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Morphological and Stomatal Guard Cell Characteristics of *in vitro* *Kaempferia rotunda* L. (Zingiberaceae) through Colchicine Induced Polyploidy

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Abstract

Kaempferia rotunda L. was introduced to plant improvement programs by polyploidy induction for developing new ornamental gingers. In order to improve some characteristics of *K. rotunda*, tetraploid plants were induced by *in vitro* culturing young shoots of *K. rotunda* on gelrite modified MS medium supplemented with 0.2 % colchicine for 4 days. After incubation, all of the explants were transferred to culture on shoot multiplication medium and subcultured until M_1V_4 generation for chimera segregation. Ploidy determinations were made by flow cytometry analysis. Stomatal guard cell size, leaf thickness, and chloroplast numbers in the stomata of polyploid plants were significantly increased when compared with diploid plants. However, difference of stomatal index was not found in all polyploid plants. Two solid lines of tetraploid plants, normal green and variegated dwarf, were generated. The leaves of the normal green group were thicker and wider than those of diploid plants. In contrast, the variegated dwarf group showed smaller leaves with chlorophyll mutation.

Keywords: Colchicine, flow cytometry, tetraploid, variegated leaf, plant improvement

Introduction

Kaempferia rotunda is a perennial herb which is widely distributed in tropical Asia [1]. Young leaves of *K. rotunda* are used as a vegetable [2], and rhizomes are used for curing stomachache, nausea, and dyspepsia in Ayurvedic medicine [3]. Rhizome extracts of *K. rotunda* are composed of several constituents, such as benzoate, *n*-pentadecane, camphene, and volatile oil [4]. Moreover, 2 antioxidant compounds, 2'-hydroxy-4,4'6'-trimethoxy-chalcone and (+)-crotepoxide, were also found [5]. Some species are also used as ornamental plants, such as *K. elegans* (Wall.) Baker and *K. pulchra* Ridl. [1]. With its attractive the leaf patterns and flowers, *K. rotunda* (**Figure 1**) has the potential to be developed as a new ornamental ginger after some improvement is made.

Colchicine is an alkaloid substance which has been found in the corm [6], leaf [7], and seed [8,9] of *Colchicum autumnale* L. The function of colchicine is to be an anti-mitotic spindle, by binding in specific sites on tubulin subunits. Thus, it inhibits microtubule polymerization. Since the 18th century, colchicine has been known to relieve inflammation resulting from gout [6]. In addition, colchicine has also been widely used in plant breeding to double the chromosomes, because the spontaneous autotetraploid formation rate is estimated at a low frequency [10]. This chromosome manipulation frequently increases

cell size and plant tissue thickness [11]. Previous results showed that the leaves and flowers of tetraploid plants were larger and more compact in growth habit, which are desirable characters for ornamental traits [12], than those of diploid plants. *In vitro* colchicine treatment was successful in generating many polyploid cultivars, such as *Lychnis senno* Siebold & Zucc. [13] *Anthurium andraeanum* cv. 'Arizona' [14], and *Gerbera jamesonii* 'Sciella' [15]. A few researches of tetraploid induction in Zingiberaceous plant have been reported, such as *Hedychium muluense* R.M.Sm. [16] and *Zingiber officinale* Roscoe [17]. However, the improvement of *K. rotunda* has never been reported. To the best of our knowledge, this study is the first *in vitro* tetraploid induction in *K. rotunda* by colchicine treatments which reports evidence of morphological and some anatomical changes and of chlorophyll mutation of polyploid *K. rotunda*.

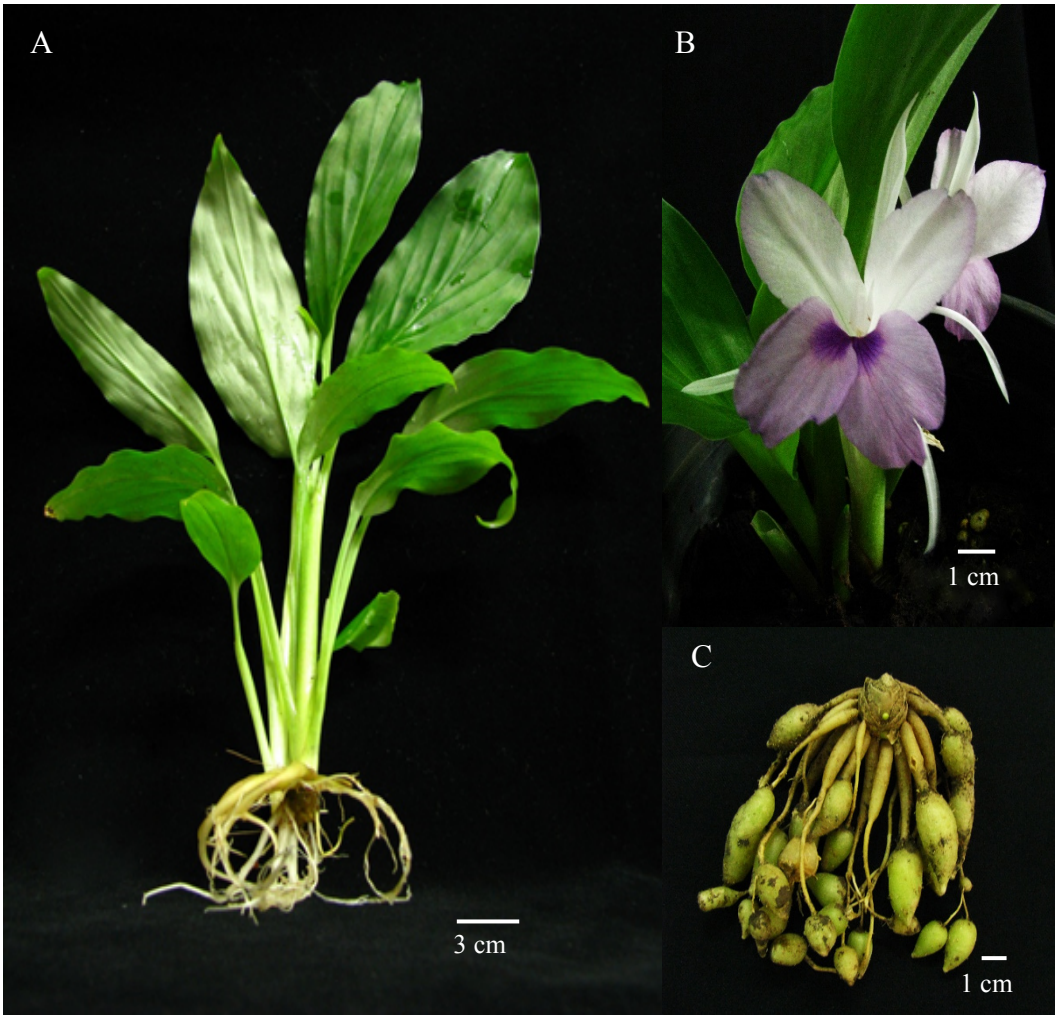

Figure 1 Various parts of *Kaempferia rotunda*; vegetative part with leaves and initial stage of storage roots (A), flowers bloomed in summer (May - June) with white lateral staminode and purplish labellum (B), and dormant rhizomes with numerous fascicle roots produced in the winter season (C).

Materials and methods

Plant materials and tetraploid induction

In vitro K. rotunda plantlets were cultured on shoot multiplication medium, which is MS medium [18] supplemented with 17.8 µM BA, 3 % sucrose, and 2 gL^{-1} Gelrite. The pH was adjusted to 5.7 - 5.8 before autoclaving. All explants were cultured at 25±2 °C under cool white fluorescent light with 37 µmol m^{-2} s^{-1} light intensity for 16 h light and 8 h dark of photoperiod. The 60 young shoots of *K. rotunda* at 1 - 1.5 cm long were cultured on solidified MS medium supplemented with 0.2 % (w/v) colchicine for 4 days in order to obtain the highest percentage of tetraploid plants [19]. All treated plantlets (M_1V_1) were subcultured monthly in shoot multiplication medium until M_1V_4 generation.

Ploidy level investigation by flow cytometry

The 2-step flow cytometry protocol [20] was used for preparing nuclei from *K. rotunda*. Fifteen *in vitro K. rotunda* plantlets of M_1V_4 generation, which originated from different lines, were randomly selected. The 1st - 3rd leaf from each shoot of 2-month-old plants were collected in aseptic conditions. About 1 cm^2 of each leaf was excised and 1 ml of cold Otto I buffer added before being chopped with a sharp razor blade in a plastic petri dish. The nuclear suspension was filtered with 46 µm pore size of nylon mesh and centrifuged at 3,000 rpm for 1 min. The supernatant was removed. Then, the precipitate was added with 100 µl of Otto I buffer at room temperature and gently shaken before being incubated for 15 min at 4 °C. One milliliter of Otto II buffer supplemented with RNase IIA, propidium iodide (at a final concentration of 50 ml ml^{-1}), and 2-mercaptoethanol (2 ml ml^{-1}) was added before being incubated for 30 min at room temperature. *In vitro* intact plants without colchicine treatment were represented as standard diploid plants. A minimum of 3,000 stained nuclei were recorded in each sample by using a BD Fluorescent activated cell sorter (BD FACSCalibur™) flow cytometry (Becton Dickinson, USA). The histogram of the DNA content was interpreted by the CellQuest software package (Becton Dickinson, USA).

Stomata characteristics and morphological observation

Ninety leaf samples from each ploidy level plant, including the control, were used for stomata investigation. The leaf scraping method was used for stomata determination. The fully expanded leaves at the 2nd - 4th positions from the shoot tip of M_1V_4 plants were excised. Then, the leaves were gently scraped at the abaxial surface. Data on stomatal guard cell size, stomata density, and chloroplast numbers were collected by observation with ×1,000 magnification under a light microscope. Stomatal index was determined with Royer's formula, SI (%) = [S × 100/(S+E)], where SI is the stomatal index, S is the number of stomata, and E is the number of epidermal cells [21].

Fifteen plants from each ploidy level of *in vitro Kaempferia rotunda* were randomly selected and morphological characteristics were determined. Data on leaf, shoot, and root number, plant height, leaf width, leaf thickness, and leaf and root length were collected. The percentage of variegated leaf emersion was displayed. The leaf color was determined by the color chart of The Royal Horticultural Society [22].

Statistical analysis

The average data on shoot numbers, plant height, leaf thickness, stomatal guard cell size, and density of each colchicine treatment were statistically compared using a completely randomized design (CRD) with 3 replications. Data were subjected to one-way analysis of variance (ANOVA) and the means were separated using Duncan's multiple range test (DMRT) at a 95 % confidence level.

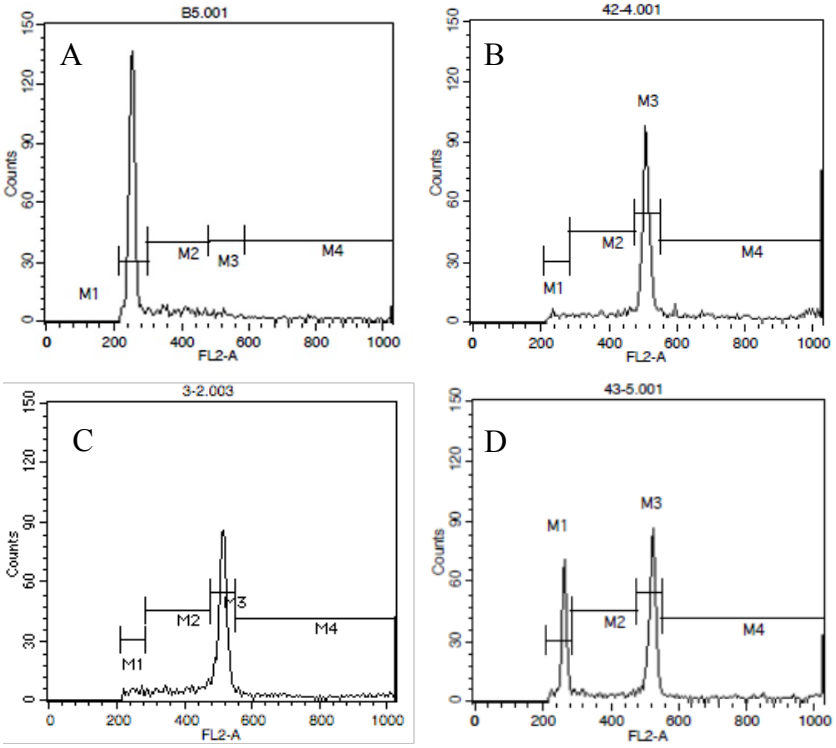

Figure 2 Flow cytometry analysis histogram of various ploidy levels; diploid (control plants) showed a single sharp peak approximate to channel 260 (A). Two groups of tetraploid plants given a single peak arose at the same position around channel 520, which were derived from normal green (B) and variegated dwarf (C) plants. Mixoploid plants showed double peaks at channels 260 and 520, and could be assumed to be ploidy chimerism plants (D).

Results and discussion

The results of the flow cytometry analysis of colchicine treated plants showed 3 groups of ploidy levels: diploid, tetraploid, and mixoploid plants. From fifteen of the colchicine-treated plants, only 3 plants showed unchanged ploidy levels and 6 plants of tetraploid and mixoploid were obtained. A histogram of diploid plants showed a single peak approximate to channel 260 (**Figure 2A**). The peak of both tetraploid groups arose at around channel 520 (**Figures 2B** and **2C**). The histogram of mixoploid plants showed double peaks, and could be assumed to be ploidy chimerism plants (**Figure 2D**).

Stomatal guard cell widths of diploid plants were significantly less than those of tetraploid and mixoploid plants. The results of stomatal guard cell length and chloroplast numbers per stomata in tetraploid and mixoploid plants were higher than in diploid plants. In contrast, the highest stomata density was found in diploid plants (**Table 1**). These characteristics would indicate that the enlargement of cells of tetraploid plants has a larger and thicker organ such as leaves or flowers as noticed in *Gaura lindheimeri* Engelm. & A.Gray [23]. Even though changes in the anatomical characteristics of stomata were found, the stomatal index of all ploidy levels of *K. rotunda* was not altered. This was in contrast to the stomatal index of tetraploid plants in *Lagerstroemia indica* L. [24] and *Centella asiatica* (L.) Urb. [25], which were less than diploid plants. The results indicated that only stomata results could not specify tetraploid plants from mixoploid plants, as found in *Citrullus lanatus* (Thunb.) Matsum. & Nakai [26]. In *K. rotunda*, it could be assumed that stomata size and stomata density could not distinguish tetraploid

plants and mixoploid plants, while chloroplast number and leaf thickness were significantly increased in polyploid plants when compared with diploid plants. The number of chloroplasts has been reported as an efficient prescreening marker to identify diploidy and polyploidy in colchicine induced poplar and black locust [27]. Increasing leaf thickness was also exhibited in tetraploid of *Lespedeza formosa* (Vogel) Koehne [28]. From these results, chloroplast numbers in stomata and leaf thickness seemed to be a good early indicator to separate diploidy and polyploidy of *K. rotunda*.

Two stable characteristics of tetraploid plants, normal green leaf plants (G) and variegated dwarf plants (VD), appeared in M_1V_3 generation. The correlated results were found in *Miscanthus sinensis* Andersson, which gave a high rate of albinism after antimitotic treatment [29]. This may be a side effect of colchicine treatments that affects chromosome [30] and chloroplast DNA abnormality [31]. The highest width and thickness were found in G plants when compared to leaves of VD and control diploid plants. The VD plants had the highest shoot proliferation. However, the lowest in plant height, leaf length, root length, and root numbers were also found (**Table 1**). A comparison of morphology of *ex vitro* plants with different ploidy level plants is shown in **Figure 3**. Generally, tetraploid plants exhibit increased height and larger organs, but some previous researches examined more robust and shorter plants, such as *Eustoma grandiflorum* 'Blue poppy' [32]. Leaf color, which was determined by an RHS color chart, showed that the green color at code number 141A is shown in the control and normal green (G) plants. The VD leaves showed many colors; the variegated part was of the yellow-green group 144A, and the green part was of the yellow-green group 145A. The color of green group 135A was found in the leaves of mixoploid plants.

Table 1 Morphological and anatomical characteristics in different ploidy levels of *Kaempferia rotunda* in M_1V_4 generation.

Characteristics	Group/Ploidy level			
	Control (2n)	Normal green (4n)	Variegated dwarf (4n)	Mixoploid (2n + 4n)
Stomatal guard cell width (μm)	22.69±0.23^{a}	28.96±0.64^{c}	29.28±0.45^{c}	25.88±1.60^{b}
Stomatal guard cell length (μm)	38.38±0.66^{a}	52.62±1.56^{b}	54.41±0.62^{b}	47.33±4.23^{b}
Stomata numbers/1 mm^2	32.60±2.00^{b}	21.70±0.80^{a}	47.40±1.61^{c}	29.79±5.35^{ab}
Chloroplast numbers/ stomatal guard cell	20.80±1.00^{a}	33.30±0.70^{b}	32.00±1.13^{b}	34.13±5.02^{b}
Stomatal index	6.87±0.40^{ns}	7.60±0.49 ^{ns}	7.91±0.30 ^{ns}	7.18±0.90 ^{ns}
Shoot numbers	1.80±0.20^{a}	2.40±0.20^{a}	12.10±0.80^{c}	4.10±0.60^{b}
Plant height (cm)	11.24±0.70^{b}	12.68±3.21^{b}	6.74±0.39^{a}	11.49±0.59^{b}
Leaf numbers	4.00±0.10^{a}	4.60±0.20^{a}	4.70±0.30^{a}	5.40±0.20^{b}
Leaf length (cm)	8.89±0.72^{b}	10.42±2.21^{b}	5.44±0.22^{a}	9.12±0.56^{b}
Leaf width (cm)	1.80±0.12^{a}	3.21±0.52^{c}	1.82±0.09^{a}	2.15±0.10^{b}
Leaf thickness (μm)	481.67±12.80^{a}	781.67±55.47^{c}	648.33±46.96^{b}	581.67±33.08^{b}
Root numbers	11.00±0.60^{bc}	12.8±0.90^{c}	5.70±0.80^{a}	9.10±0.80^{b}
Root length (cm)	4.19±0.26^{b}	4.09±0.23^{b}	2.87±0.31^{a}	3.99±0.33^{b}

Means± SE within each row with different superscript letters are significantly different at P = 0.05 (by DMRT)
ns: not significant

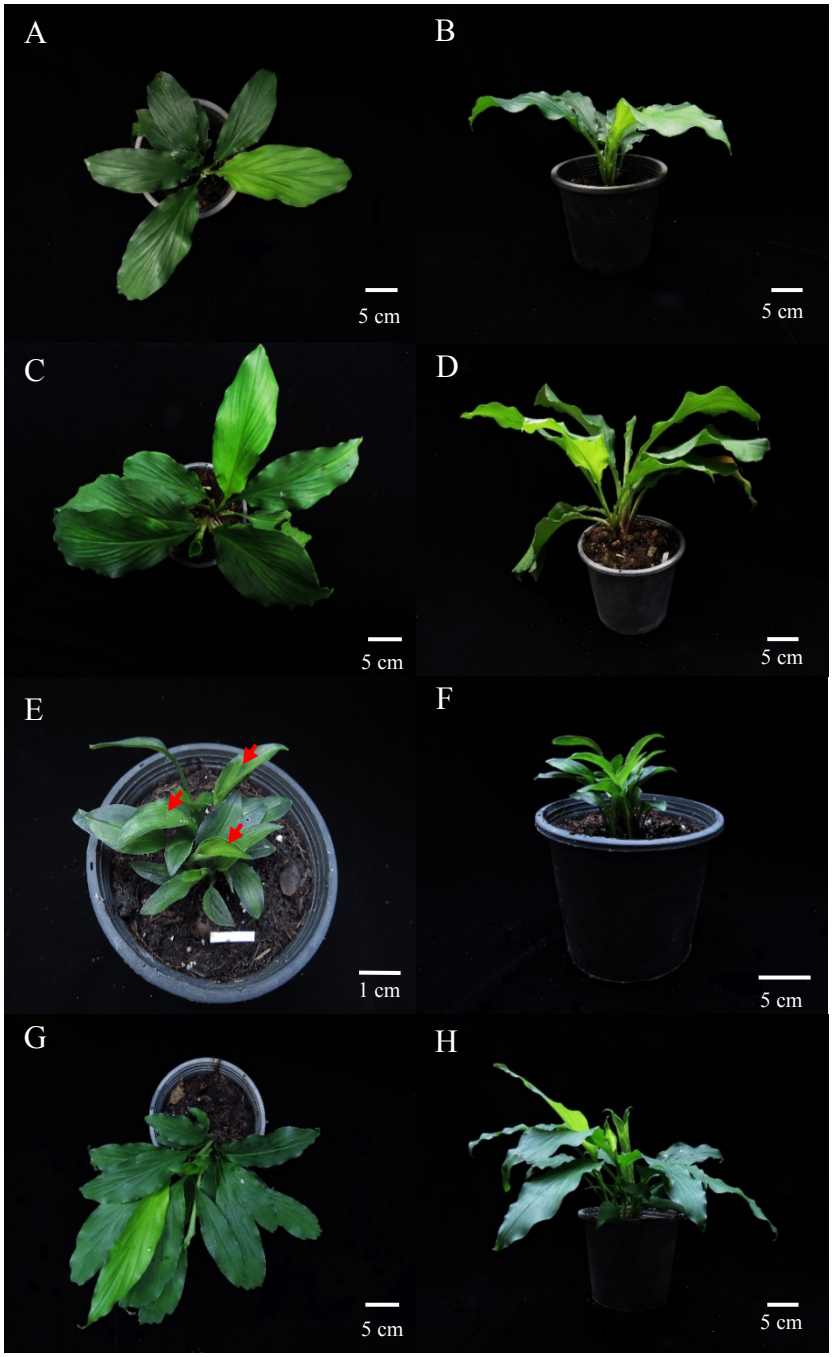

Figure 3 Morphology of 6-month-old *ex vitro* plants of diploid control plants (A-B), tetraploid normal green plants (C-D), tetraploid variegated dwarf plants (E-F), and arrow heads indicating variegated parts of leaves and mixoploid plants (G-H).

Conclusions

In conclusion, the present experiments showed that stomatal guard cell size, chloroplast numbers in stomata, and leaf thickness could be introduced as an early marker to predict ploidy levels in *K. rotunda*. This is the first report which demonstrates that colchicine treatment has the potential to produce dwarf lines and induce chlorophyll mutation in *K. rotunda*.

Acknowledgements

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