Morphological characteristics and identification of new monosomic stocks for cotton (*Gossypium hirsutum* L.)

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ABSTRACT

The presence of distinct morphological markers in monosomics is important for selection and maintenance of the monosomic plants in subsequent generations and for a well-targeted chromosome substitutions. Here we present cytological and morphological features of the cotton (*Gossypium hirsutum* L.) monosomic lines developed in Uzbekistan, and their identification by means of translocation tests. We report "reduced" stigma as a new phenotypic marker for cotton monosomics, which makes it possible to distinguish cytotypes without cytological analyses. We identified eleven cotton monosomes by translocation tests using our 28 translocation cotton lines. We determined such features of the cotton monosomic lines as significant lowering of the pollen fertility, genetic determination of variation in pollen fertility in different flowers of the same monosomic plants and variation of both meiotic index and tetrads with micronuclei in different buds. New features of cotton monosomic lines, described herein, should be useful for future cotton genome investigation and development of new chromosome substitution lines.

Keywords: cotton Monosomic Stocks; Morphological Markers; Translocation Test; Identification of Monosome; Reduced Stigma

1. INTRODUCTION

The development of monosomic stocks for one of the widely-grown fiber crops, cotton (*Gossypium hirsutum* L.), has taken place over many years. The current inventory of monosomics lacks deficiencies for five chromosomes 8, 11, 13, 19 and 24. Therefore, development of monosomics for one or more of these aforementioned chromosomes is a task of high priority. Although the aneuploid lines provide incomplete cotton genome coverage [1], the chromosome assignments of many molecular markers and candidate genes have been successfully accomplished [2-7]. Use of F_1 hypoaneuploid hybrids resulting from the crosses of *G. hirsutum* aneuploids and *G. barbadense* L. species in molecular genetic analyses has facilitated the localization of different molecular markers on specific cotton chromosomes [8-11]. However, some loci were not assigned using the aneuploids due to the lack of a full set of cotton aneuploids [11-14]. During the past several decades, we extensively worked on the development of aneuploid cotton lines from common genetic background of highly inbred line L-458 of *G. hirsutum* using radioactive irradiation techniques that resulted in creation of novel sets of monosomic and translocation lines for cotton. The preliminary cytogenetic and morphological characteristics of this new collection were partially reported previously [15-21]. Cytogenetic details of this new monosomic collection are also studied (Sanamyan *et al.* 2010, unpublished, submitted for publication elsewhere). Here we report the details of morphological characteristics of the cotton monosomic stocks and the results of identification of some of our monosomic line by using translocation lines.

2. MATERIALS AND METHODS

2.1. Morphological Analyses

All aberrant plants were analyzed morphologically. Vegetative and generative plant organs were studied to reveal new morphological markers. We studied plant architecture, brunching type, leaf plate, stem and leaf pubescence, detailed flower morphology including number of stamens and ovules, as well as structural features of all plant organs.

2.2. Identification of the Monosomics

Identification of the monosomes was carried out using

the translocation test. For this purpose, the homozygous translocation lines from Uzbek Cytogenetic Collection [17-19] were crossed with the monosomics as males. Hybrids were analyzed to identify 2n-1 translocation heterozygotes. To reveal "critical configurations" and detect common chromosomes among the chromosomes involved in interchanges with monosomes, a meiotic metaphase I analysis was carried out in heterozygotes of monosomic translocation. All cytological observations were carried out with the microscopes Biomed (Leica, Heerburg, Switzerland) and Laboval (Carl Zeiss, Germany). Monosomics were numbered in detection order (Mo1-Mo92). Monosomic lines were maintained vegitatively in the greenhouse of the National University of Uzbekistan.

3. RESULTS AND DISCUSSION

3.1. Cytology, Pollen Fertility and Plant Morphology of Cotton Monosomic Lines

In cytogenetic analysis, 24 out of 46 cotton monosomic lines showed modal chromosome pairing with 25 bivalents plus one univalent at metaphase-1 of meiosis. The remaining 21 monosomic lines were characterized by the presence of additional univalents in some pollen mother cells (PMCs); moreover, three lines (Mo10, Mo11 and Mo39) had highest frequencies of such univalents (from 1.21 ± 0.10 to 1.33 ± 0.08 in average per cell, respectively) (**Table 1**). The line Mo4 was characterized by the presence of rare trivalents in some PMCs that suggested pairing of the monosomic chromosome with homoeologous chromosome. Appearance of additional univalents in the monosomic lines was seen previously in cotton. In that, six monosomes from the USA Cytogenetic Collection, isolated from the progenies of monosomics, involved other chromosomes [22]. Homozygotization of daughter monosomic genotype led to meiosis stabilization and absence of additional univalents in subsequent generations. In wheat, the monosomic phenomena known as of univalent shift were seen on several occasions [23].

Monosomic lines were also distinguished by sizes of the univalents. Thus, 8 lines were characterized with univalents of large sizes, 26 monosomic lines had univalents of medium sizes, 9 had small univalents. The remaining 3 monosomic lines had extremely small univalents that suggested, a different sub-genome origin and

Table 1. Cytogenetic characteristics of some cotton monosomic lines.

Monosomic line	Size of univalent	Total no. of cells in MI	Chromosome associations		Microsporocytes			Pollen fertility	
			Univalents	Bivalents	Total no. of microsporocytes	Meiotic index	Tetrads with micronuclei (%)	Total no. of pollen grains	Fertility (%)
Mo4	Small	34	$0,88 \pm 0,06$	$24,88 \pm 0,06$	3967	$96,24 \pm 0,30$	$0.55\pm0,12$	1084	$95,39 \pm 0,64$
Mo10	Small	39	1.21 ± 0.10	24.90 ± 0.05	1224	96.16 ± 0.55	1.80 ± 0.38	279	19.35 ± 2.37
Mo11	Small	90	1.33 ± 0.08	24.39 ± 0.22	3609	98.25 ± 0.22	0.58 ± 0.13	1922	96.41 ± 0.42
Mo19	Large	150	1.00 ± 0.00	25.00 ± 0.00	7361	92.50 ± 0.31	3.00 ± 0.20	3801	94.53 ± 0.37
Mo22	Small	42	1.14 ± 0.08	24.93 ± 0.04	3735	96.39 ± 0.31	0.80 ± 0.15	895-830	48.33 ± 1.67- 88.40 ± 1.11
Mo34	Small	27	1.00 ± 0.00	25.00 ± 0.00	1373	96.72 ± 0.48	1.82 ± 0.36	320-588	3.44 ± 1.02- 49.32 ± 2.06
Mo39	Small	41	1.29 ± 0.11	24.85 ± 0.05	3965	97.25 ± 0.26	1.16 ± 0.17	497-1017	12.27 ± 1.47- 72.84 ± 1.39
Mo46	Small	31	1.00 ± 0.00	25.00 ± 0.00	1380	97.10 ± 0.45	1.67 ± 0.34	724-518	28.59 ± 1.68- 80.50 ± 1.74
Mo84	Extremely small	85	1.07 ± 0.05	24.96 ± 0.03	1986-5841	49.40 ± 1.12- 95.48 ± 0.27	12.44 ± 0.74- 0.53 ± 0.10	1020-3957	65.14 ± 1.45- 94.46 ± 0.36
Mo89	Small	78	1.05 ± 0.04	24.97 ± 0.02	7287	98.19 ± 0.16	0.77 ± 0.10	569-3240	74.69 ± 1.82- 95.86 ± 0.35

genetic non-uniformity. In three monosomic lines (Mo1, Mo9 and Mo46), the sizes of univalents differed various in the parental and daughter monosomics, underlining the possibility of univalent shifts in progeny.

Analysis of tetrads of microspores showed a high meiotic index in the majority of the monosomic lines with the exception of the line Mo84 which varied in both meiotic index (from $49.90 \pm 1.12\%$ to $95.48 \pm 0.27\%$) and tetrads with micronuclei (from 12.44 ± 0.74 to $0.53 \pm 0.10\%$) in different buds (**Table 1**). The meiotic index variation led to variation in pollen fertility (from $65.14 \pm 1.45\%$ to $94.46 \pm 0.36\%$) within individual flowers of the same plant. It should be noted that lower meiotic index was recorded in wheat monosomic lines and a high percentage of tetrads with micronuclei confirmed that univalents frequently lagged during chromosome disjunction [24].

Pollen fertility analysis of cotton monosomic lines after acetocarmine staining showed high pollen fertility in the majority of the lines. Only line Mo10 was characterized with strong lowering of the character (to $19.35 \pm 2.37\%$) that suggested its partial sterility with chromosome deficient pollen (**Table 1**). Six other monosomic lines (Mo22, Mo34, Mo39, Mo46, Mo84 and Mo89) showed variation in pollen fertility in different flowers within the same monosomic plants (Table 1). In the three parental monosomics (Mo22, Mo39 and Mo46), variation in pollen fertility among different flowers within the same plants also was observed (13.43-91.37%; 2.53-34.21%; 2.10-92.46%, respectively). The ranges of variation in pollen fertility were wider in two monosomics (Mo22 and Mo46). A similar effect, detected in daughter monosomics, confirmed the genetic determination of such variation and suggested chromosome localization of the gene(s) for male gametophyte viability in the deficient chromosomes. It is known that the majority of cotton chromosome deficiencies are not transmissible via pollen due to non-functionality of chromatin-deficient pollen [25]. Besides, Kakani *et al.* [6] indicated that gene(s) responsible for pollen spine development were located on long arm of chromosome 12 using the advanced technique of confocal laser scanning microscopy and substitution lines.

A study of the morphology of cotton monosomic plants revealed the specific influence of monosomy on many characters that differentiated them from disomic sibs. Such characters were thin stem, feeble leafing, small leaves, short internodes, crooked sympodia, small flowers and bolls, as well as deformed and obligospermous bolls. At the same time, 4 monosomic lines (Mo35, Mo36, Mo40 and Mo50 (e.g. **Figure 1(b)**) looked like disomic sibs. Although the majority of the monosomic lines had a compact bush, 10 lines (Mo3, Mo7, Mo11,

Mo31 (e.g. **Figure 1(c)**), Mo35, Mo39, Mo60, Mo69, Mo73 and Mo89) were characterized by a scattered bush. Two lines (Mo7 and Mo56) differed by having a crooked sympodia and 3 other lines (Mo75, Mo76 (e.g. **Figure 1(d)**) and Mo82) had elongated internodes. In three lines (Mo13, Mo34 and Mo66), a dense stem pubescence was observed whereas leaf pubescence was feeble. Three monosomic lines (Mo16, Mo31 and Mo48) had difference in leaf sizes within the same plant and two other lines (Mo9 and Mo76) had leaf folding in the area of the main rib or lobe division, respectively (**Figure 2**).

Four monosomic lines (Mo4, Mo10, Mo46 and Mo67) differed by having feeble budding and flowering (to 10-15 flowers during the summer) whereas three other lines (Mo22, Mo39 and Mo56) had strong budding and flowering (to 40-60 flowers during the summer) but low seed and boll set (from 10.10 ± 0.78 to 20.71 ± 0.52 per one boll). Many monosomic lines were characterized by small flowers and bracts; however, six lines (Mo4, Mo10, Mo16, Mo34, Mo46 and Mo48) were distinguished by a strong reduction in flower sizes (from 38 mm to 48 mm). Taken together, seven monosomic lines (Mo9, Mo31, Mo39, Mo71, Mo72, Mo73 and Mo76) had large bracts (to 65x67 mm for Mo9) and 5 monosomics (Mo4, Mo10, Mo34, Mo46 and Mo80) had small bracts (to 25x21mm for Mo10). Some chromosome deficient lines (Mo31, Mo72 and Mo76) differed by having a large number of bract teeth (from 14 to 18) whereas other lines had small number of bract teeth (Mo4, Mo10, Mo19, Mo34, Mo46 and Mo80) (from 8 to 12) (**Figure 3**). In the Mo39 line additional bracts were present, in the Mo17 the bracts were asymmetrical and in the Mo27 the bracts were deformed with feebly expressed teeth. The most variability was observed for the character "presence/absence of nectary" where in 15 monosomic lines not all bracts had nectarines (**Figure 4**), and Mo66 lacked any external nectaries. Nectaries of different sizes within a single flower were presented in 6 monosomic lines (Mo9, Mo27, Mo31, Mo39, Mo84 and Mo89).

Monosomy had an influence on the stigma structure and sizes in a flower. Thus, there were shorter stigmata in 3 lines (Mo17, Mo19 and Mo28) and a broad "reverting" stigma in Mo39. A new phenotypic marker for cotton monosomy—"reduced" stigma was detected in Mo62. Analysis of Mo62 progeny revealed the presence of reduced stigma only in monosomic cytotypes whereas disomic ones had normal stigmas as did the control (**Figure 5**). This trait makes it possible to distinguish cytotypes within the progeny without cytological analysis. However, stigma reduction rate was varied in different flowers within the same plant (**Figure 6**). Thus, there were three basic reduction ranges: a little reduction (stigma to 7-9 mm), medium reduction (stigma to 2-6

Figure 1. Some examples of morphology of cotton monosomic plants compared to original parental line: (a) parental line L-458; (b) Mo50; (c) Mo31; (d) Mo76.
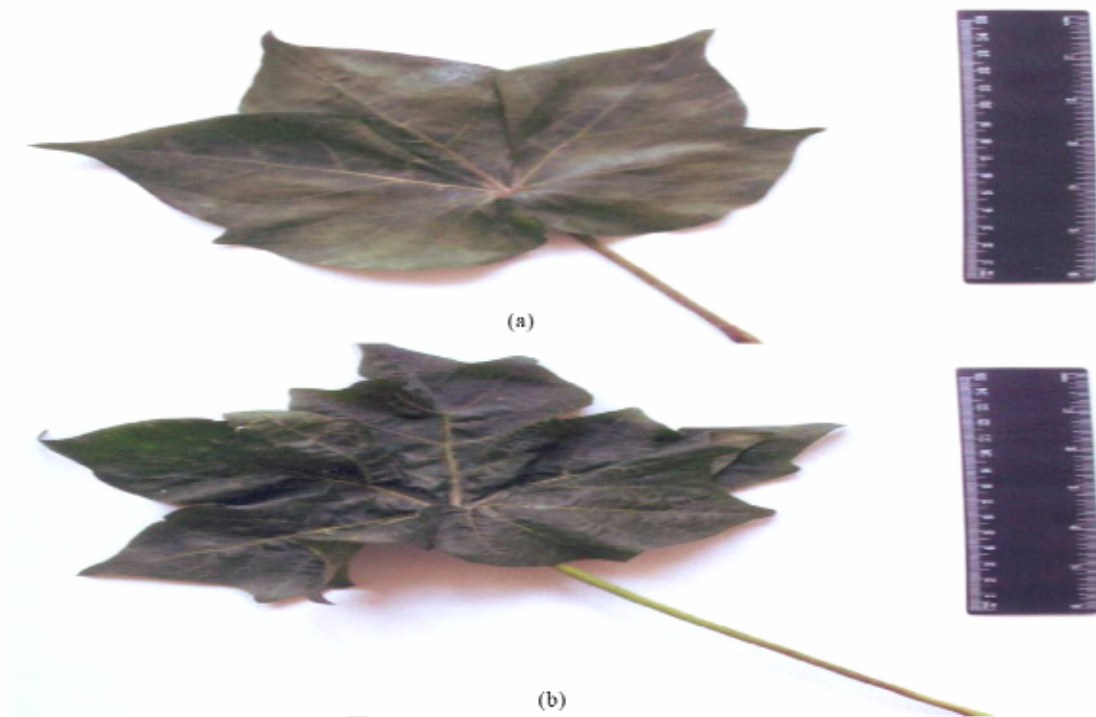
Figure 2. Changed leafs in cotton monosomic lines with leaf folding in the area of the main rib: (a) Mo9 or lobe division (b) Mo76.
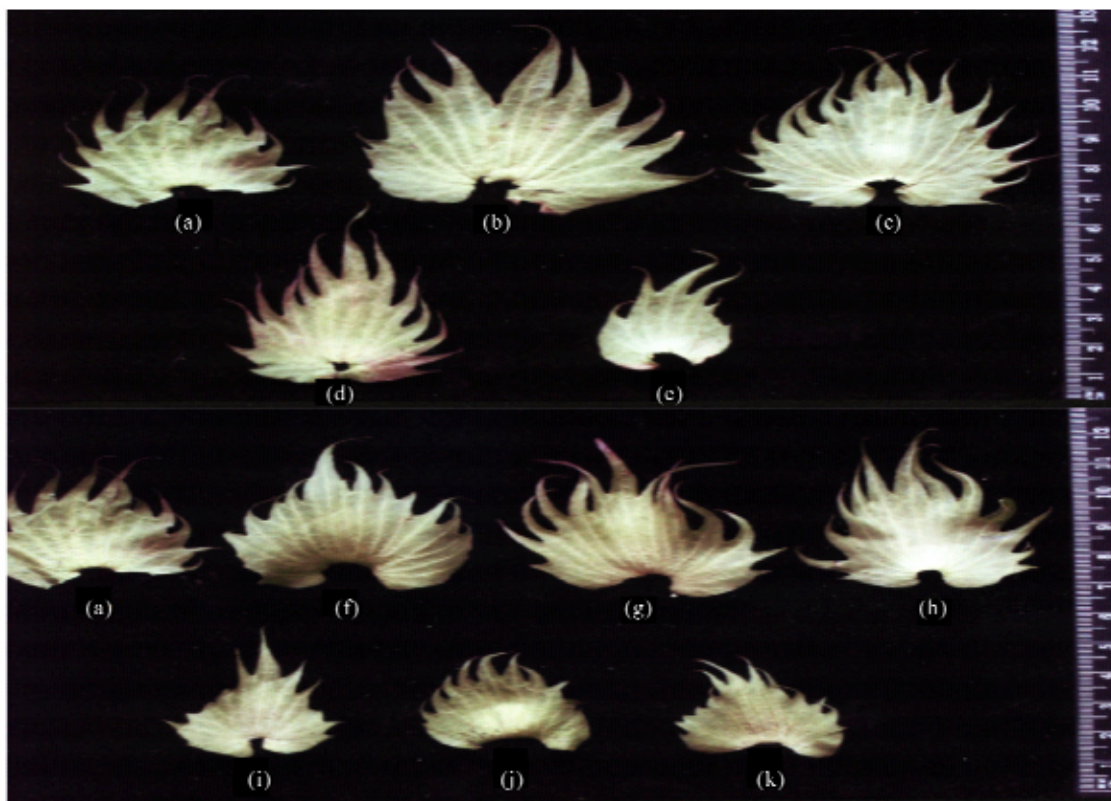

Figure 3. The bracts in the different cotton monosomic lines: (a) parental line L-458; (b) Mo39; (c) Mo72; (d) Mo31; (d) Mo66; and (f to k) Mo84, Mo89, Mo81, Mo88, Mo4, Mo92.
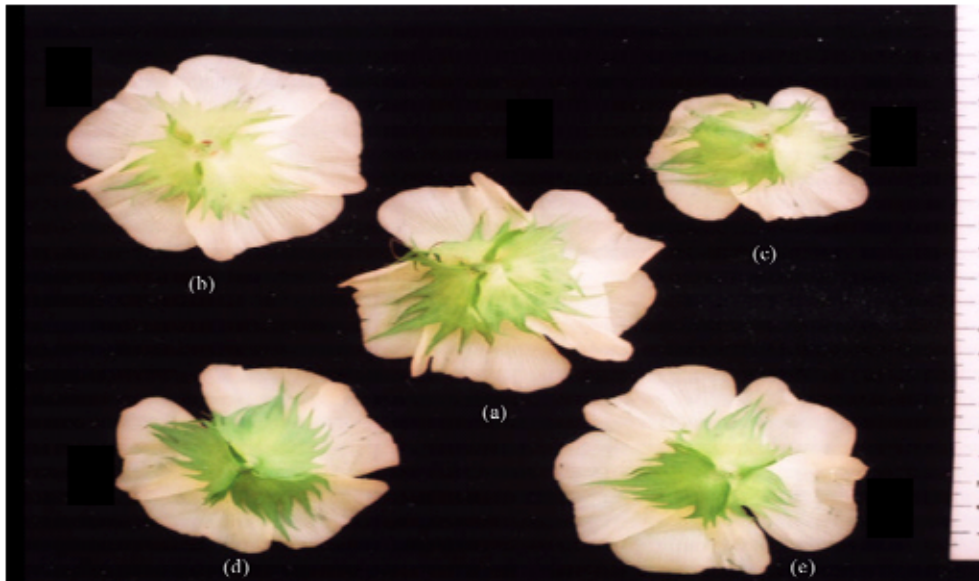
Figure 4. Outside view of nectaries in some cotton monosomics: (a) L-458 parental line; (b) Mo13; (c) Mo39; (d) Mo71; (e) Mo72.

Figure 5. The flowers of the different cytotypes from progeny the monosomic line Mo62: (a) L-458 - parental line; (b) disomic cytotype with normal stigma; (c) monosomic cytotype with medium reduction of the stigma; (d) monosomic cytotype with strong reduction of the stigma.
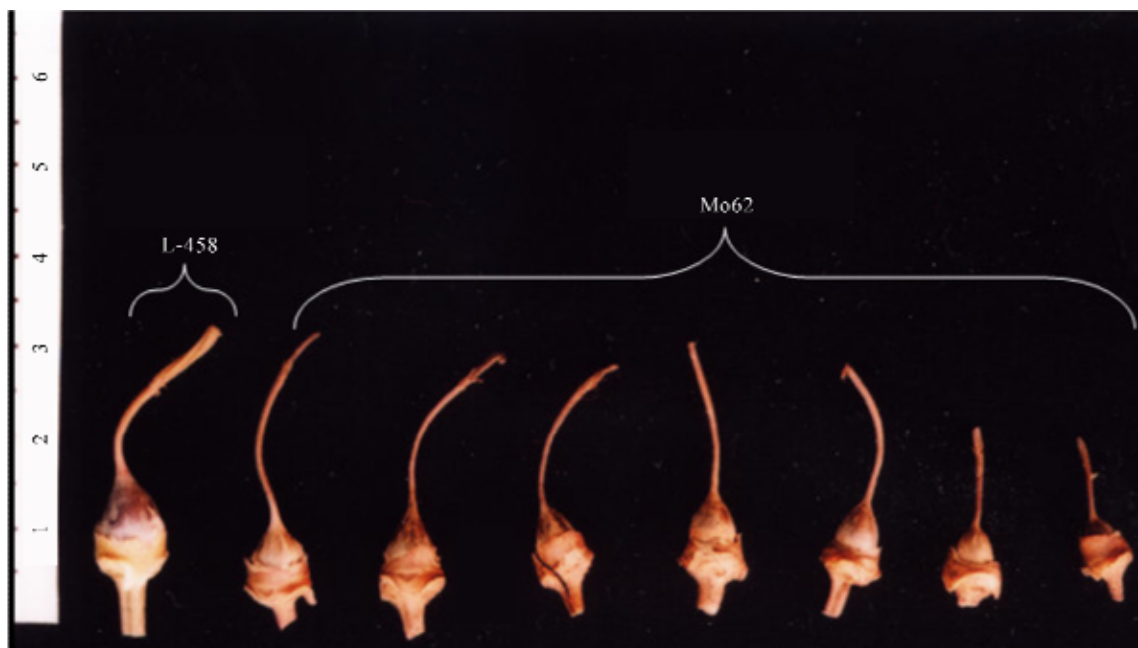
Figure 6. Reduced stigma in the cotton monosomic line Mo62: different range of the reduction of the stigma in Mo62 compared to parental line L-458.

mm), and strong reduction (stigma to 1 mm; **Figure 6**). Moreover, as a rule, strongly reduced stigmas were located inside the staminate columns. Besides flowers with reduced stigmas, there were flowers in which the stigma was closed inside the stylar tissue. A dependence of stigma reduction rates related to the seasons of a year was also established.

All daughter monosomics of Mo62 were fertile both as males and females but had lower seed number per a boll (22.30 ± 1.83) and lower seed set (76.90 ± 2.47 %) in comparison with the parental line L-458 (34.40 ± 0.62 and 89.81 ± 1.55, respectively). A monosome of *G. hirsutum* with a strong stigma reduction but still fertile, has not been described. Thus the monosome in Mo62 for the chromosome of cotton genome could be new. In *G. hirsutum*, miniature stigma were previously designated as "cryptic" because they were usually hidden by the androecium [26] and "club" stigma where homozygous recessive has an extremely small stigmatic surface located at the tip of the style [27]. Both produced completely viable pollen. Due to the lack of a functional stigmatic area "club" stigma plants were completely female sterile and formed a small number of seeds only after manual pollination. A mutant with "rudimentary" stigma also was described in other tetraploid cotton *G. barbadense* L. The styles and stigmas were so dwarfed that they did not emerge from androecium and availability of fertile pollen was such that the numerous attempts to produce seeds by self-pollination or cross-pollination failed [28].

The strongest changes due to monosomy concerned sizes and shapes of bolls as most of the lines formed smaller bolls from round almost spherical to elongated bolls with beaks or without beaks compared to control line. Many of the bolls of monosomics were ribbed or deformed due to a number of abortive ovules and immature seeds (**Figure 7**). As a result, the number of seeds per boll and seed set were lower in all monosomic lines (9.50 ± 1.62 in Mo13 and 32.61 ± 3.99 % in Mo76, respectively) in comparison with the parental line (34.40 ± 0.62 and 89.81 ± 1.55, respectively). Mo4 was characterized with variation of boll sizes within the same monosomic plant and also the fruit occurred in clusters. Flowers and fruit clusters were also observed in Mo19. Mo66 was distinguished by a large broad beak at the top of an ovoid boll (**Figure 7(d)**). Thus, it was shown that an individual chromosome deficiency had a specific influence in plant morphology and that some of them had unique marker characters. However, the clear similarity both morphological and cytogenetic features in some monosomics of our collection suggested probable redundancy of some monosomics.

3.2. Identification of Monosomes by Means of Translocation Test

A lot of small chromosomes occur in the karyotype of tetraploid cotton *G. hirsutum* and the absence of distinctive morphological markers for the chromosomes make it impossible to distinguish and identify chromosomes with the help of standard techniques of karyologic analy-
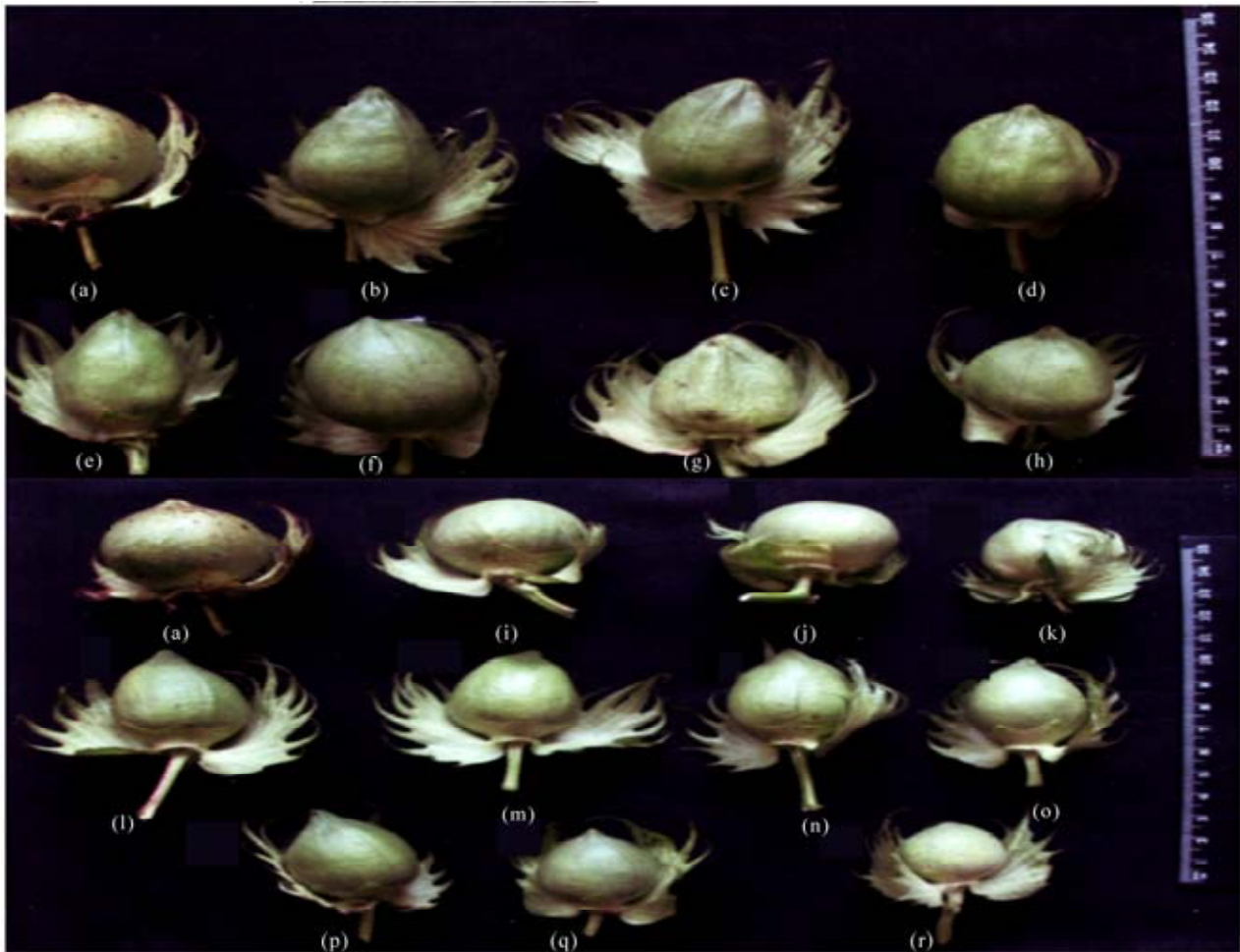
Figure 7. The bolls of the different cotton monosomic lines: (a) L-458 - parental line; (b to h) Mo72, Mo31, Mo66, Mo60, Mo50, Mo39, Mo16 and (I to r) Mo80, Mo4, Mo92, Mo89, Mo81, Mo76, Mo62, Mo75, Mo87, Mo88.

sis. Therefore, we identified monosomes to be specific chromosomes of the cotton genome using the translocation tests on hybrids of monosomics with translocation lines from the Uzbek Cytogenetic Collection. Analysis of hybrid chromosome pairing was used to reveal monosomic translocation F_1 hybrids and to study "critical configurations". The recently developed 28 translocation lines (Tr1-Tr28) from our collection [17,18] were used for monosome identification according to the method described previously [29]. Our initial efforts to monosome identification were presented in previous article [16]. Here we present identification of 15 new monosomics in addition to 20 the monosomics identified previously.

According to **Table 2** eleven monosomics from our collection (Mo3, Mo10, Mo11, Mo19, Mo27, Mo39, Mo48, Mo53, Mo56, Mo73 and Mo85) were associated with the chromosomes of seven translocation lines (Tr1, Tr3, Tr5, Tr8, Tr11, Tr12 and Tr16) as chromosome pairing of 24 bivalents plus one trivalent was observed

in PMCs of the F_1 monosomic hybrid plants (Figure 8a). In this study, we also identified four monosome pairs (Mo10 and Mo73; Mo39 and Mo56; Mo48 and Mo53; Mo11 and Mo19) that were associated with the translocation lines Tr3, Tr5, Tr12 and Tr16, respectively. Thus, three of the above-mentioned monosome pairs (Mo10 and Mo73, Mo39 and Mo56, Mo 11 and Mo19) involved the same chromosomes with the each pair. In future analyses, hybrids from the crosses of the monosomics and other translocation lines, involving the same chromosomes, will confirm our interpretation and identification. However, there is evidence for monosomes Mo48 and Mo53 that nonhomologous as the chromosomes from two different sub-genomes all involved with translocation line Tr12. According to the preliminary numeration that was used in this investigation, differed from the numeration published by Brown [30], chromosome 5 is from the A_t-genome and chromosome 14 from D_t-genome [31]. In nonhomology of the monosome and chromosomes involved in interchange of cross of Mo48

Table 2. Cytological test for identification of the monosomes with the help of translocation lines.

Monosomics	Translocation lines							Total number of crosses tested
	Tr1	Tr3	Tr5	Tr8	Tr 11	Tr 12	Tr 16	
Mo3			-		+	-		4
Mo7			-	-		-	-	7
Mo10		+	-	-				3
Mo11	-	-	-	-	-	-	+	14
Mo13	-					-	-	13
Mo19		-	-				+	5
Mo27			-	+				5
Mo31	-	-	-	-	-	-	-	25
Mo35				-				3
Mo36								3
Mo38	-		-	-				9
Mo39			+	-				6
Mo41					-			1
Mo48						+		4
Mo50	-	-	-	-		-		19
Mo53				-		+		2
Mo56			+	-		-	-	8
Mo60			-	-			-	9
Mo62	-		-					6
Mo66	-	-	-	-	-	-	-	12
Mo67	-							2
Mo69	-		-	-	-	-	-	21
Mo70		-	-		-	-	-	11
Mo71	-	-			-	-	-	12
Mo72	-	-						9
Mo73	-	+	-	-	-	-	-	14
Mo75	-	-	-	-	-		-	18
Mo76	--			-				9
Mo77								6
Mo79	-		-	-			-	10
Mo80			-		-			4
Mo81	-		-		-	-	-	12
Mo84								1
Mo85	+					-		4
Mo89							-	5

(+ associated, – independent)

and Tr2, one of the interchanged chromosomes was the chromosome 14 D_t-genome. The cross Mo53 and the line Tr8 revealed that in nonhomology of the monosome and the chromosomes in the interchange involved chromosome 5 of A_t-genome thus showed nonhomology of Mo48 and Mo53.

We have isolated 4 monosomics (Mo70-Mo73) from the progeny of the same desynaptic plant and proposed possible monosomy for different nonhomologous chromosomes of the cotton genome. Indirect confirmation was available with the detection of monosome Mo73 homology and one of the chromosomes involved in interchanges in the line Tr3 whereas the other three monosomes from the progeny of the same desynaptic plant (Mo70, Mo71 and Mo72) did not has any chromosomes in common in the Tr3 interchange. Another monosome (Mo85), isolated from the other desynaptic progeny, showed homology with a chromosome involved in an interchange with Tr1. This test revealed that the chromosomes of Tr1 were rarely involved in translocations. Tr1 had common chromosomes only with two lines—Tr2 and Tr20 with multiple interchanges [31]. This verified our assumption that new or rare monosomes would occur in progenies of desynaptic forms of cotton [15].

Translocation tests involving other 24 monosomic lines have not yet revealed any homology of the monosomes and the chromosomes involved in interchanges because they showed detections of chromosome pairing with 23 bivalents plus one univalent plus one quadrivalent (**Figures 8(b)-(d)**). However they did demonstrate the differences in the studying level of the lines as well as depended on transmission rates of the monosomics in hybrid progenies. There is an evidence of the comparative rareness of other monosomes from our collection (**Table 2**). For instance, we confirmed homology of the monosomes and the chromosomes in interchanges in 4 monosomics (Mo10, Mo27, Mo48 and Mo53) with analysis of 2-5 hybrid crosses while the absence homology was detected in the 8 monosomics (Mo13, Mo31, Mo50, Mo66, Mo69, Mo71, Mo75 and Mo81) in analysis of 12-25 hybrids. Assignment of the chromosomes involved in interchanges with Tr1, Tr8 and Tr16 the A_t-genome and with Tr2 the At- and D_t-genomes [31] allowed six monosomes (Mo11, Mo19, Mo27, Mo39, Mo56 and Mo85) to be assigned to the A_t-genome of cotton.

Use of the translocation tests for monosome identification revealed some differences among the lines in the frequency of each monosome and the chromosomes involved in interchanges. Monosome transmission rates were different in self-pollination progenies and hybrids owing to differences of transmission rates of haplo-de-
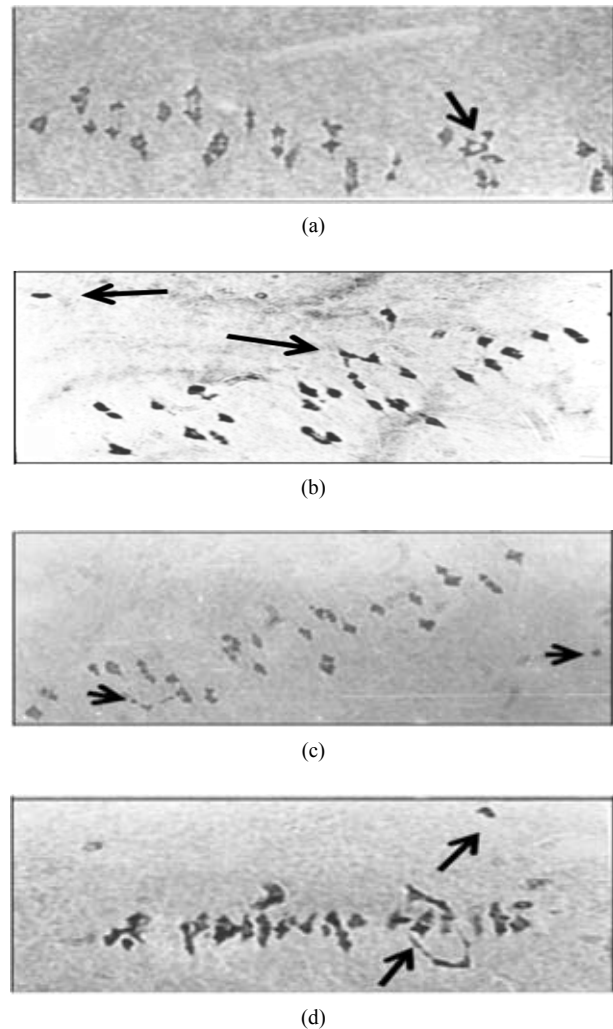
(a)

(b)

(c)

(d)

Figure 8. "Critical configurations" of the chromosomes at the meiotic metaphase I cells in cotton F1 plants from crosses the monosomic x translocation lines: (a) Mo85xTr1 (24II + 1III); (b) Mo75xTr5; (c) Mo75xTr16; (d) Mo77xTr21 (23II + 1I + 1IV). The arrows point to the univalents and quadrivalents. Magnification x 1000.

ficient gametes in monosomic translocation hybrids. The results showed interesting "rareness" of some monosomes with respect to the chromosomes involved in interchanges due to the absence of homology among them. These results suggested the need for more complete coverage of cotton genome with interchanges and deficiencies.

A comparative analysis of monosomic frequencies in the USA Cytogenetic Collection revealed more frequent occurrence of monosome A2 from the A_t – genome (28 times), characterized by a more frequent transmission rate (45%) and chromosomal interchange frequency (12 translocations) [32,33]. However, sometimes monosomic transmission rates, detection of the monosomes as dou-

bles, and/or chromosome involved in translocations were not in correspondence. For example, the transmission rate of the chromosome D18 was 43%. It was revealed 7 times from different sources and involved in only one chromosome interchange that showed differences among various chromosomes, participating in interchanges and deficiencies.

4. CONCLUSIONS

The results presented in this report suggested a detection of "reduced" stigma as a new unique phenotypic marker for cotton monosomics which makes it possible to distinguish cytotypes without cytological analyses. Our cotton monosomic lines are unique and should be a valuable cytogenetic tool not only for chromosome assignment of new marker genes and genome enrichment with new chromosome deficient plants, but also for a development of new cotton chromosome substitution lines and germplasm introgression. In future, we will identify our cotton monosomic stocks using a well-defined tester-set of translocation lines of the USA Cytogenetic Collection, kindly provided by Dr. D. M. Stelly, Texas A&M University, USA, under USDA germplasm exchange program. Moreover, research is underway to develop chromosome substitution lines via interspecific hybridization of monosomic stocks and *G. barbadense* (Pima 3-79 and 5904-I variety) for effective use of monosomics in cotton breeding programs. An effort toward identification of specific chromosomes for our collection using *a priori* chromosome-associated DNA markers is also in progress.

5. ACKNOWLEDGEMENTS

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