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ANALYSIS OF PLOIDY LEVEL OF *ARTEMISIA ANNUA* L. BASED ON FLOW CYTOMETRY AND CONFOCAL LASER SCANNING MICROSCOPY

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Abstract

Eighty nine Artemisia samples treated with different concentrations of colchicine were used as breeding samples, with diploid Artemisia as the control. The ploidy levels of samples were determined by flow cytometry and confocal laser scanning microscopy (CLSM). An analysis of the flow cytometry results identified three suspected tetraploid plants and seven suspected triploid plants. The results of this study may be useful for breeding new Artemisia lines.

Introduction

Artemisia annua L. commonly known as "Artemisia" has been used to produce traditional Chinese medicine for treating yin deficiency, hectic fever, inflammation, jaundice and malaria (Udaykumar 2014). There is growing interest in the utility of Artemisia in Europe and North America (Dhingra *et al*. 1999). Artemisia contains hydroxylated and polymethoxylated flavonoids, including eupatin, cirsilineol, chrysoplenetin, chrysosplenol D, casticin and artemetin (Bhakuni *et al*. 2001). Flavonoids from Artemisia possess biological activities relevant for medical applications (such as anti-oxidant, anti-cancer and anti-malarial properties) (Iqbal *et al*. 2012, Kim *et al*. 2014, 2015). As the interest in artemisinin has increased, a growing number of workers have attempted to domesticate Artemisia as a cultivated crop (Laughlin 1993, 1994). However, Artemisia is a strictly self-incompatible plant that is highly sensitive to environmental stimuli and the available diploid Artemisia genotype (2n = 18) produces very little artemisinin (0.01 - 0.5%) (Lestari *et al*. 2011), which is insufficient for the commercial production of this compound. Consequently, there is considerable interest in polyploid Artemisia genotypes exhibiting stable characteristics through breeding program. Increasing ploidy level may enable plants to grow under diverse environmental conditions and the associated genome-dosage effects may be relevant for crop improvement (Yildiz 2013, Fort *et al*. 2015). During plant breeding to increase ploidy, it is very important to accurately determine the ploidy levels of the progeny. The ploidy level and chromosome structure can be analyzed by flow cytometry method (FCM), which has increased the use of FCM in plant breeding programs (Dolezel and Bartos 2005). FCM can be utilized for the rapid screening and earlier detection of the aneuploids in hybrid seedlings in the species (Phurailatpam *et al*. 2018). Additionally, the application of FCM for studying nuclear DNA content has recently become a new trend and has been used to analyze diverse plant species (Alatar *et al*. 2017, Girma *et al*. 2017, Wang *et al*. 2018).

Fluorescence confocal laser scanning microscopy (CLSM) is being increasingly acknowledged as a suitable technique for investigating the ploidy level of animals and plants (Hamilton and Johnston 2015). Moreover, combined CLSM and FCM is an ideal method for conducting plant ploidy investigations (Gilissen *et al*. 1994), this method also has been used in the identification of ploidy of pollen grains (Cheng *et al*. 2012). Therefore, authors applied FCM and CLSM to determine the ploidy levels of Artemisia hybrids, thereby generating potentially useful information for breeding new Artemisia varieties.

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Materials and Methods

For the diploid samples, tender leaves and pollen grains were collected from conventionally grown Artemisia plants at the Guangxi Botanical Garden of Medicinal Plants from September to October 2016. For breeding samples, 89 Artemisia samples were treated with different concentrations of colchicine (Table 1). All the samples were planted and harvested at the Guangxi Botanical Garden of Medicinal Plants from September to October 2016.

Table 1. The different concentrations of colchicine on induction of stem of *Artemisia annua*.

Treatment	Concentrations (mg/l)	Time (day)	Treatment number	induction rate (%)
N1	0	30	30	0.00
N2	10	30	30	3.33
N3	20	30	30	6.67
N4	30	30	30	6.67
N5	40	30	30	3.33
N6	50	30	30	3.33
N7	60	30	30	6.67
N8	70	30	30	13.33
N9	80	30	30	10.00
N10	90	30	30	6.67
N11	100	30	30	6.67

The FCM experiment was conducted according to a modified version of a method described by Galbraith *et al*. (1983). The extraction buffer consisted of 15 mol/l Tris-HCl (pH 7.5), 80 mol/l KCl, 20 mol/l NaCl, 20 mol/l EDTA-Na_2, 15 mol/l mercaptoethanol and 0.05% Triton X-100. The staining buffer comprised the extraction buffer supplemented with 20 mg/l RNase A and 20 mg/l propidium iodide (PI). To prepare nuclear suspensions, 500 mg young leaf samples were cut into small pieces with a blade in culture plates containing 2 ml extraction buffer. Samples were filtered through a sterilized 260 mesh nylon net and centrifuged (1,000 rpm). After discarding the supernatant, the precipitate was washed three times with extraction buffer, after which the precipitated cells were treated with the DNA-specific staining buffer. Specifically, the precipitate was mixed with 2 ml staining buffer and then incubated in darkness at room temperature for 30 min. The samples were filtered through a 500 mesh nylon net, and the resulting filtrate was collected in a standard tube for an analysis by FCM with the LSRFortessa™ cell analyzer (BD Biosciences) at the Science Experiment Centre, Guangxi University of Chinese Medicine. Diploid *Artemisia apiacea* L. was used as the control. The nuclear DNA contents of all analyzed samples were quantified relative to the control content as follows: Relative DNA content (%) = M_n of tested sample/M_1 of control × 100%. At least 10,000 nuclei were collected for each sample and each sample was analyzed twice.

The CLSM experiment was conducted according to a modified version of a method described by Guo *et al*. (2006). Diploid and breeding samples were placed in separate 5 ml centrifuge tubes and then treated as follows: (1) Pollen was fixed with 2.5% pentanediol for 1 - 2 hrs; (2) samples were carefully washed three times (10 min each) with phosphate-buffered saline (pH 7.2), (3) samples were digested with a mixture of 3% cellulase and 5% pectinase for 40 min in a water bath set at 35°C, (4) digested pollen was washed as described in step 2, mixed with 1% Triton-100, and

incubated for 1 hr (i.e., extraction), (5) after the extraction, the pollen was washed as described in step 2, mixed with 2 µg/ml DAPI staining solution, and incubated in darkness at 4°C for 30 min. Ordinary slides and cover slides were used to prepare the CLSM film, which was used immediately or stored in darkness at 4°C.

The prepared film was placed in a Leica laser scanning confocal microscope. All samples were analyzed under the same conditions (with the same exposure intensity), and three visual fields were photographed for each sample. The resulting images were analyzed with Image-Pro Plus.

Results and Discussion

Using untreated diploid Artemisia as a control, 89 selected Artemisia samples treated with different concentrations of colchicine were analyzed by FCM. In Fig. 1, the horizontal axis represents the channel fluorescence intensity, while the vertical axis represents the relative number of cells. The ploidy levels of the breeding samples were determined based on the channel fluorescence intensities. The peak relative value for the diploid DNA content was about 50, while the peak relative value for the suspected tetraploid DNA content was about 100. Additionally, the peak relative value for the suspected triploid DNA content was about 75. We identified three suspected tetraploid plants and seven suspected triploid plants (Table 2) were identified. Among plants with the same ploidy level, the relative DNA content of each sample differed. The coefficient of variation for the suspected tetraploid DNA content was 6.1%, while that of the suspected triploid DNA content was 4.0%.

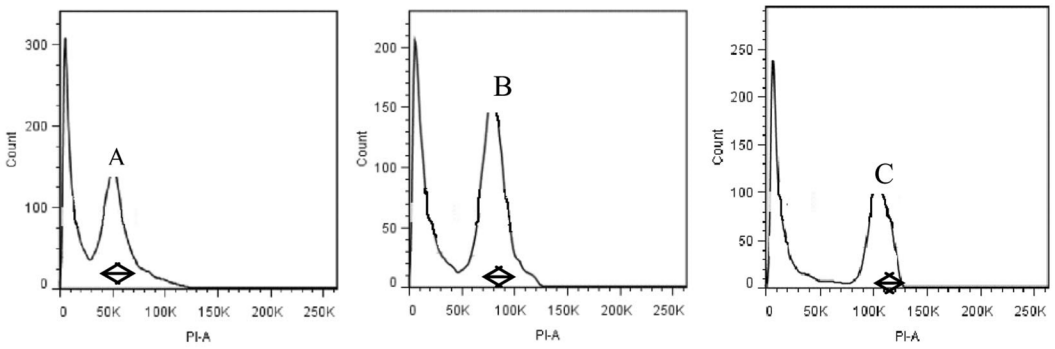

Fig. 1. Distribution curves of Artemisia DNA contents as measured by FCM. A. indicates G1 peak of the diploid DNA content. B. indicates G1 peak of the suspected triploid DNA content, C. indicates G1 peak of the suspected tetraploid DNA content.

Theoretically, plant cell size and DNA content undergo fold-increases together with chromosomal multiplication. On the basis of the Artemisia ploidy levels predicted by FCM, the control samples, suspected tetraploids and suspected triploids were further analyzed by CLSM. Specifically, three fields of vision were photographed for each sample. Representative pollen grains were selected in each photograph for a subsequent analysis. Fig. 2 presents examples of the Artemisia pollen grain photographs resulting from the CLSM analysis. Table 3 provides details regarding the analysis of the visual field area for the control and breeding samples. The areas of the suspected tetraploid pollen and suspected triploid pollen were considerably greater than that of the diploid pollen. Table 4 lists the results of the quantitative analysis of the DNA contents (nuclear fluorescence) in the control and breeding samples. Present data revealed that DNA contents were greater in the suspected tetraploid pollen and suspected triploid pollen than in the

diploid pollen. Table 5 presents the data for the analysis of the variance in the visual field areas of Artemisia diploid control and breeding samples. The difference between the suspected tetraploid and suspected triploid breeding samples was very significant (the $p = 0.0001$, $p < 0.01$ indicated that the difference was very significant). The results of the analysis of the variance in the DNA contents in the Artemisia control and breeding samples (Table 6) revealed that the difference between the suspected tetraploid and the suspected triploid breeding samples was very significant ($p = 0.0002$). These results implied that the pollen grains of the suspected tetraploid and suspected triploid plants were significantly different from those of the control diploid plants.

Table 2. Relative DNA contents in Artemisia nuclei.

No.	Ploidy	Mean G_1	Relative DNA content	Ratio
Control	2X	32.9	50.0	1.0
18	4X	65.6	99.7	2.0
19	"	61.9	94.1	1.9
80	"	70.0	106.4	2.1
1	3X	52.8	80.2	1.6
4	"	55.0	83.6	1.7
5	"	52.8	80.2	1.6
7	"	51.0	77.5	1.6
39	"	56.5	85.9	1.7
56	"	51.6	78.4	1.6
59	"	56.0	85.1	1.7

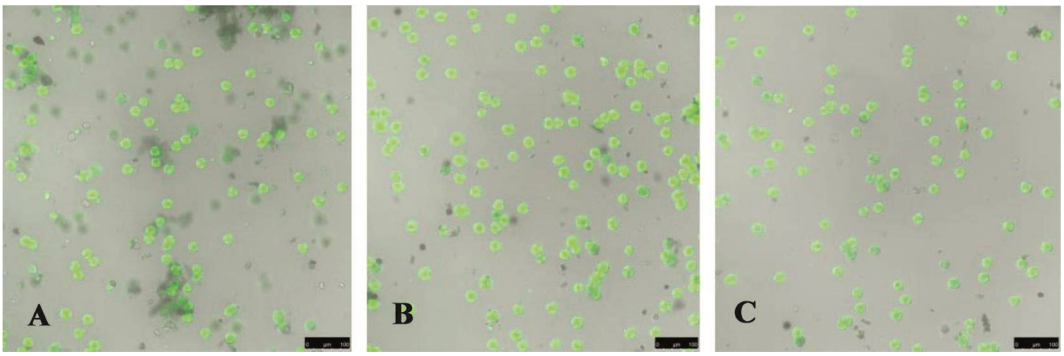

Fig. 2. Representative images of Artemisia pollen grains captured by CLSM. A. indicates control diploid pollen, B. indicates suspected tetraploid pollen, C. indicates suspected triploid pollen.

Advantages of using FCM to investigate plant ploidy levels include rapid detection and the production of accurate results. Therefore, FCM has recently been widely and quickly applied to replace the traditional chromosome counting method (Alatar *et al.* 2017, Girma *et al.* 2017, Wang *et al*. 2018). In this study, authors used FCM to analyze Artemisia ploidy levels. They examined the factors influencing FCM, including the proportion of the extraction buffer and the concentration of PI in the staining buffer. Additionally, they also evaluated the effects of the

fragmentation of Artemisia leaves because fragments that are too small result in too many incomplete cells, while fragments that are too large lead to cell adhesion. Moreover, in addition to nuclear DNA, PI also binds to RNA. Thus, to ensure the accuracy of the present results, RNase was added to the suspensions before PI was added. Furthermore, the presence of cell walls may inhibit the entry of PI into the nucleus. In this study, FCM results indicated that the peak fluorescence intensities of diploid, suspected tetraploid, and suspected triploid Artemisia plants are about 50, 100 and 75, respectively. In the present study, three suspected tetraploid plants and seven suspected triploid plants were detected.

Table 3. Visual field areas of Artemisia control and breeding samples.

Group	Number	Sum	Mean	Variance	Sd
Control	21	95683	90808	5009429.2	4875820.4
Suspected tetraploid	21	181209	172331	9455745.3	9203546.6
Suspected triploid	21	136134	130288	7257150.7	7063591.8

Table 4. DNA contents (nuclear fluorescence) in Artemisia control and breeding samples.

Group	Number	Sum	Mean	Variance	Sd
Control	21	44572803.7	16150499.2	4889913.7	4766098.3
Suspected tetraploid	21	84179159.9	30565250.2	9226133.7	8992522.8
Suspected triploid	21	64465862.7	23490216.2	7084164.8	6904789.7

Table 5. Analysis of the variance in the pollen visual field areas of Artemisia control and breeding samples.

Group	Sum of squares	Number of degrees of freedom	Mean square	F	Significance
Intra-group	47040780.43	2	23520390.22	77.30	0.0001
Inter-group	9127715.81	30	304257.19		
Total	56168496.24	32			

Table 6. Analysis of the variance in the DNA contents (nuclear fluorescence) in Artemisia control and breeding samples.

Group	Sum of squares	Number of degrees of freedom	Mean square	F	Significance
Intra-group	1.29×10^{12}	2	6.93×10^{11}	55.65	0.0002
Inter-group	3.74×10^{11}	30	1.24×10^{10}		
Total	1.76×10^{12}	32			

Confocal laser scanning microscopy combines laser and UV scanning, and the combination of the functions of fluorescence and ordinary microscopes results in high sensitivity, high specificity, the generation of clear images, and the production of accurate quantitative data for individual

cells. The DAPI solution is a fluorescent dye that binds strongly to DNA. Additionally, it can cross an intact cell membrane, and thus can be used for staining living and fixed cells. However, DAPI can also bind to RNA, although the fluorescence intensity is not as high as that resulting from the binding to DNA. To minimize experimental errors, RNase was added to the suspensions before adding DAPI. Meanwhile, to enhance the entry of DAPI into cells to stain DNA, the cell wall was digested with 3% cellulase and 5% pectinase after pollen grains were fixed. Moreover, because fluorescence can be quenched to varying extents when samples are illuminated, the CLSM films prepared in this study were stored in darkness at 4°C if they were not used immediately. Because different exposure intensities can influence the final results, all samples were analyzed under the same conditions (including the same exposure intensity). In this study, the CLSM results indicated that the pollen areas and DNA contents were greater for the suspected tetraploid and suspected triploid Artemisia plants than for the diploid control plants. Analyses of the variances in the visual field areas and DNA contents revealed significant differences among the diploid, suspected tetraploid, and suspected triploid pollen grains. Furthermore, the results of the CLSM identification were consistent with those of the FCM analysis.

The FCM and CLSM analyses confirmed that the leaf DNA contents were greater in the suspected tetraploid and the suspected triploid Artemisia plants than in the diploid Artemisia, as were the pollen areas and pollen DNA contents. Authors identified three suspected tetraploid plants and seven suspected triploid plants, the new finding indicates the necessity of further study on ploidy evolution in this species since it was observed that ploidy differentiation is related to polyploidy. So these method of ploidy determination by FCM and CLSM are rapid and accurate and can be utilized in conjunction with molecular markers for characterization of interspecific hybrids and their derivatives.

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