Induction of *2n* Gametes for Overcoming F1-Sterility in Lily and Tulip

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Abstract

For overcoming F1-sterility in interspecific hybrids, mitotic and meiotic polyploidisation is applied in lily and can result in fertile allopolyploids. The mechanism of viable pollen production of mitotic and meiotic polyploidisation is quite different. Mitotic polyploids are obtained by artificial chromosome doubling and results in normal pairing of homologous chromosome set, which enables the formation of 2x gametes during the meiosis. Meiotic polyploidisation, on the other hand, leads in rare cases of restitutional chromosome division, to the formation of unreduced gametes (*2n* gametes). In contrast to mitotic doubling, homoeologous recombination can occur in these gametes. Genomic in situ hybridization (GISH) was used to discriminate parental chromosomes in the F1 hybrids and backcross progenies to detect homoeologous recombination. Mitotic polyploidisation showed no homoeologous recombinations between the parental genomes whereas in meiotic polyploids it was detected in a high frequency. The use of *2n* gametes is therefore the most promising approach for the introgression of desirable characters in using interspecific hybrids in breeding. In both cases, the frequency of viable gametes appeared to be low and limited to a few F1-hybrids. A new and promising method is developed in which *2n*-gametes can be induced by the application of laughing gas (N_2O). This method proved to be successful in lily and the occurrence of homoeologous recombination, a characteristic of meiotic polyploidisation, was detected. In tulip where the production of mitotic polyploids requires at least five years, it will speed up breeding with interspecific hybrids enormously.

INTRODUCTION

One of the main objectives in ornamental plant breeding is to introgress desirable characters (disease resistances and quality traits) from one species into another through interspecific hybridization to achieve genetic variation. Interspecific hybrids of the genera *Tulipa* and *Lilium* have been generated through normal pollination and with the use of special pollination techniques combined with ovary-, ovule- and embryo-rescue (van Tuyl et al., 1991) respectively. The major drawback of these hybrids is their high sterility. The traditional method to restore fertility is the generation of allopolyploids using spindle inhibitors such as colchicine (Arisumi, 1973; Asano, 1982), oryzalin (van Tuyl et al., 1992) and N_2O (Ostergren, 1954) among others. However, this is a time consuming method, especially in the case of *Tulipa*, where the production of mitotic polyploids takes up to five years. An alternative is the use of *2n* gametes, which are produced spontaneously by the F1 hybrid but in low frequencies (reviews by Harlan and De Wet, 1975; Veilleux, 1985; Ramanna and Jacobsen, 2003). The use of unreduced gametes (*2n* gametes) is advantageous over the use of mitotic chromosome doubling because recombination can occur between the parental chromosomes, which is not the case in mitotic polyploids in which autosyndetic chromosome pairing predominates (Karlov et al., 1999; Lim et al., 2001; Ramanna et al., 2003). There have been some attempts to

induce *2n* gametes with the use of chemicals such as caffeine and N_2O during microsporogenesis (Lim et al., 2005; Okazaki et al., 2005; Barba-Gonzalez et al., in press), the last among these chemicals being more successful due to the administration method. N_2O is used as a gas under pressure, it can be absorbed by all the tissues and the toxic effects, if any, are mitigated by removing the tissues from the gas chamber. If the appropriate tissues are treated at the right stage, *2n* gametes can be induced with the use of this gas. In a previous investigation the occurrence of recombination between the parental chromosomes in sterile interspecific F1 lily hybrids was demonstrated after the use of N_2O to induce *2n* gametes (Barba-González et al., in press). This method was applied to more sterile lily and tulip hybrids with the aim of restoring fertility and inducing *2n* gametes, the effectiveness and the implications of the use of laughing gas are discussed.

MATERIAL AND METHODS

Plant Material and N_2O Treatments

1. *Lilium*. Whole lily plants with bulbs ranging 0.5 to 1 cm from nine different genotypes of Oriental × Asiatic F1 lily hybrids and four BC1 progeny plants (Table 1) were placed in gas chamber and treated with N_2O at a pressure of 6 bars as described by Zeilinga and Schouten (1966). After treatment, the N_2O was slowly released to prevent cell rupture. Lily plants where grown in greenhouse under standard conditions.

2. *Tulipa*. Tulip bulbs were harvested in summer and stored according to standard procedures. After 15 weeks of storage, five to seven bulbs (depending on stock quantity) of several tulip cultivars and F1 hybrids were randomly selected for N_2O treatments twice weekly intervals (Table 2). Bulbs were placed in plastic nylon mesh bags, sealed and placed inside a gas chamber which was slowly filled with N_2O to a pressure of 6 bars and held for 48 hours, according to Zeilinga and Schouten (1966). After treatment, the N_2O was slowly released. The bulbs were then placed back into storage conditions to satisfy the necessary cold requirement and stored. After receiving the necessary cold requirement, the tulip bulbs were placed into the greenhouse and forced under standard conditions.

Fertility

The criteria used to determine the fertility were 1) in vitro pollen germination, carried out in artificial agar media containing per liter: 100 g sucrose, 5 g bacteriological agar, 20 mg boric acid and 200 mg calcium nitrate, the pollen was cultured at 25ºC during 24 h; and 2) embryo formation after crossing.

1. *Lilium*. I) Fresh pollen was utilized for the in vitro pollen germination test and II) the treated plants were used both as female and male parents in crosses with recurrent Asiatic cultivars and in one case with a *L. longiflorum* × *L. dauricum* F1 hybrid (Table 1). 40-60 days after pollination the swollen fruits were collected, surface sterilized by submersion in 80% ethanol and flamed. The embryos were excised from the swollen ovules and cultured in enriched media (van Tuyl et al., 1991).

2. *Tulipa.* I) Flowers were emasculated as the tepals began to show color and the anthers were dried in a desiccation box. II) The F1 hybrids that showed germination percent of 10% or greater were backcrossed to *T. gesneriana* plants. Seed pods were left to develop naturally and the number of seeds was recorded.

In Situ Hybridization

In situ hybridization was performed in the progeny of treated lilies as follows: root tips were collected early in the morning, pretreated in saturated α-bromonaphtalene solution in ice-water overnight, fixed in ethanol:acetic acid (3:1) and stored at -20ºC until use. Cell wall digestion was performed by rinsing the root tips in citrate buffer during 2 h followed by incubation in a pectolytic enzyme mixture containing 0.2% (w/v) pectolyase Y23, 0.2% (w/v) cellulase RS and 0.2% (w/v) cytohelicase in 10 mM of citrate buffer (pH 4.5). Squish preparations were made in a drop of 40% acetic acid; frozen in liquid

nitrogen to remove the coverslip with a razor blade, dehydrated in absolute ethanol and air-dried. Slides were incubated 1 h in RNAse A (100 µg/ml) at 37ºC; followed by 10 min in pepsin (5 µg/ml) at 37ºC and Para formaldehyde (4%) at room temperature, between every incubation the slides were rinsed in 2x SSC. The slides were dehydrated in 70%, 90% and absolute ethanol series.

Hybridization followed using a mixture consisting of 20× SSC, 50% formamide, 10% sodium dextran sulphate, 10% SDS, 25-50 ng of probe DNA (sonicated genomic DNA (1-10 kb) from the Oriental cultivar 'Sorbonne' labelled with Biotin-16-dUTP by nick translation according to manufacturer instructions (Boehringer Mannheim, Germany)) and 3 µg per slide of autoclaved DNA (100-500 bp) from the Asiatic cultivar 'Connecticut King'. The hybridization mixture was heated at 70ºC during 10 min and directly placed on ice for at least 10 min. 40 µl of hybridization mixture were applied to each slide. Slides were denatured at 80ºC for 10 min. After overnight incubation in a humid chamber at 37ºC, the slides were washed for 15 min at room temperature in 2 × SSC and 30 min in 0.1 × SSC at 42ºC. The probe was detected with anti-digoxigenin-fluorescein (Amersham Biosciences, UK). Chromosomes were counterstained with 1 µg/ml DAPI (4,6-diamidino-2-phenylindole) and a drop of Vectashield anti-fade (Vector Laboratories, Brulingame, CA) was added for its examination under a Zeiss Axioplan 2 Photomicroscope equipped with epi-fluorescent illumination, filter sets of DAPI and Cy3. Images were captured by a Photometrics Sensys 1,305 × 1,024 pixel CCD camera, processed with Genus Image Analysis Workstation software (Applied Imaging Corporation) and sharpened with a 7×7 Gauss spatial filter. DAPI fluorescence was pseudo-coloured in blue and the Oriental DNA probe fluorescence in green. Optimal brightness and contrast were achieved with Adobe Photoshop image processing.

RESULTS AND DISCUSSION

The different genotypes of *Lilium* and *Tulipa* that were treated with N_2O were tested for fertility and crossability. The pollen fertility was assessed by counting only the large and well filled pollen grains that germinated in vitro, the abnormal and aborted pollen grains were ignored. In all cases there was little or no germination in the controls (non-treated plants or bulbs). Based in these counts the higher germination percentage was observed in the case of lily in the F1 hybrids 951301-5 and 951502-1 (70% and 73% respectively) (data not included). Four other genotypes had an intermediate germination percentage (5.7-36.8%) whereas the remaining three had little or no germination (data not included). In the case of tulip, the 17 treated genotypes responded in a positive way and the germination percentages ranged from 3 to 88% (Table 2). However, in both lily and tulip, it was not possible to determine whether all the treated flower buds had the same or similar meiotic cell division stage at the time of treatment.

In the case of lily all plants that showed 5% or more pollen germination were used as male and female parents to be crossed to Asiatic fertile genotypes and in one case to an F1 *L. longiflorum* ×*L. dauricum* in order to determine their ability to produce embryos and progenies. In three F1 genotypes, 951502-1, 951301-5 and 969023-2, embryos were obtained when used as male and female parents (Table 1). 951502-1 was previously known by its ability to produce *2n* gametes and progeny. However, the production of *2n* gametes of 951501-1 increased from 16.6% to 73% after N_2O treatments (Barba-González et al., 2004).

Remarkably not only the sterile F1 lily hybrids were able to produce "fertile" pollen but also sterile triploid hybrids from mitotic and meiotic polyploidisation origin were able to produce both, fertile pollen and progenies. In three genotypes, 012048-1, 022612-1, 012046-1, embryos were produced when they were used as male parent and in one genotype, 012611-10, embryos were produced when used as female parent. In two cases, embryos were produced when triploid cultivars were backcrossed to interspecific F1 hybrids (Table 1).

In the case of tulip, only those genotypes that showed higher than 10% germination were backcrosses to *T. gesneriana* plants. 13 out of 17 genotypes that produced

"fertile" pollen were able to set seeds. The seed production ranged from 0.38 to 13.6, estimated on the basis of embryos per pollination. There was no direct relation between the germination percentage and the seed set. In the case of the genotype 111 it had a germination percentage of 88%, when 37 flowers were pollinated there was no seed set, while the genotype 9 had a germination percentage of 10%, 8 flowers were pollinated and 109 seeds were produced (Table 2). About 80% of the 858 seeds obtained from the N_2O treated tulip bulbs have germinated (data not included).

A total of 41 lily BC1 (A × OA) progeny plants were analyzed for their ploidy level; 36 were triploid ($2n=2x=36$) and seven were tetraploid ($2n=4x=48$). The notable feature is that all the progeny plants had euploid chromosome complements. Tetraploid progenies were the result of functioning of the *2n* eggs from the Asiatic cultivar. The occurrence of euploid progenies implied that the F1 OA hybrids contributed with 12O + 12A genome chromosomes to the progenies. 12 progeny plants were analyzed through GISH to verify this. With the exception of one triploid and one tetraploid, GISH analysis confirmed that the N_2O treated F1 OA hybrids contributed 12 chromosomes each from the O and A genomes and recombination among the parental genomes was observed (Fig. 1). The two exceptional progeny plants (042923-1, 042924-1) possessed variable chromosome numbers of O and A genomes (data not included), obviously produced by indeterminate meiotic restitution, while the rest of progeny plants originated through the functioning first division restitution gametes.

We can conclude from this investigation that "fertility" can be restored in the sterile F1 and triploid interspecific hybrids. Production of a considerable number of embryos was achieved (248 in the case of lily and 858 in the case of tulip). The application of N_2O treatments to sterile hybrids can reduce the breeding time considerably. The fact that not all the genotypes produced positive results can be due to the different meiotic stages when the N_2O was applied. Meiosis in anthers of *Lililum longiflorum* has a duration of 50 days (Taylor and McMaster, 1954); in such cases, it might be possible to determine the optimum stage for N_2O treatments to maximize the chances of inducing fertility (Barba-Gonzalez et al., in press).

This method to restore fertility is advantageous over mitotic polyploidisation, because the flower bud and bulbs can be treated with N_2O to restore fertility, while the process of mitotic polyploidisation takes several years. Recombination is not present in the gametes of polyploids produced by mitotic chromosome doubling while it is present in unreduced gametes. By this method it was possible to prevent sterility in many genotypes of lily and tulip, recombination among the parental genomes is achieved in their chromosomes and introgression is accomplished in the first backcross generations.

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Tables

Table 1. Number of embryos obtained after N_2O treatment of sterile F1 Oriental × Asiatic (OA) lily hybrids and triploid BC1 (AOA) interspecific lily hybrids.

Genotype	Cross ♀	x	♂	# pollinations	# embryos
042923	AA*		951301-5	19	179
042924			OA		
	AA		951502-1	3	35
			OA		
	AA		952059-9	4	1
			OA		
	AA		969023-2	6	28
			OA		
052121	980072		012611-10	6	14
	AA		AOA		
052124	031028		951502-1	3	20
	AA		OA		
	951301-5		AA	10	32
	OA				
	969023-2		AA	10	1
	OA				
052002	012048-1		950181	4	2
	AOA		LD		
052109	022612-1		051064	2	1
	AOA		AA		
052188	012046		022356-4	2	3
	AOA		OA		

*AA = Asiatic, OA = F1 hybrid Oriental × Asiatic, LD = *L. longiflorum* × *L. dauricum*, AOA = Triploid hybrid obtained by the cross (Oriental × Asiatic) × Asiatic.

Table 2. Number of seeds obtained after N_2O treatment of sterile F1 *T. gesneriana* × *T. fosteriana* hybrids.

Genotype	Parents		Pollen germination (%)	# pollinations	# seeds
	T. gesneriana ♀	*T. fosteriana* ♂			
6	'Christmas Marvel'	'Cantata'	23	12	10
111	'Christmas Marvel'	'Princeps'	88	37	0
164	'Christmas Marvel'	'Princeps'	45	29	0
125	'Kees Nelis'	'Cantata'	39	16	169
126	'Kees Nelis'	'Cantata'	12	12	26
129	'Kees Nelis'	'Cantata'	27	15	32
131	'Kees Nelis'	'Cantata'	45	14	137
134	'Kees Nelis'	'Cantata'	13	12	0
19	'Kees Nelis'	'Cantata'	33	14	0
64	'Kees Nelis'	'Cantata'	20	21	8
65	'Kees Nelis'	'Cantata'	30	17	211
89191-34	'Kees Nelis'	'Cantata'	21	23	10
9	'Kees Nelis'	'Cantata'	10	8	109
140	'Lustige Witwe'	'Cantata'	80	19	16
26	'Lustige Witwe'	'Cantata'	3	6	10
83	'Lustige Witwe'	'Cantata'	11	21	116
PrEi	*T. praestans*	*T. eichleri*	15	8	4

Figures

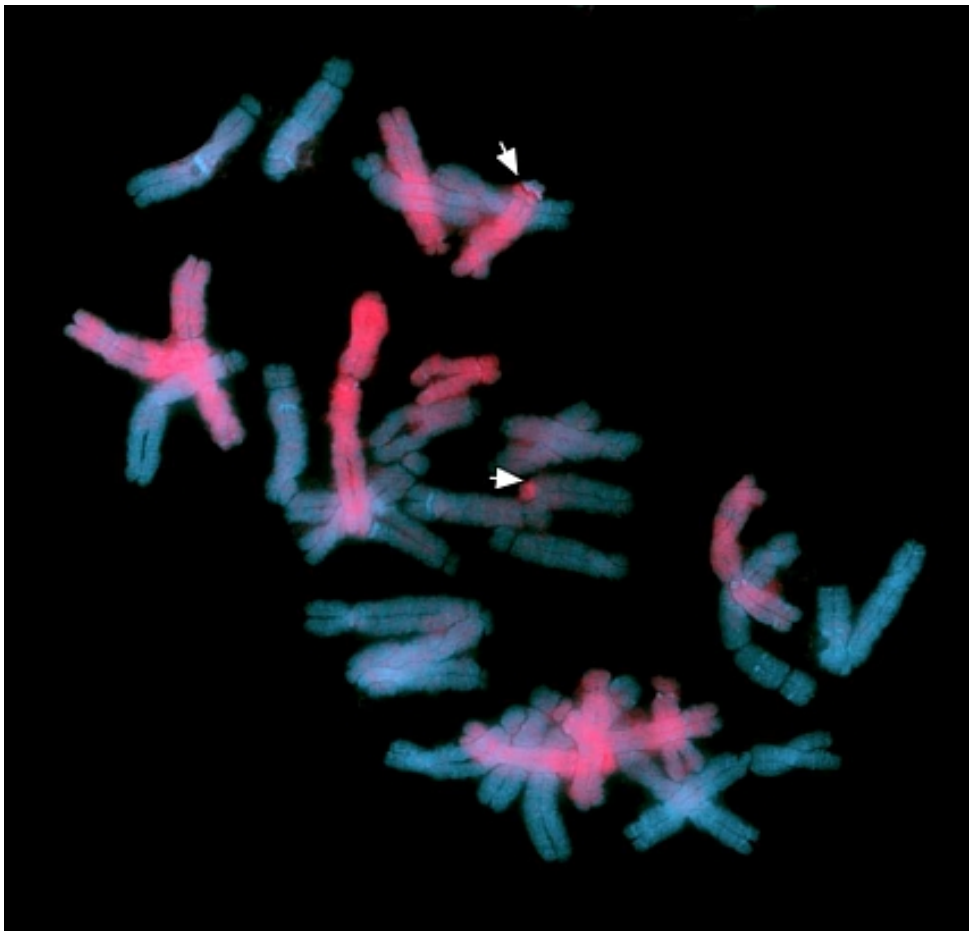

Fig. 1. Chromosome complement of the BC1 progeny 042928-2 showing a triploid chromosome number with two recombinant chromosomes (arrows). The Biotin-labelled Oriental DNA of 12 chromosomes was detected with the Cy3-streptavidin system (pink fluorescence) and the 24 Asiatic chromosomes were counter stained with DAPI (blue fluorescence).