

Full Length Research Paper

Comparative genomic *in situ* hybridization analysis on the chromosomes of five grass species with rice genomic DNA probe

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Comparative genomic *in situ* hybridization (cGISH) with biotin-labeled rice genomic DNA to the chromosomes of *Zea mays*, *Hordeum vulgare*, *Sorghum bicolor*, *Setaria italic* and *Secale cereale* were conducted to analyze genomic homology between rice and other grass (Gramineae) species. At 75% stringency, the rice DNA probe generated large numbers of signals dispersedly distributed over all chromosomes in all target species. The nucleolar organizing regions (NORs), a few telomeres, most centromeric regions and numerous interstitial sites were detected. The signals in small genomes were relatively sparse and unevenly distributed along chromosomes, whereas those in large genomes were dense and basically evenly distributed. Chromosomal *in situ* suppression (CISS) experiments indicated that the signals represented the hybridization of repetitive DNAs is common between rice and the target genome. cGISH conducted at low and high stringency revealed that repetitive DNAs with ~65% homology are rich and those with ~90% homology are rare between rice and maize or barley. cGISH using maize genomic DNA probe to the rice chromosomes generated relatively sparse and unevenly distributed signals. Our results reveal that there exist besides rDNA and telomeric repeats, some other conserved repetitive DNAs among grass species of different subfamilies. Our results also suggest that ancient conserved repetitive DNAs had undergone considerable amplification in large grass genomes during evolution.

Key words: Comparative genomic *in situ* hybridization (cGISH), genomic homology, chromosome, grass genome, repetitive DNA.

INTRODUCTION

Comparative genome analysis is essential in elucidating the mechanisms of genome evolution in plants. Comparative mapping of related plant species by using a set of restriction fragment length polymorphism (RFLP) markers became available to plant genetics (Tanksley et al., 1988; Bonierbale et al., 1988) more than two decades

ago. Such investigations demonstrated that gene content and gene order among related plant species remained largely conserved over millions of years of evolution (Gale and Devos, 1998; Schmidt, 2000). The focus of comparative genomics has recently shifted from comparisons at the gross map level to studies of gene organization in small chromosomal regions and finally to the DNA sequences itself with the aim of revealing the microcolinearity, and determines the mechanisms and rates of plant genome evolution (Bennetzen, 2000).

Fluorescence *in situ* hybridization (FISH) has also been adopted in comparative genome analysis in plants because of its ability of physical localization of DNA

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Abbreviations: cGISH, Comparative genomic *in situ* hybridization; NORs, nucleolar organizing regions; CISS, chromosomal *in situ* suppression.

sequences. Localization by FISH of multiple repetitive sequences, each representing a substantial fraction of the genome, can reveal the distribution characteristic of repetitive DNAs along chromosomes in different species (Brandes et al., 1997; Kumar et al., 1997; Friesen et al., 2001). Genomic *in situ* hybridization (GISH), a modification of the FISH technique, allows chromosomes from different parents/ancestors/genomes in hybrid plants to be distinguished (Raina and Rani, 2001). In conventional GISH experiments, labeled genomic DNA from one species and unlabeled blocking DNA from another species were simultaneously applied, allowing genome-specific sequences remain exposed as sites for probe hybridization. A new GISH approach, comparative genomic *in situ* hybridization (cGISH) technology, has been developed in plants (Takahashi et al., 1999; Zoller et al., 2001; Falistocco et al., 2002). In cGISH experiments the labeled total genomic DNA of one species hybridizes to chromosomes of related or distantly related species without application of blocking DNA, thus the hybridization signals represent the hybridization of DNA sequences in common between the two species. Therefore, cGISH technology is a useful tool for comparative genome analysis in plants to show genomic homology between related or distantly related plants as well as the chromosomal distribution of conserved DNA sequences, providing valuable information on the genomic organization and genome evolution in plants (Zoller et al., 2001).

Higher plants exhibit extraordinary variation in nuclear genome size. Molecular investigations of plant nuclear DNA content have shown that genome size variability is largely caused by differences in amount of repetitive DNAs (Flavell et al., 1974). This makes repetitive DNA of great interest for studying the molecular mechanisms of shaping architecture and function of complex plant genomes. Repetitive DNAs in plants can be approximately divided into two classes: tandemly repeated and dispersedly repeated DNAs. The former mainly include satellite DNA, rRNA genes and telomeric repeat, and the later mainly comprise trans-posable elements. In most flowering plant species investigated so far, the majority of repetitive DNA is composed of various families of retrotransposons, primarily the LTR- (long terminal repeat-) retrotransposons (Rabinowicz and Bennetzen, 2006). Recent investigations have shown that plant genomes undergo genome size increases through bursts of retrotransposition, while there is a counteracting process that tends to eliminate the transposed copies from the genomes by unequal homologous recombination and illegitimate recombination (Bennetzen et al., 2005; Vitte and Bennetzen, 2006).

Rice (*Oryza sativa* L.) is a model plant for monocots, and has important syntenic relationships with other cereal species (Gale and Devos, 1998). Rice genome (~400 Mbp) is the smallest cereal genomes and has lower content of repetitive DNAs compared with other cereal

genomes (International Rice Genome Sequencing Project, 2005). Such characteristics of the rice genome enable us to perform large-scale comparative genome analysis in plants by cGISH with rice genomic DNA probe. In this paper, biotin-labeled genomic DNA of *O. sativa* subsp. *indica* is hybridized to the chromosomes of *Zea mays*, *Hordeum vulgare*, *Sorghum bicolor*, *Setaria italica* and *Secale cereale* in order to reveal *in situ* the similarity of genomic DNA between rice and other grass (Gramineae) species and show the distribution characteristics of conserved repetitive DNAs along chromosomes.

MATERIALS AND METHODS

Plant materials and chromosome preparation

Plant species used in the present study were *O. sativa* ssp. *indica* Kato (2n = 24, ~430 Mbp), *Z. mays* L. (2n=20, ~2,600 Mbp), *H. vulgare* L. (2n=14, ~5,400 Mbp), *S. bicolor* L. Pers (2n=20, ~750 Mbp), *S. italica* (L.) Beauv. (2n=18, 490 Mbp) and *S. cereale* L. (2n=14, ~7,660 Mbp). The data of the genome size were adopted from Bennett et al. (2000). According to Song et al. (1994), mitotic chromosomes were prepared from the root tips harvested from the germinated seeds. In brief, root-tips were treated with saturated α -bromonaphthalene for 1-3 h or ice cold water for 24 h and then fixed in methanol acetic acid (3:1) overnight. For chromosome preparations, root tips were washed in enzyme buffer (150 mM citric acid/sodium citrate, pH 4.5) for 30 min, and then digested in a mixture of pectolytic enzymes containing 0.3% pectolyase Y23, 0.3% cellulase RS, 0.3% cytohellicase in the citrate buffer for 2-4 h at 28°C. One or a few root tips were transferred to a glass slide and dissected thoroughly with methanol acetic acid (3:1) using fine-pointed forceps. Finally, the slides were dried by a flame.

Preparation of probes and rice *Cot-1* DNA

Total genomic DNA was extracted from young actively growing leaves of *O. sativa* ssp. *indica* and *Z. mays* using the CTAB procedure (Murray and Thompson, 1980) and labeled with biotin-11-dUTP (Roche Diagnostics Ltd., Shanghai, CN) by nick translation. Rice *Cot-1* DNA was prepared according to the procedure described by Zwick et al. (1997).

In situ hybridization

In situ hybridization using biotin-labeled genomic DNA from *O. sativa* ssp. *indica* to the chromosomes of *Z. mays*, *H. vulgare*, *S. bicolor*, *S. italica* and *S. cereale*, and *in situ* hybridization using biotin-labeled genomic DNA from *Z. mays* to the chromosomes of *O. sativa* ssp. *indica* was performed using a modified version of the protocol described by Rayburn and Gill (1985). After fixation at 60°C for 1h, the slides were treated with 100 μ g/mL of RNase A in 2x SSC (0.3 M NaCl, 0.03 M sodium citrate) for 1 h at 37°C and washed three times in 2x SSC. Chromosomal DNA was then denatured by immersing the slides in 70% formamide at 70°C for 3 min and dehydrated in a graded ethanol series and air-dried. The hybridization solution consisting of 4 ng μ L of DNA probe, 50% (v/v) formamide, 10% (w/v) dextran sulphate, 0.1% (w/v) SDS (sodium dodecyl sulfate), 100 ng/ μ L of sheared salmon sperm DNA and 2x SSC, was incubated for 10 min at 100°C and chilled on ice for more than 5 min. Fifty microlitres of hybridization mixture were

applied to each chromosome preparation and covered with a glass coverslip. The hybridization mixture and the chromosomes were denatured together on a hotplate for 2.5 min at 80°C, and then the slides were transferred to a humid chamber for 3 days of hybridization at 37°C. After hybridization, slides were washed at 42°C for 15 min each in 20% formamide (v/v, in 2× SSC) and 2× SSC. Such post-hybridization washing together with the concentration of 50% of formamide in the hybridization mixture provided about 75% stringency, which was defined as normal stringency in this paper. For detection of the biotinylated DNA, slides were transferred to 0.1% TritonX-100 for 5 min and 1× PBS (130 mM NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄) for 2× 5 min at room temperature, and then incubated in 8 µg/mL of Cy3-conjugated streptavidin (Amersham Biosciences Europe GmbH, Freiburg, Germany) in detection buffer containing 1% (w/v) BSA (Bovine serum albumin) for 1 h at 37°C. After incubation the slides were washed two times in 1× PBS at room temperature.

Chromosome DNA were counterstained with 3 µg/mL of DAPI (4',6-diamidino-2-phenylindole) in antifade solution Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA). The slides were examined with an Olympus BX60 epifluorescence microscope equipped with a SenSys 1401E cooled CCD (charge coupled device) camera (Roper Scientific Inc., Trenton, NJ). The CCD camera was controlled using MetaMorph software (Universal Imaging Corp., Buckinghamshire, UK). Grey-scale images were captured with UV (for DAPI) and G (for Cy3) excitation filters and then pseudo-colored and merged. Final image adjustments were completed using Adobe Photoshop software.

In situ hybridization using rice genomic DNA probe to the chromosomes of *Z. mays* and *H. vulgare* was also conducted at low (~65%) and high (~90%) stringency. The stringency conditions were decided by the concentration of formamide in hybridization mixture together with the condition of post-hybridization washing according to the calculations by Schwarzscher and Heslop-Harrison (2000). The formamide in the hybridization mixture was 30% and 60% for low and high stringency hybridizations, respectively. Correspondingly, washing was conducted at low (20% formamide and 2× SSC at 42°C for 15 min each) and high (55% formamide and 1× SSC at 42°C for 15 min each) stringency.

Chromosomal *in situ* suppression (CISS) control experiments were performed using unlabeled *Cot-1* DNA from *O. sativa* ssp. *indica* in cGISH using rice genomic DNA to chromosomes of *Z. mays* and *H. vulgare*, in which the hybridization mixture included unlabeled *Cot-1* DNA and labeled genomic DNA with 20:1 ratio and hybridized according to the above protocol.

RESULTS

cGISH using rice genomic DNA probe to the chromosomes of five grass plants

The hybridization patterns obtained with biotin-labeled total genomic DNA from *O. sativa* ssp. *indica* and chromosomes of *Z. mays*, *H. vulgare*, *S. bicolor*, *S. italica* and *S. cereale* were shown in Figures 1 and 2A-2F. It was obvious that at normal (~75%) stringency, the rice DNA probe generated large numbers of signals dispersedly distributed over all chromosomes in all target species after Cy3-streptavidin detection without signal amplification. The hybridization patterns varied to a certain extent among different target species.

The metaphase chromosomes of *Z. mays* and *H. vulgare* displayed a large number of dispersed

signals which were basically evenly distributed along the lengths when probed by the rice genomic DNA at normal stringency (Figure 1A-1B, 1G-1H). The signals in maize and barley metaphase diploid complement counted up to 212 and 296, respectively. The nucleolar organizing regions (NORs), most centromeric regions, some telomeres and numerous interstitial sites were labeled by the probe. The heterochromatic knobs of maize with strong positive DAPI staining were devoid of signals. At low (~65%) stringency, the number and density of the signals both in maize and barley increased considerably (Figure 1C-1D, 1I-1J), but the knobs of maize were still devoid of signals, which was also evident in the interphase nuclei (Figure 1C-1D). In contrast, at high (~90%) stringency, the number of signals both in maize and barley decreased sharply, dropping to 33 and 31, respectively; only the NOR signals as well as a few other signals detected with low reproducibility were being shown (Figure 1E-1F, 1K-1L).

Like in maize and barley, most centromeric regions, some telomeres and numerous interstitial sites were detected in *S. bicolor*, *S. italica* and *S. cereale* by the rice DNA probe at normal stringency (Figure 2A-2F). The number of signals in their metaphase diploid complement was 137, 119, and 370, respectively. All the chromosomes of *S. bicolor* display uneven signal distribution with the distal regions of most chromosomes showing intense and dense signals, and the proximal regions of all chromosomes and the short arms of some chromosomes, which are strongly stained by DAPI, showing no signals or a few faint signals (Figure 2A-2B). In *S. italica*, the signals were less dense and relatively unevenly distributed along chromosomes and most of the centromeric signals were prominent (Figure 2C-2D). The signals in *S. cereale* were the densest among the five target species and displayed relatively even distribution (Figure 2E-2F).

The hybridization characteristics of the rice DNA probe was tested by hybridization to the chromosomes of *indica* rice which revealed strong and dense signals along the chromosomes with predominant hybridization in pericentromeric and other heterochromatic regions (Figure 2G-2H). The CISS experiments showed that the hybridization of labeled rice DNA to the chromosomes of maize and barley were almost blocked by the *Cot-1* DNA from *indica* rice (images not shown).

cGISH using maize genomic DNA probe to the chromosomes of *indica* rice

cGISH using maize genomic DNA probe to the chromosomes of *indica* rice was used for characterization of the chromosomal distribution in *indica* rice of the repetitive DNAs in common between maize and rice. Dispersed signals were generated over all the rice chromosomes when probed by biotin-labeled maize

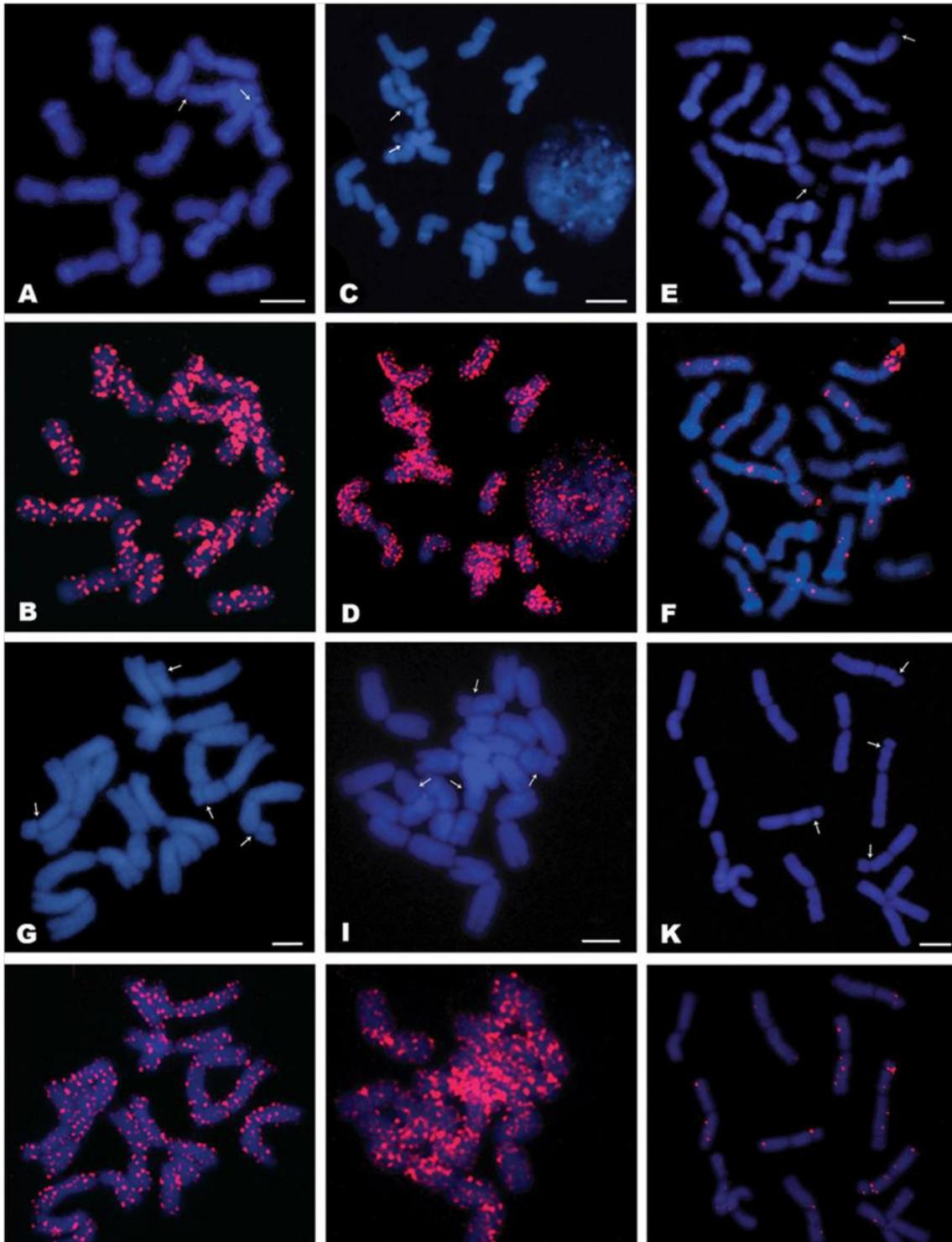


Figure 1. *In situ* hybridization with biotin-labeled genomic DNA from *indica* rice to the mitotic chromosomes of *Z. mays* and *H. vulgare*. A-B, C-D and E-F: DAPI-stained metaphase chromosomes and hybridization signals of *Z. mays* in normal, low and high stringency hybridizations, respectively. G-H, I-J and K-L: DAPI-stained metaphase chromosomes and hybridization signals of *H. vulgare* in normal, low and high stringency hybridizations, respectively. Arrows indicate the NORs. Bar=5 μ m (H,J,L).

genomic DNA (Figure 2I-2L). The number of signals in rice metaphase diploid complement was 115. The NORs,

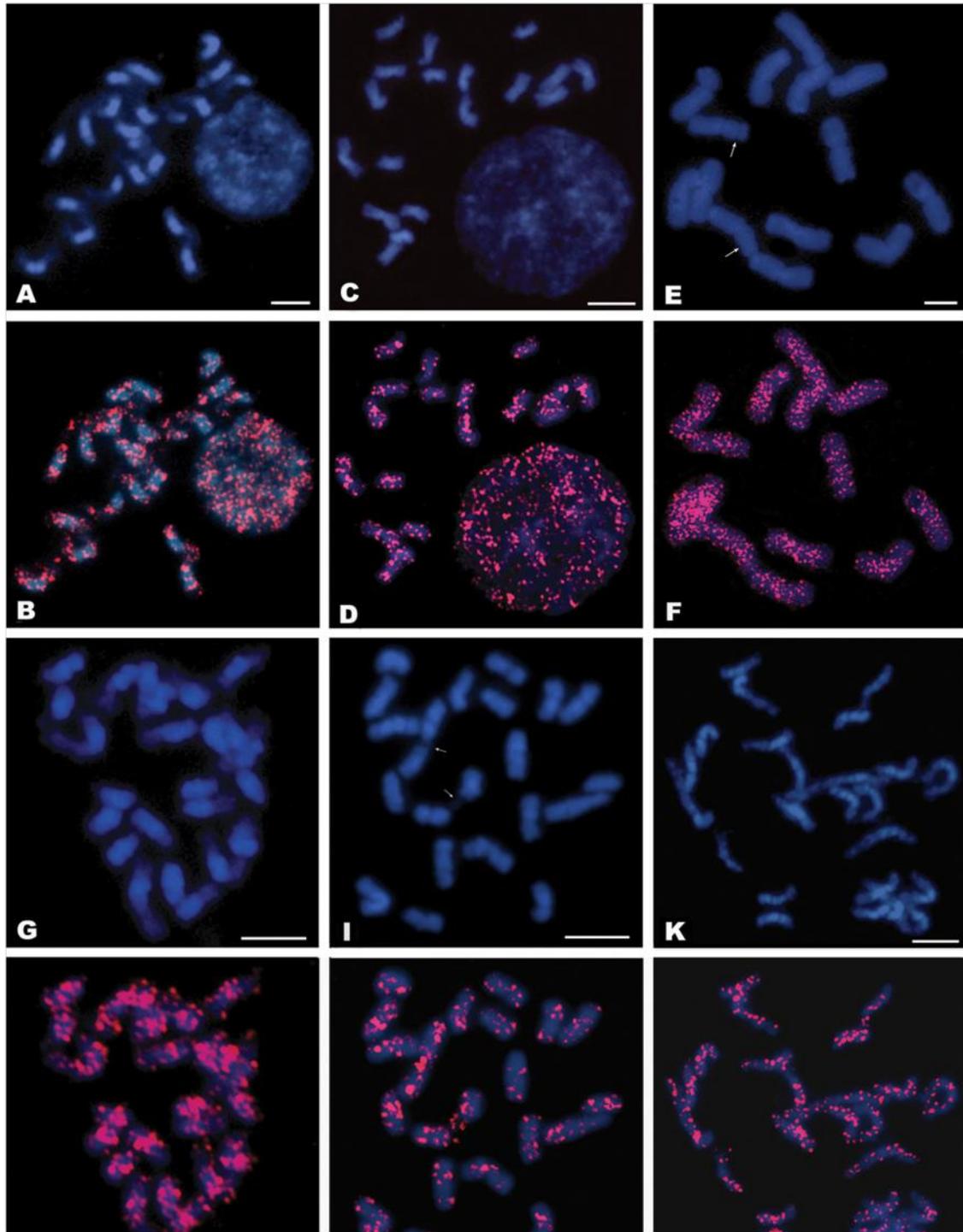


Figure 2. *In situ* hybridization with biotin-labeled genomic DNA from *indica* rice to the mitotic chromosomes of *S. bicolor*, *S. italica*, *S. cereale* and *indica* rice (A-H), and *in situ* hybridization with biotin-labeled maize genomic DNA to the mitotic chromosomes of *indica* rice (I-L) at normal stringency. A-B: *S. bicolor*; C-D: *S. italica*; E-F: *S. cereale*; G-L: *indica* rice.. A, C, E, G, I, K: DAPI-stained mitotic chromosomes and interphase cells; B, D, F, H, J, L: Hybridization signals. Arrows indicate the NORs. Bar=5 μ m (H,J,L).

most centromeres, a few telomeric regions and many interstitial sites were labeled by the maize DNA probe. The signals varied in size and intensity, and displayed

very unevenly distribution along the lengths. As revealed in late prophase cells, hybridization signals appeared not only in the proximal regions of all chromosomes and

some heterochromatic arms that were strongly DAPI-stained but also in the distal euchromatic regions that were lightly DAPI-stained (Figure 2K-2L). The signals on the rice chromosomes probed by the maize genomic DNA were less dense and more unevenly distributed in comparison with those on the maize chromosomes probed by the rice genomic DNA at normal stringency.

DISCUSSION

The homology of repetitive DNAs among grass genomes

In our cGISH experiments, the labeled total genomic DNA from *indica* rice was hybridized to the chromosomes of five grass plants without application of blocking DNA. Such hybridization was based upon the DNA homology across the whole genome between the probe and the target species. Our CISS experiments demonstrated that the cGISH signals represented the hybridization of repetitive DNAs in common between the probe and the target species.

It is well known that the coding regions of rDNA repeats are highly conserved across the whole plant kingdom (Lapitan, 1991). The NORs consisting of 17S–5.8S–25S ribosomal DNA coding units were detected by the rice DNA probe. The *Arabidopsis*-type telomeric repeats are known to be conserved across almost all plants except *Allium* and a few other genera (Schwarzacher and Heslop-Harrison, 1991; Fuchs et al., 1995; Pich and Schubert, 1998; Weiss and Scherthan, 2002). The arrays of telomeric repeat can differ in length between species and between chromosomes within a species. The telomere with less repeats could not usually be detected even using the telomere repeat as probe if without signal amplification (Schwarzacher and Heslop-Harrison, 1991; Fuchs et al., 1995). Therefore, the telomeric signals detected in all target species in our cGISH experiments must be the telomeres with long arrays of repeat.

Rice and the five target species are of grass family (Gramineae). They belong to three different subfamilies: *O. sativum* belongs to Ehrhartoideae, *Z. mays*, *S. bicolor* and *S. italica* belong to Panicoideae, *S. cereale* and *H. vulgare* belongs to Pooideae (Kellogg, 2001). It has been revealed that grasses originated 70–55 million years ago (mya), and rice diverged from the ancestor of maize and sorghum 50 mya (Kellogg, 2001). The fact that large numbers of signals were observed on the chromosomes of the five grass species by the rice DNA probe at normal stringency indicated that besides rDNA and telomeric repeats, there exists some other conserved repetitive DNAs with ~75% homology across species of different Gramineae subfamilies. This was also demonstrated by results of cGISH using maize genomic DNA to the chromosomes of *indica* rice. cGISH at low and high stringency revealed that the conserved repetitive DNAs

with ~65% homology are rich but the conserved repetitive DNAs with ~90% homology are rare among different Gramineae subfamilies.

Whole genome sequencing has revealed that 42% of the rice genome consists of repetitive DNAs including satellites and transposable elements (International Rice Genome Sequencing Project, 2005). Only a few satellite DNAs have been characterized in the rice genome. The highly repetitive 155–165 bp CentO satellite DNA together with centromere-specific retrotransposons (CRR) is located within the functional domain of the rice centromere and are found basically conserved across *Oryza* species (Jiang et al., 2003). Other satellite DNAs investigated such as TrsA, TrsB and Os48 are genome-specific or even chromosome-specific (Uozu et al., 1997; Cheng et al., 2001). Similarly, the satellite DNAs conserved across species of a genus or a tribe (example, the pAs1 family exists in the tribe Triticeae) have been characterized in other plant genomes but the majority of satellite DNAs investigated so far are found to be highly variable, usually showing species, genome, or even chromosome specific (Sharma and Raina, 2005).

The transposon content of *indica* rice is at least 35% and is populated by representatives from all known transposon superfamilies (International Rice Genome Sequencing Project, 2005). The LTR retrotransposons mainly including Ty3/*gypsy* and Ty1/*copla* account for 26% of the rice genome (Paterson et al., 2009). It has been revealed that each of the major clades of rice LTR retrotransposons are more closely related to elements present in other species than to other clades of rice elements (McCarthy et al., 2002). For examples, the RTs of the clade including high-copy *gypsy*-like families *Osr33/Rire8* and *Osr34* and low-copy families *Osr32* and *Osr56/Rire3* show high sequence similarity to an LTR retrotransposon in pineapple (~70% to Acr-1) and to one in *S. bicolor* (~77% to Retrosor3) (McCarthy et al., 2002). Phylogenetic analysis has also demonstrated that rice and maize retrotransposon families are frequently more closely related to each other than to families within the same species (Meyer et al., 2001). Examination of the distribution of seven wheat retrotransposon families in Gramineae species has shown that the representatives of two families have homologues in the species out of Pooideae subfamily including rice. For example, there is 82% nucleotide sequence homology between the family 1 representative clone and a rice retrotransposon fragment named *TOSRT4* (Matsuoka and Tsunewaki, 1997). The centromeric retrotransposon (CR) belonging to Ty3/*gypsy* group is highly conserved across the grass species and highly specific to the centromeric regions of grass chromosomes (Langdon., 2000; Jiang et al., 2003). Recent phylogenetic analysis has shown that *copla* elements from barley, wheat, rice and *Arabidopsis* can be classified into six ancient lineages that existed before the divergence of monocots and dicots. The six lineages show a surprising degree of conservation in sequence

organization and other characteristics across species (Wicker and Keller, 2007). These facts indicate that a lot of retrotransposon families are of ancient origin and have maintained high conservation during long-time evolution of plant genomes.

In all plants investigated, the most significant contributions to genome size have been retrotransposons, primarily the LTR-retrotransposons (SanMiguel et al., 1996; Vicient et al., 1999; Rabinowicz and Bennetzen, 2006). In maize, retrotransposons make up over 75% of the nuclear genome (Baucom et al., 2009), and many LTR-retrotransposons are intermixed with genes, often as nested structures of LTR-retrotransposons inserted into LTR-retrotransposons (SanMiguel et al., 1996; Rabinowicz and Bennetzen, 2006). It is believed that retrotransposons are major contributors to all other large plant genomes (Bennetzen et al., 2005; Vitte and Bennetzen, 2006).

In summary, it is reasonable to suggest that most of the dispersed cGISH signals represent the hybridization of conserved retrotransposon families between rice and the target species. As an example, the signals shown in most centromeric regions should represent the hybridization of the conserved CR.

Conserved repetitive DNAs and genome evolution in grass

Our results show that the abundance of signal generated by the rice DNA probe was mainly related to the genome size of target species instead of the degree of relationship between species. For example, the signal abundance of rye was much higher than that of barley.

Compared with middle and large angiosperm genomes, the rice genome has much lower content of repetitive DNAs, and hence the conserved repetitive DNAs in the rice genome shared with other distantly related species should account for relatively small portion of the rice genome. The fact that large numbers of relatively evenly distributed signals were generated on the chromosomes of the middle and large target genomes such as maize, barley and rye indicated that the conserved repetitive DNAs have undergone considerable amplification and homogenization of distribution in middle and large plant genomes during evolution. This is further supported by the fact that the signals on the rice chromosomes probed by maize genomic DNA displayed much lower density and much more uneven distribution compared with the signals on the maize chromosomes probed by rice genomic DNA.

Many reports have shown that amplification of repetitive DNAs, primarily LTR retrotransposons, is the main cause of genome expansion in plants, and amplification of retrotransposon have appeared to occur mainly in recent evolutionary history (SanMiguel et al., 1996; Bennetzen et al., 1998; Vicient et al., 1999; Gaut et al., 2000; Meyers et al., 2001). However, our investigation

indicated that the repetitive DNAs established in the ancient progenitor not only have been still conserved across species of different grass subfamilies, but also have undergone amplification and accumulation during evolution of genome expansion. This suggests that like generation of new repetitive DNAs, the amplification of ancient conserved repetitive DNAs should also play an important role in the expansion of genome size in grass plants.

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