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Changes in the endopolyploidy pattern of different tissues in diploid and tetraploid *Phalaenopsis aphrodite* subsp. *formosana* (Orchidaceae)

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ABSTRACT

Endopolyploidy is frequently observed during development in plant species. Patterns of endopolyploidy are diverse in the various organs of different plant species. However, little is known about the role of endopolyploidization and its significance in orchids. This study was undertaken to determine the extent of endopolyploidy in different tissues of the diploid and tetraploid genotypes of *Phalaenopsis aphrodite* subsp. *formosana* and to examine the factors that contribute to increased ploidy levels. Endopolyploidy occurs in various tissues of diploid and tetraploid orchids, at different developmental stages and under different culture conditions, as determined by flow cytometry. In this study, different patterns of endopolyploidy were observed in parts of the protocorms, leaves, roots and flowers. Endopolyploidy was found in all tissues studied except the pollinia and the tetraploid ovaries. A higher degree of endopolyploidy was observed in mature tissues compared to young tissues, greenhouse-grown plants compared to *in vitro* plants and diploid plants compared to tetraploid plants. We discuss the relationships between endopolyploidization and several factors related to plant growth, as well as some practical considerations of these findings.

1. Introduction

Endopolyploidy is the existence of cells of different ploidy levels within tissues of a species due to either endoreduplication or endomitosis [1–4]. It is frequently observed in plants, concomitant with development [2,5]. Endopolyploidy occurs commonly in angiosperms and is more common in species with a relatively small genome size. Even within species, endopolyploidy is organ-specific [1,2]. A high degree of endopolyploidy has been detected in the mature organs of *Arabidopsis* [6] and other plant species [2]. The pattern of endopolyploidy is usually expressed as variation of the frequencies of cells having different nuclear DNA contents, which can be determined by flow cytometry. Generally, mixtures of endopolyploid cells with different DNA contents, ranging from 4C to 64C (where C represents the nuclear DNA content of gametes) [2] were observed. However, extremely high levels of endopolyploidy have been reported in different plants species [2,7,8]. Endopolyploidy is commonly generated by endoreduplication, a process in which DNA replication occurs in the absence of mitosis [2,5,8–11]. An increase in ploidy levels can boost metabolism, increase gene redundancy, improve crop yield and quality [12], accelerate growth and enhance physiological functions [2].

Orchidaceae is one of the most variable angiosperm families with respect to genome sizes [13]. Diverse endopolyploidy patterns have been observed in different tissues of several genera and species of Orchidaceae, where endopolyploidization is concurrent with maturation or aging [14–19]. In *Vanda* Miss Joaquim, distinctive endopolyploidy patterns were detected in terrestrial and aerial roots under various stages of differentiation [16]. Patterns of endopolyploidy even differ within species of Orchidaceae. For example, nuclei with DNA contents ranging from 2C to 8C were found in the basal part of leaves and root tips of *Spathoglottis plicata* [18], but no endopolyploidy has been observed in root segments with root hairs. Thus, the patterns of endopolyploidy in the root sections of different species of orchids require further characterisation. Endoreduplication is a factor that contributes to cell growth during the development of flowers in several orchid species because the fresh weight of cells is positively correlated to the endopolyploidy level [17]. Because the endoreduplication transition rate can be reduced by low temperatures [19], a compensation-balance may exist between endoreduplication and growth rates. Therefore, the role of endoreduplication in plant development and how it is affected by environmental factors are interesting issues that need to be addressed.

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Comparisons of endopolyploidy patterns in plants of different ploidy states have been made in several plant species. In four organs of tomatoes, a similar pattern of endopolyploidy was found in diploid and tetraploid plants [20]. Conversely, a higher degree of endopolyploidy was observed in diploid maize [21] and portulaca [22] than those in tetraploids. Little is known about the variation in endopolyploidy at different developmental stages or ploidy states, including diploid and tetraploid, among the species of Orchidaceae. The goals of this study were to determine the extent of endopolyploidy in different tissues of the diploid and tetraploid genotypes at various developmental stages in *Phalaenopsis aphrodite* subsp. *formosana* and to examine the factors that cause an increase in ploidy levels.

2. Materials and methods

2.1. Plant materials and growth conditions

Greenhouse-grown diploid (2x, provided by Pony Orchids, Pingtung, Taiwan) and tetraploid (4x, purchased from Taiwan Sugar Corporation, Tainan, Taiwan) *P. aphrodite* subsp. *formosana* (Miwa) E.A. Christ individuals were used in this study. The chromosome number of this species is $2n = 2x = 38$. The tetraploid plant used in this study originated from an autotetraploid line that was discovered in the 1970s [23]. Two-year-old plants were grown in pots in the growing room at $22 \pm 2\,^{\circ}C$ and 16-h light/8-h dark photoperiod with light irradiance of 60 $\mu mol\ m^{-2}\ s^{-1}$ provided by white fluorescent light tubes (FL40D, TOA Lighting, Taiwan) to induce flowering and the production of capsules. To produce *in vitro* seedlings, seeds obtained by self-crossing the above plants were surface sterilised with a 5% (v/v) Clorox^{TM} solution for 7 min. Sterilised seeds were rinsed three times with autoclaved distilled water and germinated in 9 cm Petri dishes containing a modified Murashige and Skoog (MS) medium (including MS salts 1.1 $g\,L^{-1}$, tryptone 1.0 $g\,L^{-1}$, sucrose 20 $g\,L^{-1}$, homogenised potato 6.5 $g\,L^{-1}$ and agar (Plant TC Grade, Phyto Technology Laboratories, Shawnee Mission, KS, USA) 8.5 $g\,L^{-1}$; pH 5.8). They were then placed in the culture room at $25 \pm 2\,^{\circ}C$ and 16-h light/8-h dark photoperiod with a light irradiance of 35 $\mu mol\ m^{-2}\ s^{-1}$ provided by white florescent light tubes (FL40D, TOA Lighting, Taiwan). After 3 months, the developed protocorms were transferred to Petri dishes containing T2 medium (Hyponex No. 1 3.5 $g\,L^{-1}$, tryptone 1.0 $g\,L^{-1}$, citric acid 0.1 $g\,L^{-1}$, activated charcoal 1 $g\,L^{-1}$, sucrose 20 $g\,L^{-1}$, homogenised potato 20 $g\,L^{-1}$, homogenised banana 25 $g\,L^{-1}$ and agar 7.5 $g\,L^{-1}$; pH 5.5) until seedlings had developed three to four leaves.

Different plant parts were used in the flow cytometric analysis, including the middle portion of leaves of 2-year-old plants and the sepals, petals, lips, columns, pollinia, pedicels and ovaries of fully opened flowers before pollination. Segments of roots approximately 2-mm long were cut from the actively growing root tip. For tissue-cultured materials, the apical meristem-containing segments and the basal segments of the 2-month-old protocorms were used. Tissues from the middle portion of leaves (0.3–0.5 cm^2) and 2-mm segments of roots of the 6-month-old *in vitro* seedlings were also used for analysis.

2.2. Determination of chromosome number

Root tips were collected and pre-treated with 2 mM 8-hydroxyquinolin for 2 h at 15 °C. After fixing them with a 3:1 mixture of freshly prepared ethanol and glacial acetic acid overnight, the samples were washed twice with distilled water and then rinsed with citrate buffer (4 mM citric acid and 6 mM sodium citrate; pH 4.5). They were then incubated in an enzyme mixture consisting of 3% (w/v) peptinase (Sigma, St. Louis, USA) and 3% (w/v) cellulase (Yakult Honsha, Tokyo, Japan) in citrate buffer for 2 h at 37 °C. Afterwards, the samples were rinsed twice with distilled water, squashed onto slides in 55% acetic acid, stained with 4′-6′-diamidino-2-phenylindole (DAPI) (Partec, Münster, Germany) in an antifade reagent (Molecular Probes, Eugene, USA) and observed under a fluorescence microscope.

2.3. Preparation of nuclear suspension and determination of DNA content by flow cytometry

A PA-I flow cytometer (Partec, Münster, Germany) equipped with a HBO-100 mercury lamp was used to determine relative fluorescence intensity of intact nuclei isolated from different plant tissues. Samples were prepared according to the manual of the Partec Cystain UV Precise P kit, which included the extraction and staining buffers. A sharp razor blade was used to chop fresh tissue (10–40 mg) into pieces with a size < 1 mm in a 6 cm glass Petri dish containing 100 μL of extracting buffer (solution A of the Cystain UV precise P kit for plant DNA, Partec, Münster, Germany). Then 400 μL of staining buffer (DAPI, solution B of the kit) was added, and the tissue was filtered through a 30-μm nylon mesh. The process of nuclear extraction and staining was carried out on ice. From each sample, 5000–10,000 particles were analysed on a linear scale using the flow cytometer. Histograms were analysed using the Partec FloMax software and the percentages of the nuclei of different peaks were calculated. These peaks represent nuclei of different ploidy levels. For each experiment, samples of young leaves from the *in vitro* plantlets of diploid *P. aphrodite* were used to adjust the gain of the flow cytometer so that the first G_0/G_1 peak was positioned approximately at channel 50 on a 1000 channel scale, representing the 2C DNA content of this species. Then all other samples were analysed relative to the reference leaf sample. The nuclear DNA content of diploid *P. aphrodite* is 2.80 pg $2C^{-1}$ [15].

Five to 15 samples from each developmental stage of the greenhouse-grown plants and 10–30 samples of *in vitro* plants were subjected to flow cytometry. The means and standard deviations of the percentage of the total amount of nuclei in all peaks of the histogram were calculated and analysed. In this study, three parameters were used to estimate the degree of endopoly-

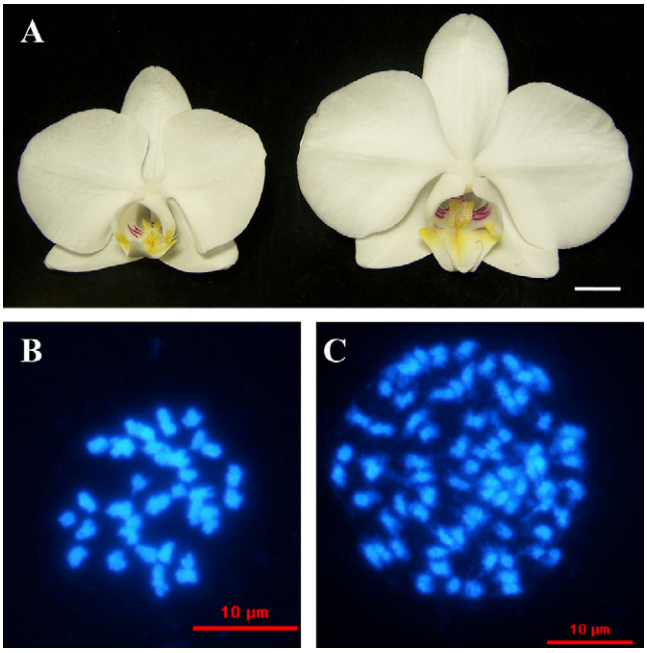

Fig. 1. Flowers and chromosomes of *P. aphrodite* subsp. *formosana*: (A) flowers of diploid (left) and tetraploid (right) (bar = 1 cm), (B) chromosomes of diploid individual, (C) chromosomes of tetraploid individual.