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Induction of Polyploid *Phalaenopsis amabilis* by N_2O Treatment

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Ploidy levels of the progenies obtained by the crossing of untreated *Phalaenopsis amabilis* (2n=2x=38) with the same species treated with N_2O for 24 or 48 hrs were determined by flow cytometry and chromosome counting. The treatment for 24 hrs resulted in 35.6% triploid and 6.7% tetraploid induction. Only 5% tetraploid progenies were obtained by the treatment for 48 hrs.

Key words: Flow cytometry, nitrous oxide, polyploidy, *Phalaenopsis*

INTRODUCTION

Polyploids have played an important role in higher plant evolution, as most flowering plants are polyploids (Leitch and Bennett, 1997; Rubuluza *et al*., 2007). Polyploidy has been used in horticulture as a breeding tool to enhance characteristics such as flower size, flower number, plant size, increased width–to–length ratio of leaves and leaf thickness (Shao *et al*., 2003; Rubuluza *et al*., 2007).

Phalaenopsis is one of the most popular orchids in the world market (Wang, 2004). The basic chromosome number of the genus is 2n=2x=38. The most popular commercial varieties/ hybrids are usually tetraploid, while the wild species are diploid. Therefore, it is hard to transfer decent genes from wild species to the most advanced varieties or hybrids. To develop a conversion procedure to double the ploidy level of the wild species of *Phalaenopsis* enables hybridization with the commercial varieties (Chen *et al*., 2009). Chromosome doubling to produce different ploidy levels in plants is usually achieved by anti–microtubule agents such as colchicines, oryzalin, trifluralin and nitrous oxide gas (N_2O). We showed in the previous report the effectiveness of N_2O application to induce unreduced gamete of *Phalaenopsis amabilis* (Wongprichachan *et al*., 2013)

Flow cytometry is a rapid and exact method for estimating nuclear DNA content (Galbraith *et al*., 1983). It can be efficiently used for ploidy determination in plants growing in the field, greenhouse and *in vitro* (Doležel *et al*., 1989; Śliwińska and Steen, 1995; Joachimiak *et al*., 2001; Thiem and Śliwńska, 2003). This technique has been proven to be faster and precise than conventional methods such as chromosome counting or measuring stomata length (Sgorbati *et al*., 1986; Ollitrautt–Sammarcelli *et al*., 1994; Tosca *et al*., 1995; O'Brien *et al*., 1996; Ozaki *et al*., 1998; Pinheiro *et al*., 2000; Vainola, 2000; Thao *et al*., 2003).

The aim of this study was to produce polyploids of *P. amabilis* by using unreduced gametes induced by N_2O treatments.

MATERIALS AND METHODS

Plant materials

Phalaenopsis amabilis (2n=2x=38) were used in this study. Plants were cultivated in 10 cm diameter pots in a greenhouse with a 16/8 h (day/night) photoperiod at the Horticulture Technology Center, National Chiayi University, Chiayi, Taiwan (R.O.C.).

Pollen grains of *P. amabilis* treated with N_2O for 0, 24 or 48 hrs were hand pollinated to untreated plants of *P. amabilis*. Ten flowers were used for each crossing. Capsules were harvested 130 days after pollination.

Three capsules per crossing were surface–sterilized for 20 min in 20% (v/v) Clorox solution and 0.05% Tween20 and subsequently rinsed 3 times with sterilized distilled water. Seeds were germinated aseptically in 90×15 mm petri dishes containing 25 mL basal medium supplemented with 2.5 g/L Hyponex #1 ($7N–6P_2O_5–19K_2O$), 200 mg/L myo–inositol, 2 g/L peptone, 1 g/L ammonium nitrate, 60 g/L Potato, 1 mg/L naphthalene acetic acid (NAA), 0.5 mg/L kinetin, 0.5 mg/L indole–3–butyric acid, 1 g/L activated charcoal, 20 g/L sucrose and 10 g/L agar. The pH value of the media was adjusted to 5.4 with 1 N KOH or HCl prior to autoclaving for 15 min at 121˚C. After 3 months, the developed protocorms were transferred to new medium supplemented with 2.5 g/L Hyponex #1, 200 mg/L myo–inositol, 2 g/L peptone, 25 g/L Potato, 30 g/L Banana, 0.5 mg/L NAA, 1 g/L activated charcoal, 20 g/L sucrose and 15 g/L agar. Plantlets were subcultured on the medium of the same constituents every 2 months. All cultures were incubated at 25±2˚C in a 16/8 hrs photoperiod at light intensity of 54 μmol m^{-2} s^{-1} with cool–white fluorescent light lamps.

Sample preparation and Giemsa staining for chromosome observation

Growing root tips were pretreated with 2 mM 8–hydroxyquinoline (Sigma–Aldrich, St. Louis) on a

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rotary shaker (100 rpm) for 5 hrs at 20˚C, and fixed in 95% ethanol–acetic acid (3:1 by vol.) for 24 hrs at 4˚C. They were digested with 1% (w/v) cellulase (Sigma–Aldrich) and 0.5% (w/v) pectinase (Fluka) in 10 mM of citrate buffer (Pedrosa *et al*., 2001). The digested samples were mounted on a slide with a droplet of 10% Giemsa (Sigma–Aldrich), covered with a cover slide, and microscopically analyzed (Axio version Rel. 4.5, Germany) with a CCD camera (Cannon A620, Japan).

Preparation of nuclear suspension for determination of ploidy levels by flow cytometry

Nuclear DNA content in young leaves (0.3–0.5 cm^2) of the plantlets were analyzed by flow cytometry (Beckman, Germany). Samples were prepared according to the manual of the Partec Cystain Cystain PI Absolut P kit, which included the extraction and staining buffers. A sharp razor blade was used to chop fresh tissue into pieces with a size of <1 mm in a 6 cm glass petri dish containing 500 μL of the extracting buffer. After 30–90 sec of incubation in the Extraction Buffer the samples were filtered through a Partec 50 μm CellTrics disposable filter. Then 2 ml of staining solution Propidium Iodide (with PI and Rnase) was added. The process of nuclear extraction and staining was carried out on ice.

Determination of ploidy levels of plantlet by flow cytometry

To determine the ploidy levels of the samples of each treatment, young leaves of *P. aphrodite* var. *formosana* with diploid level (2n=2x=38) were used as reference for the 2C DNA value which was set to the channel at 50 (G_0/G_1 peak) of the machine using linear scale. The ploidy levels of the samples were determined by comparison with these reference 2C DNA values. During analyses, samples of *P. aphrodite* var. *formosana* (diploid) were regularly run, in order to check the stability of peak position corresponding to the diploid level. Results were displayed in the histograms showing number of nuclei according to relative fluorescence intensity, which is proportional to DNA content. Diploids progenies showed 2C peak at 50 (G_0/G_1 peak) of the machine using linear scale and tetraploid progenies will show first peak position on 100 of the machine. In each sample the DNA content of at least 5,000 nuclei was analyzed and percentage of ploidy levels of progenies was calculated based on at least 20 observations of each treatment.

RESULTS

Some progenies from the crossing of untreated parents with N_2O–treated parents and *P. aphrodite* var. *formosana* as internal standards exhibited G_0/G_1 peak at the same channel, indicating that these progenies are diploid (Fig. 1). The flow cytometry histograms of the diploid progenies (Figs. 2B and 3B) were clearly different from those of other progenies, and they were considered to be triploid (Fig. 2C) and tetraploid (Figs. 2D and 3C). The ratio of genome values in the first peak of diploid: triploid: tetraploid progenies was about 1 : 1.5 : 2.0, which indicates that the genome sizes of triploid and tetraploid progenies were about 1.5– and 2–fold bigger than those of diploid progenies, respectively (Figs. 2 and 3).

N_2O treatment for 24 hrs induced triploid (36%) and tetraploid (7%), whereas that for 48 hrs induced only 5% tetraploid (Table 1).

The chromosome observation confirmed the ploidies of the progenies. Chromosome numbers of the diploid, triploid and tetraploid progenies were 2n=2x=38, 2n=3x=57 and 2n=4x=76, respectively (Fig. 4).

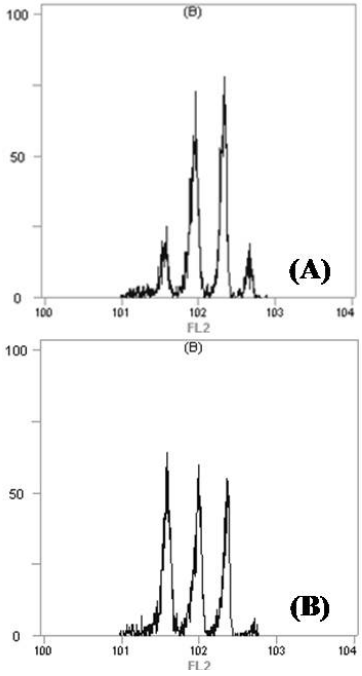

Fig. 1. Flow cytometry histograms of a diploid *P. aphrodite* var. *formosana* (A) and progenies of untreated *P. amabilis* crossed with 24 h N_2O-treated *P. amabilis* (B).

Table 1. Effects of N_2O on ploidy levels in the progenies of untreated × treated *P. amabilis*

N_2O treatment (hr)	No. of plantlets examined	% ploidy (No.)		
		Diploid	Triploid	Tetraploid
0	20	100 (20)		
24	45	57.8 (26)	35.6 (16)	6.7 (3)
48	20	95.0 (19)		5.0 (1)

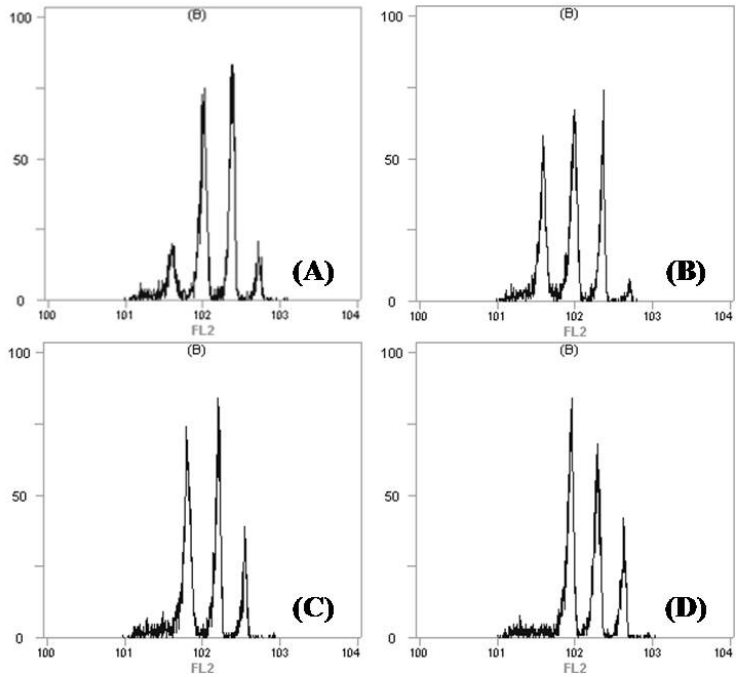

Fig. 2. Flow cytometry histograms of the progenies of untreated *P. amabilis* crossed with 24 hrs N_2O-treated *P. amabilis*. (A) Untreated parent, (B) diploid progenies, (C) triploid progenies and (D) tetraploid progenies.

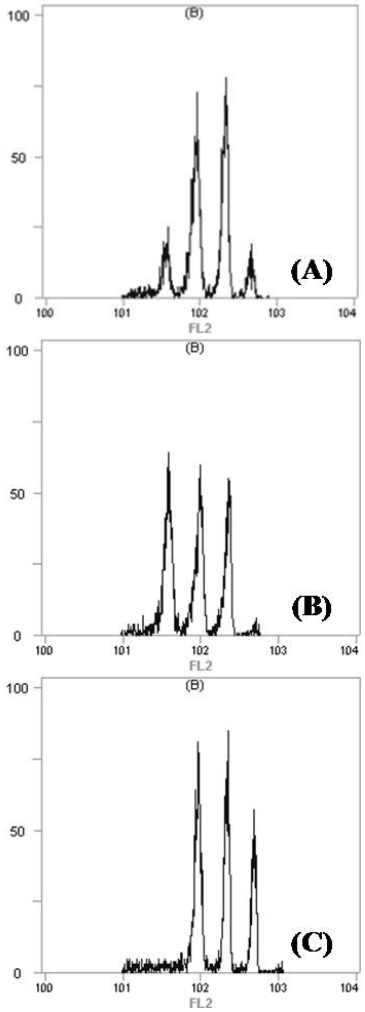

Fig. 3. Flow cytometry histograms of the progenies of untreated *P. amabilis* crossed with 48 hrs N_2O-treated *P. amabilis*. (A) Untreated parent, (B) diploid progenies and (C) tetraploid progenies.

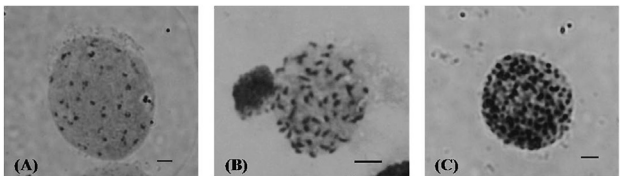

Fig. 4. Chromosomes of root tip cells of (A) diploid (2n=2x=38) (B) triploid (2n=3x=57) and (C) tetraploid (2n=4x=76) progenies. bar=10 μm.

DISCUSSION

Polyploidy induction has been successfully applied in ornamental, vegetable and medicinal plants by polyploidizing agents such as colchicines, oryzalin, trifluralin and N_2O (Petersen *et al*., 2003; Thao *et al*., 2003, 2004; Rubuluza *et al*., 2007; Praça *et al*., 2009). N_2O has been used previously in tulips (Okazaki *et al*., 2005), lilies (Barba–Gonzalez *et al*., 2006; Akutsu *et al*., 2007) and *Begonia* (Dewitte *et al*., 2010) for polyploidy breeding by inducing unreduced gametes and to restore fertility in sterile genotypes.

Our results showed that N_2O treatments are effective methods to induce polyploidy in *P. amabilis*. The histrograms in Figs. 2C, 2D and 3C are representative fluorescence profiles for nuclei from the diploid, triploid and tetraploid plants, which are similar to the results of Petersen *et al*. (2003), Thao *et al*. (2003, 2004) and Rubuluza *et al*. (2007). Our results indicate that the procedure was adequate for induction polyploidy P. amabilis plantlets. Induction of tetraploid progenies in this study suggests that the plant also produced 2n eggs in the crossings without N_2O treatment. The agreement of the results of chromosome count in root tips and flow cytometric values demonstrates the reliability of flow

cytrometry as a practical and rapid tool for screening the ploidy level of the progenies as shown by Galbraith *et al*. (1983) and Thao *et al*. (2004), since a plant can be analyzed when the first leaf appears in vitro before acclimatization to greenhouses.

In conclusion, successful induction of polyploids in *P. amabilis* confirmed the effectiveness by N_2O as a polyploidizing agent. The polyploids that we obtained, will be used in further breeding works.

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