Research Article

Induction and Flow Cytometry Identification of Tetraploids from Seed-Derived Explants through Colchicine Treatments in *Catharanthus roseus* (L.) G. Don

Shi-Hai Xing,[1] Xin-Bo Guo,[2] Quan Wang,[1] Qi-Fang Pan,[1] Yue-Sheng Tian,[1] Pin Liu,[1] Jing-Ya Zhao,[1] Guo-Feng Wang,[1] Xiao-Fen Sun,[2] and Ke-Xuan Tang[1, 2]

[1] *Plant Biotechnology Research Center, Fudan-SJTU-Nottingham Plant Biotechnology R&D Center, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, China*
[2] *State Key Laboratory of Genetic Engineering, Morgan-Tan International Center for Life Sciences, School of Life Sciences, Fudan University, Shanghai 200433, China*

Correspondence should be addressed to Ke-Xuan Tang, kxtang1@yahoo.com

The tetraploid plants of *Catharanthus roseus* (L.) G. Don was obtained by colchicine induction from seeds explants, and the ploidy of the plants was identified by flow cytometry. The optimal treatment is 0.2% colchicine solution treated for 24 hours, and the induction rate reaches up to 30%. Comparing with morphological characteristics and growth habits between tetraploids and the control, we found that tetraploids of *C. roseus* had larger stoma and more branches and leaves. HPLC analysis showed tetraploidization could increase the contents of terpenoid indole alkaloids in *C. roseus*. Thus, tetraploidization could be used to produce higher alkaloids lines for commercial use. QRT-PCR results showed that the expression of enzymes involved in terpenoid indole alkaloids biosynthesis pathway had increased in the tetraploid plants. To our knowledge, this was the first paper to explore the secondary metabolism in autotetraploid *C. roseus* induced by colchicine.

1. Introduction

Polyploidy is an important genomic feature for all eukaryotes, especially in plants where most or all angiosperms are polyploids or have polyploidy origins [1, 2]. Polyploidy, a widespread phenomenon in the evolution of flowering plant and an element in plant speciation and diversification [3], has played a key role in plant evolution and breeding and it can also cause much variation in plant phenotype. Generally speaking, polyploids may differ from their progenitors in morphological, ecological, physiological, and cytological characteristics [4], such as broader leaves, good-quality, high-yielding, and enhanced resistance to environment stress and diseases [5–7].

The traditional method to obtain polyploid plants is to use colchicine ($C_{22}H_{25}NO_6$) [8–13], which is an alkaloid contained in seeds and bulbs of *Colchicum autumnale* L. Colchicine has affinity for tubulin, a microtubule-subunit protein, and inhibits spindle function, thereby preventing both cell and nuclear division, during the chromosomes replicate and divide to form separate sister chromosomes. Blakeslee and Avery [14] were the first to treat in vivo seeds, axillary buds, and shoots with colchicine to produce tetraploid plants of *Datura stramonium* L. Colchicine treatments are frequently applied in vitro. Colchicine is added to the culture media, and polyploid induction and plant regeneration are obtained by organogenesis or somatic embryogenesis. Classical types of explants are shoot tips and axillary shoot buds obtained by tissue culture [15–17] although seeds have also been used [11]. Oryzalin (3,5-dinitro-$N4$, $N4$-dipropylsulfanilamide) is a dinitroaniline herbicide with strong antimitotic activity which has also been used to obtain polyploid plants [12]; it binds to tubulin and inhibits polymerization of microtubules leading to chromosome doubling [18]. It has been used as polyploidization agent less frequently than colchicine. Some other substances

were also used for polyploidy induction such as dihydroxylated monochlorobiphenyls [19] and nitrous oxide gas [20].

Traditionally, all of botanic ploidy of chromosome had been determined by microspectrophotometry [21]. While the state of some plants karyology is rather poor and difficult for preparing good metaphase spreads due to the rigid cell walls of the plants; therefore, it is difficult to determine the ploidy by microscopy method. Flow cytometry (FCM) is a technique extensively used from 1980s on estimating nuclear DNA content rapidly [22] and has been already found very useful in plant taxonomy to screen ploidy levels and to determine genome size [23–26]. This method was originally developed for the analysis of blood cells. The range of applications has continued to increase and encompass the analysis of ploidy of animals and plants, cell-cycle kinetics, and presence of specific antigens. FCM has become one of most useful methods due to it is convenient, fast, and reliable. Sample preparation usually occupies only a few minutes and rarely requires expensive reagents. Analysis is rapid, and representative numbers of nuclei are measured in a short time [27].

In most plant species, doubling the chromosome number may lead to larger cell sizes and subsequently larger plant organs. The chromosome number can also have a positive influence on the levels and composition of the constituents in plants. For example, the production of bioactive secondary metabolites has conspicuous changes [28]. Rapid progress has been made in crops, such as wheat, cotton, and rape, meanwhile, the yields of which were doubled when the genomes were duplicated [5, 29, 30]. Polyploidy has also effect on gene expression either at the transcriptome level or at the proteome level [31]. So, extensive studies have carried out with microarray and two-dimensional electrophoresis in gene expression variance resulted from polyploidization [8, 32].

Catharanthus roseus (L.) G. Don (*C. roseus*) which belongs to the family of Apocynaceae is an ornamental and a medicinal plant species [31]. *C. roseus* has more than 130 terpenoid indole alkaloids (TIAs) [33, 34]. Among them, vinblastine and vincristine are the most widely used and most famous anticancer TIAs drugs [35]. Many of the genes involved in TIAs biosynthesic pathway of *C. roseus* have been cloned and sequenced for the analysis of their expression in various plant organs [36–38] (Figure 1). The wild type of *C. roseus* is a diploid ($2n = 16$; 1500 Mbp = $12 \times$ *Arabidopsis thaliana* genome) plant [35]. Kulkarni and Ravindra had found that *Pythium aphanidermatum* (which causes die-back and collar and root rot) infecting can induce autotetraploid lines in 1988 [39]. The autotetraploid plants appeared higher resistance to *Pythium aphanidermatum* and appeared some unique phenotypes.

In this study, tetraploid plants of *C. roseus* were obtained by using colchicine, and the ploidy of the *C. roseus* plants induced were determined by FCM. The morphological and physiological phenotypes of the tetraploids were observed comparing with diploids. The content of three alkaloids, vindoline, catharanthine, and vinblastine, was compared with the controls. The expression level of nine genes (Figure 1) involved in the biosynthesis of TIAs and a jasmonate-responsive APETALA2 (AP2)-domain transcription factor (ORCA3) was analyzed by QRT-PCR.

2. Materials and Methods

2.1. Plant Materials and Colchicine Treatments for Tetraploid-Induction. *Catharanthus roseus* (L.) G. Don seeds (Pacifica cherry red) were purchased from Pan American Seed Co. (Ill, USA; its website is http://www.panamseed.com/).

Colchicine which was purchased from Beijing Dingguo biological Co., LTD (Beijing, China) was dissolved in ethanol to gain stock with a concentration of 1% (w/w). The working solution was prepared by diluting the stocks in water and sterilized by filtration (0.22 μm). *C. roseus* seeds were immersed in the stocks in 2 mL Eppendorf tubes with different concentrations and exposure time (Table 1). The treated seeds were rinsed with sterile water for 3-4 times in laminar flow cabinet and germinated in Petri dishes with MS (Murashige and Shoog, 1962) agar solid medium in tissue culture room, during this period the number of contaminated and dead seeds of each treatment was counted. After germination, the seedlings were transferred into 60-cell plug tray containing soil and organic manure mixture in Shanghai Jiao Tong University glass greenhouse for 2 weeks average temperature at 25°C ± 3°C, relative humidity varied between 65.3–73.1% and photoperiod at about 16 h for acclimation. Then, they were shifted to the outside field of the botanical garden.

The control seeds, the seeds of wild-type *C. roseus* consisted of the same process with the treatments and were identified as diploid by FCM. Each treatment was set up with 50 replicates.

2.2. Tetraploid Detection by Cytometry. Ploidy level of the plants of *C. roseus* treated with different colchicine concentrations and different time of exposure were determined by BD FACS calibur cell sorting System flow cytometry (FCM) (Becton Dickinson Bioscience, San José, USA) equipped with two lasers. Nuclei suspensions were obtained after chopping approximately 100 mg of fully developed fresh leaf tissue by a sharp razor blade in a specific buffer on ice according to Galbraith et al. [40]. The most popular isolation buffer Otto's buffers [41] was modified and used to prepare chromosome samples, the modified buffer contained 5 mM $MgSO_4$, 25 mM KCl, 2.5 mM HEPES, 0.125% Triton X-100 and 6.5 mM DTT, pH = 8.0. Nuclear suspensions were filtered through a 50 μm nylon filter and RNase A (TIANGEN BIOTECH Co., Ltd., Beijing, China) at a concentration of 1 μg/mL was added to each sample. All leaves chopped for samples must be fresh, and all processes must operate on ice; samples must also be kept on ice until analysis by FCM. Histograms were analyzed using the internal software of the FCM (BD FAC Station data processing system), which determines peak position and the relative ploidy index of the samples.
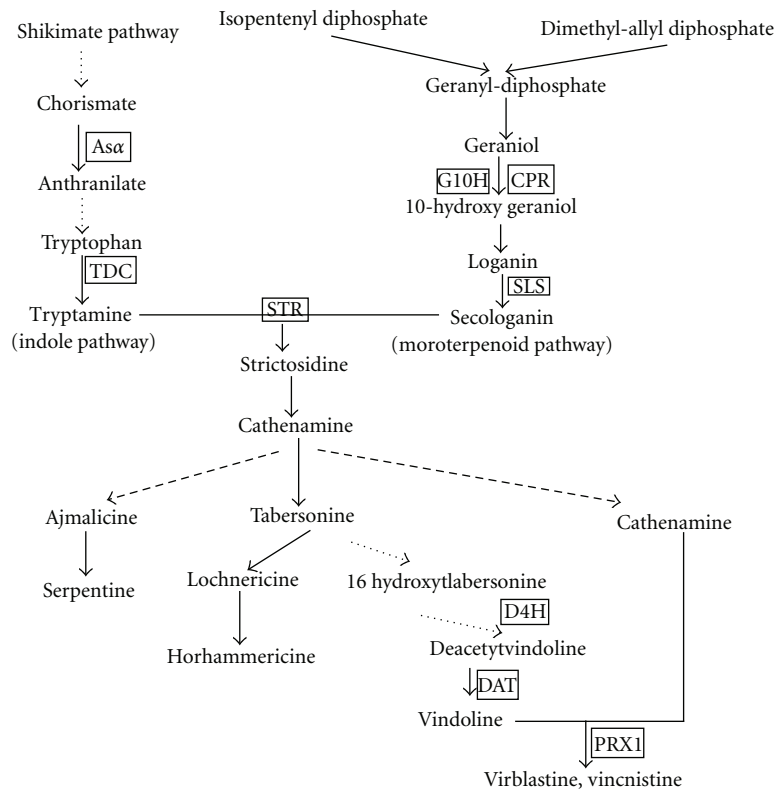
FIGURE 1: TIAs biosynthetic pathway and relative genes in *C. roseus*. Unbroken arrows indicate single enzymatic conversions and broken arrows indicate multiple enzymatic steps.

Nuclei suspensions were centrifuged at 3000 rpm for 5 min two times. Discarding the supernatant, we resuspended pellet in isolation buffer with 0.1 mg/mL of Propidium Iodide (PI) (Sigma, USA) at 37°C for 15 min. Then, they were analyzed using the FACS cytometer. The first laser was tuned to multiline UV (333.6–363.8 nm), and a power output of 300 mW and mithramycin fluorescence was stimulated with the second laser emitting 200 mW at 457 nm. A solution of 50 mM NaCl was used as a sheath fluid and the nuclei suspensions were analyzed at rate of 200–400 particles s^{-1}. Approximately 20000–50000 chromosomes were analyzed in each sample as the FCM analyses nuclei DNA content.

Fluorescence emission was measured through a 488 nm long pass filter in front of FL1 photomultiplier. Relative fluorescence intensities were acquired on a histogram of FL1 fluorescence pulse area. For bivariate analysis, the suspensions of isolated nuclei were stained with PI (propidium iodide) at 0.1 mg/mL. The fluorescence of a complex is considerably increased in the presence of Mg^{2+} ions, and $MgSO_4$ was added to nuclei suspension at the final concentration of 10 mM prior to staining. Due to the spatial separation of 200 μm of the laser interception points with the liquid jet, a half mirror was used to split PI fluorescence to FL1 photomultiplier through a 475 nm long pass filter. Relative fluorescence intensities were acquired on histograms of FL1 and FL4 fluorescence pulse area.

2.3. QRT-PCR.

Total mRNAs of *C. roseus* leaves was isolated using the DP437-RNA plant_plus Plant Total RNA Kit (TIANGEN BIOTECH Co., Ltd., Beijing) according to the manufacturer's recommendations and treated with DNase I (Sigma-Aldrich) to remove any traces of DNA. Extracted total mRNAs was quantified by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) with absorbance at 260 nm and an ethidium bromide (EB) stained test agarose gel electrophoresis used to verify the quality. Reverse transcription reaction was performed using the PrimeScript RT reagent Kit (Perfect Real Time) (Takara Co. Ltd., Japan) following manufacturer's instructions. 500 ng total RNA, 2 μL 5 × PrimeScript Buffer (for Real Time), 0.5 μL Prime-Script RT Enzyme Mix I, 0.5 μL Oligo dT Primer (50 μM) $*$1, 0.5 μL Random 6 mers (100 μM) $*$1 and RNase Free dH_2O were added up to volume of 10 μL for reverse transcription reaction according to the manufacturer's instructions. The mixture was obtained on ice. The reverse transcription reaction was carried out at 42°C for 15 min.

After the synthesis of first-strand cDNA had finished and subsequently diluted six-fold, PCR was performed to analyze the expression pattern of nine genes and a transcript factor gene (Figure 1), and gene *rps9* (40S ribosomal protein S9 gene) was used as a quantitative internal control. All the nuclear sequences of the designed gene-specific primers were designed using Primer 3.0 software (http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) as seen in Table 2.

Table 1: Effect of direct application of colchicine solutions on the seeds of *C. roseus* and the number of tetraploid plants regenerated (50 seeds for each treatment).

Concentration (%)	Time (hours)			
	12	24	36	48
0	0 (50)	0 (50)	0 (50)	0 (50)
0.05	0 (50)	2 (50)	2 (50)	5 (50)
0.1	3 (50)	7 (50)	9 (50)	3 (50)
0.2	8 (50)	15 (50)	12 (50)	5 (50)
0.3	11 (50)	7 (50)	3 (50)	0 (50)
0.4	0 (50)	0 (50)	0 (50)	0 (50)

PCR amplification was carried out in a final volume of $25\,\mu L$ reaction mixture containing $2\,\mu L$ of previous diluted cDNA products as template, $2.5\,\mu L$ SYBR *Premix Ex Taq* (Perfect Real Time) (2 × Conc.) with ROX (Takara) and 200 nM of each gene-specific primer in each run. PCRs with no cDNA and three controls were also done for each primer pair. The PCR reaction of each gene must include internal control gene *rps9*. The QRT-PCRs were performed on a BioRad CFX96 Real-Time System and BioRad CFX Manager software (BioRad). All the Real-time PCRs were performed under the following conditions: 5 min at 95°C, and 40 cycles of 15 s at 95°C and 30 s at 58°C in 96-well reaction plates (BioRad). The specificity of amplicons was verified by melting curve (disassociation) analysis (60–95°C) after 40 cycles. All reactions were performed in triplicate. The relative gene expression was quantified by using the comparative C_T (threshold cycle) method as described (Shalel-Levanon et al. 2005) [42].

2.4. Phenotype Observing of Colchicine Solution Induction Plants. The abaxial epidermises of mature leaf parts were peeled from fresh leaves of confirmed tetraploid and the control *C. roseus* plants. The epidermises were mounted on glass slides and photographed under a fluorescence microscope (Olympus BX51, Olympus Inc., Tokyo, Japan) with an ocular scale, and the visual field area of the ocular is 1 mm^2 under magnification of 40×. Stomata length and density were also measured under magnification of 40×, and the stomatal density (d), stomatal apparatus length (l), and width (w) were measured. The stomatal apparatus area (As) was calculated as follows: As $= 1/4 \times \Pi \times l \times w$, and total stoma area (At) was calculated as follows: At $=$ As $\times d \times 100\%$ [43]. Digital images were manually analyzed with Adobe Photoshop CS 8.0. Some other morphological characteristics and growth habits were also compared with tetraploid plants and diploid status of *C. roseus*. For leaf phenotype observations, ten leaves from each part of five different individuals randomly were examined for tetraploid plants and controls, and more than six images per leaf were examined.

2.5. Leaves Samples Preparation and Alkaloids Extraction. The leaves samples were collected from each part of the tetraploids and control plants. The samples were dried at 45°C for 48 h to 60 h and pulverized in a mortar. Pulverized samples (100 mg) were immersed in 1.5 mL Eppendorf tubes in 1 mL methanol overnight at 4°C. Then, the samples in the tubes were put into ultrasonic aqueous bath (DL-60D) with the power of 80 W for 30 min and centrifuged at 12000 rpm/min for 10 min at room temperature. The precipitated samples which had been shaken were put into ultrasonic aqueous bath for 30 min and centrifuged once again. The supernatant was filtered into new 1.5 mL Eppendorf tubes with $4\,\mu m$ organic filter membrane for HPLC assay. The processed samples were stored at 4°C for later determination.

2.6. Quantification of Alkaloids by HPLC. For HPLC analysis, individual stock solutions of standard samples, catharanthine, vindoline and vinblastine (Sigma-Aldrich, USA), were prepared at a concentration of $1\,\text{mg}\cdot\text{L}^{-1}$ in methanol, and stored at −20°C. The HPLC analysis was performed using a Sapphire-C18 (4.6 mm × 250 mm, 5 μm) column at a column temperature of 35°C and Hitachi L-2000 series HPLC system. This system is consisted of a L-2000 Organizer, a L-2130 Pump, a L-2200 AutoSamplera L-2300 Column Oven and a L-2455 Diode Array Detector. The injection volume was $10\,\mu L$. The mobile phase (acetonitrile : diethylamine buffer solution = 1 : 1) was used at a constant flow rate of 1 mL per minute. The DAD detection wavelength was 220 nm. A mixture of standards and alkaloids were detected. Alkaloids vindoline, catharanthine, and vinblastine were identified after UV analysis of absorbance chromatograms [44]. The TIAs levels were determined by the areas of peaks in chromatographic profiles at 14.41 min for vindoline, 19.73 min for catharanthine, and 31.26 min for vinblastine.

Quantification analysis was repeated for three replication of each tetraploid plant, and the controls which also treated with colchicine and identified as diploids by FCM in parallel of catharanthine, vindoline, and vinblastine, the means, and standard deviations were calculated. The alkaloids content of control was adopted the average value of three independent diploid plants which also treated with colchicine, while the content of the tetraploid was from the average of ten tetraploid lines. The alkaloids were quantified by using regression equation of calibration curve.

2.7. Statistical Analysis. All data in this work were obtained from three independent replicates. Data were analyzed with one and two dimension analysis of variance and followed by the F value test. The values are mean ± SD for three samples in each group and difference between treatments was considered as significant when $P \leq .05$ or .5.

3. Results

3.1. Tetraploid of C. roseus Induction and Identification. *C. roseus* seeds were treated with different concentrations of colchicine solution for different time intervals. After germination, seedlings were grown in greenhouse for two months, and then, their ploidy were determined by FCM

TABLE 2: Genes and their primer sequences used for real-time PCR.

accession no. (gene)	Primers	Primer sequences (5′–3′)	Amplicon length (bp)	Annealing temperature (°C)
AM236089.1 (*prx1*)	rtprx1-f	TAGCTCAAACAACTCGGCCACC	195	62
	rtprx1-r	GCAGCACTGATGAATCGCACC		
AJ250008.1 (*asα*)	rtasα-f	GGCGGCGAAGCATGGGAACT	329	59
	rtasα-r	CCTCGGTCCGCTGG GGTTTC		
X69791.1 (*cpr*)	rtcpr-f	TCGGCCCTGGTACTGGACTAGC	306	65
	rtcpr-r	TGGCATCACCGCAGACGTAAAC		
L10081.1 (*sls*)	rtsls-f	CTGCCAGCTTTTGCCATATGC	191	60
	rtsls-r	GAGTCCATGAGTTCTTCAATAG		
M25151.1 (*tdc*)	rttdc-f	TCGGCATCTCACCTCAAGTTCT	207	60
	rttdc-r	TCGGGACATATACAGGCGCTT		
U71605.1 (*d4h*)	rtd4h-f	TTATGATCGAAAAAGTGAATTA	223	58
	rtd4h-r	TTTCTTGATGATCTGAGTGCGT		
AF053307.1 (*dat*)	rtdat-f	CTTCTTCTCATCACGTACCAACTC	172	60
	rtdat-r	ATACCAAACTCAACGGCCTTAG		
X53602.1 (*str*)	rtstr-f	GCCTTCACCTTCGATTCAACTG	287	62
	rtstr-r	GTGGCTAGTTGTGTGGCATACC		
AJ251269.1 (*g10h*)	rtg10h-f	ATAGCCAGAGCGGAGAAGCG	163	55
	rtg10h-r	TTTCCCCGCCTCAACCATTA		
AJ251249.1 (*orca3*)	rtocar3-f	CCGGACCCGTTAGAGTAAACC	212	58
	rtoca3-r	CGTCTCTTCTTCCTTCCTCCAC		
AJ749993.1 (*rps9*)	rtrsp9-f	GCGTTTGGATGCTGAGTTGAAG	257	62
	rtrsp9-r	GGCGCTCAAGGAAGTTCTCTAC		

(Figure 2). The number of tetraploid plants induced in each groups was different (Table 1).

By two dimension variance analysis with the factors of time interval and colchicine concentration, we found different concentration had significant effect on tetraploid plants production (Table 3). During the period of seeds germination there were no contaminated seeds basically in this experiment. The seeds treated with 0.4% colchicine solution were difficult to germinate, and their seedlings showed abnormal appearance and died rapidly. So, there were no plantlets surviving at last after treated with 0.4% colchicine solution. The same phenomenon happened in the treatment of 0.3% colchicine solution for more than 48 hours. For other time of 0.3% colchicine treatments, we could also find some plantlets which died or grew worse than normal level. It could be concluded that too high concentration of colchicine may be poison to the seeds, resulting in dying of plantlets.

From the results, low concentration of colchicine produced few tetraploid plants. There were more tetraploid plants arise after long time of low concentration treatments. The treatments of 0.2% and 0.3% colchicine with appropriate treating time could induce much more tetraploid plants than other treatments. The highest induction rate was up to 30% with 0.2% colchicine solution treated for 24 hours.

3.2. Phenotype Variation of Tetraploid C. roseus. More branches and leaves were found in tetraploid plantlets by comparing the morphological characteristics of tetraploid lines with the control in the study of Kulkarni and Ravindra in 1998 [39]. There was no significant difference in leaf area, color, and thickness between them as different to other plants tetraploidizational study. Stomas were found on the abaxial and adaxial leaf surface in both tetraploid *C. roseus* and the controls. By microscopy observation, we found the significant difference in stomata size and density (Table 4 and Figure 3). The average size of stomatal apparatus, and the total stoma area was larger in tetraploid lines than in controls. The average stomatal length, width, stomatal apparatus, and total stoma area were up to $28.26 \pm 2.51\,\mu$m, $20.35 \pm 1.80\,\mu$m, $451.56 \pm 56.32\,\mu$m^2, and $1.76\% \pm 0.01\%$ in tetraploid lines respectively, while these indexes were only $23.71 \pm 1.83\,\mu$m, $17.11 \pm 1.84\,\mu$m, $320.58 \pm 51.33\,\mu$m^2, and $1.24\% \pm 0.02\%$ in controls, respectively. The result that tetraploidization of *C. roseus* brought large stoma was consistent with most of other studies [45, 46].

3.3. Analysis of Alkaloids Concentration in the Tetraploid Plants C. roseus by HPLC. Three kinds of TIAs (vindoline, catharanthine, and vinblastine) in both tetraploid lines and the controls were determined by HPLC. Identification of alkaloids from the controls and tetraploid lines extract were operated by the comparison of the retention time and the UV spectra with those of authentic standards [47, 48]. The purity peaks were determined using the D-2000 Elite
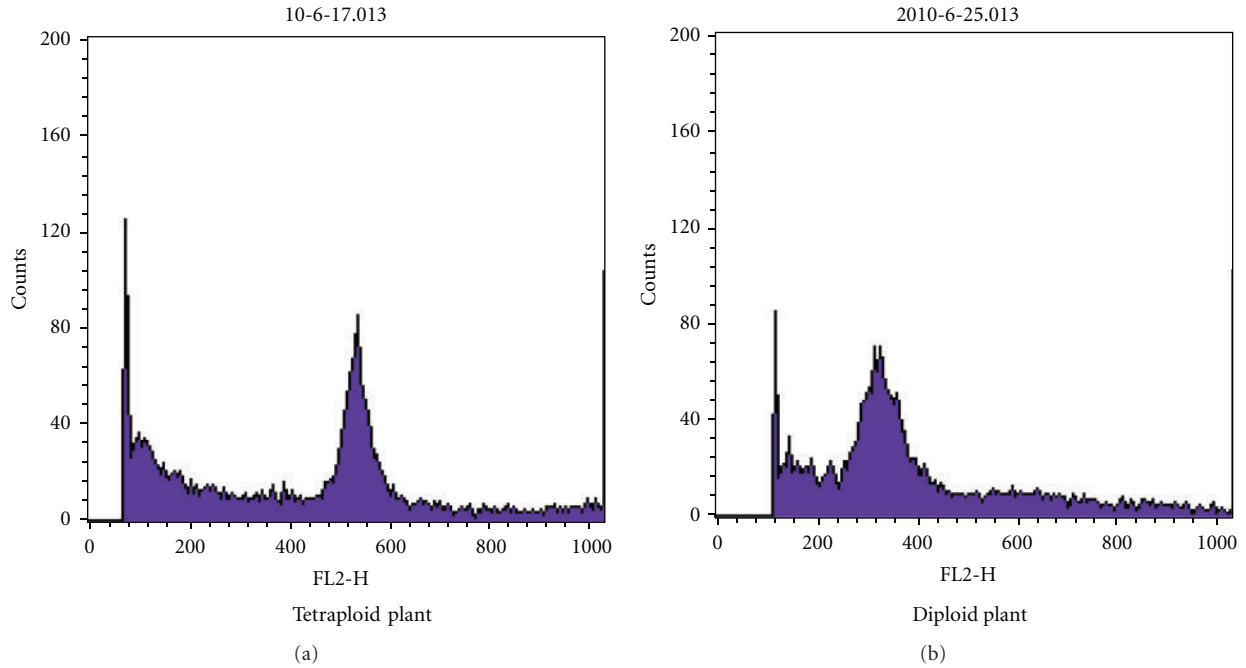
Figure 2: Histogram of the relative fluorescence intensity of nuclei isolated from the leaves of diploid and tetraploid *C. roseus* plant. (a) The control diploid plant of *C. roseus* and (b) a tetraploid plant of *C. roseus*.

Table 3: Two dimension variance analysis of different time and concentration of colchicine treatments on *C. roseus* tetraploid plants production.

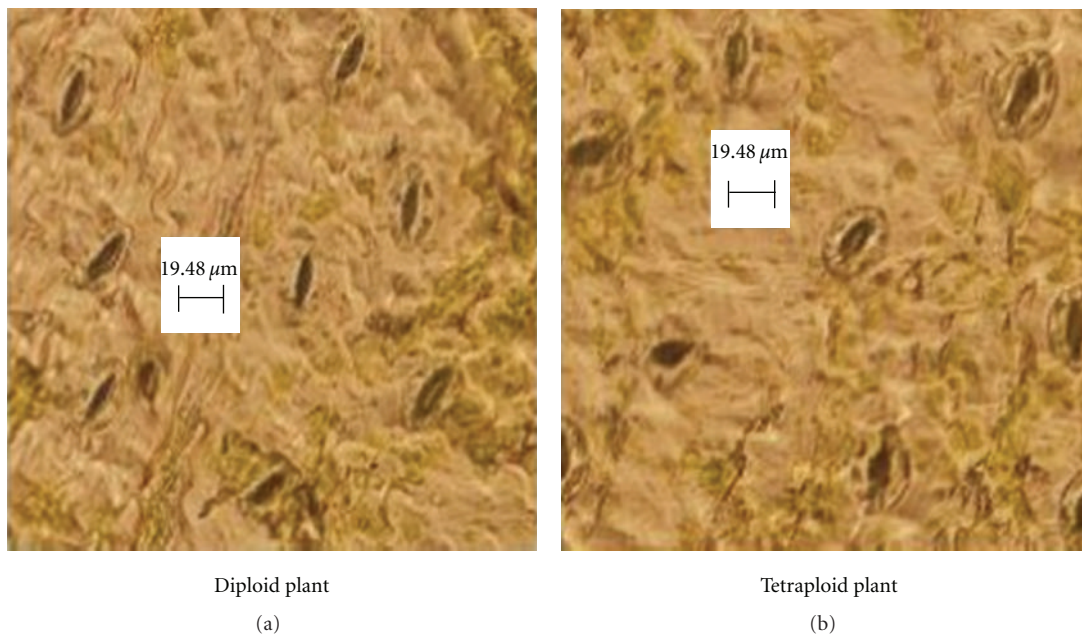| Sources of variation	Sum of square	Freedom	MS mean square	F value	Critical value
Concentration	0.029883	5	0.005976	5.757**	$F_{0.005} = 5.17$
Time	0.001933	3	0.000644	0.62–	$F_{0.05} = 3.098$
Random error	0.015572	15	0.001038		
Total variation	0.186133	23			

Diploid plant

(a)

Tetraploid plant

(b)

Figure 3: Stomata in abaxial leaf epidermis under fluorescence microscope of diploid (a) and tetraploid (b) of *C. roseus*. Scale bar is 19.48 μm.

TABLE 4: Difference of leaf traits between diploid and tetraploid of *C. roseus*.

Parameters	Diploids	Tetraploids
d (mm^{-2})*	43.82 ± 1.91^a	38.96 ± 2.52^b
l (μm)*	23.71 ± 1.83^a	28.26 ± 2.51^b
w (μm)	17.11 ± 1.84^a	20.35 ± 1.80^b
As (μm^2)*	320.58 ± 51.33^a	451.56 ± 56.32^b
At (%)*	1.24 ± 0.02^a	1.76 ± 0.01^b

Different letters (as (a) and (b) in the table) in the same row indicate significant statistical difference $P < .05$, *indicate $P < .5$ (ANOVA).
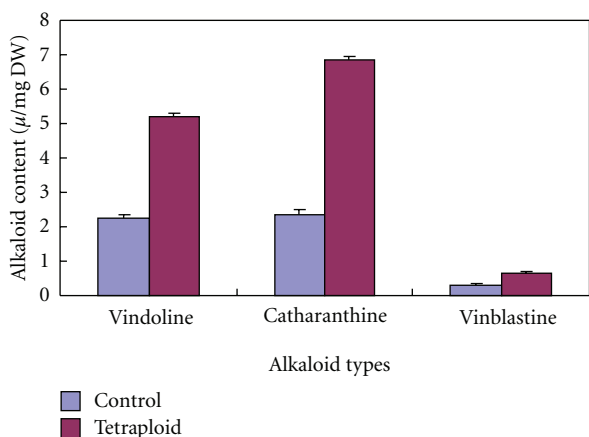
FIGURE 4: Difference of the average content of TIAs alkaloids among tetraploid plants and controls in *C. roseus*.

internal software to make sure that a peak contained only one compound.

Our results showed that all of the three TIAs accumulation increased in the tetraploid lines (Figure 4). Under the same conditions, the average amount of vindoline, catharanthine, and vinblastine was 0.226 ± 0.0072%, 0.237 ± 0.0111%, and 0.031 ± 0.0019% on a dry weight leaves basis in the controls, respectively, while their average amount was up to 0.522 ± 0.0079%, 0.684 ± 0.0123%, and 0.067 ± 0.0021% on a dry weight leaves basis in tetraploid lines. By one dimension analysis, we found there were significant variance between tetraploid lines and the diploid controls in the alkaloids content in *C. roseus*. Tetraploidization increase vindoline, catharanthine, and vinblastine content in *C. roseus* leaves, and they increased 130.9%, 188.6%, and 122.6% above control, respectively.

3.4. QRT-PCR. To explore the variance of genes expression between tetraploid plants and the control plants induced by colchicine solution, total RNA was extracted from the leaves of both tetraploid lines and three control plants cultured under the same conditions. Meanwhile, in this study, we wanted to find the relationship between TIAs accumulation and the expression of TIAs biosynthesis-related genes and transcript factor. By this reason, QRT-PCR analyses were performed (Figure 5).

Compared with the tetraploid line 1, 2, 3, 4, and 5 and the control plants, the genes of *tdc*, *g10h*, *sls*, *str*, *dat*, and *prx1* in the tetraploid lines showed much higher level of relative expression than in the controls. However, the expression of *asα*, *cpr*, and *d4h* had no obviously difference among them. The relative expression of transcript factor gene *orca3* showed few higher in the tetraploidy lines than in the controls. There were no significant difference in the relative expression of all genes among the different tetraploid line 1, 2, 3, 4, and 5, as the same phenomenon was in the three control plants.

4. Discussion

Tetraploid *C. roseus* could be induced by colchicine treatment. Our research indicated that suitable concentration of colchicine could get well results, and this phenomenon was consistent with many other agents being applied for plant growth and development.

Cytochimeras might arise during the tetraploid plants induction period [15]. Even though *C. roseus* is a single-embryo plant (by anatomical analysis) which may not appear cytochimeras induced by colchicine, we identified the tetraploid lines by FCM. Each sample comprised an adult small leaf piece ($\sim$0.5 mm^2) collected from each branch of each detecting plant, with a similar leaf piece from three diploid control plants. Meanwhile, we determined each branch individually and all the adult leaves pertaining to the branch were analyzed for each plant.

There was a plant distribution pattern which considered that polyploids are better adapted than their diploid relatives [49, 50] to more extreme ecological environments. Tetraploids of *C. roseus* have better tolerance to the presses than other plant polyploids. Wild-type *C. roseus* is a tropical diploid plant which originated from South Asia, East Africa, and tropical American and only distributed in topical and subtropical area. As the above hypothesis, tetraploids of *C. roseus* may grow in cold area better than wild-type plants, then the cultivate range of *C. roseus* will expand to many extreme ecological areas by planting tetraploids. From this idea, the plant will be cultivated in pan areas, and then more biomass will be obtained. It can partly resolve the current problem of lacking of *C. roseus* TIAs.

Tetraploidization of *C. roseus* can increase the contents of TIAs in *C. roseus* as transgenic method and spraying plant growth regulators on leaves in previous studies [47, 48, 51, 52]. Tetraploids of *C. roseus* which have higher content of TIAs are of great value as a new variety with an increased TIAs production per m^2. Furthermore, we will tetraploidize the transgenic *C. roseus* lines and hybridize them to get a tetraploidizational transgenic *C. roseus* variety which may possess much higher TIAs content and some good growth habits.

The effect of polyploidization on genes expression had been studied extensively, either with microarray technology at the transcriptome level or with two-dimension electrophoresis at the proteome level [8, 32]. All of these studies found polyploidization might induce variance in some genes expression. This phenomenon may be reasoned
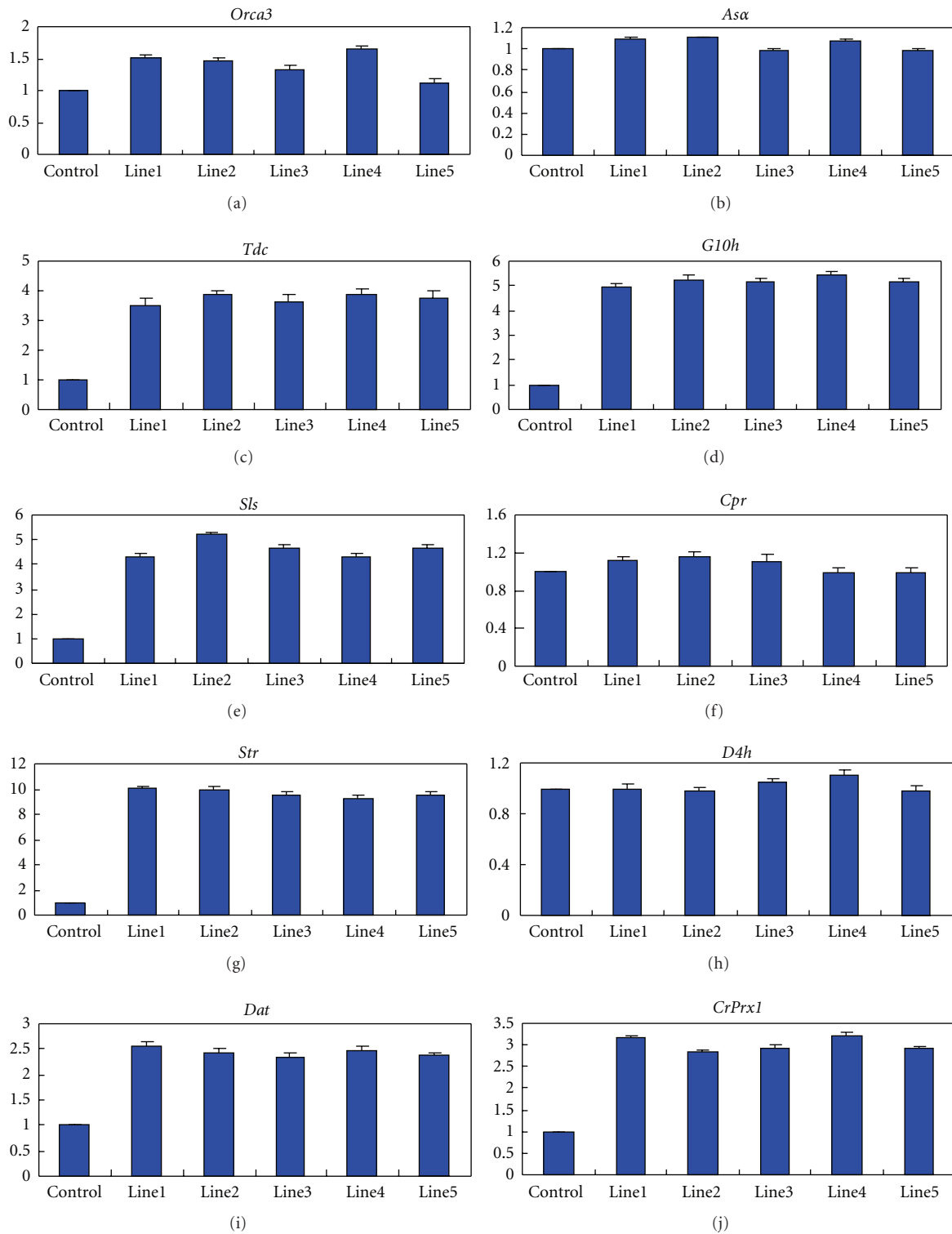
FIGURE 5: Quantitative real-time PCR analysis for the expression of ten genes in five tetraploid lines and three diploid lines which were also treated with colchicine solution in *C. roseus*. The average value relative expression of the controls was set to 1 level. The genes analyzed are *Orca3* (a), *Asα* (b), *Tdc* (c), *G10h* (d), *Sls* (e), *Cpr* (f), *Str* (g), *D4h* (h), *Dat* (i), and *CrPrx1* (j). The data of each line represented is average value of three replicates in each experiment, while the data of control represented is average value of three independent control plants with three replicates in each experiment.

from that more chromatin coming into contact with the nuclear membrane after polyploidization, thus elevating genes activities [53]. To explain the molecular mechanism of TIAs contents increased in autotetraploid *C. roseus*, transcription qualities of nine genes and a transcript factor related to biosynthesis of TIAs were analyzed by QRT-PCR. In view of our results, autotetraploid affects gene expression, and significant upregulation was observed in the transcription of *orca3, tdc, g10h, sls, str, dat,* and *prx1* in *C. roseus* tetraploid lines, while there was no obvious difference in the expression of *asα, cpr,* and *d4h*.

In view of our results, we concluded that tetraploid lines increased the average level of TIAs in *C. roseus* by modulating the expression of genes involved in their biosynthesis pathway, especially genes of *orca3, tdc, g10h, sls, str, dat,* and *prx1*.

Abbreviations

TIAs:	Terpenoid indole alkaloids
C. roseus:	*Catharanthus roseus* (L.) G. Don
PRX 1:	Peroxidase 1
SLS:	Secologanin synthase
ASα:	Anthranilate synthase alpha subunit
CPR:	Cytochrome P450-reductase
D4H:	Desacetoxyvindoline 4-hydroxylase
DAT:	Deacetylvindoline 4-O-acetyltransferase
G10H:	Geraniol 10-hydroxylase
STR:	Strictosidine synthase
TDC:	Tryptophan decarboxylase
HPLC:	High-performance liquid chromatography
ORCA3:	Octadecaniod-derivative responsive *Catharanthus* AP2-domain protein 3
RPS9:	40S ribosomal protein S9
FCM:	Flow cytometry
PI:	Propidium iodide.

Acknowledgments

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