Sugar Cane Genome Numbers Assumption by Ribosomal DNA FISH Techniques

Sarut Thumjamras
Kasetsart University, Kampeang Sean Campus/Bioproduct Science, Department of Science, Faculty of Liberal Arts and Science/Nakorn Pathom, Thailand
Kasetsart University Institute for Advanced Studies/Center for Advanced Studies in Tropical Natural Resources/Bangkok, Thailand
Email: g5328600029@mail.kps.ku.ac.th

Hans de Jong
Wageningen University/Laboratory of Genetics, Plant Science Group/Wageningen, the Netherlands
Email: hans.dejong@wur.nl

Sililuck Iamtham[1] and Siripatr Prammanee[2]
[1]Kasetsart University, Kampeang Sean Campus/Genetics, Department of Science, Faculty of Liberal Arts and Science/Nakorn Pathom, Thailand
[2]Kasetsart University, Kampeang Sean Campus/ Biology, Department of Science, Faculty of Liberal Arts and Science/Nakorn Pathom, Thailand
[1&2]Kasetsart University Institute for Advanced Studies, Kasetsart University/Center for Advanced Studies in Tropical Natural Resources/Bangkok, Thailand
Email: {faassli, faasspp}@mail.kps.ku.ac.th

***Abstract*—Conventional cytological method is limited for polyploidy plant genome study, especially sugar cane chromosomes that show unstable numbers of each cultivar. Molecular cytogenetic as fluorescent *in situ* hybridization (FISH) techniques were used in this study. A basic chromosome number of sugar cane was estimated with three information; 1) number of 18S rDNA sites, 2) number of 5S rDNA sites and 3) total number of chromosomes. 18S and 5S rDNA were located by FISH techniques, the number of hom (e) ologous sites were illustrated in range of 7 to 9 and 13 to 15 sites. 110 chromosome numbers were shown in tapetal cells of flower buds of sugar cane. The implications of these results can predict about 14 basic chromosomes numbers but 5S rDNA seem reliable indicate for basic chromosome number and 18S rDNA were discussed about nucleolar dominance phenomenon of the sugar cane "KPS 00-25" cultivar.**

***Index Terms*—basic chromosome number, 45S rDNA, 5S rDNA, FISH**

I. Introduction

Sugar cane is a major crop grass having sugar content in the stem that is the main sugar crop production of the world. Sugar cane belongs to the Poaceae family, *Andropogonae* tribe and the genus is *Saccharum* L. There are many species such as *S. officinarum* is a kind of juicy cane and sweet softness, Chinese cane as *S. sinense*, Indian canes as *S. barberi*, *S. robustum*. and *S. spontaneum* are wild species that sugar free but they are disease resistant and abiotic tolerant ability [1]. All of them are polyploidy plant. The results of natural hybridization and conventional breeding programs have reached the modern sugar cane genome more complexity.

Modern sugar cane cultivars are inter-specific hybrid crossed species. Molecular cytogenetic can help for locate mapping the genome by fluorescent *in situ* hybridization (FISH). FISH is a fluorescent-labeled probes technique that has allowed researchers look into the target cells and chromosomes content by specific sequenced probed. FISH techniques have usually used to locate single copy genes, repetitive sequences, selectively identify genes [2], [3]. The techniques can be investigated markers on chromosomes and guide plant-genome research [4]-[6].

The ribosomal gene clusters are used as source of FISH markers. The 18S-5.8S-25S rRNA and 5S rRNA genes are located in long tandem repeat unit. Generally, the 18S-5.8S-25S rRNA genes are usually known as proportional activity of rRNA genes on secondary constrictions and nucleolar organizer regions (NORs) [7]. Therefore, the ribosomal genes have used to study of the genome of sugar cane. The modern sugar cane cultivars were hybridized between *S. officinarum*, *S. spontaneum* and partial hybrid forms. They were verified by genomic *in situ* hybridization (GISH) and FISH [8]-[10]. The basic chromosome set were also found x = 8 for *S. spontaneum* [11], x = 10 for *S. officinarum* and *S. robustum* by FISH technologies [10]. According to the recent researches, the results showed that modern cultivars have a genome size

Manuscript received May 3, 2013; revised July 6, 2013;

doi: 10.12720/jomb.2.4.248-251

around 10 Gb and 120 chromosomes, 70–80% of which are entirely derived from *S. officinarum*, 10–20% from *S. spontaneum* and few from interspecific recombinations [8], [12]- [14].

Sugar cane genome information is important key for conventional breeding program and sequencing. The 18S rDNA, 5S rDNA sites, and total chromosome numbers may be used to estimate basic chromosome of new cultivated sugar cane in this study.

II. Materials and Methods

A. Chromosome Preparation

Sugar cane cultivar namely "KPS 00-25" from Thailand were used as materials. Young flower buds were soaked with fresh solution of ethanol and acetic acid (3:1 v/v) 1-2 hr. Then samples were transfer and kept in 70% ethanol. Anthers were then equilibrated with MiliQ-water and citrate buffer for 3 min 2 times of each. They were digested with enzyme solution, CellulaseRS Yakult 203027, Pectolylase Y23 Sigma P-3026 and Cytohelicase Bio Sepra 249701, for 2 hr at 37˚C. The cells were spread on slide with a drop of 45% acetic acid for 2 min at 50˚C. The slides were rinsed again with fixed solution (3:1 v/v of ethanol and acetic acid) and were dried again at 50˚C. Cell division stages were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and slide were checked with a fluorescent microscope.

B. Probes

The 18S rRNA genes (sub-unit of 45S rDNA) probes were made by PCR amplification of sugar cane genomic. The genes specific primer were set 5'-CGAACTGTGAA ACTGCGAATGGC-3' and 5'-TAGGAGCGACGGGCG GTGT-3' [15]. The amplification reaction mixture consisted of 10 ng sugar cane genomic DNA, 10 µM of each primer, 5 mM dNTP, 1XPCR buffer (10 mM Tris-HCl (pH8.3), 50 mM KCl, 1.5 mM $MgCl_2$), and 1U of *Taq* polymerase (Fermentus), in a final volume of 25 µL. The PCR was carried out as follows: one cycle of 15 min at 95˚C; and 35 cycles of 1 min at 95˚C, 1 min at 68˚C and 1.5 min at 72˚C. The probe was directly labeled by Nick Translation Mix with DEAC-dUTP (Roche). The 5S rDNA probes, we used PCR amplification of sugar cane genomic DNA with primer set 5'-GATCCCATCAGAA CTTC-3' and 5'-GGTGCTTTAGTGCTGGTAT-3' [16]. The amplification reaction mixture of 5S rDNA PCR reaction was the same as above. The PCR was carried out as follows: one cycle of 5 min at 94˚C; and 35 cycles of 1 min at 94˚C, 1 min at 55˚C and 1 min at 72˚C. The probe was indirect labeled by Biotin Nick Translation Mix with biotin-16-dUTP (Roche) that revealed by Alexa 647 stain.

C. Fluorescence in Situ Hybridization

The selected slides were incubated in 100 µg/mL RnaseA in 2X saline sodium citrate (SSC) (0.03 sodium citrate and 0.3 M NaCl) for 1 hr at 37˚C and washed in 2xSSC for 5 min 3 times, fixed in freshly 1% formaldehyde buffer (10xPBS, 10x$MgCl_2$ and 37% formaldehyde) for 10 min, washed in 2xSSC for 5 min 3 times, soaked in 70%, 90%, 100% freshly ethanol for 3 min of each, and air-dried. Hybridization mixed probes consisted of 10% dextran sulfate, HB50, and probes in a final volume of 20 µL/slide. Then the mixtures were denatured in boiling water for 10 min and immediately put on ice for 5 min before applied mixture on slide, baked for 3 min at 80˚C. The slides were incubated in moist chamber at 37˚C overnight. The post-hybridized slides were washed in 2xSSC for 5 min 3 times, stringency washed in 50% formamide for 5 min 3 times at 42˚C. The slides were detected of biotin-strepavidine-Alexa 647 and biotin-anti-strepavidine was used to detect biotin probes for 1 hr at 37˚C of each. The slides were washed in 4T (4xSSC and tween20), TNT (NaCl, Tris-HCl pH 7.5 and Tween-20) and 2XSSC for 5 min of each, respectively. The slides were then dehydrated in ethanol series and air-dried. The chromosomes were counterstained with DAPI. The slides were mounted in Vectashield antifade solution and photographed on Zeiss Axioplan microscope equipped with Plan Apochromatic objectives and epifluorescence illumination.

D. Images Procession and Analysis

The images were colored and merged with Adobe Photoshop software. Chromosome numbers, signals of rDNA sites and members of chromosome chiasma were assumed the basic chromosome number by dividing the total number of chromosome by the number of homologous rDNA sites.

III. Results

A. Cytological Observation

110 chromosomes were counted in tapetal mitotic cells of young flower buds of "KPS 00-25" cultivar (Fig. 1a), using DAPI fluorescent stain.

B. 18S and 5S rDNA Mapping

18S rDNA and 5S rDNA were located on interphase cells of meiosis for *in situ* hybridization mapping, using PCR probes of 18S rDNA and 5S rDNA labelled with Nick translation for DEAC (purple color) and biotin for Alexa647 (red color), respectively. Both of gene families, the 18S rDNA showed hom(e)ologous sites for 7 to 9 signals (Fig. 1b). Besides, the 5S rDNA showed hom(e)ologous sites for 12 to 15 signals on chromosomes (Fig. 1c).

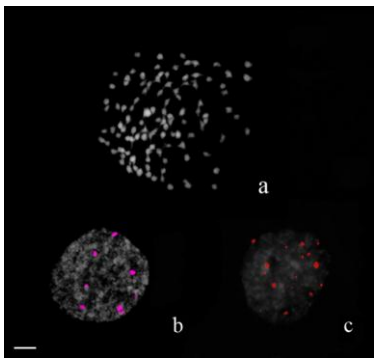

Figure 1. a. 110 chromosome numbers of a tapetal cell, b. 8 signals of 18S rDNA of FISH mapping and c. 15 signals of 5S rDNA of FISH mapping. Bar stands for 10 µM.

Data collection of related literatures showed locus numbers of 45S rDNA and 5S rDNA using FISH techniques. After calculation, the basic chromosome numbers for 10 of *S. officinarum* and *S. robustum*, total chromosome number were observed for 80 or 60. The basic chromosome numbers of *S. spontaneum* were reported of 8, total chromosome numbers were reported in range of 103-115 [8], [10]. Hybrid sugar cane was known as modern cultivars that showed the total chromosome in range of 103-115 and the basic chromosome numbers were varied from 8 to 11 [8], [17] (Table I).

TABLE I. DATA COLLECTION OF RELATED LITERATURES SHOWED LOCUS NUMBERS OF 45S RDNA AND 5S RDNA USING FISH TECHNIQUES.

Cultivars	2n	45S rDNA	5S rDNA	Authors
-*S. officinarum*				
Black Cheribon	80	8	8	D'Hont *et al.*,1998
Cristalina	80	8	8	Cuadrado *et al.*, 2004
-*S. spontaneum*				
SES 106B	64	8	8	D'Hont *et al.*, 1998
NG 51-2	80	10	10	D'Hont *et al.*, 1998
Mandalay	96	12	12	D'Hont *et al.*, 1998
-S. robustum				
NG 77230	80	8	8	D'Hont *et al.*, 1998
Mol 4503	60	6	6	D'Hont *et al.*, 1998
IM 76234	60	6	6	D'Hont *et al.*, 1998
-Hybrids				Jankin *et al.*, 1995
85N904	110	11	-	
MQ66-14	112	14	-	Jankin *et al.*, 1995
My 5514	~103	10	10	Cuadrado *et al.*, 2004
B 42231	~110	10	10	Cuadrado *et al.*, 2004
C 236-51	~115	12	12	Cuadrado *et al.*, 2004
KPS 00-25	110	7-9	13-15	This study

IV. DISCUSSION

The 18S rDNA sites are sub-units of 45S rDNA that are arranged in long tandem arrays of repeat unit and are associated with secondary constrictions and area of NORs. The 5S rDNA sites are also smaller tandem repeat unit [7], [18]. The presence of locus of the 18S and 5S rDNA sites allowed the basic chromosome numbers to be predicted by dividing the total chromosome numbers by the number of sites of rDNAs for each gene locus. The results revealed a basic chromosome number of X = ~14 or ~8, respectively. According to the consensus of basic chromosome numbers of 8 or 10 for modern sugar cane was determined [8], [17]. For ~8 basic chromosomes of the 5S rDNA seem reliable indicate for genome numbers. However, many researches have used 45S rDNA sites to determine the basic chromosome of sugar cane and polyploidy plants [9], [10], [17]. In this case, the ~14 basic chromosome number of 7-9 sites of 18S rDNA showed different number of the compared literatures. This phenomenon can be found in hybrids and allopolyploid. The rRNA genes can expressed only one of two parents which the rRNA gene of one parental species dominates over the rRNA genes of another species, called "amphiplasty" or "nucleolar dominance" [19]-[21]. The present locus was possibly deleted of 18S rDNA signals as discussed above.

V. CONCLUSION

The implications of 45S rDNA and 5S rDNA sites could be used to predict the basic chromosome number by FISH techniques. The results showed the basic chromosome numbers around 14 of the sugar cane cultivar KPS 00-25. The suggestion, unclear 18S rDNA sites were shown of the experiments. This is the challenge to be confirmed the genome numbers by cross species FISH technique with chromosome sequences from other related species. However, the method as described in this paper was the initial method as a rough estimate the basic chromosome numbers of new hybrid sugar cane cultivar of Thailand.

ACKNOWLEDGEMENT

The authors wish to thank Assoc. Prof. Dr. Rewat Lersrutaiyotin from Cane and Sugar Research and Development Center of Kasetsar University for the sugar cane flower bud samples. This work was partially supported by the Center for Advanced Studies in Tropical Natural Resources Institute for Advanced studies, Kasetsart University, under the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, Ministry of Education, Thailand.

REFERENCES

[1] B. T. Roach, "Nobilisation of sugarcane," in *Proc Int. Soc. Sugar Cane Technol*, vol. 14, 1972, pp. 206-216.
[2] A. Kato, J. C. Lamb, and J. A. Birchler, "Chromosome painting using repetitive DNA sequences as probes for somatic chromosome identification in maize," in *Proc. Natl Acad Sci USA*, vol. 101, 2004, pp. 13554-13559.
[3] M. A. Lysak, A. Pecinka, and I. Schubert, "Recent progress in chromosome painting of arabidopsis and related species," *Chromosome Res*, vol. 11, pp. 195-204, 2003.
[4] K. W. Chang, S. A. Fang, F. C. Chang, and M. C Chuang, "Chromosomal conservation and sequence diversity of ribosomal RNA genes of two distant Oryza species," *Genomics*, vol. 96, pp. 181-190, 2010.
[5] D. M. Figueroa and H. W. Bass, "A historical and modern perspective on plant cytogenetics," *Briefings Functional Genomics*, vol. 9, pp. 95-102, 2010.
[6] Y. C. Song and J. P. Gustafson, "The physical location of 14 Rflp markers in rice (Oryza Sativa L.)," *Theor. Appl. Genet.*, vol. 90, pp. 113-119, 1995.
[7] R. Flavell and D. Smith, "Variation in nuclear organizer rRNA gene multiplicity in wheat and rye," *Chromosoma*, vol. 47, pp. 327-334, 1974.
[8] A. Cuadrado, R. Acevedo, S. M. D áz de la Espina, N. Jouve, *et al.*, "Genome remodelling in three modern S. officinarum × S.

spontaneum sugarcane cultivars," *Journal of Experimental Botany*, vol. 55, pp. 847-854, 2004.
[9] A. D' Hont, "Unraveling the genome structure of polyploids using fish and gish; examples of sugarcane and banana," *Cytogenet Genome Res*, vol. 109, pp. 27-33, 2005.
[10] A. D'Hont, D. Ison, K. Alix, C. Roux, *et al.*, "Determination of basic chromosome numbers in the genus saccharum by physical mapping of ribosomal RNA genes," *Genome*, vol. 41, pp. 221-225, 1998.
[11] S. Ha, P. Moore, D. Heinz, S. Kato, *et al.*, "Quantitative chromosome map of the polyploid saccharum spontaneum by multicolor fluorescence in situ hybridization and imaging methods," *Plant Molecular Biology*, vol. 39, pp. 1165-1173, 1999.
[12] A. D'Hont, L. Grivet, P. Feldmann, S. Rao, *et al.*, "Characterisation of the double genome structure of modern sugarcane cultivars (Saccharum spp.) by molecular cytogenetics," *Mol. Gen. Genet*, vol. 250, pp. 405-413, 1996.
[13] A. Piperidis and A. D'Hont, "Chromosome composition analysis of various *Saccharum* interspecific hybrids by genomic in situ hybridisation (GISH)," in *Proc. Int. Soc. Sugarcane Technol*, vol. 24, 2001, pp. 556-559.
[14] A. D'Hont, and J. C. Glaszmann, "Sugarcane genome analysis with molecular markers, a first decade of research," in *Proc. Int. Soc. Sugarcane Technol.*, vol. 24, 2001, pp. 556–559.
[15] K. W. Chang, S. A. Fang, F. C. Chang, and C. M. C hung, "Chromosomal conservation and sequence diversity of ribosomal RNA genes of two distant Oryza species," *Genomics*, vol. 96, pp. 181-190, 2010.
[16] D. H. Koo, Y. Hur, D. C. Jin, and J. W. Bang, "Karyotype analysis of a Korean cucumber cultivar (C. Sativus L. Cv. Winter Long) using C-banding and bicolor fluorescence in situ hybridization," *Mol Cells*, vol. 13, pp. 413-418, 2002.
[17] M. J. Jenkin, S. M. Reader, K. A. Purdie, and T. E. Miller, "Detection of rDNA sites in sugarcane by FISH," *Chromosome Res*, vol. 3, pp. 444-445, 1995.
[18] Y. Mukai, T. Endo, and B. Gill, "Physical mapping of the 18S.26S rRNA multigene family in common wheat: identification of a new locus," *Chromosoma*, vol. 100, pp. 71-78, 1991.
[19] M. S. Navashin, "Amphiplastie-Eine neue karyologische Erscheinung," in *Proc Int Conf Genet*, vol. 5, 1982, pp. 1148-1152.
[20] C. S. Pikaard, "Nucleolar dominance: Uniparental gene silencing on a multi-megabase scale in genetic hybrids," *Plant Mol. Biol.*, vol. 43, pp. 163-177, 2000.
[21] S. Tucker, A. Vitins, and C. S. Pikaard, "Nucleolar dominance and ribosomal RNA gene silencing," *Current Opinion in Cell Biology*, vol. 22, pp. 351-356, 2010.

Mr. Sarut Thumjamras was born Nov. 3, 1981. He is a Ph.D. Student and the student ID is 5328600029. He is majored in Bio-products science, Department of Science, Faculty of Liberal Arts and Science. He got his Master degree: Master of Science (Economic Botany) 2010, Kasetsart University, Kamphaeng Sean Campus, Nakorn Pathom, Thailand. He got his Bachelor's degree: Bachelor of Science (Biology) 2004, Burapha University, Chonburi, Thailand. His contact address is Biology, Faculty of Liberal Arts and Science, Kasetsart University, Kamphaeng Sean Campus, Nakorn Pathom
.