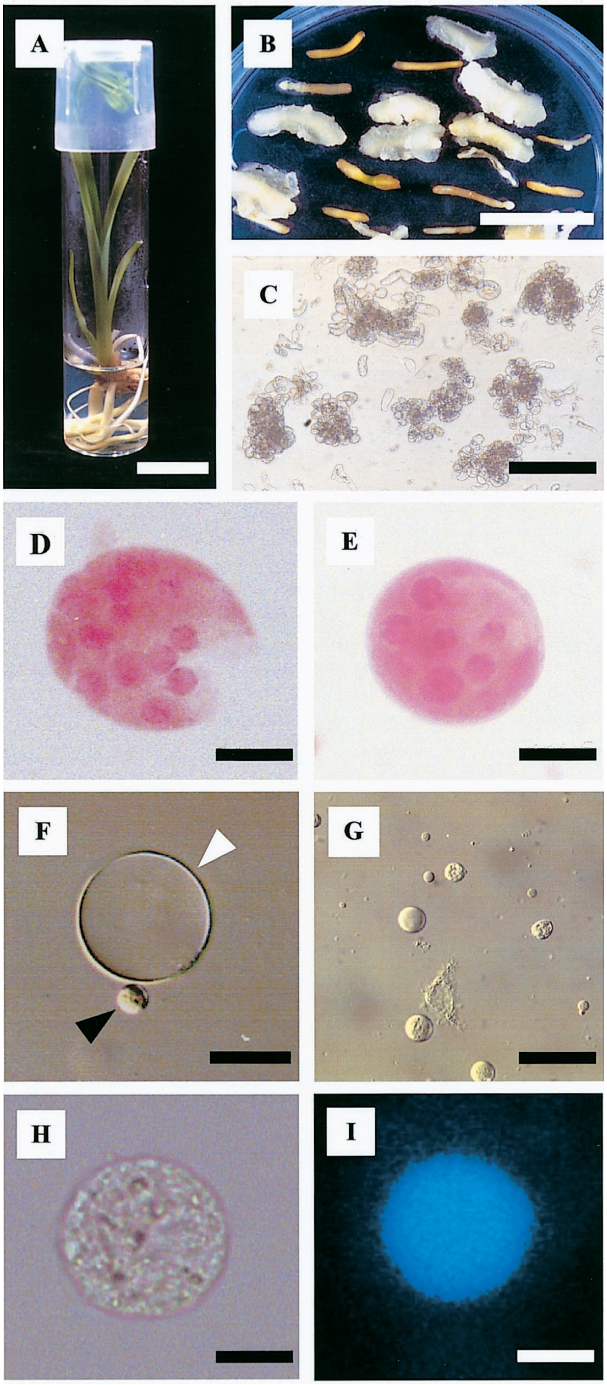

Fig. 2. Isolation of somatic microprotoplasts from cell suspension cultures of *Hemerocallis hybrida* 'Stella d'Oro'

A: *In vitro*-grown plantlet. Bar = 2 cm. B: Creamy-white calli. Bar = 2 cm. C: Fine cell clumps in the suspension culture. Bar = 200 µm. D: Suspension cell with several micronuclei. Bar = 20 µm. E: Protoplast with several micronuclei. Bar = 20 µm. F: Vacuoplast (white arrow head) and microprotoplast (black arrow head) obtained after ultra-centrifugation. Bar = 20 µm. G: Microprotoplasts purified by sequential filtration. Bar = 20 µm. H and I: DAPI-stained microprotoplast under light and UV microscopy, respectively. Bars = 5 µm.

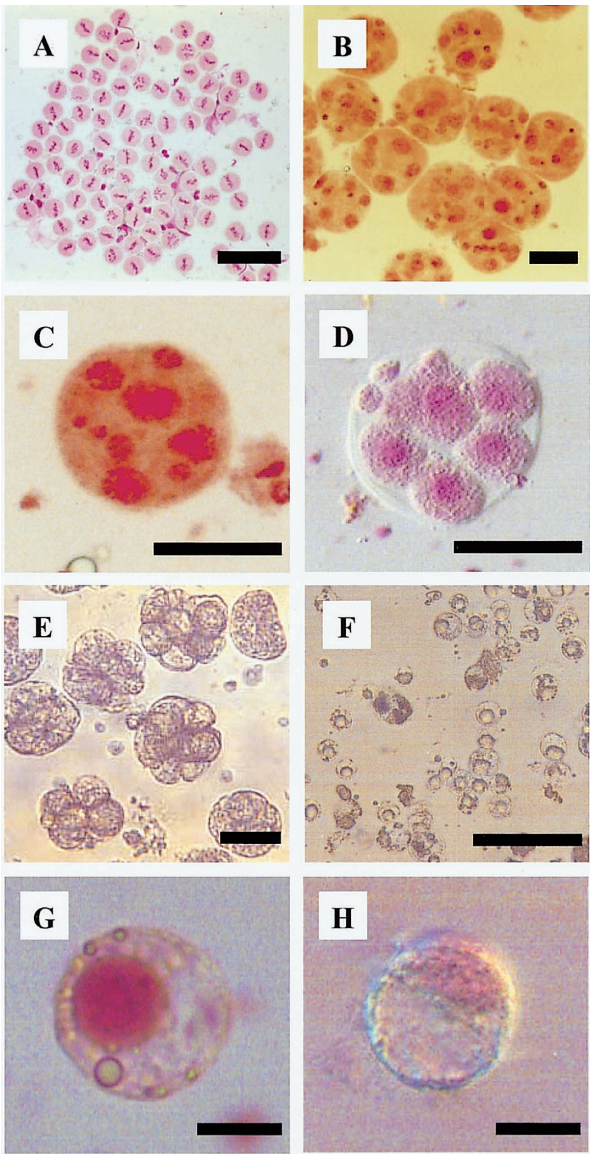

Fig. 4. Isolation of gametic microprotoplasts from developing microspores of *Lilium longiflorum* 'Hinomoto'

A: Microsporocytes at metaphase I. Bar = 100 µm. B and C: Meiocytes with several micronuclei. Bars = 50 µm. D: Meiocytes with several microcells. Bar = 50 µm. E: Microcell-formed meiocytes at middle to late tetrad stages. Bar = 50 µm. F: Gametic microprotoplasts purified by sequential filtration. Bar = 50 µm. G: Gametic microprotoplast with a micronucleus surrounded by a thick rim of cytoplasm. Bar = 5 µm. H: Gametic microprotoplast with a micronucleus and a vacuole. Bar = 5 µm.

Preparation of gametic microprotoplasts from developing microspores of *Lilium* spp. (unpublished data)

1. Plant materials

Six *Lilium* genotypes (2n = 2x = 24), *L. regale*, *L. longiflorum* 'Georgia' and 'Hinomoto', *L. speciosum* 'Uchida', the Asiatic hybrid lily 'Connecticut King' and the Aurelian hybrid lily 'Golden Splendor', were used. They were grown in the greenhouse without heating.

2. Micronucleation

Flower buds containing anthers with microsporocytes at the diakinesis to metaphase I of the meiosis (Fig. 4A) were harvested. The stage of the microsporocyte development was estimated based on the bud length for each *Lilium* genotype. Anthers were isolated from the buds and transferred to micronucleation media containing half-strength MS salts, double-strength MS vitamins, 1 g/L casamino acid, 100 g/L sucrose, and 10 μM of the spindle toxin, isopropyl *N*-(3-chlorophenyl)carbamate (CIPC), and cultured for 3 to 4 days at 25°C in the dark on a rotary shaker (100 rpm). CIPC efficiently induced micronucleation in *L. longiflorum* 'Hinomoto' (Fig. 4B, C), and nuclei ranging from 1 to 20 per meiocyte (mean number: 7.5) were obtained. About 90% of the CIPC-treated microsporocytes formed more than 4 nuclei, and meiocytes with 7 or 8 nuclei were most frequently obtained. Until now, amiprophos-methyl had mainly been used for inducing micronucleation in microsporocytes of *Solanum tuberosum*[5], and in suspension-cultured cells of several Solanaceous species[14,17] and *Helianthus giganteus*[2]. On the other hand, micronucleation of suspension-cultured cells of *Hemerocallis hybrida* was efficiently induced by propyzamide[21,22]. However, the efficiency of these spindle toxins on the induction of micronucleation in microsporocytes of *L. longiflorum* 'Hinomoto' was rather limited.

The CIPC treatment also efficiently induced micronucleation in the other 5 *Lilium* genotypes, and mean numbers of nuclei per meiocyte ranging from 5.4 to 11.7

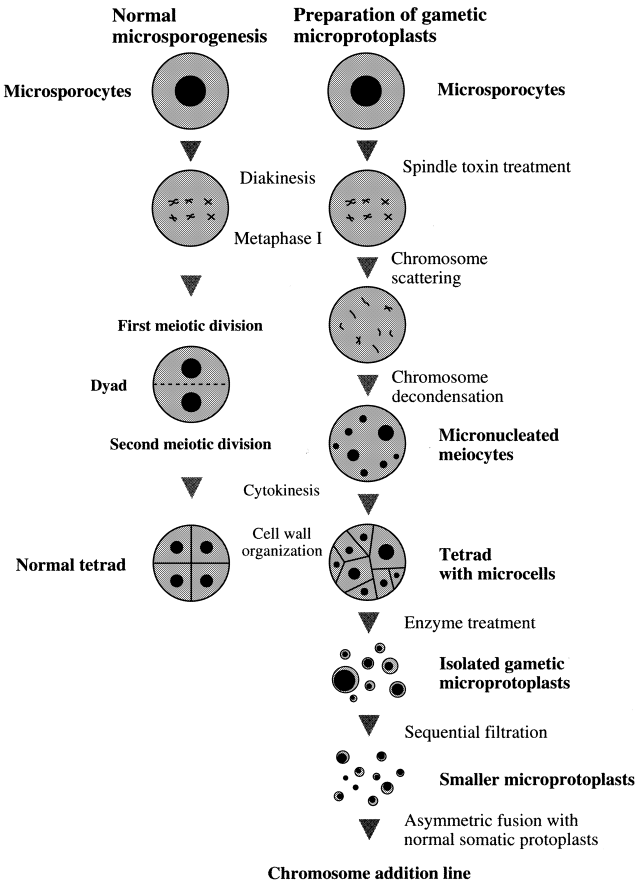

Fig. 3. Scheme for the preparation of gametic microprotoplasts from developing microspores in higher plants

For inducing micronuclei, microsporocytes at the diakinesis to metaphase I are treated with a spindle toxin. Micronucleated meiocytes containing several nuclei with different sizes formed microcells during spindle toxin treatment via cytokinesis as in the case of normal tetrad formation. Gametic microprotoplasts are isolated from microcell-formed meiocytes by enzyme treatment and enriched by sequential filtration using nylon sieves with decreasing pore sizes.

and maximum numbers of nuclei per meiocyte ranging from 9 to 20 were obtained depending on the genotype. Among the genotypes examined, the Aurelian hybrid lily 'Golden Splendor' gave the highest (11.7) mean number of nuclei per meiocyte.

3. Isolation of gametic microprotoplasts of *L. longiflorum* 'Hinomoto'

Four to 5 days after the initiation of the CIPC treatment of anthers, micronucleated meiocytes formed microcells via cytokinesis (Fig. 4D) as in the case of normal tetrad formation. Anthers containing micronucleated meiocytes at the middle to late tetrad stages (Fig. 4E) were transversely cut into several sections in order to extrude the meiocytes into a cell wall-digesting enzyme solution which consisted of 1% Cellulase Onozuka RS, 1% Macerozyme R10, 5 mM MES and 0.6 M sorbitol. After being subjected to the enzyme treatment at 25°C in the dark for 2 h, protoplast suspensions were filtered through a nylon sieve (pore size 50 µm) and the protoplasts were washed twice with a 0.5 M sorbitol solution. Meiocyte-derived gametic (micro)protoplasts less than 10, 10–20 and 20–50 µm in diameter were obtained with yields of 5.5×10^4, 6.6×10^4 and 4.9×10^4 per anther, respectively, and thus more than 70% of the (micro)protoplasts were less than 20 µm in diameter.

4. Enrichment and characterization of gametic microprotoplasts of *L. longiflorum* 'Hinomoto'

In order to enrich smaller gametic microprotoplasts, sequential filtration using nylon sieves with decreasing pore sizes (50, 20 and 10 µm) was carried out as in the case of the somatic microprotoplasts described above. Smaller microprotoplasts (Fig. 4F–H) with DNA contents below the 2C level, as indicated by flow cytometry analysis, were predominantly obtained by sequential filtration. Each of these gametic microprotoplasts had a micronucleus surrounded by a thick rim of cytoplasm (Fig. 4G), and some of them also contained vacuole(s) (Fig. 4H). The size of the gametic microprotoplasts appeared to depend upon that of the micronucleus. Flow cytometry analysis indicated that the majority of the gametic microprotoplasts obtained after sequential filtration had a micronucleus with DNA contents equivalent to one or a few chromosomes.

Conclusion

We have established efficient systems for preparing somatic microprotoplasts from cell suspension cultures of *Hemerocallis hybrida* 'Stella d'Oro' and gametic microprotoplasts from developing microspores of *Lilium longiflorum* 'Hinomoto'. Compared with the somatic microprotoplast system, the gametic system appeared to be more practical in Liliaceous ornamental plants. Microsporocytes have several advantages over cell suspension cultures as a starting material for microprotoplast preparation: no requirement for time- and labor-consuming processes for the establishment and maintenance of suspension cultures, no requirement for additional synchronization treatments of cell division, no requirement for ultra-centrifugation for isolating microprotoplasts, and a higher efficiency on the induction of micronucleation. Although the system for gametic microprotoplasts is applicable only to the restricted stage of flower development, developing microspores can be obtained by regulating the flowering time.

The systems described here may pave the way for the transfer of one or a few chromosomes via microprotoplast fusion from *Hemerocallis hybrida* 'Stella d'Oro' or *Lilium longiflorum* 'Hinomoto' to other Liliaceous ornamental plants, for example, *Lilium* × *formolongi*[3,7], *Agapanthus praecox* (unpublished) and *Muscari armeniacum* (unpublished), in which protoplast-to-plant systems have so far been established. Chromosome addition lines produced via microprotoplast fusion may contribute to genetic improvement as well as chromosome studies in Liliaceous ornamental plants.

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