DNAMETHYLATION ANALYSIS OF CHROMOSOME 6 AND 8 IN *ALLIUM FISTULOSUM* L. USING AN ANTIBODY AGAINST 5-METHYLCITOSINE

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Abstract: the DNA methylationpattern of 6 and 8Allium fistulosum L. metaphase chromosomes wasexaminedwith a specific antibodies against 5-methylcytosine. Immunodetection of5-methylcytosine (5mC) has shown that distal regions of the chromosomes were highly methylated, and differences may be present between corresponding regions of homologues. The stable methylation patterns have appeared to be co localized with both localization subtelomeric tandem repeat and C-bands.

Key words: methylation, chromosomes, Allium fistulosum.

Much information about DNA methylation in animals and fungi has been collected so far, but the data on DNA methylation in plants is scant, while it is known that the plant DNA is highly methylated [14]. The origin and function of such a high level of methylation in plants are still not completely clear. The level of DNA methylation in plants is 10 times higher than in vertebrates [8,9]. There are significant differences in the level of DNA methylation in different species of plants. In *Arabidopsis, 6%* cytosines are methylated, in most other plants - 20-30%. These differences are related both to the number of repeated sequences, and different levels of methylation CNG sequences [4].

DNA methylation controls all genetic processes in a cell, such as replication, transcription, DNA repair, recombination, transposition of genes, and it is a mechanism of cellular and sexual differentiation and genomic imprinting [2, 9]. It was shown that the methylation in animals controls the gene expression during development and cell differentiation. However, it is unclear, if such correlation takes place in plants. The correlation seems to be present in the zein gene expression in maize, but it is absent in the alcohol dehydrogenase gene [20, 25]. In any case, it is difficult to imagine that all 5-methylcytosine in plants are involved in controlling gene expression.

The methylation in Arabidopsis thaliana is well-studied. The genes responsible for the methylation are known [15]. The stable inheritance of the methylation was shown [17]. Reduced DNA methylation in Arabidopsis thaliana after treatment with 5-azacytidine results in abnormal plant development [11]. Research conducted in 2010 on hybrids between Raphanus and Brassica showed significant differences in the methylation sites in comparison with the parental forms, which may indicate the important role of methylation for the stabilization of the hybrid in the process of its formation, when methylation sites coordinate interaction of nuclear genes with cytoplasmatic genes [16].

DNA of prokaryotes contains modified bases N6-methyladenine and 5-methylcytosine, while the DNA of higher eukaryotes mainly contains 5-methylcytosine [1, 24, 26, 27]. Specific antibodies for 5-methylcytosine (5-mC) allow localizing methylated segments of DNA to the physical chromosome [17]. Detection of methylated regions in the 5-mC antibody was performed on the chromosomes of humans and animals [19,23], as well as on the chromosomes of plants: the polytene chromosomes of Phaseolus coccineus [5] and mitotic metaphase chromosomes of Allium cepa [6].

We have been investigating the sites of DNA methylation in mitotic chromosomes of A. fistulosum. Now enough data has been collected that stable methylation sites do not contain genes, this fact served as a prerequisite for these studies [1]. The questions arise: firstly, do exist stable sites of methylation in the genome of A. fistulosum and, secondly, will they coincide with the localization of non-coding DNA? Despite the fact that the phenomenon of DNA methylation is an epigenetic process and reflects both the structure and functional activity of chromosomes, the analysis of the distribution of methylation sites in humans and animals showed the presence of stable methylation sites [19, 23].

Material and methods

Seeds *oi Allium fistulosum* L. (2n=16), cultivar Parado, were germinated at 22°C. Root tips were pretreated in the *a* - Bromnaphthalin saturated solution, then they were fixed in ethanol-acetic acid (3:1). Chromosome preparations were made according to the standard method by spreading [21].

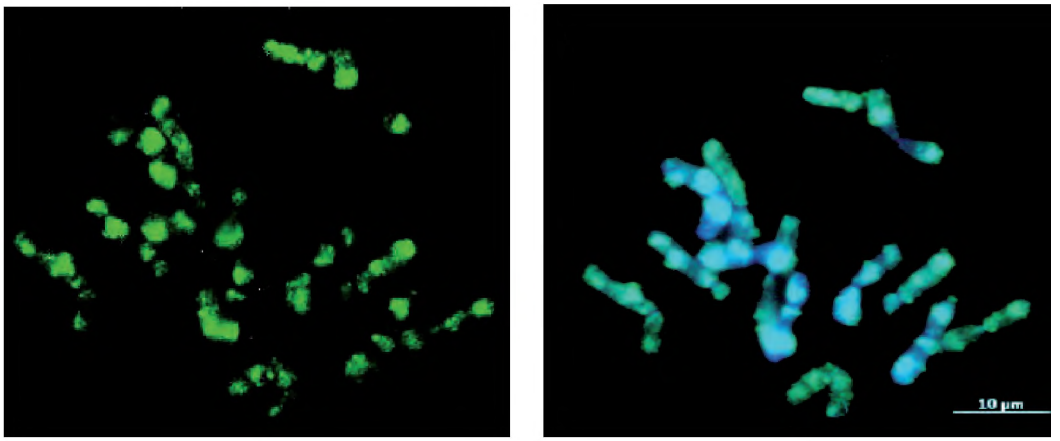
Immunodetection. For 5mC detection slides were incubated in 4% paraformaldehyde/PBS **(IOмM** sodium phosphate, 143 mM NaCl) for 10 min, then washed in lxPBS two times for 5 min, then dehydrated for 3 min each 70% (at -20°C), 90% and 100% ethanol. The air-dry slides were denatured in HB50 (2xSSC, 50% formamide, 50mM sodium phosphate pH 7.0) at 80°C for 2 min, then washed in an ice 2 x SSC two times for 5 min, then slides were treated with the 1% BSA, lx PBS solution at 37°C for 30 min in a moisture chamber, and then incubated with a mouse monoclonal antibody against 5mC (Calbiochem Cat. No. NA81), diluted 1/200 with 1% BSA, at 37°C for lh in a moisture chamber. After three washes with lx PBS for 5 min, the slides were incubated with anti-mouse FITC (Calbiochem Cat. No. 401219), diluted 1/200 with 1% BSA, at 37°C for lh in a dark moisture chamber, and washed in lxPBS with 0.1% Tween20 two times for 5 min. The slides were counter stained with DAPI and mounted in an anti-fade solution (Vectashield).

Microscopy was performed on a fluorescent microscope Carl Zeise Axiolmager (http://www.zeiss.com/), equipped with a mercury-vapor lamp, a set of filters for viewing DAPI and FITC fluorochrome, digital camera Axio Cam MRm. Images were produced and merged using Axio Vision version 4.6.3.

Analysis of the methylation sites. The size of the methylation sites were measured using the Micro Measure [22]. We measured the length of the chromosome, centromeric index, the size of each distinct site of methylation. The data were processed using Excel MS 2003.

Results

The analysis of distribution DNA methylation pattern was conducted in the mitotic metaphase chromosomes of A. fistulosum. Figure 1 shows mitotic metaphase chromosomes after immunodetection of DNA methylation using antibodies against 5 mC. For analysis we selected chromosomes 6 and 8 as these chromosomes are easily identified in the chromosome set without karyotyping: chromosome 6 is subakrocentric chromosome with a satel-



a b

Fig. 1. Mitotic metaphase of A. fistulosum after detection of the methylation pattern with antibodies against 5-mC. a) photomicrography with only FITC-filter(green), b) microphotography using DAPI(blue) -and FITC-filter followed by a combination of images in the program AxioVision, version 4.6

lite having a relative length 11,9 ± 0,3 and centromeric index 18,2 ± 2 , 6; chromosome 8 is the smallest chromosome of set having a relative length 10,3 ± 0,3 and centromeric index 38,2 ±3,0.

Chromosome 6. Analysis of chromosome 6 has shown that distal regions were stably methylated in both arms. The average length of the methylation site was 18.0% ± 3.2 on the long arm and 9.6% ± 1.9 on the short arm of the chromosome. Some of the analyzed chromosomes 6 on the long arm were methylated in the pericentromeric region with the average size 14.5%± 6.6 ofthe length ofthe chromosome. The differences in the distribution of methylation sites in homologous chromosomes should be noted. In some cases, the sites in the homologous chromosomes can be methylated in one homolog and unmethylated in the other. The measurement results for the chromosomes carrying the methylation sites only at the distal regions are shown in table 1. Based on the experimental data, ideogram of chromosome 6 indicating the size and position of stable and rarely methylated sites was constructed (fig. 2).

Table 1

The stable methylation sites in the distal region of chromosome 6 Allium fistulosum

Chromosomes	Long arm			Short arm			Satellite **	The total length of the methylation sites in the chromosome, %.
	the length of arm	the length of the distal methylated site	the length of unmethylated site	the length of arm	the length of the distal methylated site	the length of unmethylated site	the length of the satellite	
1	2	3	4	5	6	7	8	9
1	85,08*	18,66	66,41	14,92	7,51	7,41	13,77	26,2
2	81,81	17,65	64,15	18,44	7,5	10,94	15,53	25,2
3	81,81	10,09	71,72	18,2	5,04	13,16	14,2	15,1

Continued

1	2	3	4	5	6	7	8	9
4	80,71	10,46	70,26	19,3	8,32	10,97	14,46	18,8
5	82,36	22,39	59,97	17,64	11,07	6,57	12,44	46,3
6	81,69	22,57	59,12	18,31	13,08	5,23	12,3	47,3
7	80,32	10,88	69,45	19,68	13,55	6,13	12,53	24,4
8	78,32	17,14	61,18	21,68	13,58	8,1	13,4	30,7
9	85,55	22,91	62,64	14,44	10,54	3,9		33,4
10	84,99	12,55	72,44	15	10,17	4,83		22,7
11	87,39	8,97	69,86	12,61	11,34	1,27	10,44	28,9
12	88,22	6,21	82,01	11,77	11,77	0		20,1
13	83,77	6,47	77,3	16,22	10,4	16,22		16,9
14	81,12	8,36	72,76	18,88	11,87	7,01	10,47	20,2
15	84,57	8,32	76,24	15,43	6,86	8,57		15,2
16	83,77	20,37	63,4	16,23	11,58	4,64		31,9
Mean value	83,09	14,00	68,68	16,80	9,61	7,18	8,10	25,81
The standard deviation of the mean, p = 0.95	1,44	3,22	3,53	1,42	1,9	2,22	3,5	5,70

* All sizes of lengths are relative to the length of the entire chromosome (excluding the size of the satellite).

** Satellites were saved from not all of the analyzed chromosomes in the preparation of cytological preparations. Methylation sites of of the satellites were not detected.

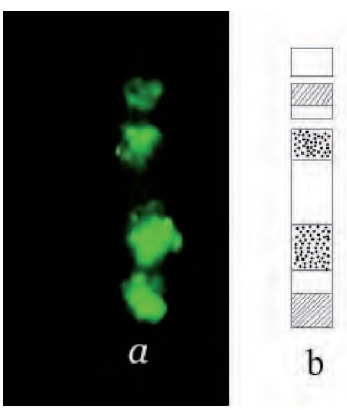


Fig. 2. The location of methylation sites on chromosome 6 Allium fistulosum: a) the landing site of antibodies to 5-mC (green); b) ideogram of chromosome 6: consistently methylated regions, rarely methylated regions, unmethylated regions

Chromosome 8. On chromosome 8 distal regions of the short and long arm were stably methylated. On the short arm the average size of the methylated site was 18.9% ± 4.1 of the length of the entire chromosome. In two cases the short arm was methylated almost along its entire length. Two variants with pericentromeric regions methylated were found, the length of the methylation site was 11.7% ± 8.05. The long arm was stably methylated in the distal region, the length of the methylation site was 19.8%±4.7of the chromosome, in three casesthe pericentromeric region was methylated, the length of the site was 19.2% ±7.1. The differences in methylation were detected between appropriate sites in homologous as it was shown for chromosome 6.

The data for the chromosomes carrying the methylation sites only at the distal region are represented in table 2. Based on the experimental data, the ideogram of chromosome 8 indicating the methylation sites has been drawn (fig. 3).

Table 2

The stable methylation sites in the distal region of chromosome 8 Allium fistulosum

Chromosomes	Long arm			Short arm			The total length of the methylation sites in the chromosome, %
	the length of arm	the length of the distal methylated site	the length of unmethylated site	the length of arm	the length of the distal methylated site	the length of unmethylated site	
1	61,4*	15,1	36,3	38,6	21,4	27,2	36,5
2	59,5	15,9	43,6	40,6	13,4	29,1	29,4
3	61,4	17,8	43,6	38,6	21,8	16,8	39,5
4	62,5	16,3	46,2	37,5	21,7	9,8	38,0
5	63,2	16,1	63,2	36,8	30,3	6,5	46,3
6	62,4	11,0	51,4	37,6	14,6	22,8	25,6
7	59,1	15,6	59,1	40,9	14,8	26,7	30,3
8	59,7	31,7	28,0	40,3	14,7	25,6	46,4
9	63,8	24,2	39,6	36,2	16,8	19,5	40,9
10	63,6	16,5	47,0	36,4	14,8	36,4	16,5
11	69,2	29,2	40,0	30,8	13,7	17,1	42,9
Mean value	62,2	19,8	45,1	38,8	18,9	26,8	28,2
The standard deviation of the mean, p = 0.95	2,2	4,7	6,8	3,0	4,1	7,3	7,8

* All sizes of lengths are relative to the length ofthe entire chromosome.

Discussion

For the first time, the distribution of DNA methylation pattern in chromosomes of *A. fistulosum* was studied. The physical organization of DNA methylation pattern in plant chromosomes has been studied to a very little extent. Most of the work concerning in situ localization of methylated DNA performed on human chromosomes and animal cells.

We conducted a comparative analysis of the localization methylation sites with the earlier data distribution of C-bands on chromosomes of *A. fistulosum* [13], which revealed the coincidence ofthe stable methylation sites with the C-bands in the distal regions of chromosomes. It should be noted that on the short arm of chromosome 8 the coincidence of location of interstitial rarely methylated sites with

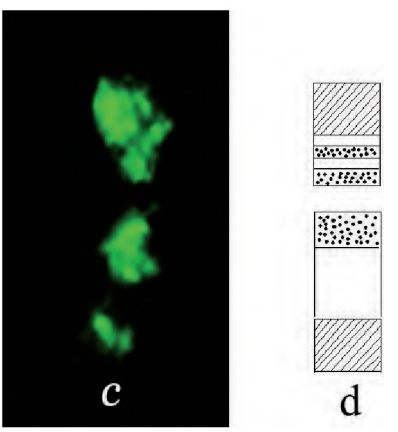


Fig. 3. The location of methylation sites on chromosome 8 onion Allium fistulosum: a) the landing site of antibodies to 5-mC (green); b) ideogram of chromosome 8: consistently methylated regions rarely methylated regions, unmethylated regions

C-bands was also observed. Our results are consistent with the data of the analysis of the chromosomal distribution of methylation sites in *A. cepa* [6]. The authors showed that methylation sites, in general, coincided with the location of heterochromatin sites - C-bands. Based on our data and research carried out on *A. cepci,* we may assume that the DNA in the C-band is likely to be in methylated state. However, in similar studies of Zingeria biebersteiniana, it was reported, that not always highly methylated regions coincided with the localization of C-band [12]. Clear C-bands are typical for most representatives of the family Poaceae, including Zingeria biebersteiniana, while the C-banding pattern in Alliums is weakly represented in the distal regions.

The comparison of the methylation site distribution with our earlier results on FISH using as a probe of PCR products obtained with primers for subtelomeric heterochromatin repeat in *A. fistulosum* [3] revealed co-localization of methylation sites and 382 bp tandem satellite repeat.

Our results showed differences in the distribution of methylation sites in homologous chromosomes, which was manifested in the presence of clear-cut methylation site in one homologue and its absence in the other. The same difference in methylation between homologous chromosomes was observed in Zingeria biebersteiniana [7]. The authors explained this phenomenon by the difference in the organization of chromatin in the relevant regions, which may reflect differences in transcriptional activity of homologous chromosomes.

Our subsequent investigations of the physical mapping of genes and noncoding DNA on the Allium chromosomes and the level of methylation will bring more clarity to understanding of the structural and functional organization of DNA in chromosome. Taking in account an important role of methylation in stabilizing of hybrids, the study may assist the interspecific selection.

This work was granted by the Ministry of Education and Science of Russian Federation within the framework of Federal Program "Scientific and scientific-pedagogical personnel of innovative Russia," State contract number P809. The authors wish to thank a graduate student A.N. Marchenko of RSAU-MTAA for her assistance.

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АНАЛИЗ РАСПРЕДЕЛЕНИЯ САЙТОВ МЕТИЛИРОВАНИЯ НА ХРОМОСОМАХ 6 И 8 *ALLIUM FISTULOSUM* L. С ИСПОЛЬЗОВАНИЕМ АНТИТЕЛ К 5-МЕТИЛЦИТОЗИНУ

Аннотация: проведены исследования распределения сайтов метилирования на хромосомах б и 8 Allium fistulosum L. *с использованием антител к 5-метилцитозину. Установлено, что стабильно были метилированы дистальные регионы хромосом. Выявлены различия в метилировании гомологичных хромосом. Показано наибольшее соответствие стабильных сайтов метилирования с локализацией субтеломерного тандемного повтора и С-бэндами.*

Ключевые слова: метилирование, хромосомы, Allium fistulosum.

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