Nuclear DNA content of ten rice species as determined by flow cytometry

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ABSTRACT

Accurate estimations of nuclear DNA content of rice are important because this crop is being used in many types of molecular studies. The main objective of this study was to estimate the nuclear DNA content in ten rice species by flow cytometry. Most of the values obtained were lower than those obtained in earlier studies using Feulgen microdensitometry. Significant differences in genome size among rice species were found. *Oryza glaberrima* had the smallest genome (0.73–0.76 pg/2C), while *O. minuta* and *O. latifolia* had the largest values (ca. 2.33 pg/2C). Tetraploid species had more nuclear DNA than diploid ones. The AA genome had less DNA (0.86–0.96 pg/2C) than the CC (1.14–1.17 pg/2C) and EE (1.99 pg/2C) genomes. The AA genome of *O. sativa* was larger than the A^aA^a genome of *O. glaberrima* and the A¹A¹ genome of *O. longistaminata*, which had mean values of 0.73–0.76 and 0.78 pg/2C, respectively. *Indica* (IR36) and *japonica* (Yukihikari) cultivars of *O. sativa* also showed significant differences. Three different nuclear DNA levels (1.93, 1.85, and 1.31 pg/2C) were found among five *O. ridleyi* plants analyzed. Flow cytometry also allowed rapid and reliable determination of the ploidy level of anther culture-derived plants.

1. INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important crop species, being a staple food for more than half the world's population (Coffman and Juliano, 1987). It is considered by many people to be an ideal species for genetic and molecular studies, and much effort and expense has recently been devoted to development of techniques for such work. Rice is diploid (2n=24), has a small genome size

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(Arumuganathan and Earle, 1991a), and can be regenerated and transformed relatively easily (e.g., Li et al., 1990; Peng et al., 1992; Cao et al., 1992). A vast germplasm reservoir of domesticated and wild rices is available to rice researchers; furthermore, the genus *Oryza* shows extensive intraspecific and interspecific variation, as measured by restriction fragment length polymorphism (Pham, 1990; McCouch and Tanksley, 1991; Barbier et al., 1991; Wang et al., 1992).

Information about genome size is helpful in assessing the difficulty of gene mapping and isolation of genes by map-based cloning. Previous studies in which Feulgen microdensitometry was used to estimate genome size of rice species show great variation in the values obtained (Katayama, 1971; Bennett and Smith, 1976, 1991; Iyengar and Sen, 1978; Nagato et al., 1981). Flow cytometric methods for determination of nuclear DNA content are exceptionally rapid, convenient, accurate, and sensitive (Galbraith et al., 1983; Arumuganathan and Earle, 1991a, b; McMurphy and Rayburn, 1991). Therefore, the objectives of this study were to use flow cytometry to estimate nuclear DNA content in several rice species and also to identify ploidy levels in anther culture-derived rice plants.

2. MATERIALS AND METHODS

Plant materials

Ten rice species (Table 1) were selected for estimation of nuclear DNA content by flow cytometry. The seeds were obtained from the International Rice Research Institute (IRRI), Manila, Philippines. Pre-germinated rice seeds were sown in plastic trays containing soil which was kept wet throughout the experiment. Trays were placed in a growth chamber with 12 hours of fluorescent light and a day/night temperature setting of 27/25°C. Samples for analysis (15–20 mg) were taken from the two youngest leaves of 20 to 30 day-old seedlings.

Young *O. sativa* plantlets (2 to 3 weeks old) obtained through anther culture were provided by Dr. T. Croughan (Louisiana State University at Crowley, LA). These plantlets were derived by culture of anthers from hybrid plants from six different crosses (crosses A-E) of various rice lines. After DNA determination, plantlets were potted and kept in the growth chamber for observations on flowering, sterility, plant height, and tiller number.

Flow cytometry

Samples were prepared for flow cytometric analysis using the procedures of Arumuganathan and Earle (1991b). Suspensions of plant nuclei stained with propidium iodide were analyzed using an EPICS profile analyzer (Coulter Electronics, Hialeah, Florida) equipped with an Argon ion laser emitting at 488 nm. Cell clumps and debris were excluded from analysis by using red fluorescence and forward angle light scatter gates. The red fluorescence emission signal of prop-

idium iodide was processed with 457–502 nm long-pass laser blocking and 610 nm long-pass absorbance filters. The resulting distributions of DNA content in particles were determined on a linear scale. Two determinations were done per isolation, and at least 2000 nuclei were examined each time. At least two different isolations were analyzed from each rice species or accession. Values for nuclear DNA content were estimated by comparison of the rice nuclear peak on the linear scale with the peak for chicken red blood cells (CRBC) included as internal standards in each run. A value of 2.33 pg/2C was used for the nuclear DNA content of CRBC (Galbraith et al., 1983).

Statistical methods

Comparisons of DNA content among rice species used a standard analysis of variance model under a completely randomized design with one factor (rice species). For comparisons of DNA content among anther culture-derived plants, a combined analysis of variance was used considering the following factors: cross, ploidy level, and individual plants (cross/ploidy).

3. RESULTS

Nuclear DNA content in the genus *Oryza*

Flow cytometric analysis of nuclei isolated from rice leaves showed only a single peak corresponding to the 2C level ($G_0 + G_1$ phase); peaks corresponding to the 4C level ($G_2 + M$ phase) were not detected (Fig. 1). This indicates that there are no dividing cells in the portions of the leaves sampled.

Fig. 1 shows histograms of relative fluorescence of nuclei from various rice species. Nuclei from test plants were mixed with nuclei of *O. sativa* cv IR36 and CRBC except in the case of *O. latifolia* and *O. minuta*. The 2C peaks of *O. latifolia* (Fig. 1c) and *O. minuta* (Fig. 1g) coincided with the CRBC peak, indicating that they have similar DNA content.

The genome sizes of the various rice species showed significant differences (Fig. 1; Table 1). *O. glaberrima* had the smallest genome size (0.73–0.76 pg/2C), while *O. minuta* and *O. latifolia* had the largest values (ca. 2.33 pg/2C). As expected, tetraploid species had more nuclear DNA than diploid species. Except for *O. australiensis*, the values found are smaller than those previously determined by Feulgen microdensitometry (Bennett and Smith, 1976; Iyengar and Sen, 1978).

In rice, 22 species and six genomes (A, B, C, D, E and F) are recognized (Vaughan and Sitch, 1991). Cultivated rice species (*O. sativa* and *O. glaberrima*) have the AA genome. The nuclear DNA content in the AA genome is smaller than in the CC and EE genomes (Table 1). Significant differences in nuclear DNA content were found within the AA genome: the AA genome of IR36 (*O. sativa*) had more DNA (0.90 pg) than the A^gA^g genome of *O. glaberrima* (0.75 ± 0.03 pg) and the A^1A^1 genome of *O. longistaminata* (0.78 ± 0.02 pg). Dif-

Figure 1. Histograms of relative propidium iodide fluorescence in nuclei isolated from different *Oryza* species. Nuclei from *Oryza* species were mixed with nuclei from *O. sativa* cv. IR36 and/or chicken red blood cells (CRBC) as standards for flow cytometric analysis. Nuclear DNA content was calculated as follows: relative fluorescence of sample/relative fluorescence of standard $\times$ DNA content of standard. The DNA content used for CRBC was 2.33 pg (Gelbraith et al., 1983).

ferences between the latter species are not significant. There were no significant differences between the 15 accessions of *O. glaberrima* examined. Apparently there is much less variation in DNA content within *O. glaberrima* than *O. sativa*, within which much variation was found by C. P. Martínez et al. (unpublished data). These results are in agreement with those of Vaughan (1989), who reported that *O. glaberrima* is not as variable as *O. sativa*, and Second (1982),

Table 1. Nuclear DNA content of 10 rice species as determined by flow cytometry

Scientific Name (IRRI Acc. No.)	Genome Group	Nuclear DNA Content			
		This study		Previous work	
		pg/2C	Mbp [†] /1C	pg/2C	Reference [‡]
<i>Oryza glaberrima</i> (15 accessions*)	A ^g A ^g	0.73–0.76	352–366	1.37 5.33	1 7
<i>Oryza longistaminata</i> (WL02)	A ¹ A ¹	0.78	376	0.78 1.25	6 [§] 1
<i>Oryza sativa</i> ssp. <i>indica</i> cv. IR36	AA			0.87–0.96 1.01 1.20 1.67 2.10 3.40	6 [§] 8 2 1 3 4
ssp. <i>japonica</i> cv. Yukihiikari	AA	0.88	424	0.86–0.91 0.80 1.20 1.55 1.90 3.30	6 [§] 8 2 1 3 4
<i>Oryza officinalis</i> (101150)	CC	1.14	550	2.02 4.7–6.07	1 5
<i>Oryza eichingeri</i> (101422)	CC	1.17	564	ND	
<i>Oryza australiensis</i> (100882)	EE	1.99	960	1.78 11.41	1 7
<i>Oryza ridleyi</i> (100821, 5 plants)	? tetraploid	1.31, 1.85, 1.93	632–931	2.65	1
<i>Oryza grandiglumis</i> (101405)	CCDD	1.99	960	ND	
<i>Oryza latifolia</i> (100965)	CCDD	2.32	1124	ND	
<i>Oryza minuta</i> (101141)	BBCC	2.33	1124	2.83	1

* IRRI accessions 103329, 103350, 103437, 103438, 103443, 103490, 103494, 103635, 103685, 103704, 1041659, 104174, 104278, 104527.

LSD Species (5%) = 0.05; MSE = 0.001; C. V. = 2.94%.

[†] 1 picogram (pg) = 965 million base pairs (Mbp) (Bennett and Smith, 1976).

[‡] Except where noted, Feulgen microdensitometry was used for previous work. ND: not determined; 1: Iyengar and Sen (1978); 2: Bennett and Smith (1976); 3: Bennett et al. (1982); 4: Nagato et al. (1981); 5: Katayama (1971); 6: Arumuganathan and Earle (1991a) with some data provided by Kikuchi and Martínez; 7: Katayama (1967); 8: Bennett and Smith (1991).

[§] Determined by flow cytometry.

who showed through isozyme analysis that *O. glaberrima* is less polymorphic than *O. sativa* and *O. breviligulata*.

O. officinalis and *O. eicheingeri*, which have the CC genome, had DNA contents of 1.14 and 1.17 pg/2C, respectively (Table 1). *O. australiensis* and *O. grandiglumis* belong to the same *O. officinalis* complex and have the same genome size (1.99 pg/2C). However, *O. australiensis* is a diploid species having the EE genome while *O. grandiglumis* is considered a CCDD allotetraploid (Morishima, 1984; Vaughan and Sitch, 1991). If these two species are correctly characterized in terms of ploidy level, the EE genome is large.

O. ridleyi is also a tetraploid ($2n=48$) but its genomic constitution is unknown (Morishima, 1984; Vaughan and Sitch, 1991). Three significantly different nuclear DNA values (1.93, 1.85 and 1.31 pg) were found among five plants of a single accession (Table 1; Figs 1e and 1f), suggesting that this species is composed of plants having different genome sizes. We did not find any such differences in nuclear DNA content among plants within the same accessions of the other rice species.

Variation in Nuclear DNA content within O. sativa

Reference diploid cultivars IR36 (*indica*), and Yukihihikari (*japonica*) had values of 0.90 ± 0.02 pg and 0.88 ± 0.01 pg, respectively, significantly different at the 5% level of probability. Iyengar and Sen (1978), Nagato et al. (1981), and Kwon and Soh (1985) also found significant differences in DNA content between *japonica* and *indica* types by Feulgen microdensitometry, although their actual values were substantially higher than ours. However, the differences in DNA content between any two cultivars of *indica* and *japonica* may not always be significant. Our previous study of 49 *indica* cultivars and 20 *japonica* cultivars found variations in DNA content among different cultivars within these subspecies (Arumuganathan and Earle, 1991a). The DNA content of *indica* cultivars ranged from 0.87 to 0.96 pg/2C, while that of *japonica* cultivars ranged from 0.86 to 0.91 pg/2C. Thus some cultivars from these subspecies have similar values for their nuclear DNA content, even though *indica* cultivars tend to have higher values.

Nuclear DNA content of anther culture-derived rice plants

Flow cytometry was also used to determine the ploidy level of rice plantlets obtained by culture of anthers from hybrids produced from six different crosses within *O. sativa*. Representative results from the crosses are presented in Table 2. Some plants from each cross had the diploid value for *O. sativa*, approximately 0.90 pg. Others had the haploid value of approximately 0.45 pg. One plant in cross D had a value of 1.80 and was apparently a tetraploid. There were significant differences in nuclear DNA content among diploid and haploid plants within crosses; more heterogeneity in DNA content was found among the diploid plants than among the haploid ones.

Table 2. Differences in nuclear DNA content and ploidy level among anther-culture derived *O. sativa* plants from 6 crosses, as determined by flow cytometry

Ploidy level	Cross A	Cross B	Cross C	Cross D	Cross E	Cross F	Total
Haploid							
of plants	5	13	11	7	14	9	59 (56%)
(pg DNA)	(0.45–0.47)	(0.42–0.46)	(0.43–0.45)	(0.45–0.47)	(0.45–0.48)	(0.42–0.45)	
Diploid							
of plants	14	4	9	9	2	8	46 (43%)
(pg DNA)	(0.91–0.98)	(0.90–0.91)	(0.88–0.95)	(0.89–0.97)	(0.92–0.94)	(0.87–0.99)	
Tetraploid							
of plants	0	0	0	1	0	0	1 (1%)
(pg DNA)				(1.80)			
Total # of plants	19	17	20	17	16	17	106

There was complete agreement between the classification of ploidy by flow cytometry and the classification based on morphological characteristics of the same plants grown to maturity under greenhouse conditions. The plants with nuclear DNA content of ca. 0.45 pg were completely sterile and had very short tillers, tiny panicles and flowers, and small narrow leaves, as expected in haploid plants. The diploid plants (ca. 0.90 pg) were completely normal and fertile. The tetraploid plant (1.80 pg) was taller than the diploids and had longer, broader leaves. It produced only a few grains, but these were larger than normal.

The flow cytometric studies showed that 56% of the plants recovered from anther culture of the six crosses were haploid; however, the percentage varied widely among crosses, from 26% (cross A) to 88% (cross E). Other workers (Chen and Li, 1978; Ying, 1986; Raina, 1989) have also reported differences in percentages of haploid, diploid and tetraploid plants as a result of the anther culture process.

4. DISCUSSION

We used flow cytometry to determine the genome size of cultivated and wild rice species and plants derived from anther culture. Previous work has shown that flow cytometry can be used to detect significant genome size variation among plant species and that the results compare favorably with those from Feulgen microdensitometry (Galbraith et al., 1983; Rayburn et al., 1989; Rayburn and Auger, 1990; Arumuganathan and Earle, 1991a, b). The rice genome sizes observed in this study were considerably smaller than most of those determined by microdensitometry results in earlier papers. By making use of better internal

standards, Bennett and Smith (1991) recently reported genome sizes for *indica* (1.0 pg/2C) and *japonica* (0.8 pg/2C) much closer to those we found (Table 1), indicating that comparable results may be obtained by both methods. However, flow cytometry is much faster and more convenient.

Significant differences in the genome size among wild rice species were found (Table 1), as in previous work (Katayama, 1967; Iyengar and Sen, 1978; Nagato et al., 1981). Our data show the lowest nuclear DNA content in the AA genome present in cultivated rice (*O. sativa* and *O. glaberrima*) and suggest that cultivated rice has a significantly lower DNA amount than wild rice (except for *O. longistaminata*). Two previous comparisons of cultivated and wild rice offer quite different interpretations of the lower value for *O. sativa*. Nagato et al. (1981) concluded that the mean DNA content in cultivated rice has decreased over the course of evolution. Iyengar and Sen (1978) suggested the opposite because in their study *O. rufipogon* (wild ancestor of *O. sativa*) had a lower DNA content (1.28 pg) than cultivated rice, *O. sativa japonica* (1.55 pg) and *O. sativa indica* (1.67 pg). Unfortunately, *O. rufipogon* was not included in our study.

Significant differences in DNA content between cultivated and wild species have been found in some other plant genera. A decrease in DNA amount per genome over the course of domestication has been claimed to occur in *Glycine* species (Yamamoto and Nagato, 1984), *Piper nigrum*, *P. betle* and *P. longum* (Samuel et al., 1986). However, the opposite trend was noted in *Capsicum annum* and *C. baccatum* (Bennett and Smith, 1976), as well as in *Phaseolus vulgaris*, at least in the meso-american center of domestication. Comparisons of the DNA content of cultivated landraces and wild *P. vulgaris* (the ancestors of the common bean) from Mexico show that the DNA content is higher in landraces than in modern varieties (Martínez et al., in preparation). In *Allium*, both gain and loss of nuclear DNA were observed (Jones and Rees, 1968). According to Bennett (1976), cereal species selected for cultivation at successively lower latitudes show a strong trend toward decreasing genome sizes.

O. ridleyi is a rare species that usually grows in shaded habitats beside rivers, streams or pools (Vaughan, 1989). Five different plants from Acc. 100281 were analyzed, and three significantly different genome sizes were found (Table 1). Wild taxa are generally heterogeneous and heterozygous, and the collected samples are usually the bulked seeds of a population (IRRI, 1991). According to Vaughan and Sitch (1991) and Morishima (1984), *O. ridleyi* is a tetraploid; however, the different genome sizes found (1.93, 1.85, and 1.31 pg) could mean that this species is an unstable tetraploid.

Anther culture-derived plants are known to present differences in ploidy level. Several techniques, such as determination of chromosome numbers through root squashes and/or observation of morphological traits (mainly spikelet sterility), can be used to determine the ploidy level of a given plant, but these procedures are time-consuming. Our results showed that flow cytometry gives a quick and

reliable determination of the ploidy level at a very early stage of growth.

In addition, statistical analysis of the flow cytometry data showed significant differences in mean nuclear DNA content among crosses and among plants within crosses and ploidy level. The differences among and within crosses could be the result of differences in nuclear DNA content between parents involved in a given cross, in which case the data suggest segregation for DNA content among plants. Unfortunately, the parental sources involved in each cross were not available for flow cytometric analysis. Somaclonal variation is another possible explanation. Quantitative variation in the amount of DNA in closely related cereal species frequently involves repetitive sequences (Flavell et al., 1977; Bedbrook et al., 1980). Variation in the copy number of such sequences during the tissue culture process (Kikuchi et al., 1987) may account for the differences in DNA content of plants derived from anther culture.

We have also used flow cytometry to determine the ploidy status of rice cell suspension cultures kept in our lab for different lengths of time. The ploidy level of cell suspension cultures changes over time, resulting in a mixture of cells having different ploidy levels (data not shown). Such information about the ploidy status of a culture can be very important if cell suspensions are used in transformation experiments aimed at regeneration of normal diploid plants.

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