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ORIGINAL ARTICLE

Endoreduplication in *Phalaenopsis* is affected by light quality from light-emitting diodes during somatic embryogenesis

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Abstract Endoreduplication is a developmental process that is unique to plants and occurs in all plants. The present study aimed to assess endoreduplication in various explant tissues and regenerated somatic embryos of *Doritaenopsis*. We further investigated the effects of light quality on endoreduplication and somatic embryo proliferation. To this end, we studied endoreduplication in leaves and root tips from regenerated plantlets and somatic embryos and in developing somatic embryos under 4 types of lighting conditions: red light, red + far-red light, red + blue light, and white light. We found that the degree of endoreduplication varied in different explants, and that the choice of explants used also influenced the ploidy levels of the newly regenerated somatic embryos. The DNA content of the leaf (2C–8C) was less than that of the root tip (2C–16C) and somatic embryo (2C–64C). In terms of light quality, the combination of red and far-red light produced the highest number of somatic embryos, while maintaining a low degree of endoreduplication. The data obtained indicate that this light combination stimulates somatic embryogenesis in *Doritaenopsis* and may exert some control on endoreduplication during cell division. These findings can be applied to achieve a reduction in somaclonal variations for the purpose of mass proliferation and genetic improvement.

Keywords *Doritaenopsis* · Somatic embryo · Endoreduplication · Light quality · Somaclonal variation

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Introduction

Endopolyploidy is common in plants, occurring in about 90% of the angiosperm species (Joubes and Chevalier 2000). This unique process is an important component of organ development and is often related to plant cell size (Larkins et al. 2001; Sugimoto-Shiraru and Roberts 2003). Endoreduplication was first detected as a polyploid mitosis within the elongation zone of onion roots subjected to hormone treatment (Levan 1939). Endoreduplication level varies depending upon the species, the tissue, age of plants, and the environment (Gendreau et al. 1999). It has been reported that, in the same plant, older tissues have a higher ploidy level than younger tissues (Joubes and Chevalier 2000; Kinoshita et al. 1991; Melaragno et al. 1993). This phenomenon of having different ploidy levels within the same tissue is known as polysomaty. This situation is well documented in the endosperm and cotyledons of developing seeds (Sun et al. 1999), but also occurs in other developmental processes of plants (Chung et al. 1998; Kladnik et al. 2006). In Orchidaceae, endoreduplication was examined during the assessment of ploidy levels and nuclear DNA content (Jones and Kuehnle 1998; Jones et al. 1998). In these studies, it was found that Orchidaceae species naturally have a very high endoreduplication level compared to the levels in other plant families (Jones et al. 1998; Jones and Kuehnle 1998; Lee et al. 2004).

In recent years, the use of light-emitting diodes (LEDs) as a radiation source for plants has attracted considerable interest because of its vast potential for developmental and photomorphogenetic studies as well for its commercial applications. In plants, development is strongly influenced by light through photoreceptors known as phytochromes (which detect red and far-red light), cryptochromes (blue and UV-B), and phototropins (blue and UV-A). All these photoreceptors are involved in one or more processes of photomorphogenesis (Ascencio-Cabral et al. 2008). Light quality at different wavelengths is known to influence plant growth and development in annual plants (Heo et al. 2006; Nhut et al. 2003), but little information exists on the effects on somatic embryo processes. The few exceptions include the description of somatic embryogenesis in quince (D'Onofrio et al. 1998) and papaya (Ascencio-Cabral et al. 2008). Studies on hypocotyl cells of *Arabidopsis thaliana* have indicated that light differentially regulates the endoreduplication cycle (Gendreau et al. 1998). Nevertheless, the relationship between light quality and somatic embryogenesis, and the effect of light quality on endoreduplication occurrence remains to be determined.

In a previous study, we observed that somatic embryo cells (often referred to as "protocorm-like body" in Orchidaceae) in *Doritaenopsis* exhibit various levels (2C–32C) of polysomaty (endoreduplicated cells), which is the condition of a cell having reduplicated chromatin in its nucleus (Park and Paek 2006). In the fully developed somatic embryo of *Doritaenopsis*, the upper part contained 2C–8C, while the middle and lower parts contained 2C–32C nuclei (Park and Paek 2006). Young somatic embryos (2-week-old) contained 2C–16C, whereas older embryos (4- to 10-week-old) contained 2C–32C nuclei (Park and Paek 2006). These experimental results suggest that polysomaty in somatic embryos may be partially responsible for the frequent occurrence of undesired polyploid variants of orchid plants (Park and Paek 1999, 2006).

In this paper, we report the endoreduplication level from the various tissues that are used as an explant to induce somatic embryos in *Doritaenopsis*. Furthermore, we assessed the light quality influencing somatic embryo formation and its endoreduplication level.

Materials and methods

Plant materials and culture conditions

To investigate the endoreduplication dynamics during somatic embryogenesis in *Doritaenopsis*, in vitro plantlets obtained from flower stalk cultures of *Doritaenopsis* "Happy Valentine" were used (Park et al. 2002). Plantlets derived from flower stalks were cultured in mHyponex medium (6.5:6.5:19 = N:P:K) supplemented with 2 g l^{-1} peptone, 3% (w/v) potato homogenate, 0.5% activated charcoal, and 30 g l^{-1} sucrose for rooting. The youngest leaf was dissected from the plantlet when 2 or 3 leaves had developed. Five to six thin leaf sections (0.5–1 mm thick) were taken from the leaf and cultured onto somatic embryo induction medium following the protocol described by Park et al. (2002). The pH of the medium was adjusted to 5.5 and all media were autoclaved for 20 min at 115°C (1.37×10^5 Pa). Cultures were incubated in a tissue culture room maintained at 25° ± 1°C under a 16-h photoperiod with light from cool white fluorescent lamps (Kumho FL40D, Korea) at 30–40 μmol m^{-2} s^{-1} photosynthetic photon flux (PPF). After 2 months of culture, somatic embryos were formed from leaf sections and used for the LED experiment. Endoreduplication was analyzed from the young leaf, root, and somatic embryo.

LED system

The LED system (GF-320s; Good Feeling, Korea) used in this study comprised of LED sticks, a panel, and a main controller for light intensity. In the first experiment, cultures were placed either under various 1:1 combinations of radiation (RB, red + blue; RFr, red + far-red) from the LEDs, red (R), cool white fluorescent lamps (Kumho FL40D) (F), or dark (D) as the control. In the second experiment, cultures were placed either under F, R, 1R + 1Fr, or 1R + 2Fr conditions. The energy ratio (%) in spectral distributions of LED used in the experiment was 1:1 or 1:2 in mixed radiations. As spectral energy sources, the emission of blue LED was 440 nm, red was at 650 nm, and far-red was at 730 nm. All cultures were maintained for 4 weeks in a growth chamber at 24 ± 1°C, with a PPF of 40 ± 10 μmol m^{-2} s^{-1} and a 16-h photoperiod.

Endoreduplication analysis

A fresh sample of 100 mg was taken from each type of tissue. Samples were examined by Ploidy Analyzer (Partec, Münster, Germany). The samples were chopped with a sharp razor blade in nuclei extraction buffer (solution A of the high resolution kit for Plant DNA; Partec). After filtration through a 30-μm nylon sieve, 2.0 ml staining solution containing the dye 4,6-diamidino-2-phenylindole-2HCl (solution B of the kit) was added. Analyses were performed with a PA flow cytometer (Partec) and data were processed by DPAC software (Partec). In each sample, a minimum of 3,000 particles (total count) were analyzed. To determine the standard peak position of 2C cells, the 1C peak was determined from the nuclei of pollinia from the flowers of mother plants grown in a greenhouse (at least 3 replications for each measurement). Data were plotted on a

semi-logarithmic scale so that the histogram peaks from 2C to 64C were evenly distributed along the abscissa. The data are presented as percentage of the total number of nuclei in all peaks of the histogram.

Light microscopy observation

For histological observations, samples collected during different culture periods were fixed in a solution containing 2.5% glutaraldehyde and 1.6% paraformaldehyde buffered with 0.05 M phosphate buffer, pH 6.8, for 24 h at 4°C. Samples were dehydrated in an alcohol series and then embedded in Technovit 7100 (Kulzer, Germany) according to the methods described by Yeung (1999). Serial 3 µm sections were cut with glass knives on a Reichert-Jung 2040 Autocut rotary microtome. The sections were stained with Periodic acid Schiff's reaction for total carbohydrates and counterstained with amido black 10B for proteins or toluidine blue O for general histological organization (Yeung 1999). The sections were examined and photographed using a Leica Aristoplan light microscope.

Results and discussion

Endoreduplication dynamics in various explant tissues

In this study, 3 tissues, including the leaf, root tip, and somatic embryo, which are used as a material for somatic embryo induction and proliferation, were sampled and analyzed to investigate their endoreduplication level. Young leaves, root tips, and somatic embryos of the *Doritaenopsis* hybrid had 2C–8C, 2C–16C, and 2C–64C nuclei, respectively (Fig. 1). Nuclei isolated from leaves, root tips, and somatic embryos appeared to have different degrees of endoreduplication, and all tissues contained mainly 4C cells, more so than 2C or 8C cells. A cell with 4C content can divide into two 2C daughter cells or it can

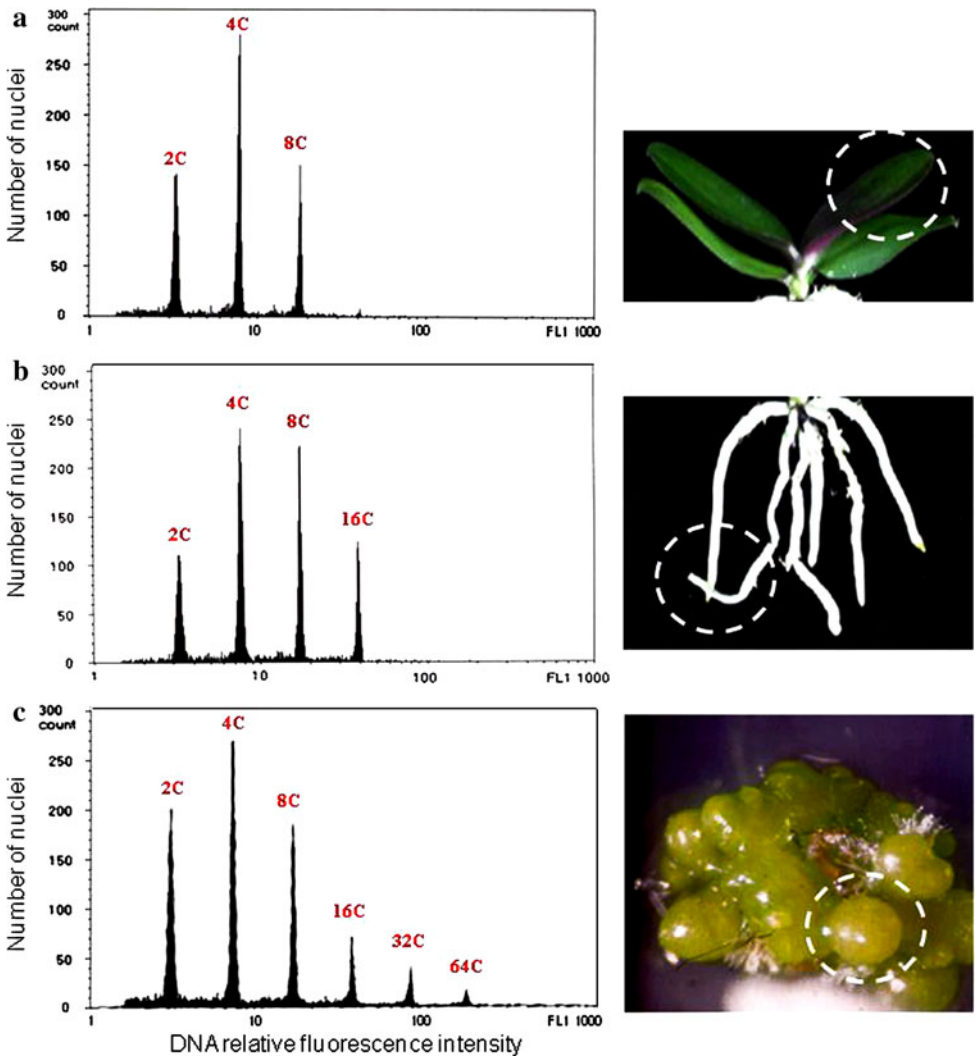

Fig. 1 Endoreduplication levels in the tissues of *Doritaenopsis* hybrid. Young leaf showing 2C–8C DNA contents in the cells (**a**), 2C–16C DNA contents in the root tip (**b**), and 2C–64C DNA content in the somatic embryo (**c**)

continue into the endoreduplication cycle. Unfortunately, the endoreduplicated 4C cells cannot be discriminated from the S phase DNA content of a normal cell cycle by flow cytometry.

Doritaenopsis, including the *Phalaenopsis* genus, is clonally proliferated in two ways, namely, by adventitious shoots or by somatic embryos (Arditti and Ernst 1993). Proliferation rate is higher via multiplication by somatic embryos (Park et al. 2000). For inducing somatic embryos, the selection of explants is considered important, as it affects the proliferation efficiency and variant occurrence (Park and Paek 2006).

In this study, we have shown that the leaf contains a lower endoreduplication level than the root tip or the somatic embryo, and we have indicated that the leaf is the best material to induce somatic embryos. For repetitive multiplication of somatic embryos, we cannot avoid using somatic embryos as material. However, in this study, the somatic embryo showed the highest endoreduplication level, 2C–64C, and based on this result, we suggest that it is better to reduce the number of multiplication times and to frequently renew the somatic embryos by leaf section culture to decrease the frequency of polyploid variant occurrence.

Several possible origins of somaclonal variations have been suggested (Bouman and De Klerk 1997). Along with several other factors, such as growth regulators, the culture period, and other cultural conditions in vitro, genetic traits of the plant species are one of the reasons for the induction of somaclonal variation (Pierik 1987). In many cases, changes of programmed DNA in the mother plant, in particular endopolyploidy, provide a chance for variation during in vitro proliferation. Polysomaty (due to endoreduplication) is a further possible reason for variation (Bouman and De Klerk 1997; Pierik 1987). If embryogenic cell division takes place from polyploid cells in the explant tissue, and if it becomes dominant over the diploid cells during successive subculture, regenerants become polyploids (Bouman and De Klerk 1997; Pierik 1987). The relationship between the endoreduplication level of the explant and polyploid variant occurrence has been reported by many researchers (Bouman and De Klerk 1997; Park and Paek 2006; Tokuhara and Mii 2001).

Among the tissues tested in our study, the somatic embryo of *Doritaenopsis* showed the highest levels of endoreduplication from 2C to 64C nuclei. Conversely, the embryos exhibit only 2C in the ploidy pattern of pakchoi, the Chinese cabbage, and the tulip, while in cotyledons, the hypocotyls and leaf show various ploidy patterns from 2C to 64C (Kudo and Kimura 2001). In our previous report on *Doritaenopsis*, the top part of the somatic embryo contained a lower endoreduplication level than the middle and lower part (Park and Paek 2006). It is assumed that the meristem is the germ line, that is, cells that duplicate carefully, so generally meristem cells are 2C (Bouman and De Klerk 1997), while the cells on the outside of the meristem contain various ploidy levels (De Veylder et al. 2002). In this study, we easily identified polyploid cells by their cell size and the size of the nucleus (Fig. 2). The epidermal cells had small size nucleus, but endoreduplicated cells, found in the cortex of the mature somatic embryo, had a large nucleus (Fig. 2). This observation supports the finding that endoreduplication of *Orchidaceae* is comparatively high, but variable depending upon tissue type, age, and location in the same plant.

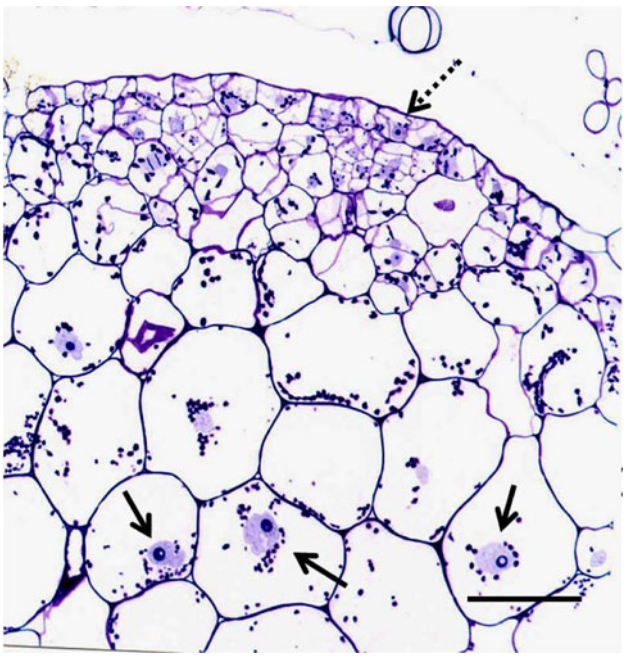

Fig. 2 Polysomaty occurrence during somatic embryo aging in *Doritaenopsis* hybrid. 2C cells in epidermal layers (*broken line arrow*) and polysomatic cells (*solid line arrows*) inside 2-week-old somatic embryo (*scale bar* 200 μm)

In general, endoreduplicated cells have been restricted to specific cell types that are highly specialized and usually large, such as root hairs, raphide crystal idioblasts, and embryo suspensors (Kudo and Kimura 2001). In most plants, endoreduplicated cells (≥4C) are relatively few compared to 2C nuclei of cells, e.g., in the onion (Bohanec and Jakse 1999), the oil palm (Rival et al. 1997), the *Rhododendron* (De Schepper et al. 2001), and the Chinese foxgloves (Park and Paek 1999). Endoreduplication in orchid species has been studied to identify ploidy level (Jones and Kuehnle 1998; Jones et al. 1998) and nuclear DNA content (De Schepper et al. 2001). In these studies, it was found that Orchidaceae species have a high endoreduplication level compared to other plant families.

Light quality effect on somatic embryo formation

In the first experiment, we investigated the effects of light quality on somatic embryo proliferation using 4 types of light, i.e., F, R, 1R + 1Fr, 1R + 1B, and D. All light conditions positively stimulated the formation of secondary somatic embryos from the embryo section used as an explant (Fig. 3) and resulted in 2 times higher somatic embryo formation than that induced by the dark condition. Interestingly, the combination of red and far-red light (1R + 1Fr) produced the highest number of somatic embryos compared to the other light treatments. By visual observation, somatic embryos formed under 1R + 1Fr were small in size, but many in number, while those induced by fluorescent light were bigger, but fewer in number.

Similar results have been reported for petal cultures of *Araujia sericifera*, where somatic embryo induction by Fr followed by R resulted in an increased number of somatic embryos (Torne et al. 2001). This finding is in contrast to observations in quince leaf culture in which somatic embryo induction was less than half in Fr that observed under monochrome R.

Far-red light is light at the extreme red end of the visible spectrum, between red and infra-red light, usually regarded as the region between 700 and 800 nm wavelengths. Many researchers have tested the effects of mixed light, e.g., red plus far-red and blue plus far-red on somatic embryo formation (Lian et al. 2006; Shohael et al. 2006). The effect of light quality on in vitro regeneration is quite controversial. For example, red light stimulated somatic embryo induction from quince (D'Onofrio et al. 1998), but not clearly in *Eleutherococcus senticosus* (Shohael et al. 2006). In other studies, shoot regeneration was decreased by red light in the lily (Lian et al. 2006), but increased in *Actinidia deliciosa* (Muleo and Morini 1990).

Based on these reports, the light at a specific wavelength plays a different role in the regeneration of various species. In this study, monochrome R did not promote somatic embryo formation in *Doritaenopsis*, but when combined with Fr, the number of somatic embryos per explant was remarkably increased. As far as we know, this study is one of the first to provide direct evidence for the effect of Fr light on somatic embryo regeneration.

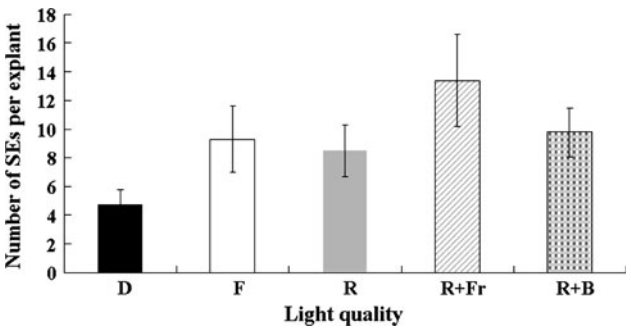

Fig. 3 Effect of light quality on somatic embryo formation from leaf segments in *Doritaenopsis* hybrid after 4 weeks of culture (mean ± SE)

In *Eleutherococcus senticosus*, red light-irradiated embryos produced high levels of the antioxidant enzymes monodehydroascorbate reductase, catalase, glutathione *S* transferase, and superoxide dismutase, which play an important role for the detoxification of harmful substances (Shohael et al. 2006). From the results of this study, we suggest that R and Fr might exert a synergetic effect on somatic embryo induction, caused by the stimulation of Fr on somatic embryo formation and the detoxification of oxidative stress caused by in vitro conditions by R, which may have resulted in an overall increase in somatic embryo production.

SE endoreduplication affected by light quality

After observing the positive effect of R + Fr irradiation on somatic embryo formation, we cultured the somatic embryo segments under 4 types of light quality, i.e., F, R, 1R + 1Fr, and 1R + 2Fr, with more focus on R and Fr light than in previous experiments.

Before culturing under various light qualities, endoreduplication of SEs that were used as explants varied from 2C to 16C; many cells contained 4C (23.5%) in their DNA content and even 16C was evident in 14% of the cells (Fig. 4). After 2 weeks of culture, secondary somatic embryos started to form on the surface of the explant somatic embryo. Endoreduplication of somatic embryos cultured under R and 1R + 1Fr irradiation showed high 2C and 4C cells compared to F and 1R + 2Fr treatments (Fig. 4b). After 4 weeks of culture, interestingly, the ratio of 2C and 4C cells was decreased in almost all treatment groups compared to that of 2 weeks endoreduplication. We postulate that most of the cells were in the G1/S phase of the cell cycle, because somatic embryos were newly formed at the time, resulting in high 2C and 4C cells. At 4 weeks, endoreduplication was high in F and R groups, with decreased 2C and increased 4C–16C cells, whereas the occurrence of 2C cells was still high in the 1R + 1Fr group (Fig. 4c). In the *Doritaenopsis* somatic embryo, epidermis cells are mainly 2C and the occurrence of 2C cells is higher in young somatic embryos than older ones (Park and Paek 2006). This fact explains that, in our study, many of the secondary embryos were formed under 1R + 1Fr conditions, while few somatic embryos were induced and aged during F, R and 1R + 2Fr culture.

The number of endoreduplication cycles appears to be under the control of developmental conditions and environmental conditions such as light (Gendreau et al. 1998; Lee et al. 2004, 2007). It is well known that

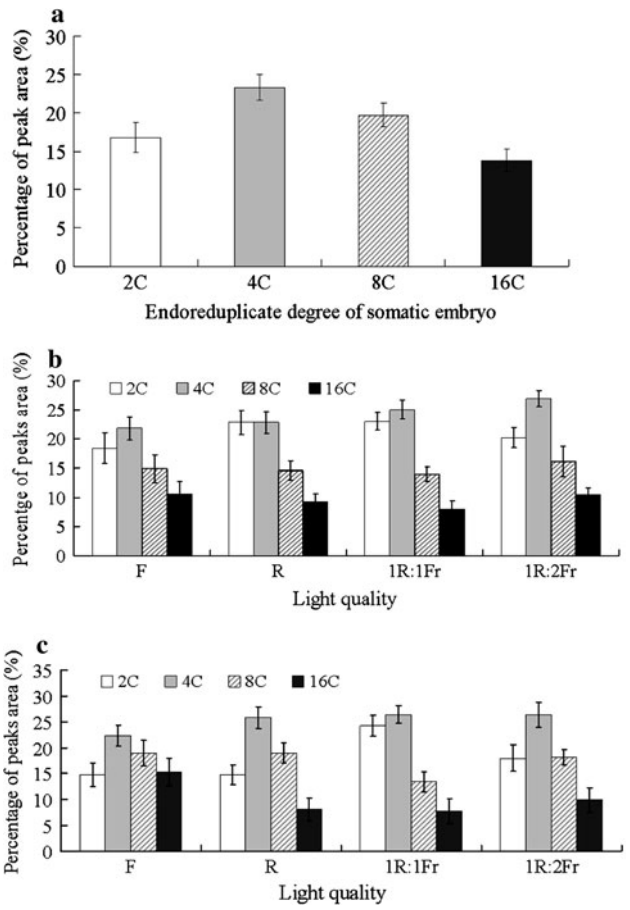

Fig. 4 Changes of endoreduplication levels during SE development under various light qualities in of *Doritaenopsis* hybrid. Endoreduplication of fully developed somatic embryo (4-week-old) under fluorescent lamp before subculturing (**a**), endoreduplication levels in somatic embryos after 2 weeks of subculture (**b**), and after 4 weeks of subculture (**c**)

endoreduplication is involved in cell extension, e.g., cell size (Sugimoto-Shiraru and Roberts 2003). In an annual plant seedling study, Fr light inhibited leaf extension (Heo et al. 2006). In our study, the balanced R and Fr combination appeared to stimulate embryogenic cell division and inhibit cell extension, resulting in high 2C and low 8C and 16C values, a change that is ideally suited to avoiding somaclonal variation.

Gendreau et al. (1998) reported that light limits both cell size and endoreduplication. High endoreduplication is correlated with organ development and cell extension. In the orchid flower, low temperature reduced the rate of progression of nuclei from lower C value to higher C value, but extended the time for organ development (Lee et al. 2007). Once the organ is fully developed, the level of endoreduplication does not increase further (Lee et al. 2007).

The findings of the present study will help obtain a high number of somatic embryos with low levels of endoreduplication, which may reduce somaclonal variation in a short period of time for the purposes of mass proliferation and genetic improvement.

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