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Tetraploid Induction and Identification of *Gossypium arboreum*

Na Yang, Erhua Rong, Qianru Li, Juan Dong, Tianqin Du, Xiaoming Zhao, Yuxiang Wu*

College of Agriculture, Shanxi Agricultural University, Taigu, China
Email: *yuxiangwu2009@hotmail.com

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Abstract

***Gossypium arboreum* (2n = 26, A_2) is a diploid species with limited production in acreage compared with *G. hirsutum* and *G. barbadense.* However, its unique traits such as insect and disease resistance contribute an important germplasm to cotton breeding. So polyploid manipulation for *G. arboreum* is an effective approach of germplasm development. This research focused on tetraploid induction of *G. arboreum* by colchicine. Morphology and cytology identifications for obtained mutants were also conducted. The seedling growth and development of all mutants was more stunted than controls. According to preliminary morphological characteristics, mutant rates in different treatment were statistically estimated and the highest mutant rate was 42.31% under the treatment of 0.1% colchicine for 24 hours. The chromosome number of most mutants was 2n = 4x = 52, while the chromosome number of diploid controls was 2n = 2x = 26 by cytology observation of root tip cells. By microscope observation of low leaf epidermis, there were significant differences for stoma area between tetraploids and diploids. The meiosis behavior of the induced tetraploid was much more complex than that of the diploid. At diakinesis, some univalent, trivalent and polyvalent were also observed besides bivalent and quadrivalent. There were different kinds of polyad in tetraspore period of mutants. The dissociate chromosomes existed during metaphase I and II, the unbalance separation of chromosomes existed during anaphase I and II. As a result, tetraploid mutants of *G. arboreum* were identified and their desirable traits would be further evaluated to incorporate into next breeding program.**

Keywords

***G. arboreum*, Tetraploid Induction, Karyotype Analysis, Cytology Identification**

*Corresponding author.

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1. Introduction

Polyploidy has been an important factor in eukaryote evolution [1]. Between 30% - 70% of angiosperm species are believed to endure polyploidization [2]. Observations showed that polyploidy is not only a rare macro evolution event but also ongoing process [3]. Recent studies have demonstrated that polyploid genomes can be highly dynamic and undergo rapid structural and functional alterations, such as diploidization [4] [5]. Plants with the same ploidy level get higher outcrossing rate [6], except for *Gossypium*.

Cotton is an important natural fiber crop traceable in tropic aridity regions, which was developed from originated a kind of perennial xylophyta into an annual crop growing in subtropical and temperate zone today [7]. *G. arboreum* (2n = 26, A_2) is a diploid species with limited production in acreage compared with *G. hirsutum* and *G. barbadense*. However, its unique traits such as insect and disease resistance contribute an important germplasm to cotton genetic and breeding studies. Hutchinson [8] [9] insisted that *G. arboreum* was the result of evolution of *G. herbaceum* under special environment in the tropical climate. However, Fryxel [10] proposed that *G. arboreum* and *G. herbaceum* formed respectively after acclimation in different environment conditions, agricultural and cultural center.

Methods using colchicine for polyploidy induction are common for a range of plant species [11] [12]. Some reports of *Gossypium* spp. by colchicine induction have also been available [13] [14]. This article aims to get tetraploid of *G. arboreum* and find the best colchicine concentration and aplication time to provide reference for further induction of *Gossypium*. This new germplasm will contribute to the research for systematic and evolution in *Gossypium* and their desirable traits will be evaluated in a further breeding program.

2. Material and Methods

2.1. Plant Material

G. arboreum came from National Wild Cotton Nursery in China. Field experiments and inside experiments were finished in Shanxi Agricultural University. The first generation M_1 acted as material for meiosis examination, while M_2 was suitable for mitosis examination in this study.

2.2. Polyploidy Inducing

Totally 102 pots were chosen for colchicine treatments and 25 pots were left as controls. All seeds were delinted by H_2SO_4 before use and approximately 3 seeds were planted in every separate pots with sunshine potting soil. When seedlings were at the cotyledon stage, the suitable plant (subsample) in each pot was treated by modified method of 1% agar with colchicine of different concentration semi-solid (26 pots for 0.1%, 27 pots for 0.2%, 24 pots for 0.3% and 25 pots for 0.4%) for 24 hours. A single drop (2 - 4 μL) of the warm (~50˚C) semi-solid was painted between cotyledons of each seedling to cover the apical bud. The treated pots were then covered by a plastic cup under a high humidity growth chamber at 25˚C. After each treatment, plastic cup was uncovered and the residue was carefully removed with tweezer. Sprinkling water and nutritive solution (0.3 g CON_2H_4 and 0.5 g KH_2PO_4 in 1 L distilled water) were applied once a day to help recovering the seedling growth for two weeks and then seedlings were transplanted in the field.

2.3. Morphological Examination

Morphological characteristics of four treatments were examined during the seedling growth period. Retardant growth, crimpled and dark green leaf were measured as mutant preliminary indices and mutant rates of different treatments were estimated.

2.4. Cytological Identification

The ploidy level of *G. arboreum* was also estimated by chromosome counting of root tips from putative mutants earlier determined by morphological characteristics. Chromosome spreads were made according to the method of [15]. Microscope slides were examined with an Olympus BX51 (Japan) using a 100× objective. The chromosome number of all treatments in root tips was counted in at least 10 cells. A plant with all root tip cells showing 26 chromosomes was classified as diploid, and with all cells showing 52 chromosomes was determined as tetraploid. Karyotype analysis was conducted according to [16]. Karyotype formula was obtained according to [17]

and karyotype category according to [18]. Index of relative length (IRL = chromosome length/total length of chromosome group × 100%) was calculated according to [19].

2.5. Stoma Measurement

The ploidy level of *G. arboreum* was estimated either by stoma measurement. Low leaf epidermis was torn from obtained tetraploids and diploid controls in the same internode (the forth) and mounted on a clean slide with a drop of distilled water respectively. Another drop of 1% I-KI solution was added to stain the stoma and covered with cover slip. Slides were examined with a OLYMPUS BX51 using a 40× objective. The density and size of stoma under the same magnified scope were measured. At least 10 scopes were chosen and mean numbers were statistically calculated.

2.6. Meiotic Studies

Flower buds for meiotic studies were collected from each individual plant in the field between 9:00 and 10:30 am and fixed in freshly prepared Carnoy's fixative solution II (ethanol: acetic acid: chloroform = 5:3:2) for 12 h, then transferred to 70% alcohol, and stored under refrigeration for use. Meiotic analysis was carried out on flower buds of a suitable size; after washing the fixed buds in distilled water, anthers were squashed on slides in carbol fuchsin solution. Photographs were taken from freshly prepared slides using an Olympus BX51 microscope with automatic camera. Meiosis was studied using a minimum of 30 PMCs. With reference to the abnormal chromosome behaviors [20], tetraploid was separated from diploid.

2.7. Data Analysis

The experimental design was completely randomized and data analyses were performed using DPS software.

3. Results and Discussion

3.1. Estimation of Mutant Rates

The growth rate of mutant plant with lower strain height, smaller internode length as well as deformed and crimpled leaves were all slower than diploid control in different period (**Figure 1**). The mutation rates of 0.1%, 0.2%, 0.3% and 0.4% were 42.31%, 14.81%, 25.00% and 4.00% respectively (**Table 1**) by statistic analysis. This study gave the suggestion that colchicine concentration of 0.1% was the most effective for mutagenesis. The higher concentration is, the lower survival rate is. The deformity of mutants was mainly caused by the destroy of spindle from colchicine poison. Colchicine mainly concentrated on the metaphase of cell mitosis, however not all metaphase cells became polyploids. The growth rate and cell volume of diploids and polyploids are different, so the combination of diploid and polyploid cells caused crimpled leaf.

3.2. Chromosome Counting

Karyotype of diploid controls was shown in **Table 2**. Karyotype formula is 2n = 2x = 26 = 18m + 6sm (4SAT) + 2st (2SAT) and karyotype type is 2B. The range of total length variation is from 5.15 to 10.90. Arm ratio range from 1.08 to 3.38 with average ratio of 1.58. Index of relative length range from 0.67 to 1.42. There are satellites

Table 1. Induction effect of colchicine on *G. arboreum* at different treatment.

Concentration	Number of treated plants	Number of survivals	Number of mutants	Survival rate (%)	Mutation rate (%)
CK	25	21	-	84.00	-
0.1%	26	11	11	42.31	42.31
0.2%	27	4	4	14.81	14.81
0.3%	24	6	6	25.00	25.00
0.4%	25	3	1	12.00	4.00

Figure 1. Seedling of M_0 control (1st) and mutants (2nd and 3rd). *bar stands for 10 cm.

Table 2. The coefficient of chromosome for diploid control.

Number	Relative Length/(%) (S + L = T)			Index of Relative Length	Centromere Index %	Arm Ratio	Category
	Long arm	Short arm	Total length				
1	5.89	5.01	10.90	1.42	45.95	1.18	m
2	6.19	3.53	9.72	1.26	36.36	1.75	sm
3	4.71	4.27	8.98	1.17	47.54	1.10	m
4	4.71	3.68	8.39	1.09	43.86	1.28	m
5	4.42	3.98	8.39	1.09	47.37	1.11	m
6	3.98	3.68	7.66	1.00	48.08	1.08	m
7	3.98	3.68	7.66	1.00	48.08	1.08	m
8	3.68	3.39	7.07	0.92	47.92	1.09	m
9	3.09	2.36	5.45	0.71	43.24	1.31	m
10	3.53	2.80	6.33	0.82	44.19	1.26	m
11*	6.04	2.80	8.84	1.15	31.67	2.16	sm (SAT)
12*	3.98	1.47	5.45	0.71	27.03	2.70	sm (SAT)
13*	3.98	1.18	5.15	0.67	22.86	3.38	st (SAT)

Note: *Sat-chromosome. The length of satellites is not included in the chromosome length.

on short arms of chromosome 11, 12, 13. IRL = 4L + $8M_2$ + $8M_1$ + 6S. L stands for that IRL of the chromosome is more than 1.26, M_2 for IRL ranges from 1.01 and 1.25, M_1 for IRL ranges from 0.76 to 1.00, S for IRL is less than 0.75.

Karyotype of tetraploids was shown in **Table 3**. Karyotype formula is 2n = 4x = 52 = 8M (4SAT) + 44m (8SAT) and karyotype Type is 1B. The range of length variation is from 5.21 to 10.73. Arm ratio range from 1.00 to 1.44 with average ratio of 1.14. Index of relative length range from 0.68 to 1.39. There are satellites on short arms of chromosome 11, 12, 13. IRL = 8L + $16M_2$ + $20M_1$ + 8S. The tetraploid had twice chromosome number more than the diploid *G. arboreum*. There were two pairs of centromere chromosomes in the mutant ones, but none in the diploid controls. There was no acrocentric chromosome or telocentric chromosome in the tetraploid, whose range of chromosome length variation got narrowed, without arm ratio more than 2. Tetraploid genome may be got rearranged and its fragments may be got lost like other studies [21] [22]. But this deduction needs other direct evidences.

Table 3. The coefficient of chromosome for tetraploid mutant.

Number	Relative Length/(%) (S + L = T)			Index of Relative Length	Centromere Index %	Arm Ratio	Category
	Long arm	Short arm	Total length				
1	5.39	5.34	10.73	1.39	49.79	1.01	m
2	5.79	4.01	9.80	1.27	40.91	1.44	m
3	4.63	4.10	8.73	1.13	46.94	1.13	m
4	4.59	4.05	8.64	1.12	46.91	1.13	m
5	4.01	3.78	7.79	1.01	48.57	1.06	m
6	3.96	3.43	7.39	0.96	46.39	1.16	m
7	3.96	3.43	7.39	0.96	46.39	1.16	m
8	3.78	3.56	7.35	0.07	48.48	1.06	m
9	3.12	3.12	6.23	0.81	50.00	1.00	M
10	3.03	2.58	5.61	0.73	46.03	1.17	m
11*	4.01	4.01	8.01	1.04	50.00	1.00	M (SAT)
12*	4.01	3.12	7.12	0.93	43.75	1.29	m (SAT)
13*	2.80	2.40	5.21	0.68	46.15	1.17	m (SAT)

Note: *Sat-chromosome. The length of satellites is not included in the chromosome length.

3.3. Stoma Measurement

Stoma studies can give a quick view to judge the ploidy level of *G. arboreum*. In this study, two epidermis from strictly comparable leaves in the same internode (the forth) showed an obvious difference in the size of stoma between the obtained tetraploids and diploid controls in **Table 4** and **Figure 2**. By microscope measurement, stomatal approximate area increased by 81.28% from 453.99 μm^2 of diploid to 822.99 μm^2 of the tetraploid. Stomatal aspect ratio changed from 1.30 of diploid to 1.41 of the tetraploid. But it was decreased by 56.17% from the diploid (14.83/200 μm × 200 μm) to the tetraploid (6.50/200 μm × 200 μm) for the stoma density. Chloroplast number per stomatal increased by 18.47% from 38.83 of the diploid to 46.00 of the tetraploid. There were significant differences between the tetraploid and diploid of *G. arboreum* at 0.01 and 0.05 level. This study suggested that polyploid mutants could be identified by stoma measurement and it is an important cytology phenomena for stoma distinction in different ploidy plants. The average chloroplast number per 200 μm × 200 μm in the tetraploid is 299, lower than that in the diploid (575.85), explaining why SPAD (Soil and Plant Analyzer Development) value in functional leaf of the tetraploid is lower than that of the diploid during the whole growth period. The smaller stomatal density and bigger area are indices of huge cell type of polyploidy. The increased chloroplast number per stomatal in tetraploid may need much more material and energy to get synthesis of substances and formation of organelles.

3.4. Meiosis Measurement for Induced Tetraploids

Observations were made on chromosome morphology and behavior during meiosis of PMCs for induced tetraploids. The steps of induced tetraploidy in meiosis were similar to diploid controls, but much more complex. It was noticed that clustering of chromosomes as unit into separated groups formed in leptotene. At diakinesis, the disorder synapsis of homology segment made homologous chromosomes appear as abnormal styles like o, v, x, ∞ (**Figure 3(a)**, **Figure 3(b)**). During metaphase I, the chromosome configurations were formulated as univalent, bivalent, trivalent, quadrivalent and multivalent, but the main types were bivalent and quadrivalent. Single chromosome or chromosome groups, forming micronucleus later probably (**Figure 3(l)**), were located outside of equatorial plate in metaphase I (**Figure 3(c)**). Chromosome comfiguration was 5.57I + 10.54II + 0.87III + 4.84IV + 0.14V + 0.11VI according to chromosome behavior of 37 PMC in metaphase I. In anaphase I, chromosome

Table 4. Comparison of stomatal in leaf between controls and mutants.

Sample	Stomatal density/ 200 μm × 200 μm	Stomatal aspect ratio	Stomatal approximate area (μm^2)	Chloroplast number per stomatal
Control group	14.83 ± 1.60aA	1.30 ± 0.18aA	453.99 ± 44.13aA	38.83 ± 3.19aA
Induced group	6.50 ± 1.38bB	1.41 ± 0.09bB	822.99 ± 76.56bB	46.00 ± 1.90bB
Rangeability	−56.17%	+8.46%	+81.28%	+18.47%

Note: Small letters stand for significance at 0.05 level and capital letters stand for significance at 0.01 level.

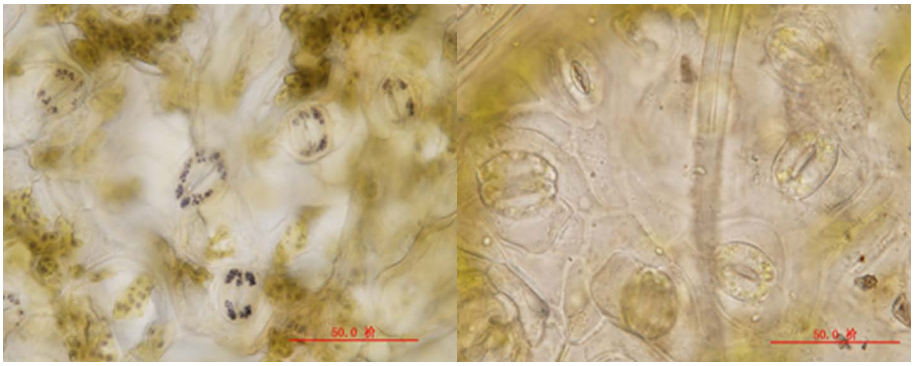

Figure 2. Stoma of diploid (left) and tetraploid (right). *bar stands for 50 μm.

cluster and laggard chromosomes were observed when the homologous chromosomes separated (**Figure 3(d)**, **Figure 3(e)**). Chromosome bridges and fragments were observed in anaphase II (**Figure 3(f)**). Abnormalities observed in telophase II are the out of synchronous segregation of genome, one half segregated earlier, and the other one later. These explain the occurrence of dyads, triads and tetrads by simultaneous cytokinesis and higher volume of polyspory (**Figure 3(g)**, **Figure 3(h)**, **Figure 3(i)**, **Figures 3(j)-(l)**).

The pollen grains of controls developed uniformly and nearly spherical with the average diameter of 83.04 ± 11.16 μm (**Figure 4**). The pollen grains of mutants developed uneven with specially big and extremely small ones. The diameter of specially big pollen grains was 200.00 μm, and that of extremely small pollens was 25.83μm, whose proportion of abnormal pollen grain is 4.28% (**Table 5**).

Induction of polyploidy may create wider amplitude of genetic variation which is necessary to study various cytogenetic backgrounds relating to its improvement as an ideal and alternative pulse crop in different geographical regions. Polyploids have immense importance in maintaining these aneuploids in population. Successful crossing between tetraploids and diploids often results in triploid plant which can produce different trisomics, 3n + 1 or 3n − 1 individuals in progeny. There are evidences that doubling the chromosome number may change magnitude of sterility, pattern of sexuality, plant habit and increase winter hardiness [23]. Polyploids are more tolerable and adaptable to a wider range of environmental conditions [21]. The ability to induce chromosome doubling, therefore, is of importance to practical as well as to theoretical genetics and in evolution. Analysis of meiotic chromosome association in autopolyploid plants can give precise information on organization of chromosome pairing and synapsis during meiosis and has practical value in understanding of undesirable effects of polyploidy on fertility and stability. Meiotic consequences of artificial polyploidization and its effect on different phenotypic traits can be studied to enhance the knowledge base of chromosome doubling in plants. The desirable traits of this tetraploid plants would be evaluated to incorporate into a further breeding program and the research for systematic and evolution in *Gossypium*. There were tetraploid cells and diploid cells observed in nearly all of the mutants. So we need much more work to get homozygous tetraploid plants.

4. Conclusion

From this study, *Gossypium arboreum* (2n = 26, A_2) was manipulated polyploid induction by colchicine. Morphology, cytology and other stoma, meiosis identifications all showed that the obtained mutants were tetraploids and their desirable traits would be further evaluated to incorporate into next breeding program.

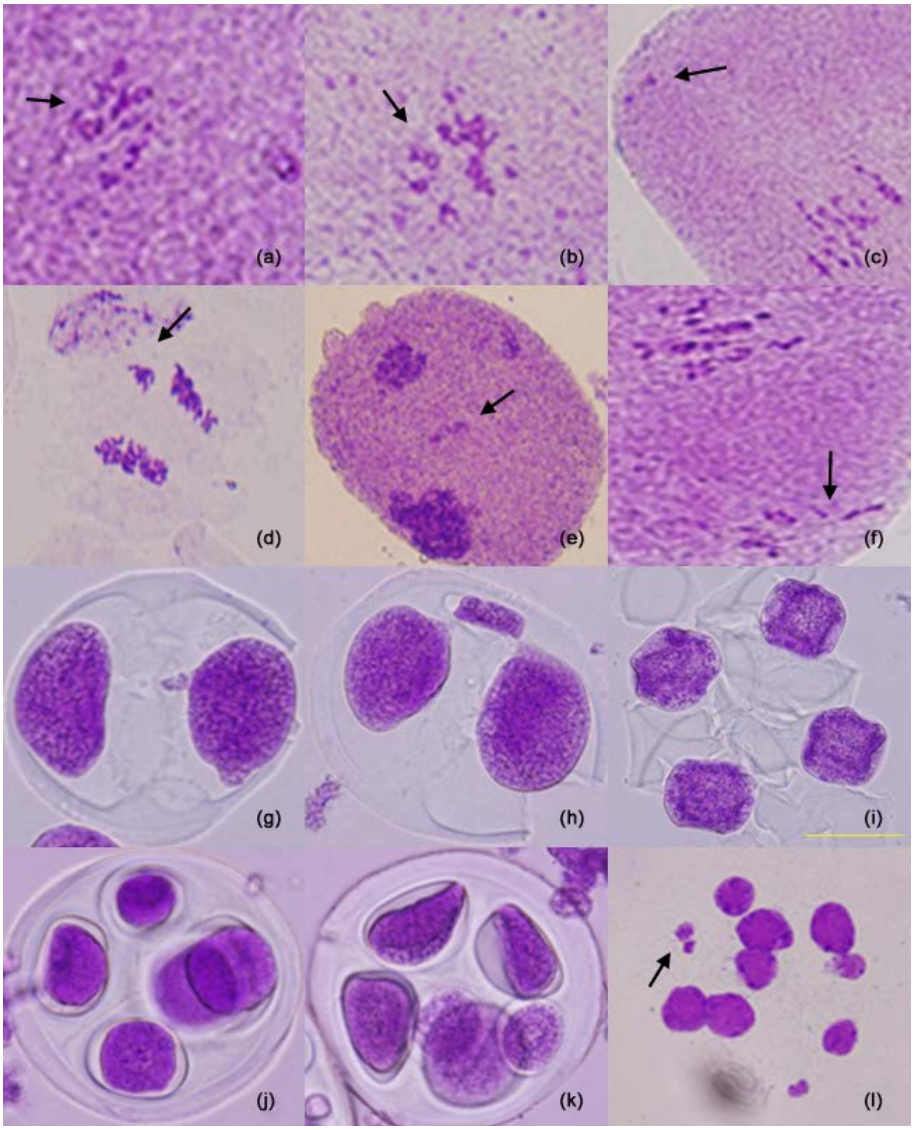

Figure 3. Abnormal meiosis of tetraploid mutants. (a), (b) o, v, x, ∞ style homologous chromosomes & univalent, bivalent, trivalent, quadrivalent and multivalent; (c) isolated chromosomes; (d) chromosome cluster in anaphase I; (e) laggard chromosomes; (f) the chromosome bridges and fragments; (g) dyad; (h) triad; (i) wizened tetrad; (j)-(l) polyspory and micronucleus.

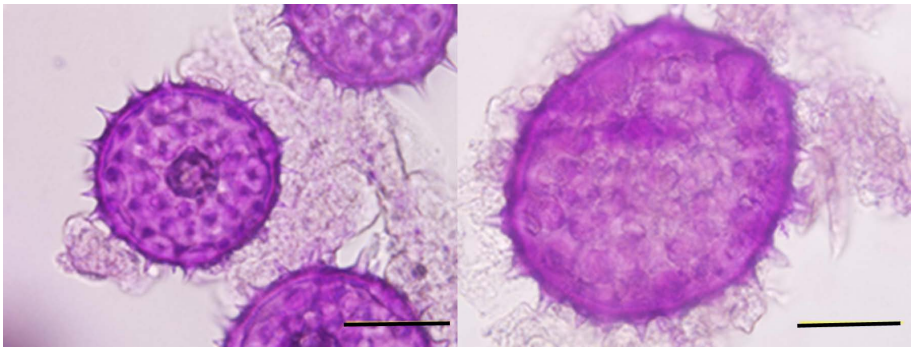

Figure 4. Pollen grain of diploid (left) and tetraploid (right). *bar stands for 50 μm.

Table 5. Comparison of pollen between controls and mutants.

Sample	Pollen grain diameter (μm)	Proportion of abnormal pollen grain (%)
Control group	83.04 ± 11.16aA	-
Induced group	116.79 ± 9.54bA	4.28
Rangeability	+40.64%	-

Note: Small letters stand for significance at 0.05 level and capital letters stand for significance at 0.01 level.

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