Front. Agric. China 2008, 2(4): 380–385
DOI 10.1007/s11703-008-0083-6

RESEARCH ARTICLE

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Effect of *in vivo* plant preservation on the fertility and chromosome configuration of a quadri-specific hybrid derived from 4 cultivated cotton species

Abstract A quadri-specific hybrid F_1 (*G.arboreum*, *G. herbaceum*, *G.hirsutum*, and *G.barbadense*) was produced by crossing the doubled-chromosome (*G.arboretum* × *G. herbaceum*) F_1 with (*G.hirsutum* × *G.barbadense*) F_1. The hybrid plants were preserved for 15 years via the method of grafting. The hybrid flowers were self-crossed and back-crossed every year, and the pollen vigor was tested as well. The results showed that the percentage of fertile pollens and weak-vigor pollens increased with the time of plant preservation, especially for those of weak-vigor that occupied about 10% of the total pollens after 15 years of growth. Meanwhile, the percentage of the sterile pollens decreased with the plant preservation, and the fertility of the quadri-specific hybrid was recovered partially. After plant preservation for more than 10 years, some back-crossed seeds were obtained via backcrossing with upland cotton parents, and a few self-crossed seeds were produced finally. The characteristics of chromosome behavior and the chromosome configuration during the meiosis were studied and the results showed that the chromosome configuration of the quadri-specific hybrid plant grown for 15 years was $2n = 52 = 4.92\text{I} + 14.62\text{II} + 2.29\text{III} + 1.56\text{IV} + 0.71\text{V} + 0.19\text{VI}$, while that from the plants of 15 years ago was $2n = 52 = 8.4\text{I} + 8.1\text{II} + 5.7\text{III} + 1.9\text{IV} + 0.6\text{V}$. It was showed that the univalents decreased to 3.5 per cell, bivalents increased up to 6.5 per cell, and the number of multivalents decreased obviously as well. It was found from this experiment that extending the growth period by the method of grafting would be helpful to harmonize the relationship among the different chromosome groups which came from different species, and balance the chromosome configuration, which led to the partially recovering in plant fertility of the interspecific hybrids.

Keywords cultivated cotton, quadri-specific hybrid, chromosome configuration, fertility

1 Introduction

The genus '*Gossypium*' is comprised of about 50 species including the world's most important fiber crop, cotton. The genus ranges from shrubs to small trees in arid regions, spreading throughout the tropics and subtropics (Wang et al., 2004). Four different cultivated species have been independently domesticated at different times in human history, including the New World allopolyploids, *Gossypium hirsutum* L. and *Gossypium barbadense* L., and the Old World diploids, *Gossypium herbaceum* L. and *Gossypium arboreum* L.. These species provide most of the world's textile fibers and are the important sources of oil and cottonseed meal. But currently, over 90% of the world's cottons derive from *G. hirsutum*, while *G. barbadense* produces most of the remaining commercial cotton. Among the four cultivated cotton species, there are many favorable characteristics such as fiber quality and yield traits, insect and pathogen resistance, tolerance to environmental stresses, etc (Valicek et al., 1985), so the four cultivated cotton species are the important germplasm sources for cotton genetic improvement.

However, there was no report about the quadri-specific hybrid from the four cultivated species until Wang et al. (1990) who crossed the cultivated diploid species of *G. arboreum* with *G. herbaceum*. After doubling the chromosomes of the interspecific hybrid, a quadri-specific hybrid was then obtained by crossing the hybrid F_1 of *G. hirsutum* and *G. barbadense* with the amphidiploid of *G. arboreum* and *G. herbaceum.* The obtained quadri-specific hybrids were highly sterile and no seed was produced when back-crossed with upland cotton. But after a long time preser-

Translated from *Cotton Science*, 2007, 19(6): 461–466 [译自: 棉花学报]

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vation by grafting, the fertility of hybrids was partially recovered (Gao et al., 2003; Gao et al., 2005). In this paper, the performance of self-crossing and back-crossing of the quadri-specific hybrid was analyzed for many years. The characteristic of chromosome behavior during meiosis and the pollen vigor of the quadri-specific hybrid were studied. Furthermore, by comparing the chromosome configuration of the quadri-specific hybrid with that of 15 years ago, the mechanism of fertility restoration was explored at the cytological level, which may provide a scientific foundation for cotton genetics and breeding.

2 Materials and methods

2.1 Plant materials

Five cotton accessions including four cultivated species and the quadri-specific hybrid F_1 were evaluated in the present study. The plants were cultivated in a growth chamber at the farm of Zhejiang University. The parents of the quadr-specific hybrid F_1 were Wanzi (*G. arboreum*), Xiancaomian (*G. herbaceum*), DPL15 (*G. hirsutum*), and Minufi (*G. barbadense*). The parent seeds were the offsprings by self-crossing for many generations.

2.2 Methods

2.2.1 Crossing and back-crossing

Two interspecific hybrids (F_1) were produced by crossing *G.arboreum* with *G.herbaceum*, and *G.hirsutum* with *G. barbadense*, respectively. The chromosomes of (*G. arboretum* × *G. herbaceum*) F_1 were then doubled by treating the branches with aqueous colchicine and dimethyl sulfoxide (DMSO). Finally a quadri-specific hybrid F_1 was obtained by crossing the hybrid (*G. hirsutum* × *G. barbadense*) F_1 with the amphidiploid hybrid of (*G. arboretum* × *G. herbaceum*) F_1. The two di-specific hybrids (F_1) were planted in the field, and the quadric-specific hybrid F_1 was planted in greenhouse and reproduced by grafting using upland cotton seedling as anvils due to the seed number and the plant sterility. The grafting was carried out every two years, and totally 7-time graftings were done until now.

The squares that petals just come out of the sepals in the maternal parents, which would be flowering next day, were selected for emasculation and the stigma was covered by a wax tube for prevention of unwanted pollination. The designed pollination was done with the paternal parent pollens next morning from 8 to 12 o'clock, and then a few drops of the mixtural solution containing 25 $mg \cdot L^{-1}$ GA_3 and 25 $mg \cdot L^{-1}$ NAA were added at the base of corollas to prevent crossed boll from shelling. Re-pollination would be done next day using the same method. Because of high sterility of quadri-specific hybrid F_1, the squares need not be emasculated before pollination, and the back-crossing could be done directly in the morning when the flowers of the qudri-specific hybrid were blooming.

2.2.2 Cytological observation

The squares for pollen mother cell (PMC) meiosis observation were sampled at 9 o'clock in the morning when the length of calyces and petals was the same (4–6 mm in length). In order to take off the bracts, calyces and corollas, they were pretreated in the saturated p-Dichlorobenzene for 2 hours, rinsed in the water, and transferred into the fixation solution (95% ethanol:acetic acid:trichloromethane = 5:3:2) for 2–24 h. Then they were kept in 70% ethanol at 4°C for PMC observation. The chromosome spreads were made using the modified enzyme digestion method according to Li and Zhang (1998).

2.2.3 Pollen vigor measurement

The pollen vigor of the hybrid and its parents was measured using water leaching method. When the pollen was leached in water, the range length of inclusion emission from fertile pollen was 5–15 times of pollen diameter, and that of weak-vigor pollens was 1–4 times of pollen diameter. The sterile pollen had no inclusion at all.

3 Results and analysis

3.1 The characterization and fertility of the quadri-specific hybrid

The growth and development of the (*G. arboretum* × *G. herbaceum*) F_1 plants were found normal, and the phenotype of the plants was the intermediated between two parents, with some heterosis in vegetative growth. The whole characteristics of the hybrid flowers were those of *G. arboretum*, which were bigger than those of *G. herbaceum* but later in flowering time.

The growth and development of the (*G. hirsutum* × *G. barbadense*) F_1 plants were normal as well, and most of the plant phenotypes resembled that of upland cotton (maternal parent). The flower parts such as the petals, bracts and stigma were bigger than those of both parents, as well as the plant size and the heterosis were significant due to their stronger growth habits. Also, the flowers of the hybrid were light amaranth in color with dark purple bases in the petals. There were more fruit branches in hybrid plants with better boll setting.

However, the growth habit of the quadri-specific hybrid F_1 was strong. The flower color presented the peculiar traits of its four parents that had light red petals and light purple spots on the base of petals. The flower and spot of

flower were bigger than any of its parents, which showed an over-parent genetic characteristic. Blooming time of the quadri-specific hybrid F_1 was apparently later than its parents, displaying a typical short photo-periodical characteristic. The structure of plant and bracts was close to those of *G. hirsutum*, but the size of bracts and the combining properties by a part of bracts were near to *G. arboreum*. The hybrid was the complex of four parents from its exterior performances.

The male and female gametes of the quadri-specific hybrid F_1 were sterile seriously. Self-crossing and back-crossing with *G. hirsutum* or *G. barbadense* were carried out every year and no seed was produced. The plants of the quadri-specific hybrid F_1 were moved into greenhouse to survive the winter, and the new sprouts were grafted on the upland seedlings every two years. The self-crossing and backcrossing were done every year, and the selfed and backcrossed bolls was protected by a mixed solution of 50 $mg \cdot L^{-1}$ GA_3 and 50 $mg \cdot L^{-1}$ NAA. Satiation seeds were acted as efficient seeds and then the boll percentage was statistically calculated. The result was showed in Table 1.

It is showed that the qudra-specific hybrid F_1 was highly sterile. There was no seed produced from back-crossing until 2000, when there was only one seed obtained from back-crossing (Table 1). Since then, the number of back-crossing seeds had increased from one seed to six seeds every year and the boll-setting percentage increased from 0.53% in 2000 to 2.58% in 2005. It showed that the fertility of female gametes in the quadri-specific hybrid F_1 was improved. Since 2004, one self-crossed seed was obtained with the boll setting percentage of 0.29% (self-crossing), which increased to two seeds in 2005. It showed that the male gametes fertility was improved as well, although it was very low. The possible reason for partial fertility recovering was that, the physiological, biochemical and genetic ingredients from different parents were gradually coincident with each other following the plant preservation.

Measurement of the pollen vigor showed that the percentage of fertile pollens in the four parents was higher, more than 90% for all 4 species (Table 2). However, the pollen vigor of the quadri-specific hybrid was very low, only 4% of pollens were fertile and more than 80% of them were sterile. Continuous analysis for the pollen vigor of the quadric-specific hybrid F_1 showed that the percentage of fertile pollens increased from 2.3% to 4.0% with an average of 2.95%, while the percentage of sterile pollen decreased from 92.2% to 80.5% with the average of 86.7% by continuous measurement from 1999 to 2004. It was evident that sterile pollens decreased and fertile pollens increased after plant preservation, although the pollen vigor was still very low. Comparing the fertile pollen with weak-vigor pollens and sterile pollens in different years, it was showed obviously that the reason for sterile pollen decreasing was the increasing of weak-vigor pollens. Female gametes fertility should have the same changing tendency as the male gametes. Although weak-vigor pollens cannot challenge with normal pollens, the weak-vigor female gametes can receive normal male gametes and then produce seeds. So by extending the growth time, the sterile gametes can become weak-vigor gametes, the percentage of the sterile pollens decreased year by year, and the fertility could be recovered gradually.

3.2 PMC meiosis observation

After the plant preservation for 15 years, the growth of the quadri-specific hybrid F_1 was normal and there were many squares which could be used to observe PMC meiosis. The split process of different PMC was different and even every meiosis phases in the same anther could be observed. So there were various performances in every phase of meiosis. On diakinesis, the chromosome became condensed and tubbiness and spread over nucleus. Most chromosomes united to be bivalents and a few chromosomes cannot pair each other. On metaphase I, nucleolus and karyotheca disappeared, and spindle in cytoplasm appeared and connected with centromere. The chromosomes in most cells centralized on the equator board, and many various chromosome configuration existed such as bivalents, trivalents, quadrivalents, and quinquevalents,

Table 1 Results of self-crossing and back-crossing for the quadri-specific hybrid F_1

year	self-crossing				back-crossing			
	flower number	boll number	seed number	boll percentage	flower number	boll number	seed number	boll percentage
1990–1996	–	–	0	0	–	–	0	0
1997	234	43	0	0	210	32	0	0
1998	394	44	0	0	180	23	0	0
1999	410	54	0	0	178	19	0	0
2000	431	43	0	0	187	45	1	0.53
2001	349	45	0	0	178	24	1	0.56
2002	340	34	0	0	109	43	2	0.92
2003	430	43	0	0	165	39	2	0.61
2004	348	28	1	0.29	145	43	5	2.07
2005	430	45	2	0.23	155	55	6	2.58

Note: Boll percentage was that the seed bolls occupied to the flowers of self-crossing and back-crossing.

Table 2 Pollen vigor for the quadri-specific hybrid and its parents

material	year	fertile pollen		weak vigor pollen		sterile pollen	
		number	%	number	%	number	%
G. arboretum	2003	561	93.50	12	2.00	27	4.50
G. herbaccum	2003	532	94.83	10	1.78	19	3.39
G. hirsutum	2003	382	87.82	27	6.21	26	5.98
G. barbadense	2003	514	87.56	39	6.64	34	5.79
quadri-specific hybrid	1999	8	2.3	19	5.5	317	92.2
quadri-specific hybrid	2000	9	2.6	19	5.5	317	91.9
quadri-specific hybrid	2001	11	3.0	34	9.2	324	87.8
quadri-specific hybrid	2002	10	2.9	41	12.0	291	85.1
quadri-specific hybrid	2003	10	2.9	51	14.7	287	82.5
quadri-specific hybrid	2004	14	4.0	55	15.5	285	80.5

but most of them were bivalents (Fig. 1). A few chromosomes in PMC dispersed in the middle of cells and did not form the equator board. Some of univalents of chromosomes were out of the board. On anaphase I, there were different split states, the homologous chromosomes splitting to two poles occasionally, and on two poles there were different numbers of chromosomes. Due to disequalized distribution on anaphase I, the chromosome number of cells on telophase was different. The second split of meiosis had various abnormalities, and chromosome bridges, multi-poles splitting and lagging chromosome fragments could be observed because of the difference in chromosome numbers during the first split. On the period of tetrad, due to abnormal meiosis, four, five, and six microspores with different sizes were formed, although most of them formed tetrad microspores.

In this study, the chromosome number in PMC varied greatly, only about 70% of PMC had 52 chromosomes, which were statistically effective ones for configuration analysis. The results were showed in Table 3. From Table 3, it could be found that, on metaphase I, there were 2–7 univalents in PMC with an average number of 4.92, 10–20 bivalents (an average of 14.62), and a few multivalents. The configuration of the qudra-specific hybrid F_1 was $2n = 52 = 4.92\text{I} + 14.62\text{II} + 2.29\text{III} + 1.56\text{IV} + 0.71\text{V} + 0.19\text{VI}$ (Table 3). However, that 15 years ago was $2n = 52 = 8.4\text{I} + 8.1\text{II} + 5.7\text{III} + 1.9\text{IV} + 0.6\text{V}$ (Wang et al., 1990). Comparing the configuration of the same hybrid plant in different times, the univalents decreased greatly, but only half of those were found in 15 years ago, the bivalents increased by about 45%, and multivalents decreased sharply as well. In conclusion, with the extending the growth time, the fertility of the quadri-specific hybrid was improved due to the balance of different chromosome groups coming from different parents, which would be possible to develop a new germplasm with the characteristic derived from different cultivated cotton species.

4 Discussion

One of the sterile reasons in interspecific plant hybrid is the chromosome unbalance, and the physilogical correspondence in hybrid plant is very important as well. Due to different parent species which did not correspond with each other in a new nucleus, the growth of hybrid is abnormal in physiological metabolization which leads to

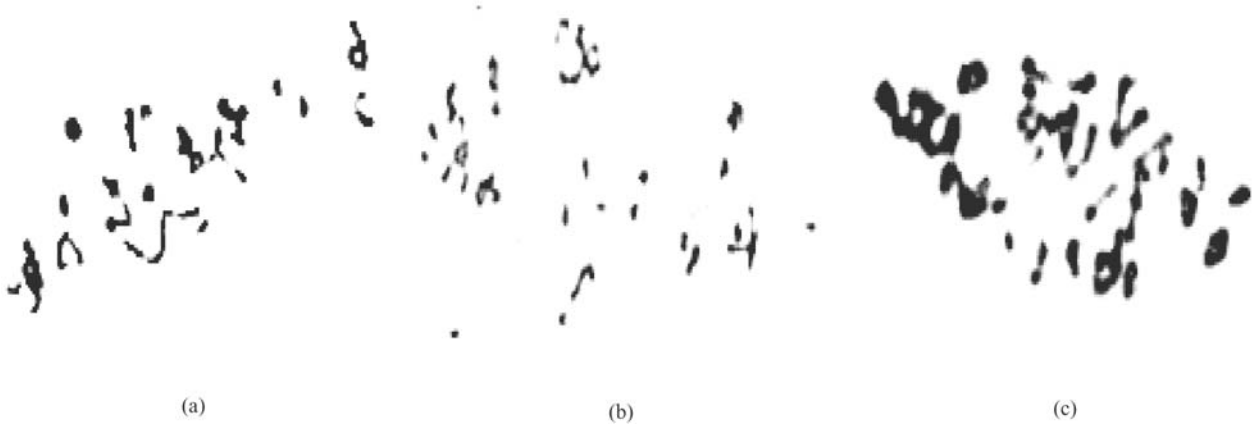

Fig. 1 Chromosome configuration at M I of the quadri-specific hybrid PMC
Note: (a) $2n = 52 = 5\text{I} + 8\text{II} + 4\text{III} + 2\text{IV} + 1\text{V} + 1\text{VI}$; (b) $2n = 52 = 10\text{I} + 8\text{II} + 1\text{III} + 3\text{IV} + 1\text{V} + 1\text{VI}$; (c) $2n = 52 = 9\text{I} + 12\text{II} + 2\text{III} + 2\text{IV} + 1\text{V}$.

Table 3 Chromosome configuration at M I of the quadric-specific hybrid PMC

no.	chromosome configuration types						PMC
	I	II	III	IV	V	VI	
1	2	14	3	2	1		2
2	3	15	2	2	1		3
3	4	19	2	1			5
4	3	19	2		1		6
5	3	20		1	1		7
6	4	11	3	3	1		8
7	4	11	4	2		1	7
8	4	12	2	3		1	6
9	4	13	3	2	1		7
10	3	18	3	1			7
11	4	19	2	1			6
12	4	12	2	2	2		6
13	5	13	3	3			2
14	5	13	4	1	1		9
15	5	14	2	2	1		7
16	5	14	3		2		7
17	5	15	3	2			7
18	5	17	3	1			6
19	5	18	1	2			7
20	6	12	1	2	1	1	8
21	6	12	3	2	1		6
22	6	13	1	3	1		5
23	6	13	2	2		1	7
24	6	13	2	1	2		7
25	6	14	1	1	1	1	8
26	6	14	3	1	1		7
27	6	17	1	1	1		5
28	6	18	2	1			4
29	7	10	4	2	1		4
30	7	16	3	1			5
31	7	17	1	2			4
total	911	2704	423	289	132	36	185
average	4.92	14.62	2.29	1.56	0.71	0.19	

abnormal growth and development, and causes high sterile (Lang, 1999). Repeat genes and repeat genomes of hybrid will interact with each other, so the genome formation and structure, gene express pattern and express level will change apparently. But different chromosomes will coincide with each other in genetics with the times proceed (Yang, 2001). Many other researchers have revealed that planting and cultivating management could promote the fertility of the interspecifc hybrids (Dewey, 1984; Liang et al., 1985; Liang et al., 1992; Yu et al., 2003), and environmental temperature could influence pollen fertility of interspecific hybrid as well (Yang et al., 2003). In our present research, it showed that hybrid had low fertility and failed to produce normal seeds by self-crossing and back-crossing. But by grafting and extending growth time for 15 years, the fertility of male and female gametes were partially recovered and a few of self-crossing and back-crossing seeds were obtained. The chromosome configuration tends to harmonize each other, and bivalents increased from 8.1 to 14.62 on the average, with the decrease of mutivalents and univalents.

The chromosome composition of the quadri-specific hybrid is $1/2(A_1A_1A_2A_2)+1/2([AD]_1[AD]_2)$ because it comes from the hybrid F_1 of *G. hirsutum* × *G. barbadense* and the amphidiploid of (*G. arboreum* × *G. herbaceum*) F_1. Theoretically, the chromosome configuration of hybrid is AAAD, and bivalents should be 13 or more and trivalents should be more. In our observation, the chromosome configuration of the quadric-specific hybrid was $2n = 52 = 4.92\text{I} + 14.62\text{II} + 2.29\text{III} + 1.56\text{IV} + 0.71\text{V} + 0.19\text{VI}$, and its chromosome configuration was $2n = 52 = 8.4\text{I} + 8.1\text{II} + 5.7\text{III} + 1.9\text{IV} + 0.6\text{V}$ 15 years ago. It is evident that A-Genome coming from different species lacks of the capability of chromosome pairing, so there are only a few trivalents in PMC of the quadri-specific hybrid during PMC meiosis, especially after 15 years. In addition, the present results showed that there are partial homologous phenomena between the chromosomes of D genome from tetraploid cotton species and A genome species, which led to more multivalents in the PMC of hybrid F_1, especially in the plant preserved for 15 years. So for the special genetic relationship among the four cultivated cotton species, a new germplasm with the advantages of four cultivated species could be developed by the interspecific hybridization.

Acknowledgements This work was supported by the National Basic Research Program of China (973 Project) (No. 2004CB117305) and the National Natural Science Foundation of China (Grant No. 30671325).

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