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ORIGINAL ARTICLE

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Isolation and characterization of gametic microprotoplasts from developing microspores of *Lilium longiflorum* for partial genome transfer in the Liliaceous ornamentals

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Abstract We have established a system for isolating gametic microprotoplasts from developing microspores of *Lilium longiflorum* 'Hinomoto' ($2n = 2\times = 24$) as a first step toward the production of intergeneric hybrid plants with one or a few alien chromosomes via microprotoplast fusion in the Liliaceous ornamentals. Anthers of this cultivar containing microsporocytes at mainly diakinesis to metaphase I were cultured for 3–4 days in a medium containing half-strength MS salts, double-strength MS vitamins, 1 g l^{-1} casamino acids, 100 g l^{-1} sucrose, and one of four spindle toxins: amiprophos-methyl, isopropyl *N*-(3-chlorophenyl)carbamate (CIPC), colchicine or propyzamide. Of the four spindle toxins examined, CIPC at concentrations of 5 or 10 μM efficiently induced micronucleation with the mean number of nuclei per meiocyte being 7.5. CIPC treatment also efficiently induced micronucleation in the other five *Lilium* genotypes evaluated (*L. regale*, *L. longiflorum* 'Georgia', *L. speciosum* 'Uchida', the Asiatic hybrid lily 'Connecticut King' and the Aurelian hybrid lily 'Golden Splendor') with the mean number of nuclei per meiocyte falling within the range 5.4–11.7. In 'Hinomoto', each meiocyte nucleus formed a microcell 4–5 days after initiation of CIPC treatment. Following isolation of such meiocytes from the anthers and their incubation in a cell-wall-digesting enzyme solution, gametic (micro)protoplasts of less than 10 μm, 10–20 μm 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. Smaller microprotoplasts with DNA content below the 2C level, as indicated by flow cytometric analysis, were enriched by sequential filtration through nylon sieves of decreasing pore size (50, 20 and 10 μm). The relative fluorescence intensity in some of their nuclei corresponded to that of one or only a few chromosomes.

Keywords Gametic microprotoplast · Micronucleus · Microsporocyte · Microcell · Spindle toxin

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Introduction

Since microprotoplasts have a micronucleus with only one or only a few intact chromosomes (Verhoeven and Ramulu 1991; Ramulu et al. 1993), the microprotoplast fusion technique, i.e., cell fusion between microprotoplasts and somatic cell protoplasts, is an attractive means of achieving partial genome transfer in higher plants (Ramulu et al. 1995, 1999). To date, asymmetric hybrid plants with alien chromosome(s) (chromosome addition lines) have been produced utilizing this technique in Solanaceous species (Ramulu et al. 1995, 1996a, b; Rutgers et al. 1997), and in the genus *Helianthus* (Binsfeld et al. 2000).

In the Liliaceous ornamentals, we aim to produce intergeneric hybrid plants via microprotoplast fusion with one or a few alien chromosomes for genetic improvement and chromosome studies. In order to apply this technique, it is first necessary to establish systems for mass-preparation of micronucleated cells followed by mass-isolation of microprotoplasts. Microprotoplasts in higher plants have generally been prepared from fast-growing cell suspension cultures by sequential treatment of the cultures initially with a DNA synthesis inhibitor to synchronize cell division, followed by a spindle toxin for micronucleation, enzyme treatment of micronucleated cells for isolating protoplasts, and subsequent ultracentrifugation of the isolated protoplasts (Ramulu et al. 1995, 1999; Saito and Nakano 2002). Recently, an alternative approach, in which microprotoplasts are isolated from developing microspores, was demonstrated for *Trillium kamtschaticum* (Ito 1990) and *Solanum tuberosum* (Matthews et al. 1999). Since meiotic cycles in higher

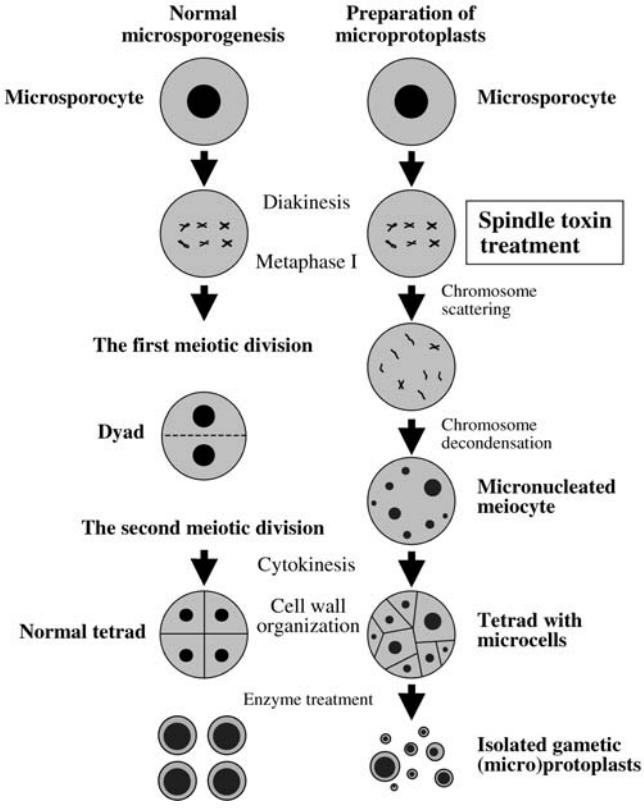

Fig. 1 A scheme for the isolation of gametic microprotoplasts in higher plants. For inducing micronuclei, microsporocytes at diakinesis to metaphase I are treated with a spindle toxin in vitro. Micronucleated meiocytes containing several nuclei of different sizes formed microcells during spindle toxin treatment via cytokinesis as in the formation of the normal tetrads. Individual gametic microprotoplasts are isolated from microcell-formed meiocytes via enzyme treatment and enriched by sequential filtration through nylon sieves of decreasing pore size. Gametic microprotoplasts are then fused with somatic cell protoplasts in order to produce chromosome addition lines

plants are generally synchronous (Ito and Stern 1967; McCormick 1993), this approach needs no additional synchronization treatments. Since each nucleus in the micronucleated meiocytes may form a microcell at the tetrad stage, gametic microprotoplasts can be directly obtained by treating such meiocytes with a cell-wall-digesting enzyme solution, omitting the ultracentrifugation process (Fig. 1).

In the present study, we describe a system for inducing micronucleation of microsporocytes in several *Lilium* genotypes. A system for isolating gametic microprotoplasts from the micronucleated meiocytes was also established for *Lilium longiflorum* 'Hinomoto', and the isolated microprotoplasts were characterized cytologically as well as by flow cytometry.

Materials and methods

Plant materials

Six *Lilium* genotypes ($2n$ =2× =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 in the present study. Bulbs were incubated at 4°C for 3 months prior to planting in plastic pots (20 cm diameter). They were then cultivated under natural day length in a greenhouse in which the temperature was maintained at around 20°C at night and 30°C during the day.

Spindle toxins

Amiprophos-methyl [APM, *O*-methyl-*O*-(4-methyl-6-nitrophenyl)-*N*-isopropyl-phosphorothioamidate] was purchased from Hayashi Pure Chemical Industries, Osaka, Japan; isopropyl *N*-(3-chlorophenyl)carbamate (CIPC) from Sigma-Aldrich, Steinheim, Germany; colchicine (COL) from Kanto Chemical, Tokyo, Japan; and propyzamide [PRO, 3,5-dichloro-*N*-(1,1-dimethylpropyl)benzamide] from Wako Pure Chemical Industries, Osaka, Japan. All stock solutions of APM (0.96 mM), CIPC (1.1 mM), COL (1.9 mM) and PRO (0.6 mM) were prepared in water-free dimethylsulfoxide and were stored at –20°C before use.

Micronucleation

In *Lilium* genotypes, each flower generally has six anthers. Flower buds containing anthers with microsporocytes at diakinesis to metaphase I of meiosis (Fig. 2A) were harvested from greenhouse-grown plants. The stage of microsporocyte development was estimated according to the bud length for each *Lilium* genotype: ca. 22.4 mm for *L. regale*, ca. 23.7 mm for *L. longiflorum* 'Georgia' and 'Hinomoto', ca. 16.2 mm for the Asiatic hybrid lily 'Connecticut King', and ca. 23.6 mm for the Aurelian hybrid lily 'Golden Splendor'. The flower buds were surface-disinfected by immersing in 70% ethanol for 20 s and then in 1% sodium hypochlorite for 20 min. After washing three times with sterilized water, anthers were isolated by dissecting flower buds and transferred to micronucleation medium containing half-strength MS (Murashige and Skoog 1962) salts, double-strength MS vitamins, 1 g l^{-1} casamino acids, 100 g l^{-1} sucrose and one of the spindle toxins at different concentrations (Table 1). All media were adjusted to pH 5.8 before autoclaving, and spindle toxins were added individually to the autoclaved medium. Erlenmeyer flasks (50 ml) were filled with 10 ml medium and three anthers from different buds were cultured per flask at 25°C in the dark on a rotary shaker (100 rpm).

Isolation of gametic protoplasts and microprotoplasts

The effect of enzyme solution composition on the yield of gametic protoplasts was initially examined using *L. longiflorum* 'Hinomoto'. Anthers containing tetrads at the middle tetrad stage were isolated from surface-disinfected flower buds and transversely cut into several sections for extrusion of the tetrads into the cell-wall-digesting enzyme solution (5 ml per anther). The enzyme solutions consisted of 5 mM 2-morpholinoethanesulfonic acid, 0.6 M sorbitol and 1% Cellulase Onozuka RS or Cellulase Onozuka R10, with or without 1% Macerozyme R10. All enzymes were purchased from Yakult Honsha, Tokyo, Japan. After 2 h of enzyme treatment of the tetrads at 25°C in the dark, the mixture was passed through a nylon sieve (pore size 50 μm), and protoplasts were freed from debris by flotation on a 0.7 M sucrose solution coupled with centrifugation (800 rpm for 3 min). The protoplasts were washed twice with a 0.5 M sorbitol solution by re-suspension and centrifugation (800 rpm for 3 min). The number

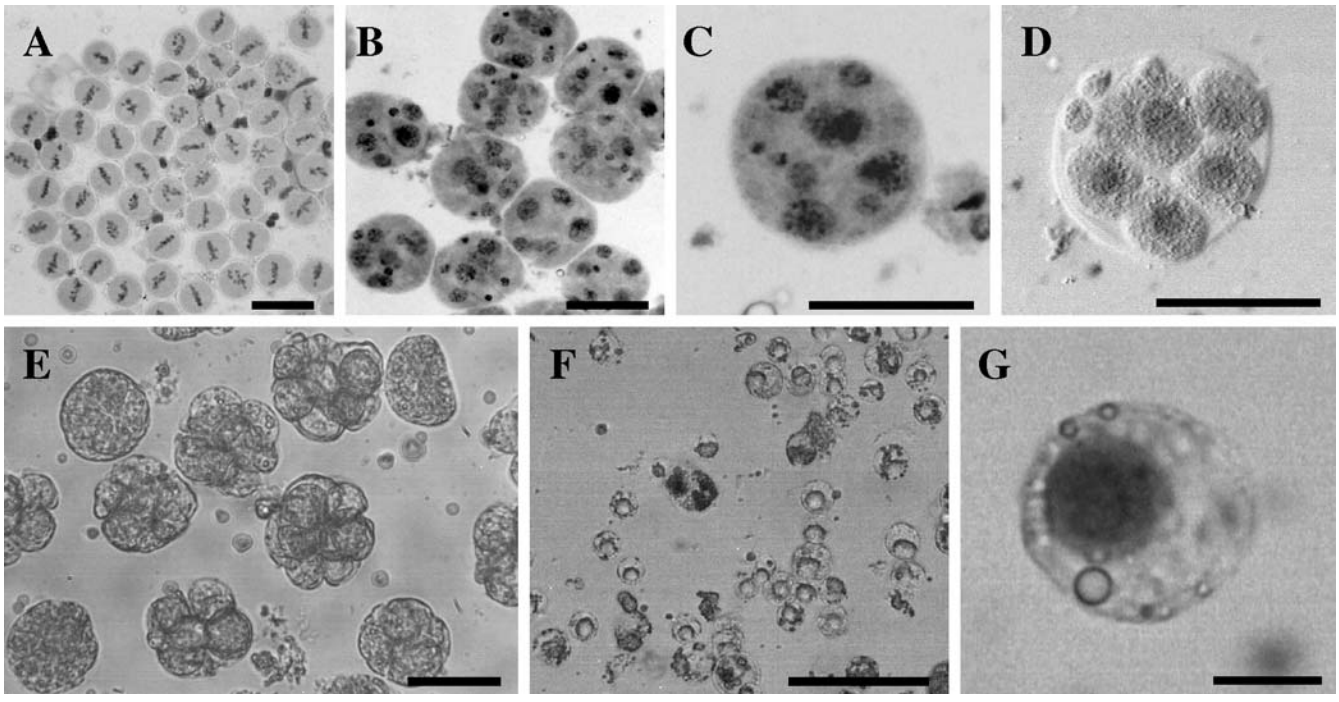

Fig. 2A–G Induction of micronucleation and isolation of gametic microprotoplasts in developing microspores of *Lilium longiflorum* 'Hinomoto'. **A** Microsporocytes at metaphase I. **B** Micronucleated meiocytes. **C** A meiocyte with several micronuclei. **D** A meiocyte with several microcells at the early tetrad stage. **E** Meiocytes with several microcells at middle to late tetrad stage. **F** Microprotoplasts purified through sequential triple filtration. **G** A microprotoplast with a micronucleus surrounded by a thick rim of cytoplasm. **D** and **F** were imaged by using differential interference contrast in transmitted light. *Bars* in **A**, **B–F**, and **G** are 100, 50 and 10 μm, respectively

Table 1 Effect of spindle toxin on the micronucleation of meiocytes of *Lilium longiflorum* 'Hinomoto'. *CIPC N*-(3-chlorophenyl)carbamate, *PRO* propyzamide, *APM* amiprophos-methyl, *COL* colchicine

Spindle toxin	Concentration (μM)	Mean no. of nuclei per meiocyte	Maximum no. of nuclei per meiocyte
CIPC	1	4.2±0.2[a]	10
	5	7.5±0.4	17
	10	7.5±0.5	20
PRO	1	3.9±0.1	11
	5	5.7±0.8	15
	10	4.2±0.2	9
APM	1	4.9±0.2	9
	5	–[b]	
	10	–	
COL	60	4.8±0.1	9
	120	–	
	240	–	

[a] Mean ± SD
[b] Few micronucleated meiocytes were obtained

of purified protoplasts per anther was determined using a hemocytometer (Kayagaki Irikakogyo, Tokyo, Japan).

For isolating gametic microprotoplasts, meiocytes extruded from the anthers 4–5 days after the initiation of spindle toxin treatment were incubated in the enzyme solution and purified as described above for the tetrads. The yields of microprotoplasts and/or protoplasts (<10 μm, 10–20 μm and 20–50 μm in diameter) obtained per anther were calculated using a hemocytometer. The gametic (micro)protoplasts were then sequentially filtered using nylon sieves of decreasing pore size (50, 20 and 10 μm).

Cytology

After 3–4 days of spindle toxin treatment, meiocytes before the tetrad stage were stained with 1% aceto-orcein for 1 min followed by gentle squashing under a glass coverslip. To determine the number of nuclei per meiocyte, at least 200 cells per anther were observed under a light microscope (Leica DMLB, Leica, Wetzlar, Germany). For gametic microprotoplasts, samples obtained after sequential filtration were stained with 1% aceto-orcein and dropped under a glass coverslip bridge prior to observation under a light microscope.

Table 2 Effect of CIPC treatment on the micronucleation of meiocytes in five *Lilium* genotypes

Genotype	CIPC concentration (μM)	Mean no. of nuclei per meiocyte	Maximum no. of nuclei per meiocyte
L. regale	5	5.4±0.1[a]	11
	10	5.2±0.2	10
L. longiflorum 'Georgia'	5	4.7±0.3	10
	10	6.2±0.1	16
L. speciosum 'Uchida'	5	8.2±1.0	17
	10	7.8±0.2	15
Asiatic hybrid lily 'Connecticut King'	5	5.6±0.1	9
	10	5.3±0.4	11
Aurelian hybrid lily 'Golden Splendor'	5	11.6±0.7	18
	10	11.7±0.3	18

[a] Mean ± SD

Flow cytometric analysis

The relative nuclear DNA content of gametic microprotoplasts was determined using a flow cytometer (PA; Partec, Münster, Germany). After sequential filtration, samples were stained for 10 min with Solution B (Partec kit) containing 4,6-diamidino-2-phenylindole prior to flow cytometric analyses. As a control, nuclei of leaf cells of mother plants ($2n$ =24 chromosomes) were also analyzed after chopping leaf tissues in 0.5 ml Solution A (Partec kit) for isolating nuclei followed by staining for 10 min with 2 ml Solution B.

Results

Micronucleation

Data on the effect of spindle toxins on the micronucleation of microsporocytes of *L. longiflorum* 'Hinomoto' are shown in Table 1. Meiocytes with a mean number of nuclei of greater than four (Fig. 2B, C) were induced by micronucleation medium supplemented with 1, 5 or 10 μM CIPC, 5 or 10 μM PRO, 1 μM APM, or 60 μM COL. On the other hand, 5 or 10 μM PRO and 120 or 240 μM COL induced only a few micronucleated meiocytes (<10%), and mononucleated meiocytes were predominantly obtained in these treatments. Of the spindle toxins examined, CIPC at concentrations of 5 and 10 μM efficiently induced micronucleation with a mean number of nuclei per meiocyte of 7.5 each and with maximum nucleus numbers of 17 and 20, respectively. Figure 3 shows the frequency distribution of the number of nuclei per meiocyte after treatment with 10 μM CIPC. The number of nuclei ranged from 1 to 20, and about 90% of the treated microsporocytes formed more than 4 nuclei; most meiocytes had 7 or 8 nuclei.

The treatments with 5 or 10 μM CIPC also efficiently induced micronucleation in the other five *Lilium* genotypes (Table 2). The mean number of nuclei per meiocyte was 5.4–11.7, and the maximum number of nuclei per meiocyte was between 9 and 18, depending on the genotype and the concentration of CIPC. At both CIPC concentrations, the Aurelian hybrid lily 'Golden Splendor' gave the highest mean number of nuclei per meiocyte of the genotypes examined, with means of 11.6 and 11.7 for 5 and 10 μM CIPC, respectively.

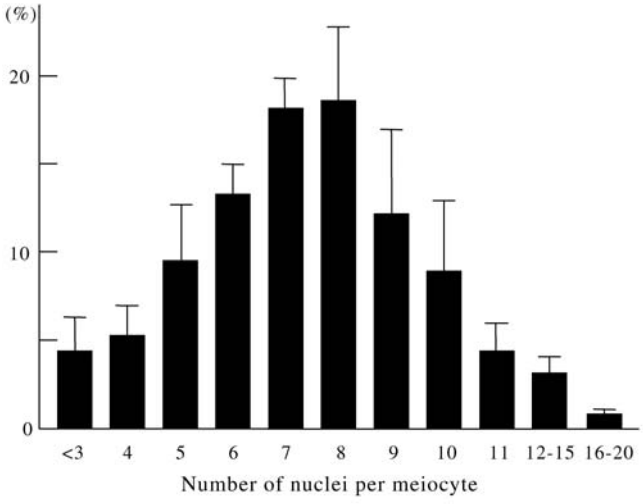

Fig. 3 Frequency distribution of the number of nuclei per meiocyte of *L. longiflorum* 'Hinomoto' treated with 10 μM *N*-(3-chlorophenyl)carbamate (CIPC). *Vertical bars* SE of the mean of five experiments

Isolation of gametic protoplasts and microprotoplasts

Gametic protoplasts could be released from the middle stage tetrads of *L. longiflorum* 'Hinomoto' by a 2-h treatment with all four enzyme solutions, but protoplast yield varied according to the composition of the enzyme solution (Table 3). A combination of 1% Cellulase Onozuka RS and 1% Macerozyme R10 gave the best result, with a yield of 9.5×10^4 gametic protoplasts per anther. In subsequent experiments, therefore, this enzyme solution was used for isolating microprotoplasts from meiocytes. Isolated gametic protoplasts were ca. 30 μm in diameter (data not shown).

Because of the asynchronous progression of microcell formation in the CIPC-treated meiocytes, those of two different developmental stages (early-to-middle tetrad stage and middle-to-late tetrad stage) were initially subjected to enzyme treatment. As a result, different stages of tetrads were found to affect the yield of gametic microprotoplasts. Meiocytes at the early-to-middle tetrad

Table 3 Effect of enzyme solution composition on the yield of gametic protoplasts per anther from tetrads of *L. longiflorum* 'Hinomoto'

Cellulase (1%)		Macerozyme (1%)	Yield ($\times 10^4$)
Cellulase Onozuka RS			6.0±0.7[a]
Cellulase Onozuka RS	+	Macerozyme R10	9.5±0.6
Cellulase Onozuka R10			1.2±0.1
Cellulase Onozuka R10	+	Macerozyme R10	5.4±0.5

[a] Mean ± SD

Table 4 The yield of (micro)protoplasts per anther isolated from CIPC-treated meiocytes of *L. longiflorum* 'Hinomoto'

	(Micro)protoplast size (μm)			Total
	<10	10–20	20–50	
Yield ($\times 10^4$)[a]	5.5±1.9	6.6±1.9	4.9±0.7	17.0±1.2
Percentage (%)	32.4	38.8	28.8	100.0

[a] Mean ± SD

stages (Fig. 2D) frequently burst during incubation in the enzyme solution, resulting in poor yields of gametic microprotoplasts. Therefore, meiocytes at the middle-to-late tetrad stage (Fig. 2E) obtained 4–5 days after initiation of CIPC treatment were subjected to enzyme treatment. Table 4 shows the yield of gametic (micro) protoplasts of different sizes per anther from meiocytes of *L. longiflorum* 'Hinomoto' treated with 10 μM CIPC. Gametic (micro)protoplasts of below 10 μm, 10–20 μm and 20–50 μm in diameter were obtained with yields of 5.5×10^4, 6.6×10^4 and 4.9×10^4 cells per anther, respectively; thus over 70% of (micro)protoplasts obtained were below 20 μm in diameter. Smaller microprotoplasts were enriched by sequential filtration through three nylon sieves of decreasing pore size (50, 20 and 10 μm) (Fig. 2F). Each of these gametic microprotoplasts had a micronucleus surrounded by a thick rim of cytoplasm (Fig. 2G), and some also had vacuole(s) (data not shown). The size of gametic microprotoplasts appeared to depend upon the size of their micronucleus.

The relative nuclear DNA content of gametic (micro) protoplasts obtained via sequential filtration was analyzed by flow cytometry. Figure 4 shows histograms of the relative fluorescence intensity of nuclei obtained from leaf tissues of *L. longiflorum* 'Hinomoto' ($2n = 2\times = 24$) as a control (Fig. 4A), and the populations obtained after single (using a sieve of pore size 50 μm) (Fig. 4B), double (50 and 20 μm) (Fig. 4C) and triple filtration (50, 20 and 10 μm) (Fig. 4D) of gametic (micro)protoplasts. The histogram from the control leaf tissues had a single peak corresponding to 2C nuclei (Fig. 4A). After single filtration, nuclei with DNA contents ranging from below the 2C level to the 4C level were observed (Fig. 4B). Almost all of the 4C nuclei were eliminated by double filtration (Fig. 4C). After triple filtration, almost all the 2C nuclei were further eliminated, and thus smaller gametic microprotoplasts with DNA contents below the 2C level were enriched (Fig. 4D). Most of the gametic microprotoplasts thus obtained had micronuclei with DNA contents equivalent to one or a few chromosomes.

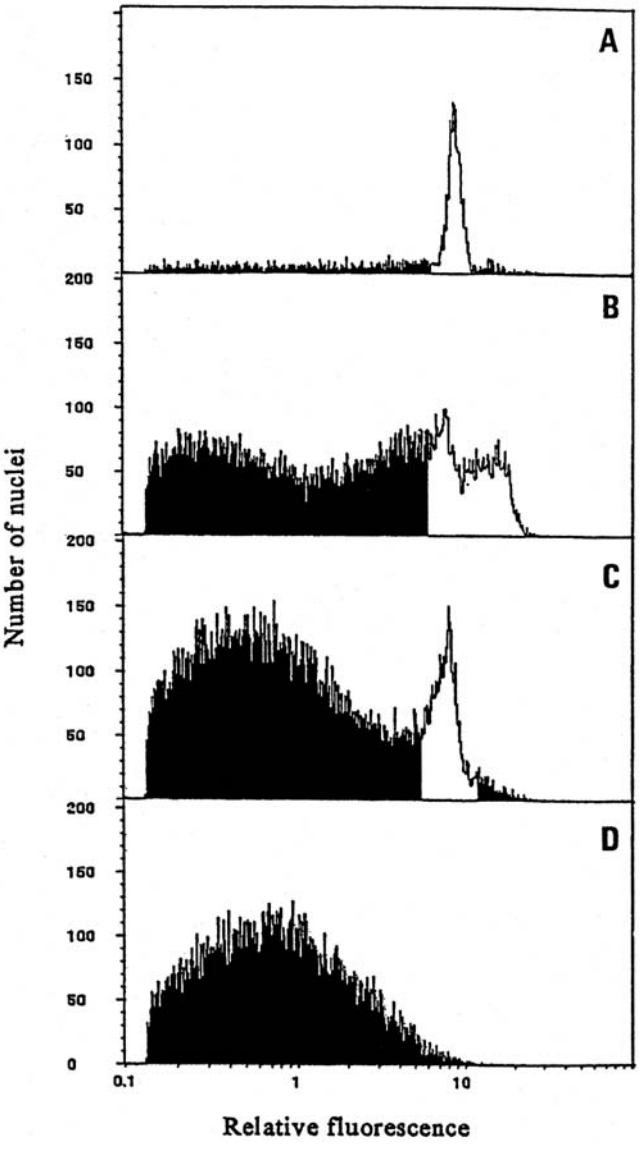

Fig. 4A–D Histograms from flow cytometric analyses of nuclear DNA contents of leaf tissues and gametic (micro)protoplasts of *L. longiflorum* 'Hinomoto'. **A** Leaf cells with 24 chromosomes. **B** A population obtained after single filtration using sieves of 50-μm pore size. **C** A population after double filtration using sieves of 50- and 20-μm pore size. **D** A population after triple filtration using sieves of 50-, 20- and 10-μm pore size

Discussion

In the present study, micronucleated meiocytes of *L. longiflorum* 'Hinomoto' were efficiently obtained by

treating the anthers containing microsporocytes with 5 or 10 μM CIPC for 3–4 days. With this treatment, the mean number of nuclei per meiocyte was 7.5 and the maximum number of nuclei per meiocyte was between 17 and 20. Such CIPC treatments also efficiently induced micronucleation in the other five *Lilium* genotypes. In 'Hinomoto', gametic microprotoplasts of less than 10 μm and 10–20 μm in diameter were successfully isolated from micronucleated meiocytes with yields of 5.5×10^4 and 6.6×10^4 per anther, respectively, 4–5 days after initiation of CIPC treatment.

Since almost all of the microsporocytes progressed synchronously through the meiotic cycle (Fig. 2A), efficient micronucleation could be achieved by treating only the anthers containing microsporocytes with a spindle toxin; thus no synchronization treatment was needed for any *Lilium* genotype examined. In addition, both the frequency of micronucleated cells and the number of micronuclei per cell obtained here by CIPC treatment of microsporocytes were much higher than those obtained in our previous study in which cell suspension cultures of the Liliaceous plant *Hemerocallis hybrida* were sequentially treated with DNA synthesis inhibitor and spindle toxin (Saito and Nakano 2001). Preparation of micronucleated cells from developing microspores, therefore, may be more practical than that from suspension cultures in the Liliaceous plants. Applicability of the system developed in the present study should be examined for other Liliaceous ornamentals.

In previous studies, the spindle toxin APM, a phosphoric amide herbicide, has mainly been used for inducing micronucleation in microsporocytes of *S. tuberosum* (Matthews et al. 1999), and in suspension-cultured cells of several Solanaceous species (Ramulu et al. 1990, 1993) and *Helianthus giganteus* (Binsfeld et al. 2000). In suspension-cultured cells of the Liliaceous ornamental plant *Hemerocallis hybrida*, micronucleation was efficiently induced by another spindle toxin, PRO, which belongs to the benzamide herbicides (Saito and Nakano 2001). However, in the present study, CIPC – a carbamate herbicide – gave the best result for inducing micronucleation in microsporocytes of *L. longiflorum* 'Hinomoto' of the four spindle toxins examined including APM and PRO. These differences may be due to some physical and/or physiological differences in the cells used for inducing micronucleation.

In the present study, micronucleated meiocytes containing several nuclei with different sizes formed microcells during CIPC treatment via cytokinesis as in the formation of normal tetrads. A similar observation has previously been reported for *S. tuberosum* (Matthews et al. 1999). Thus, gametic microprotoplasts could be directly obtained from the microcell-formed meiocytes via enzyme treatment, unlike somatic microprotoplasts, which can be obtained only after ultracentrifugation of enzymatically-isolated, micronucleated protoplasts. Isolation of microprotoplasts from developing microspores, therefore, may be more simple and practical than that from cell suspension cultures.

The enzyme solution containing both Cellulase Onozuka RS and Macerozyme R10, which had initially been established for efficient isolation of gametic protoplasts from normal tetrads, was successfully applied to the isolation of gametic microprotoplasts from microcell-formed meiocytes in *L. longiflorum* 'Hinomoto'. However, some of the microcell-formed meiocytes at the middle-to-late tetrad stages, which were used for isolating microprotoplasts, remained partly undigested after enzyme treatment. This might be due to the deposition of sporopollenin, which is known to be resistant to enzymatic degradation, at the later stages of tetrads (Bedinger 1992; Nepi and Franchi 2000). A detailed examination is needed to clarify the effects of the developmental stage of tetrads on the isolation of gametic microprotoplasts.

As in the cases of cultured cell-derived somatic microprotoplasts of *Nicotiana plumbaginifolia* (Ramulu et al. 1993, 1999), *Helianthus giganteus* (Binsfeld et al. 2000) and *Hemerocallis hybrida* (Saito and Nakano 2002), smaller gametic microprotoplasts of *L. longiflorum* 'Hinomoto' could be purified successfully from a miscellaneous population by sequential filtration through nylon sieves of decreasing pore size. Flow cytometric analyses indicated that gametic microprotoplasts with DNA contents equivalent to those of small numbers (ca. 1–5) of chromosomes were enriched after sequential filtration. Although cultured cell-derived somatic microprotoplasts have generally been reported to have a thin layer of cytoplasm around a micronucleus (Ramulu et al. 1993, 1999; Binsfeld et al. 2000; Saito and Nakano 2002), gametic protoplasts obtained in the present study had a relatively thick rim of cytoplasm around a micronucleus and, in addition, some of them also had vacuole(s). These differences may have resulted from different isolation processes of somatic and gametic microprotoplasts: somatic microprotoplasts are extruded from micronucleated somatic protoplasts during ultra-centrifugation, while gametic microprotoplasts are released directly from microcell-formed meiocytes via enzyme treatment. Ultrastructural characterization of gametic as well as somatic microprotoplasts is now in progress using transmission electron microscopy.

In the present study, we have established a system for obtaining gametic microprotoplasts for the Liliaceous ornamental *L. longiflorum* 'Hinomoto'. This preparation system may be applicable to other *Lilium* genotypes because frequent micronucleation was observed in all genotypes examined. As compared with the somatic microprotoplast system developed in our previous studies (Saito and Nakano 2001, 2002), the gametic system established here seems to be more practical in the Liliaceous ornamental plants. Microsporocytes have several advantages over cell suspension cultures as a starting material for microprotoplast preparation: (1) no need for time- and labor-consuming processes for the establishment and maintenance of suspension cultures, (2) no need for additional synchronization treatments of cell division, (3) no need for ultra-centrifugation to isolate microprotoplasts, and (4) a higher efficiency of micronu-

cleation. Although our system is applicable only within a restricted stage of flower development, developing microspores can be obtained by regulating flowering time. This system for gametic microprotoplasts, together with our previously-reported system for somatic microprotoplasts, may offer the opportunity of producing intergeneric chromosome addition lines via microprotoplast fusion in the Liliaceous ornamentals.

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