Theor Appl Genet (1997) 95: 1300–1304 © Springer-Verlag 1997

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DNA content of wheat monosomics at interphase estimated by flow cytometry

Received: 21 January 1997 / Accepted: 23 June 1997

Abstract Two complete, independently maintained sets of 21 monosomic wheat lines derived from cv. 'Chinese Spring' were analyzed for their DNA content at the G1 stage with flow cytometry. The DNA content of individual chromosomes was estimated by subtracting the value of a monosomic line from that of euploid wheat. Our data show that the estimated 2C DNA of individual wheat chromosomes in 21 monosomics at the G1 stage ranges from about 0.58 pg in chromosome 1D to approximately 1.12 pg in chromosome 3A. The A genome (2C = 6.15 pg) seems to contain more DNA than the B (2C = 6.09 pg) and D (2C = 5.05 pg) genomes. Analysis of variance showed significant differences ($\alpha = 0.01$) in DNA content both among homoeologous groups and among genomes. Our estimates of interphase DNA content of wheat chromosomes from monosomic lines were poorly correlated to the chromosome sizes at metaphase ($r = 0.622$, $P \leq 0.01$). This poor correlation might be due to differential coiling among chromosomes during cell division, possible bias of fluorochrome binding to heterochromatin, or heterogeneity among monosomic lines. Finally, flow cytometry may aid but cannot replace cytological checks in aneuploid maintenance.

Key words Flow cytometry · *Triticum aestivum* · DNA analysis · Monosomics

This research was partly supported by the University Nebraska Center for Biotechnology, and USDA National Research Initiative Competitive Grant Number 93-37300-8817. Contribution from South Dakota Agricultural Experiment Station as Journal Series Number 2939

Communicated by B. S. Gill

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Introduction

The flow-sorting of individual chromosomes following synchronized cell division is a particularly promising technique for use in developing chromosome-specific genomic DNA libraries for the mapping and isolation of agronomically important genes, particularly for species with a large genome size such as bread wheat (*Triticum aestivum* L.) (Bennett and Smith 1976; Yen 1988; Wang et al. 1992). Flow cytometry sorts individual chromosomes on the basis of the strength of the fluorescent signal, which in turn reflects the DNA content of the fluorochrome-stained chromosomes. DNA contents for individual wheat chromosomes have been estimated on the basis of Feulgen microspectrophotometry (Nishikawa 1971; Nishikawa and Furuta 1978; Furuta et al. 1988). However, these estimates have not been verified by DNA content measurements based on flow cytometry. Therefore, the latter information is needed for flow sorting of wheat chromosomes.

Pfosser et al. (1995a) reported that flow cytometry can detect differences as small as 1.84% in the nuclear DNA content. The DNA content of individual rye chromosomes or chromosome arms has been determined by this techniques (Bashir et al. 1993: Pfosser et al. 1995b). Apparently, the sensitivity of flow cytometry is sufficient to analyze DNA variations caused by a missing chromosome from wheat nuclei. Whether flow cytometry is reliable for identifying wheat monosomic lines remains untested.

The objectives of this study were: (1) to determine the DNA content of each bread wheat chromosome by

flow-cytometric DNA analysis of a complete set of monosomic wheat lines derived from cv 'Chinese Spring', and (2) to study the reliability of flow cytometry in monosomic identification.

Materials and methods

Plant materials

A complete set of 21 monosomic lines derived from bread wheat (*Triticum aestivum* L.) cultivar 'Chinese Spring' (CS) were assayed for their DNA content. To avoid errors caused by any potential monosomic shift, we obtained and analyzed two independently maintained sets of the CS monosomic lines from Dr A. Lukaszewski of the University of California-Riverside and Dr. B. S. Gill of Kansas State University. In addition, techniques of mitotic chromosome counting (Yen and Liu 1987) and N-banding (Endo and Gill 1984) or C-banding (Gill et al. 1991) were applied to confirm monosomic identity. Euploid 'Chinese Spring' was used as the standard control, and barley (*Hordeum vulgar* L.) line NE86954 was used as the internal control. Seeds were allowed to germinate in petri dishes at room temperature for 4 days. Since leaf age has been found to affect the accurate estimation of nuclear DNA content (Blondon et al. 1993; Lee et al. 1996), both disomic and monosomic lines were germinated at four different intervals to make sure that young leaves at a similar developmental stage were used for flow-cytometry analysis.

Flow cytometry

Nuclei were isolated from 1-week-old young leaves about 2 cm in length by the chopping method described by Arumuganathan and Earle (1991). Leaf materials from individual plants were put together with internal control barley leaf material into a plastic petri dish containing 0.5 ml ice-cold nucleus isolation buffer [10 m*M* $MgSO_4 \cdot 7H_2O$, 50 m*M* KCl, 5 m*M* Hepes, 3 m*M* dithiothreitol, 0.25% Triton X-100, and 100 μg/ml propidium iodide (PI)]. The leaf materials were chopped with a sharp scalpel. The nucleus suspension was filtered through a 80-μm nylon mesh, and incubated on ice for 30 min, and then analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.). Each line was analyzed four times, and duplicated samples were run per experiment (i.e., a total of eight replicates were analyzed for each line). In addition, samples from each filtered nuclei suspension were also treated with DNase-free RNase (at a final concentration of 1.25 μg/ml, Boehringer Mannheim, Indianapolis, Ind.) and incubated at 37°C for 15 min before flow-cytometric analyses to determine the effect of the presence of double-stranded RNA on the estimation of nuclear DNA content.

The wheat nuclear DNA content (2C) of 34.6 pg (Bennett and Smith 1976), which was recommended by Price and Johnson (1996) as a standard in flow cytometric DNA analysis, was used in this study to calculate the DNA content of each wheat chromosome. To avoid any possible variation of fluorescent intensity between assays, we mixed barley nuclei as an internal control with wheat nuclei in every experiment. The mean G1 fluorescent intensity (pulse area) of each wheat line was adjusted based on the variance between the mean barley G1 fluorescent intensity in each assay to the grand barley mean G1 fluorescent intensity of all the assays. A mono/disomic index (i.e., the percentage of the adjusted mean wheat fluorescent intensity for each monosomic line in relation to that of euploid 'Chinese Spring') was then calculated for each monosomic lines. The nuclear DNA content for a monosomic line was estimated by multiplying the mono/disomic index of that line by 34.635 pg. The DNA content of each chromosome was then obtained by subtracting the nuclear DNA content of a monosomic line from that of the euploid. The *t*-statistic test was applied to test the difference between dual monosomic lines, between RNA-free and non-RNA-free samples, and between disomic and monosomic CS lines. Analysis of variance and multiple comparison were also conducted to test differences among monosomic lines, among genomes, and among homoeologous groups.

Results and discussion

No significant difference was observed on the mean PI fluorescence intensities between the dual lines of each monosomics (data not shown), suggesting that no monosomic shift has occurred. Cytological observations also ruled out monosomic shift in all the monosomic lines. Therefore, the means for each chromosome were pooled for calculating DNA content. Flow-cytometric DNA analysis showed that all of the monosomic lines contain less DNA than euploid 'Chinese Spring' (Table 1). For instance, the linear PI fluorescence intensities of barley and disomic and monosomic 3A 'Chinese Spring' were 145.87, 493.52, and 477.58, respectively (Fig. 1).

Since PI will also bind to double-stranded RNA, it is necessary to determine the influence of existing RNA on the estimation of DNA content. For this purpose,

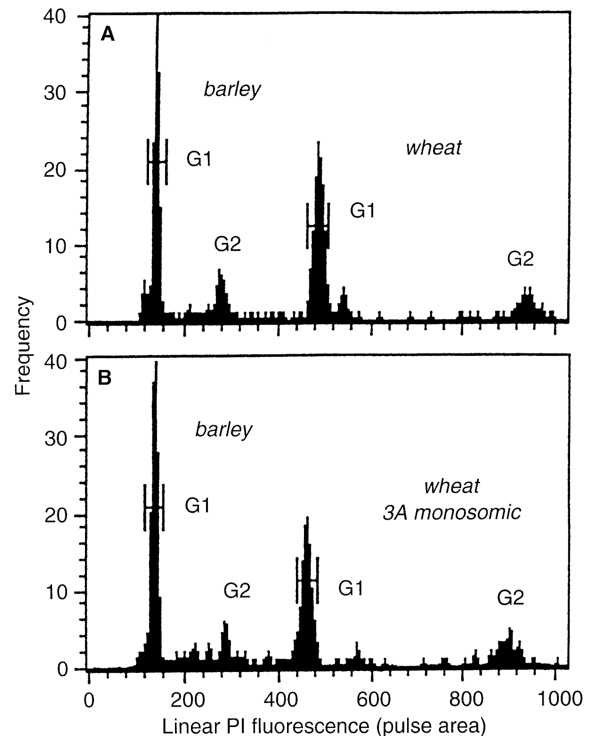

Fig. 1A,B Flow cytometric histograms showing relative DNA content on linear scale. Linear PI fluorescent intensity of G1 nuclei was used for calculating nuclear DNA content. Barley nuclei were used as an internal control. **A** 'Chinese Spring' (CS) and barley line NE 86954, **B** CS monosomic 3A and barley line NE 86954. Note that the linear PI fluorescence intensity of barley was 140.59 in both **A** and **B** and that the linear PI fluorescence intensity of CS monosomic 3A (453.55) was lower than that of disomic CS (484.88)

samples of RNA-free nuclei suspensions were made for each wheat line assayed, and their DNA content was estimated and compared with the estimated DNA content of the non-RNA-free samples of the same wheat lines. No significant difference was observed between the two estimations for any of the wheat lines assayed (data not shown). Obviously, the existence of a relatively tiny amount of double-stranded RNA at the G1 stage will have little impact on the accurate estimation of DNA content at this stage.

Table 1 shows the results of flow cytometric DNA analysis of all the monosomic and euploid 'Chinese Spring' lines. The estimated DNA content for each wheat chromosome at the G1 stage was calculated by subtracting the monosomic value from the euploid value and ranged from $2C = 0.58$ pg in 1D to $2C = 1.11$ pg in 3A. Our estimates are different from those obtained by Furuta and his colleagues who used Feulgen cytophotometry (Nishikawa and Furuta 1978; Furuta et al. 1988). In their studies, relative DNA content (in an arbitrary unit) was estimated for each univalent monosomic or monotelosomic chromosome at meiotic metaphase I of the pollen mother cells (PMC). They reported different chromosomes as having the highest and lowest DNA contents in different studies (Nishikawa 1971; Nishikawa and Furuta 1978; Furuta et al. 1988), although the same technical procedure was used. The tightness of chromatin coiling changes during meiosis, and the degree of DNA hydrolysis in the Feulgen staining procedure varies among PMCs and may account for the differences in DNA content estimated by the two techniques and among different studies from their laboratory. Direct estimates of the DNA content of isolated metaphase chromosomes

Table 1 DNA content of individual chromosomes of common wheat cultivar 'Chinese Spring'[a]

Monosomics	Mean nuclear DNA content (pg/2C)	Mean DNA content at G1 per chromosome (pg)	Range of G1 chromosome DNA content (pg)	Significant difference[b]
1A	33.80	0.84	0.82–0.87	hg
2A	33.68	0.95	0.93–0.98	lm
3A	33.52	1.12	1.08–1.15	n
4A[c]	33.86	0.77	0.75–0.80	de
5A	33.84	0.80	0.78–0.84	ef
6A	33.94	0.70	0.68–0.72	c
7A	33.67	0.97	0.94–1.00	m
Subtotal		6.15	5.98–6.36	
1B	33.77	0.87	0.85–0.91	hi
2B	33.71	0.92	0.89–0.96	kl
3B	33.72	0.92	0.90–0.97	k
4B[c]	33.84	0.80	0.77–0.84	ef
5B	33.76	0.87	0.84–0.91	ij
6B	33.73	0.90	0.88–0.95	jk
7B	33.83	0.81	0.79–0.85	fg
Subtotal		6.09	5.92–6.41	
1D	34.05	0.58	0.57–0.61	a
2D	34.00	0.64	0.62–0.66	b
3D	34.00	0.64	0.61–0.65	b
4D	33.95	0.68	0.67–0.70	c
5D	33.88	0.76	0.74–0.79	d
6D	33.73	0.91	0.88–0.93	k
7D	33.79	0.84	0.82–0.86	hi
Subtotal		5.05	4.91–5.20	
Total ($n = 21$)		17.31	16.53–17.97	
Chinese Spring	34.635			
Barley (NE86954)	10.24			

[a] The nuclear DNA content ($2C = 34.635$ pg) reported by Bennett and Smith (1976) for 'Chinese Spring' wheat was used as a reference. The barley line NE 86954 was used as the internal control. The means were calculated from eight duplicates per line. The DNA content for each monosomic was calculated by subtracting the nuclear DNA content of the corresponding monosomics from that of 'Chinese Spring'

[b] Base on mean nuclear DNA content. Lines with the same letter are not significantly different ($\alpha = 0.05$, LSD = 0.03, $df = 63$, critical $t = 2.00$)

[c] 4A and 4B were designated according to the resolution passed in the 7th International Wheat Genetic Symposium

by flow cytometry are needed to further analyze this problem.

It should also be noted that heterogeneity for protein content among monosomic lines has been demonstrated (Stein et al. 1992). If cytological heterogeneity also exists, it may affect the accurate comparison between a monosomic and euploid lines and, thus, the precise estimation of the DNA content for each monosomic chromosome with flow cytometry. Therefore, for estimating the DNA content of a monosomic chromosome, a disomic plant obtained from the same monosomic parent plant from which the assayed monosomic plants were derived should be used as a control (Yen and Baenziger 1992).

It is generally believed that the B genome has the highest and the D genome the lowest DNA content of the three wheat genomes. However, our data suggested that slightly more DNA was contained in the A genome than the B genome (Table 1). This is due to chromosomes 2A, 3A, and 7A, which contain more DNA than any B-genome chromosome. Our data also indicated that wheat chromosomes generally have their interphase DNA content poorly correlated ($r = 0.622$, $P \leq 0.01$) to their relative mitotic metaphase chromosome size (Fig. 2). As shown in Fig. 2, the poor correlation was mainly due to 3A, 4A, 7A, 3B, 4D, 6D, and 7D. Poor correlation between interphase DNA content and metaphase chromosome size was previously observed in the flow cytometric DNA analysis of some rye chromosomes (Bashir et al. 1993; Pfosser et al. 1995a,b). It seems that the poor correlation reported in all these studies may, at least partly, reflect differential coiling of chromatin during cell division. Variations in the relative chromosome size of wheat chromosomes among different cell division stages have been reported previously (see review by Schlegel and Mettin 1978). For example, Sears (1954) measured monosomic wheat chromosomes at metaphase I and anaphase II and found chromosome 5B to be the longest of all the wheat chromosomes. However, when N- and C-banded mitotic metaphase wheat chromosomes were measured, chromosome 3B was observed to be the longest (Gill 1987). Also, Larsen and Kimber (1973) reported that wheat chromosome 5B had different arm ratios at mitosis and meiosis. Apparently, two different chromosomes may not have the same degree of coiling at a certain cell division stage, and the process of coiling and uncoiling may not be synchronized among chromosomes in a cell.

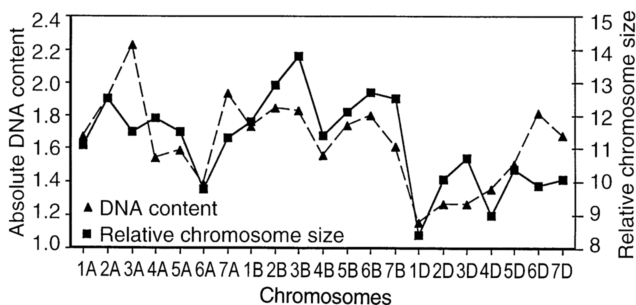

Fig. 2 A comparison between the relative chromosome sizes (Gill et al. 1991) and the estimated interphase DNA content (pg) of 21 CS wheat chromosomes at mitotic metaphase

As in the majority of flow cytometric DNA analyses in plants, PI was used in this study, because it is the best fluorochrome available for plant DNA analysis. PI is an intercalating fluorochrome. It can produce relatively bright fluorescence with low coefficients of variation (CVs) and is also stain-reliable under most conditions. PI can also preserve chromosome structure during the assay (Metezeau et al. 1993). PI is generally considered to be both chromosome-condensation- and base-composition-independent (see review by Gray and Langlois 1986) and to have the most precise stoichiometric response to DNA content (Price and Johnson 1996). However, since PI is a intercalate-binding fluorochrome, PI staining is not believed to be totally free from bias (Price and Johnson 1996). Price and Johnson (1996) suggested that bias from the binding stoichiometry of DNA stains could be the most consistent source of error in determining DNA content. If this is true, the poor correlation between the DNA content at the G1 stage and relative chromosome size at the mitotic metaphase for some wheat chromosomes might also result from differential binding of the fluorochrome PI to heterochromatin.

With the DNA content for each wheat chromosome, known, flow-cytometric DNA analysis may be used to distinguish the disomic, monosomic, and nullisomic offsprings without cytological check. Our analysis of variance showed significant differences ($\alpha \leq 0.01$) in DNA content both among homoeologous groups and among genomes (Tables 2 and 3). Wheat chromosomes can be classified into five DNA-content groups (namely, 1D, 2D and 3D, 4D and 6A, 3A, and all the others), with significantly different DNA content ($\alpha \leq 0.05$) among groups (Table 1). Our statistical analyses showed that, while significant difference exists between a monosomic and a disomic CS lines ($\alpha \leq 0.0000$) and among monosomics ($\alpha \leq 0.001$) (Table 3), some homoeologues are indistinguishable by flow-cytometric DNA analysis (Table 1). Generally, the A- and the B-genome chromosomes are clearly distinguishable from

Table 2 Analysis of variance for DNA content among homoeologous groups and among genomes in 'Chinese Spring' wheat[a]

Source	*df*	ANOVA SS	Mean square	*F* value	Pr > *F*
Group	6	0.1981	0.0330	54.38	0.0001
Genome	2	0.4354	0.2177	358.61	0.0001
Group*Genome	12	0.6976	0.0581	95.76	0.0001

[a] Based on nuclear DNA content of monosomic lines

Table 3 Analysis of variance for DNA content among monosomic 'Chinese Spring' wheat lines

Source	*df*	Sum of squares	Mean square	*F* value	Pr > *F*
Genotype	20	1.3310	0.0666	109.63	0.0001
Error	63	0.0382	0.0006		
Corrected total	83	1.3693			

their D-genome homoeologues, except for 6B which is indistinguishable from 6D. Also, chromosomes 3A, 5A, 6A, and 7A have a significantly ($\alpha \leq 0.05$) different DNA content from their B-genome counterparts. However, chromosomes 1A, 2A, and 4A are not distinguishable from their B-genome homoeologues by flow-cytometric DNA analysis. Therefore, our results suggested that flow-cytometric DNA analysis cannot completely replace cytological checks in aneuploid identification, but only aid it.

References

Arumuganathan K, Earle E (1991) Estimation of nuclear DNA content of plants by flow cytometry. Plant Mol Biol Rep 9: 229–233

Bashir A, Auger JA, Rayburn AI (1993) Flow cytometric DNA analysis of wheat-rye addition lines. Cytometry 14: 843–847

Bennett MD, Smith JB (1976) Nuclear DNA amounts in angiosperms. Philos Trans R Soc London Ser B 274: 227–274

Blondon F, Marie D, Brown S (1993) Genome size and base composition in *Medicago sativa* and *M. truncatula* species. Genome 37: 264–270

Endo TR, Gill BS (1984) Somatic karyotype, heterochromatin distribution, and nature of chromosome differentiation in common wheat, *Triticum aestivum* L. em. Tell. Chromosoma 89: 361–369

Furuta Y, Nishikawa K, Shimokawa K (1988) Relative DNA content of the individual telocentric chromosomes in 'Chinese Spring' wheat. In: Miller, TE, Koebner RMD (eds), Proc 7th Int Wheat Genet Symp. Bath Press, Bath, UK, pp 281–286

Gill BS (1987) Chromosome banding methods, standard chromosome nomenclature and applications in cytogenetic analysis. In: Heyne EG (ed) Wheat and wheat improvement, 2nd edn. ASA, Madison, Wis., pp. 243–254

Gill BS, Friebe B, Endo TR (1991) Standard karyotype and nomenclature system for description of chromosome bands and structural aberrations in wheat (*Triticum aestivum*). Genome 34: 830–839

Gray JW, Langlois RG (1986) Chromosome classification and purification using flow cytometry and sorting. Annu Rev Biophys Chem 15: 195–235

Lee JH, Arumuganathan K, Kaeppler SM, Kaeppler HF, Papa CM (1996) Cell synchronization and isolation of metaphase chromosomes from maize (*Zea mays* L.) root tips for flow cytometric analysis and sorting. Genome 39: 697–703

Larsen J, Kimber G (1973) The arm ratio of chromosome 5B from *Triticum aestivum* var 'Chinese Spring' at mitosis and meiosis. Hereditas 73: 316–318

Metezeau P, Schmitz A, Frelat G (1993) Analysis and sorting of chromosomes by flow cytometry: new trends. Biol Cell 78: 31–39

Nishikawa K (1971) DNA content of the individual chromosomes and genomes in wheat and its relatives. Seiken Ziho 22: 57–65

Nishikawa K, Furuta Y (1978) DNA content of nucleus and individual chromosomes and its evolutionary significance. In: Ramanujam S (ed.) Proc 5th Int Wheat Genet Symp. Kapoor Art Press, New Delhi, India, pp 133–138

Pfosser M, Amon A, Lafferty J, Heberle-Bors E, Lelley T (1995a) Gain or loss of single chromosomes in wheat-rye addition lines and in 6x triticale detected by flow cytometry. Plant Breed 114: 555–557

Pfosser M, Amon A, Lelley T, Heberle-Bors E (1995b) Evaluation of sensitivity of flow cytometry in detecting aneuploidy in wheat using disomic and ditelosomic wheat-rye addition lines. Cytometry 21: 387–393

Price HJ, Johnson JS (1996) Analysis of plant DNA content by Feulgen microspectrophotometry and flow cytometry. In: Jauhar PP (ed) Methods of genome analysis in plants. CRC Press, New York, pp 115–132

Schlegel R, Mettin D (1978) Bemerkungen zur karyotypanalyse beim saatweizen (*Triticum aestivum L.*). Arch Zuchtungsforsch 8: 333–344

Sears ER (1954) The aneuploids of common wheat. Research Bulletin 572, University of Missouri, Columbia, Mo.

Stein IS, Sears RG, Gill BS, Hoseney RC, Cox TS (1992) Heterogeneity of the 'Wichita' wheat monosomic set for grain quality and agronomic traits. Crop Sci 32: 581–584

Wang ML, Leitch AR, Schwarzacher T, Heslop-Harrison JS, Moore G (1992) Construction of a chromosome-enriched HpaII library from flow-sorted wheat chromosomes. Nucleic Acids Res 20: 1897–1901

Yen Y (1988) Potential value of aneuploids in chromosome isolation and molecular genetics in plant. Genome 30 [Suppl. 1]: 459

Yen Y, Baenziger SP (1992) A better way to construct recombinant chromosome lines and their controls. Genome 35: 827–830

Yen Y, Liu DJ (1987) Production, morphology, and cytogenetics of intergeneric hybrids of *Elymus* L. species with *Triticum aestivum* L. and their backcross derivatives. Genome 29: 689–694