N₂O induces mitotic polyploidization in anther somatic cells and restores fertility in sterile interspecific hybrid lilies

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Fertile plants undergoing male gametogenesis can be treated with nitrous oxide (N₂O) gas to obtain 2n male gametes. N₂O treatment is also expected to restore the fertility of interspecific hybrids through meiotic restitution or mitotic amphidiploidization. However, this technique has few applications to date, and it is unknown how N₂O treatment restores fertility in sterile hybrids. To establish optimal N₂O treatment conditions and determine its cytological mechanism of action, we treated various sized floral buds with N₂O gas at different anther developmental stages from fertile and sterile hybrid lilies. N₂O treatment using the optimal 1–4 mm floral buds induced mitotic polyploidization of male archesporial cells to produce 2n pollen in fertile hybrid lilies. In sterile hybrid lilies, N₂O treatment doubled the chromosome number in male archesporial cells followed by homologous chromosome pairing and normal meiosis in pollen mother cells (PMC), resulting in restoration of pollen fertility. Backcrossing the resultant fertile pollen to Lilium × formolongi produced many triploid BC₁ plants. Thus N₂O treatment at the archesporial cell proliferating stage effectively overcame pollen sterility in hybrid lilies, resulting in fertile, 2n pollen grains that could produce progeny. The procedure presented here will promote interspecific or interploidy hybridization of lilies.

Key Words: chromosome doubling, diploid male gamete, hybrid sterility, interspecific hybridization, Lilium, nitrous oxide, polyploid.

Introduction

Interspecific hybridization is useful for increasing genetic diversity. In ornamental flower breeding, interspecific hybridization is an essential technique that produces novel cultivars, as driven by consumer demand. Many such cultivars are used as ornamental crops (Fukai and Tsuji 2004, Okazaki et al. 1992, 1994, 1995, Saruwatri et al. 2008), whereas hybrids obtained from remote interspecific crosses are mostly sterile and cannot be used in cross breeding (Choudhary et al. 2000, Gangadevi et al. 1985, Pysa 1990). The traditional method of overcoming hybrid sterility is to double chromosome number using polyploidizing agents such as colchicine to produce allopolyploids that may be fertile (Asano 1982, Nimura et al. 2006).

Nitrous oxide (N₂O) has been applied to zygotes and seedlings in many crops as a polyploidizing agent in lieu of colchicine treatment (Berdahl and Barker 1991, Dvorak et al. 1973, Kato 2002, Kato and Birchler 2006, Nygren 1955, Òstergren 1954, 1957, Taylor et al. 1976, Zeilinga and Schouten 1968). Meiotic metaphase stage anthers of male-fertile cultivars in tulips and lilies are optimal for N₂O treatment to produce fertile 2n pollen grains (Akutsu et al. 2007, Okazaki et al. 2001, 2005). Compared to other chemicals that mostly induce mitotic polyploidization by arresting cell division, N₂O is not damaging and leads to first division restitution (FDR) or second division restitution (SDR) in meiosis in male-fertile plants, producing 2n gametes that are functional and available for crossing. Some polyploid plants have been produced using 2n gametes obtained by N₂O treatment (Akutsu et al. 2007, Okazaki et al. 2005).

Lilies (Lilium spp., 2n = 2x = 24) are one of the most important horticultural crops, with about 100 species...
widespread in the northern hemisphere (Nishikawa et al. 1999). The most important modern commercial lilies mainly belong to the Asiatic, Oriental, and Longiflorum hybrid groups, and over 10,000 cultivars, including many sterile interspecific hybrids, are registered (Matthews 2007). Barba-Gonzalez et al. (2006) obtained fertile 2n pollen from sterile lily hybrids by using N_2O treatment. Postulating that N_2O treatment could bring about meiotic restitution in sterile as well as fertile lily cultivars, they concluded from cytological analyses of progeny that N_2O had induced FDR gametes in most cases. However, the particular FDR mechanism by which hybrid sterility can be overcome does not explain the finding of Kitamura et al. (2009). In their histological study on pollen meiosis in fertile plants treated with N_2O gas for 24 h, microtubules were effectively depolymerized, which prevented chromosomes from moving to the poles and resulted in chromosome retention in the center of N_2O-treated cells. Cell plate formation took place without delay, however, yielding one daughter cell with a diploid genome and another daughter cell without chromosomes. As a result, a 2n male gamete was produced during meiotic metaphase. In sterile interspecific hybrids where chromosomes are scattered in the cytoplasm owing to non-homologous parental genomes, the cell plate divides the chromosomes unequally to make aneuploid daughter cells with or without N_2O treatment (Kitamura et al. 2009). This suggests that N_2O treatment during meiotic division does not lead to chromosome doubling in a daughter cell through the FDR mechanism in sterile hybrids. The cytological mechanism of action of N_2O treatment for overcoming hybrid sterility remains to be determined.

Therefore, this study investigated how N_2O gas leads to the development of fertile gametes in sterile interspecific hybrid lilies and determined the optimal PMC developmental stage for overcoming hybrid sterility. In addition, to verify whether the resulting fertile pollen could produce progeny, we attempted to produce BC_1 progeny using the fertile pollen obtained from completely sterile interspecific hybrids.

### Materials and Methods

#### Plant materials

The cultivars used in this study were collected from Yamaki Noen Co., Ltd. (Niigata, Japan). The cultivars used are listed in Table 1: four fertile cultivars belonging to *Lilium* spp. Asiatic hybrid lilies, a fertile Oriental hybrid lily, two LA hybrids, an OT hybrid, and an LO hybrid. Two cultivars of *L. × formolongi* were crossed with pollen from N_2O-treated hybrids. Single bulbs of each cultivar were planted in 10-cm pots in October or November and grown in an unheated greenhouse at Niigata University, Niigata, Japan.

**N_2O treatment**

N_2O treatment (modified from Akutsu et al. 2007) was performed at room temperature for 48 h in a pressure-tolerant cylinder, with N_2O gas applied at 6 atm without oxygen. Prophase I and meiotic metaphase I in Asiatic hybrid lilies occur in 15- or 20-mm floral buds, respectively (Akutsu et al. 2007). In *L. longiflorum*, premeiotic mitosis occurs in 10-mm floral buds (Taylor and McMaster 1954), and N_2O treatment during prophase I does not produce 2n pollen grains at flowering (Akutsu et al. 2007). Based on these results, we treated hybrid lilies with attached 1–10 mm floral buds, which presumably contained the archesporial cell proliferating stage. Buds were small at the time of treatment, making direct length measurements difficult. Hence, longitudinal lengths of detached floral buds from plants grown under similar conditions were measured with a caliper or ruler to estimate the floral bud sizes of treated plants at the time of N_2O treatment. The treated plants were grown in a greenhouse. N_2O treatments were conducted for Asiatic hybrid lilies, Oriental hybrid lilies and LA

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**Table 1.** Ploidy level, pollen fertility and origin of cultivars used in the study

| Cultivars         | Horticultural groups | Ploidy level | Chromosome number | Pollen fertility         | Origin                                                                 |
|-------------------|----------------------|--------------|-------------------|--------------------------|----------------------------------------------------------------------|
| Regata            | Asiatic hybrids      | 2×           | 24                | fertile                  | Intra-sectional hybrid (*Sinomartagon*)                                |
| Mona              | Asiatic hybrids      | 2×           | 24                | fertile                  | Intra-sectional hybrid (*Sinomartagon*)                                |
| Gran Paradiso     | Asiatic hybrids      | 4×           | 48                | fertile                  | Intra-sectional hybrid (*Sinomartagon*)                                |
| Loreto            | Asiatic hybrids      | 4×           | 48                | fertile                  | Intra-sectional hybrid (*Sinomartagon*)                                |
| Willeke Alberti   | Oriental hybrids     | 2×           | 24                | fertile                  | Intra-sectional hybrid (*Archeilirion*)                                |
| Serrada           | LA hybrids           | 3×           | 36                | almost sterile           | Inter-sectional hybrid; *L. longiflorum* × Asiatic hybrids (*Sinomartagon*) |
| Royal Trinity     | LA hybrids           | 3×           | 36                | low fertile              | Inter-sectional hybrid; *L. longiflorum* × Asiatic hybrids (*Sinomartagon*) |
| Yelloween         | OT hybrids           | 2×           | 24                | completely sterile       | Unknown                                                                |
| Otome no Sugata   | LO hybrids           | 2×           | 24                | completely sterile       | Inter-sectional hybrid; *L. × formolongi* × *L. rubellum* (*Archeilirion*) |
| Katsuki           | *L. × formolongi*     | 2×           | 24                | fertile                  | Intra-sectional hybrid (*Leucolirion*); *L. formosanum* × *L. longiflorum* |
| Original Ougo     | *L. × formolongi*     | 2×           | 24                | fertile                  | Intra-sectional hybrid (*Leucolirion*); *L. formosanum* × *L. longiflorum* |
hybrids in 2007, ‘Yelloween’ in 2007 and 2008 and ‘Otome no Sugata’ in 2008.

**Pollen assessments and statistical analysis**

At flowering time, a small amount of pollen was collected from six anthers of each bud, mixed, and then stained with 1% (w/v) aceto-orcein solution. Lengths of short axes of pollen grains (100–400) were microscopically measured using Image-Pro PLUS (Media Cybernetics Co., Ltd., Bethesda, MD, USA). In the control and N\(_2\)O-treated plants of ‘Yelloween’, microspore tetrad sizes were measured from young anthers of 30–35 mm floral buds. Pollen fertility was determined using the aceto-orcein method, whereby aceto-orcein-stained pollen was regarded as fertile pollen. Before pollination, the germinability of the pollen obtained by N\(_2\)O treatment was assessed after culture for 24 h at 25°C in agar medium containing 10% (w/v) sucrose, 0.7% (w/v) agar, 0.3 g/L calcium nitrate and 10 mg/L boric acid.

For data analysis, a non-parametric multiple comparison method (Steel-Dwass test) was performed using Microsoft Excel 2003 and statistical add-in software (Excel Toukei method (Steel-Dwass test) was performed using Microsoft Excel 2003 and statistical add-in software (Excel Toukei Co., Ltd., Tokyo, Japan).

**Crossing and hybrid embryo rescue**

For intraspecific hybridization of Asiatic hybrid lilies, control crosses were made as follows: 2x × 2x; 4x × 2x; and 4x × 4x. Tetraploid ‘Gran Paradiso’ was pollinated with pollen masses including putative 2n grains. Before flowering, flowers were emasculated in each cross. Capsules were harvested 2 months after pollination, and the seed number was counted for each cross.

For interspecific hybridization using fertile pollen restored from the sterile hybrid lilies, the capsules were collected about 40 days after pollination and embryo culture was performed as described by Okazaki *et al.* (1994).

**Histological observation**

PMC developmental stages of the Asiatic hybrid lily ‘Regata’ were observed. Floral buds of different sizes were collected and fixed for 24 h in Carnoy’s fluid, dehydrated in an alcohol gradient series, and embedded in paraffin. Paraffin-embedded samples were cut into 6-µm sections, stained with 0.05% (w/v) toluidine blue, mounted in balsam jelly, and observed by light microscopy (BX-60; Olympus, Tokyo, Japan).

**Determination of ploidy and hybridity**

Ploidy of seedlings and pollen grains was measured using flow cytometry as described previously (Akutsu *et al.* 2007, Okazaki *et al.* 2005). To verify the hybridity of the progeny from the cross between L. × formolongi and the N\(_2\)O-treated ‘Otome no Sugata’, genomic DNA was extracted from the leaves using the CTAB method (Murray and Thompson 1980), and the internal transcribed spacer (ITS) region of rDNA was amplified according to the methods of Nishikawa *et al.* (1999, 2001). The amplified fragments were digested by MspI and analyzed on polyacrylamide gels (Nagaoka *et al.* 2010).

**Genomic in situ hybridization (GISH)**

For mitotic metaphase chromosome analysis, the root tips were collected early in the morning. The root tips were incubated in 0.05% (w/v) colchicine solution for 2–3 h and then fixed in ethanol-acetic acid (3:1) solution for 24 h and stored at 4°C until use. The root tips were washed in distilled water and incubated in a pectolytic enzyme mixture containing 2% (w/v) cellulose Onozuka-RS (Yakult Pharmaceutical Industry Co. Ltd., Tokyo, Japan) and 1% (w/v) pectolyase Y-23 (Kanto Chemical Co., Inc.) in 10 mM citrate buffer (pH 4.5) at 37°C for 1–2 h. Squash preparations were made in a drop of 45% (w/v) acetic acid and frozen using liquid nitrogen. The cover slips were removed using a razor blade.

Genomic DNA of L. × formolongi was used as a probe and labeled with digoxigenin-11-dUTP by a standard nick translation protocol (Roche, Basel, Switzerland). GISH was performed as described by Marasek *et al.* (2006). The probe was detected with fluorescein-conjugated anti-digoxigenin. The preparations were analyzed with a microscope (BX60, Olympus) and photographed with a digital camera (DP70, Olympus). For each plant, the total number of chromosomes and the number of crossover points were determined.

**Results**

**Mitotic polyploidization of archesporial cells in fertile hybrid lilies**

Control pollen grain diameters ranged 51–71 µm (59 ± 4 µm, mean ± SD) in diploid Asiatic hybrid lily ‘Regata’ (Fig. 1A, 1B) and 50–84 µm (60 ± 6 µm) in diploid Oriental hybrid lily ‘Willeke Alberti’ (Fig. 1D, 1E). Plants with small (1–10 mm) floral buds treated with N\(_2\)O produced giant pollen in ‘Regata’ (Fig. 1C) and in ‘Willeke Alberti’ (Fig. 1F), whereas treated pollen shape was the same as the oval-shaped control pollen. For example, a 3-mm floral bud of ‘Regata’ and a 4-mm ‘Willeke Alberti’ bud treated with N\(_2\)O gas resulted in a pollen size distribution of 59–119 µm (79 ± 15 µm) and 53–110 µm (81 ± 12 µm), respectively (Fig. 1A, 1D).

Flow cytometric analysis of untreated pollen of ‘Regata’ revealed two peaks corresponding to 1C and 2C DNA content because the binucleate-type lily pollen contains one 2C generative and one 1C vegetative cell (Fig. 1G). In contrast, the N\(_2\)O-induced pollen gave rise to peaks at 1C, 2C and additional 4C levels, indicating both n and 2n pollen (Fig. 1H). These results indicated that N\(_2\)O treatment was able to induce chromosome doubling in male gametes. Based on the difference in pollen size distribution between the control and N\(_2\)O-induced pollen, we considered grains with a diameter of >75 or 85 µm diameter as putative 2n pollen grains in ‘Regata’ or ‘Willeke Alberti’, respectively.

To determine the most suitable floral bud stage for...
mitotic chromosome doubling of male archesporial cells, 1–
10 mm floral buds were treated with N\textsubscript{2}O. Mean production
rates of giant pollen in a treated flower were classified into
five categories (Fig. 2). The efficacy of giant pollen forma-
tion fluctuated among treated flowers of ‘Regata’, with up to
83\% (n = 128) of flowers having more than 3\% giant pollen
among the 155 treated flowers (Fig. 2). Flowers producing
giant pollen grains with a frequency of higher than 50\% oc-
curred in 1.0–1.9 mm and 2.0–3.9 mm floral buds. The aver-
age rate of giant pollen production in the 1.0–1.9 mm cate-
gory was significantly higher than in the other categories.
N\textsubscript{2}O treatment using smaller floral buds tended to produce
more giant pollen grains.

Similarly, in Oriental hybrid lily ‘Willeke Alberti’, N\textsubscript{2}O

Fig. 1. Size distribution, appearance, and flow cytometry histograms of pollen grains in diploid cultivars. Pollen size distributions were obtained from control pollen (shaded bars) and pollen obtained from plants treated with N\textsubscript{2}O gas for 48 h (open bars) in ‘Regata’ (A) and ‘Willeke Alberti’ (D). The appearance of untreated (B, E) and N\textsubscript{2}O-induced (C, F) pollen grains are shown for ‘Regata’ (B, C) and ‘Willeke Alberti’ (E, F). The his-
tograms show pollen samples for untreated (G) and N\textsubscript{2}O-induced (H) pollen grains in ‘Regata’. Bars = 50 \(\mu\)m (B, C, E, F).

Fig. 2. Frequency (%) of giant pollen production classes in floral buds of fertile hybrid lilies after 48 h of N\textsubscript{2}O treatment of floral buds of different
lengths. Ave., mean percentage of giant pollen grains produced in each floral bud size, with number of N\textsubscript{2}O-treated floral buds in parentheses. Different letters between cultivars represent significant differences at \(P = 0.05\), as determined by the Steel-Dwass test.
N$_2$O-induced mitotic polyploidization in anther somatic cells

1–7.9 mm (Fig. 2). The average rate of giant pollen production in the 4.0–5.9 mm category was significantly higher than in the 1.0–1.9 mm category. Although there were no significant differences on giant pollen production rate among the 2.0–3.9 mm, 4.0–5.9 mm and 6.0–7.9 mm categories, we found flowers producing giant pollen with a frequency of higher than 30% in the 4.0–5.9 mm category. Therefore, floral buds ca. 5 mm long at the time of N$_2$O treatment were likely to be suitable for induction of giant pollen. Thus, the optimal bud size of ‘Willeke Alberti’ was larger than that of ‘Regata’. The efficacy of giant pollen formation fluctuated among the treated flowers, as well as among the anthers within a given flower, in ‘Willeke Alberti’ and ‘Regata’ (data not shown).

Restoration of fertility in sterile and partially sterile hybrid lilies

OT hybrid ‘Yelloween’ was completely pollen sterile (Fig. 3A). LA hybrid ‘Serrada’ displayed low pollen fertility ranging from 0–20% (10 ± 9.7%) (Fig. 3C). To examine whether N$_2$O treatment could restore pollen fertility in sterile interspecific hybrid lilies, ‘Yelloween’ and ‘Serrada’ plants were treated with N$_2$O in 2007. A partially sterile LA hybrid ‘Royal Torinity’ also was included. N$_2$O treatment restored pollen fertility in both sterile hybrid lilies (Fig. 3B, 3D). Although acetocarmine-stained pollen is not always fertile, 76% of pollen on average was stained in the untreated plants of LA hybrid ‘Royal Trinity’ (Fig. 3E), whereas N$_2$O-treated plants produced larger, positively stained pollen (Fig. 3F). The smaller-sized, positively stained pollen likely came from normal meiosis.

To determine the optimal growth stage for overcoming pollen sterility, the aforementioned sterile and partially sterile cultivars were treated at various stages (1–7.9 mm) of floral buds in 2007. N$_2$O treatment of 1–3.9 mm floral buds of ‘Yelloween’ produced more fertile pollen grains, with the highest pollen fertility (50%) achieved from 2-mm floral buds (Fig. 4). In ‘Serrada’, fertile pollen grains were most effectively produced using 1.0–3.9 mm floral buds, with the most fertile pollen (71%) obtained from 4-mm floral buds (Fig. 4). Untreated plants of LA hybrid ‘Royal Trinity’ showed normal meiosis, with the diameter of pollen grain ranging between 57–97 µm (83 ± 10 µm). Based on this control data for pollen grain diameter in ‘Royal Trinity’, to examine the effectiveness of N$_2$O treatment, we scored the frequency of giant pollen grains as that of pollen grains with a diameter of >100 µm in the N$_2$O-treated plants. Although there were no significant differences among the 2.0–3.9 mm, 4.0–5.9 mm and 6.0–7.9 mm categories, the 2.0–5.9 mm floral buds produced more giant pollen (>100 µm) in ‘Royal Trinity’ (Fig. 4).

To confirm the reproducibility and reliability of these results, we repeated the experiments to overcome hybrid sterility using ‘Yelloween’ and a completely sterile LO hybrid ‘Otome no Sugata’ in 2008. ‘Otome no Sugata’ produced highly fertile pollen upon N$_2$O treatment (Fig. 3G, 3H). In ‘Yelloween’, the efficacy of recovery of fertile pollen varied among the treated flowers (Fig. 4) and ranged 1–67% (23% average in the 1.0–1.9 mm floral buds; 11% average in the 2.0–3.9 mm floral buds). The efficacy of fertile pollen formation fluctuated among the six anthers within a flower. For example, four of six anthers of ‘Yelloween’ had <10% recovery of fertile pollen, whereas the remaining two anthers
Nukui, Kitamura, Hioki, Ootsuka, Miyoshi, Satou, Takatori, Oomiya and Okazaki had 55% and 50% recovery. ‘Otome no Sugata’ also had variable efficacy of fertile pollen recovery among the $N_2O$-treated flowers (21–90% range; 72% average in 1.0–1.9 mm floral buds; 76% average in 2.0–3.9 mm floral buds).

Histological observations of archesporial cells in anther primordia

We observed cell development in anther primordia at the optimal floral bud developmental stage (1–4 mm) after $N_2O$ treatment in ‘Regata’. Transverse sections of the 1–3 mm floral buds showed actively dividing archesporial cells inside the peripheral layers of the stamen (Fig. 5A, 5B). As a result of the cell proliferation along the longitudinal axis of anther locules, the oblong locules were filled with archesporial cells in 3–5 mm floral buds (Fig. 5C). Cell division in another primordia was almost finished at the 7-mm floral bud stage, with 0.6% mitotic index of the archesporial cells (Fig. 5D). In 10-mm floral buds, tapetum cells surrounded PMCs, and no mitotic divisions were observed (data not shown). In the 32-mm floral buds of ‘Yelloween’ undergoing meiotic division, untreated control microspore tetrads had 20–29 $\mu$m (25 ± 1.8 $\mu$m) diameters, whereas floral buds treated with $N_2O$ had some larger microspore tetrads with 19–41 $\mu$m (29 ± 4.2 $\mu$m) diameters (Fig. 6). Among them, 29% were >30 $\mu$m in diameter, which was similar to the recovery percentage of fertile pollen at anthesis. These results suggest that $N_2O$ treatment overcame hybrid sterility in the interspecific hybrid lilies owing to chromosome doubling of archesporial cells of PMCs.

Fig. 4. Frequency (%) of floral buds in each fertile or giant pollen production class in the sterile or partially sterile hybrid lily floral buds treated with $N_2O$ for 48 h. Ave., mean percentage of fertile or giant pollen grains in each floral bud size, with number of floral buds treated with $N_2O$ in parentheses. The fertile pollen production rate at flowering (‘Yelloween’, ‘Serrada’ and ‘Otome no Sugata’) and the giant pollen production rate (‘Royal Trinity’) were measured. Different letters between cultivars represent significant differences at $P = 0.05$, as determined by the Steel-Dwass test.

Fig. 5. Transverse sections of young anthers of ‘Regata’. The floral buds were sampled at lengths of 1 mm (A), 3 mm (B), 5 mm (C) and 7 mm (D). Ep, epidermis; En, endothecium; T, tapetum; PMC, pollen mother cell. The archesporial cell undergoing mitotic division is marked with arrowheads. Bars = 50 $\mu$m.

Floral bud length (mm) at the time of $N_2O$ treatment

had 55% and 50% recovery. ‘Otome no Sugata’ also had variable efficacy of fertile pollen recovery among the $N_2O$-treated flowers (21–90% range; 72% average in 1.0–1.9 mm floral buds; 76% average in 2.0–3.9 mm floral buds).
N$_2$O-induced mitotic polyploidization in anther somatic cells

Crosses with putative 2n pollen obtained from the N$_2$O-treated plants

In the 2x-level control cross (between 2x Asiatic hybrid lilies ‘Mona’ and ‘Regata’), all capsules set normally with about 120 normal seeds per capsule (Table 1). In the 4x-level control cross (between 4x Asiatic hybrid lilies ‘Gran Paradiso’ and ‘Loreto’), normal seeds were obtained with 34 seeds per capsule. The ploidy levels of embryos excised from the resulting seeds were determined using flow cytometry (Table 2). All seeds obtained from the 2x×2x and 4x×4x crosses were diploid and tetraploid, respectively. These data indicate that the diploids and tetraploids used have normal fertility in male and female gametes. Nevertheless, no seeds were obtained in the interploidy cross between the tetraploid ‘Gran Paradiso’ and the diploid ‘Regata’, owing to triploid block. This phenomenon can act as a selective barrier against triploids in crosses of tetraploid cultivars with mixed n and 2n pollen (Akutsu et al. 2007). In such crosses, the resulting progeny seeds will be tetraploids. In fact, when the tetraploid cultivar ‘Gran Paradiso’ was crossed with 2n grain including pollen from N$_2$O-treated plants of the diploid cultivar ‘Regata’, 59% of the flowers pollinated set capsule and the resulting embryos were tetraploid. In the crosses using the 2n grain including pollen from N$_2$O-treated 3x LA hybrid ‘Royal Torinity’, the examined embryos were pentaploid. When L. × formolongi was pollinated with pollen obtained from the N$_2$O-treated ‘Yelloween’ (OT hybrid) and ‘Otome no Sugata’ (LO hybrid), some hybrid embryos were obtained. These were diploid, triploid or tetraploid (Table 3). However, no embryos were obtained using the control pollen that contained no viable grains.

Verification of hybridity

‘Otome no Sugata’ was the diploid hybrid developed by crossing L. × formolongi with L. rubellum (Okazaki et al. 1992). Crossing L. × formolongi with 2n grain including pollen of ‘Otome no Sugata’ produced in this study was regarded as a backcross, which would lead to production of triploids having the diploid genomes of L. × formolongi and a haploid genome of L. rubellum. When genomic DNA of L. × formolongi was used as a probe, 24 chromosomes were detected in the triploid progeny (Fig. 7A), indicating that these 24 chromosomes came from L. × formolongi and the remaining 12 from L. rubellum. The GISH analysis revealed that no chromosome recombination occurred between the genomes of L. × formolongi and L. rubellum in either of the three hybrid plants examined. The ITS regions of rDNA in L. × formolongi, L. rubellum, ‘Otome no Sugata’, and the backcrossed progeny were amplified by PCR, and the resulting PCR products were

Table 2. Results of interploidy crosses between diploid and tetraploid lily varieties and crosses between tetraploids and N$_2$O-treated plants

| Female parent | Male parent | No. of flowers pollinated | No. of ovaries developed | No. of seeds obtained per capsule (mean ± SD) | Ploidy level of seeds (%) |
|---------------|-------------|--------------------------|--------------------------|-----------------------------------------------|--------------------------|
| Diploid × diploid | Regata (2x) | 5                        | 5                        | 125.8 ± 18.2                                  | 100                      |
| Tetraploid × tetraploid | Loreto (4x) | 9                        | 9                        | 33.7 ± 16.7                                  | 0                        |
| Tetraploid × diploid | Regata (2x) | 10                       | 0                        | –                                             | 100                      |
| Tetraploid × N$_2$O-treated plants | Regata (2x) | 71                       | 42                       | 3.1 ± 2.7                                    | 0                        |
| Gran Paradiso (4x) | Royal Torinity (3x) | 11                       | 8                        | 5.5                                          | 0                        |

*Ploidy of mature seeds was determined by flow cytometry (n = the number of seeds examined).
<700 bp in size, and were cleaved byMspI (Fig. 7B). The ca. 160 bp restriction fragment, specific toL. rubellum genome, was transmitted to the backcrossed triploid and tetraploid progeny, indicating that the triploid and tetraploid plants derived from crossingL. ×formolongi withN2O-treated ‘Otome no Sugata’ were true hybrids, whereas the diploids were false hybrids (data not shown). The two triploid plants derived from the cross ofL. ×formolongi and ‘Yelloween’ died after transplant. Appearance of diploids and tetraploids was an unexpected result; the tetraploid BC1 plants were arisen from the fusion of spontaneous 2n female gametes and the N2O-induced 2n pollen. The diploids might be developed by spontaneous 2n female gamete formation, followed by pseudogamy.

Characteristics of hybrids

Four plants from the cross ofL. ×formolongi with N2O-treated ‘Otome no Sugata’ flowered in the 2 years after pollination. L. ×formolongi has white, trumpet-shaped, upright flowers with yellow pollen (Fig. 7C). ‘Otome no Sugata’ has pink, trumpet-shaped flowers that are slightly drooping with brownish anthers (Fig. 7D). The hybrid floral sizes were intermediate between both parents, whereas flower color and anther color were similar to ‘Otome no Sugata’ (Fig. 7E–7H). The appearance of flowers varied among hybrids, with upright positioned (Fig. 7E), slightly drooping flower positions and trumpet-shaped (Fig. 7F, 7H) or bowl-shaped (Fig. 7G) pink flowers. The appearance of the trumpet-shaped flowers of the hybrids was more likeL. ×formolongi than ‘Otome no Sugata’.

Discussion

The mechanism of action of N2O for overcoming hybrid sterility

Barba-Gonzalez et al. (2006) reported that N2O treatment overcame pollen sterility through the FDR mechanism in 5–10 mm floral buds. At this developmental stage, however, the archesporial cells in anthers are thought to undergo premeiotic mitosis. In general, microspore developmental stages correlate with floral bud lengths (Goldberg et al. 1993). InL. longiflorum, floral buds <10 mm or 10–22 mm have the proliferating stage of archesporial cells or meiotic prophase 1 PMCs, respectively (Taylor and McMaster 1954). Our histological study confirmed a similar correlation between microspore developmental stage and floral bud length in Asiatic hybrids, although these lilies have smaller floral buds thanL. longiflorum. Therefore, in the 5–10 mm floral buds used for N2O treatment by Barba-Gonzalez et al.

| Male parent          | No. of flowers pollinated | No. of capsules set | No. of plantlets obtained | Ploidy level of progeny |
|----------------------|---------------------------|---------------------|---------------------------|-------------------------|
| Untreated control    |                           |                     |                           |                         |
| ‘Otome no Sugata’ (2×) | 14                        | 5                   | 0                         | 2× 3× 4×                 |
| ‘Yelloween’ (2×)     | 37                        | 7                   | 0                         | 2× 3× 4×                 |
| Pollinated with N2O-treated plants | 32                        | 21                  | 24                        | 2× 3× 4×                 |
| ‘Otome no Sugata’ (2×) | 39                        | 22                  | 17                        | 2× 3× 4×                 |

*Ploidy of progeny was determined by flow cytometry.

Table 3. Results of interspecific crosses betweenL. ×formolongi and interspecific hybrids.
(2006), archesporial cells should undergo mitosis, indicating that production of 2n gametes in 5–10 mm floral buds does not occur via FDR during meiosis. Furthermore, if fertile pollen was produced in sterile hybrid lilies through the FDR mechanism, the resulting meiotic products should be dyads (unreduced microspores) having the same size as tetrads. In our study, however, larger microspore tetrads were observed in plants treated with N₂O. Therefore, our results suggest that the FDR mechanism is not involved in restoration of pollen fertility, and chromosome doubling in archesporial cells followed by formation of amphidiploid PMCs (2n = 4x) is the mechanism for the recovery of hybrid fertility.

Kato (2002) obtained doubled haploid lines by treating haploid maize floral primordia with N₂O, suggesting that applying N₂O to the floral primordial stage might overcome hybrid sterility. Unlike the treatment of floral primordia by Kato (2002), we treated anther archesporial cells. Thus, the use of floral tissues at different developmental stages may overcome interspecific sterility through mitotic polyploidization.

Application of N₂O treatment

The proportion of 2n pollen varied in each anther within a bud for cultivars treated with N₂O gas. This may be due to incomplete synchronized mitotic division of archesporial cells in the different anthers within a flower. Nevertheless, the developmental stages of archesporial cells correlated to some extent with floral bud lengths. As a result, the measurement of floral bud length could be used to determine the optimal timing for N₂O treatment: 1–4 mm for Asiatic hybrid lilies; 2–6 mm for Oriental hybrid lilies and LA hybrid lilies; and 1–4 mm for completely sterile OT hybrid ‘Yelloween’ and LO hybrid ‘Otome no Sugata’. This size-based criterion is not absolute because the developmental stages of floral buds and archesporial cells are affected by environmental and/or culture conditions.

Treatment using smaller floral buds tended to produce greater numbers of giant pollen grains. This indicates that if some archesporial cells undergo chromosome doubling at an early developmental stage with N₂O treatment, the resulting 4x archesporial cells could predominate, with preferential cell proliferation of the archesporial cell lineage. Additionally, in contrast to the colchicine-induced chimeric somatic tissues where 2x cells proliferate better than 4x cells, the N₂O-induced 4x archesporial cells effectively proliferated along the oblong anther locules (Fig. 5) because this narrow oblong locule allowed a certain space for 4x archesporial cells to outcompete 2x archesporial cells with 2n ploidy levels.

Chromosome doubling in archesporial cells with N₂O can shorten the period of polyploidy breeding in lilies compared to the traditional method of soaking bulb scales in colchicine, which requires a long duration to flowering. Taken together, compared to soaking in colchicine, chromosome doubling in archesporial cells treated with N₂O can save time and enhance production of polyploids.

Two methods for obtaining 2n pollen in fertile cultivars

Okazaki et al. (2005) and Akutsu et al. (2007) reported that N₂O treatment at metaphase I produces 2n pollen in *Tulipa* and *Lilium*, respectively. In the present study, N₂O treatment at the archesporial cell proliferating stage allowed mitotic diploidization of male gametes of fertile cultivars. Thus, to obtain 2n pollen in fertile cultivars, chromosome doubling by N₂O treatment has two optimal stages—the meiotic division stage and the archesporial cell proliferating stage. N₂O treatment during the short duration of meiotic metaphase I stage requires skillful techniques for optimizing timing during meiotic divisions. However, it can produce more 2n pollen than in the archesporial cell proliferating stage because PMCs at meiotic metaphase I have more synchronized cell divisions at the time of N₂O treatment than archesporial cells. On the other hand, treatment is easier at the archesporial cell proliferating stage because plants in premeiotic stage are smaller and easier to handle. Moreover, the duration applicable for N₂O treatment at the archesporial cell proliferating stage is longer than for the meiotic stage.

Characteristics of the BC₁ plants

In the backcrosses using the resultant fertile pollen, production of progeny is evidence of functional pollen obtained by overcoming pollen sterility. Although the resultant pollen is functional, only a limited number of the BC₁ plants were obtained due to interspecific incongruity in the BC₁ progeny. We did not find any chromosome recombination between the genomes of *L. × formolongi* and *L. rubellum*, probably because of the preferential homologous chromosome pairing in the amphidiploid PMCs formed by N₂O treatment. This result is consistent with previous studies by Barba-Gonzalez et al. (2004) and Lim et al. (2000), who reported that fewer chromosomal recombinations occur in progenies obtained from crosses using fertile pollen of amphidiploids restored by somatic chromosome doubling compared to crosses using fertile pollen obtained from meiotic polyploidization. In this study, we found some phenotypic variations in the BC₁ progeny, such as in the flower direction, an important trait for lily breeding (Fig. 7E–7H). This probably resulted from genetic variation between the parental plants of *L. × formolongi* because chromosome recombinations which would be a potential source of phenotypic variation in the progeny did not occur between the genomes of *L. × formolongi* and *L. rubellum*.

Overcoming hybrid sterility

Hybrid sterility derived from remote hybridization between two different species is a common phenomenon in plants and is a key problem in interspecific breeding. The selection of 2n gamete producers has contributed to overcoming hybrid sterility in crop breeding (Hanneman and Pelouquin 1968, Khan et al. 2009, Marasek et al. 2006, Ramanna et al. 2003). The diploid LA hybrid lilies derived from the cross of *L. longiflorum* and Asiatic hybrid lilies showed intermediate morphology between the parental species. This
intermediate morphology is esthetically undesirable. To improve this undesirable trait, 2n gamete producers were selected for further backcrossing of diploid LA hybrids with Asiatic hybrid lilies because diploid LA hybrids were basically pollen sterile. As a result, triploid LA hybrids have been developed for commercial distribution. Similarly, other hybrid lilies such as OT, OA and LO have been derived from more remote interspecific hybridization than LA hybrids, and hence, they have serious hybrid sterility problems that require breeding involving selection of 2n gamete producers. However, 2n gametes may not be widely applicable in breeding because their occurrence is not always predictable (Asano 1984, Barba-Gonzalez et al. 2004, Lim et al. 2001). Thus, techniques that can overcome hybrid sterility of lilies and perhaps other crops are eagerly awaited. N$_2$O treatment would be a useful technique in this regard, and the procedure presented here will promote interspecific or interploidy hybridization of lilies and other crops.

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