Chromosomal conservation and sequence diversity of ribosomal RNA genes of two distant Oryza species

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Contrary to the chromosomal polymorphism of 45 S ribosomal genes (45 S rDNA) loci in other Oryza species, each of Oryza australiensis and Oryza brachyantha has only one 45 S rDNA locus at the most conserved position of 45 S rDNAs in Oryza. O. australiensis and O. brachyantha are known phylogenetically distant and have extremely different genome sizes among diploid Oryza species. This study reveals that the sequences and organizations of intergenic spacer (IGS) for 45 S rDNA of both O. australiensis and O. brachyantha are different from other Oryza species. The IGS of O. australiensis contains 13 tandem repeats and only one transcriptional initiation site, while there are four tandem repeats and three transcriptional initiation sites in the IGS of O. brachyantha. Our results suggest different evolution processes of orthologous rDNA loci in the genus Oryza. Here we also demonstrate an efficient strategy to study locus-specific IGS before whole genome sequences data are available.

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Introduction

The transcribed region of a 45 S ribosomal RNA gene (rDNA) contains sequences encoding 16(–18)S, 5.8 S, and 25(–28)S rRNA and two internal transcribed spacers (ITS1 and ITS2). In higher eukaryotes, highly repeated 45 S rDNA units are arranged in tandem at one or several chromosomal loci. An intergenic spacer (IGS) separates two adjacent rDNAs in the tandem arrays and consists of a nontranscribed region (NTS) flanked with two external transcribed spacers (ETS). Contrary to the high conservation in the coding regions, IGSs vary in both length and sequence among closely related species [1,2]. Like other highly repeated gene families, rDNA repeats undergo a process of concerted evolution, homogenizing the sequences of each unit in an array. Therefore, units within a species are more similar to each other than to those in related species [3,4]. However, IGS variants are common in a genome with multiple rDNA loci because the occurrence of homogenization across chromosomes is infrequent [5,6].

Highly similar and repetitive sequences such as rDNAs usually present a technical challenge in an automatic assembly. A lack of genomic sequence information hampers the progress of identifying the rDNA variants (v-rDNA) with computational and bioinformatic methods. To date, only one mouse rDNA transcription unit has been sequenced in its entirety [7] and in GenBank Release 163, December 2007. Methods other than whole genome sequencing are applied to study v-rDNAs. In human, rDNA variant on a specific chromosome was cloned after PCR amplification from a rodent–human somatic cell hybrid containing that chromosome [8]. In mouse, considerable restriction fragment length polymorphisms (RFLP) and a variable number of repeats in the nontranscribed spacer were detected in rDNA units [9]. Seven mouse v-rDNAs on different chromosome loci were cloned based on RFLPs, showing unequal activities in different cell types [10]. In Arabidopsis thaliana, rDNAs clustered in two nucleolar organizing regions (NORs) on chromosome 2 (NOR2) and chromosome 4 (NOR4). Both could be differentiated by RFLP [11].

In the genus Oryza, 45 S rDNA loci are polymorphic in both number and location. From one to four rDNA loci have been detected on Oryza chromosomes by fluorescence in situ hybridization (FISH) analyses [12]. Oryza australiensis (EE) as well as Oryza brachyantha (FF) has only one 45 S rDNA locus at the end of the short arm of chromosome 9 (9 S), which is the most conserved position of the 45 S rDNA locus in the genus Oryza [12,13]. The length polymorphism of IGSs in the genus Oryza has been well documented [14–17]. The integrated rDNA array at the end of chromosome 9 S in Oryza sativa ssp. japonica cv. Nipponbare were analyzed [18] soon after the completion of the whole rice genome sequencing project [19]. Three types of DNAs ranging from 7928 bp to 8934 bp long were contained in this region. The length heterogeneity in these three types of rDNAs is due to the fact that they contain different numbers of a 254-bp subrepet in their IGS [18]. Such 254-bp subrepeats in IGS were detected in most of the Oryza genome except for EE and CCDD [21]. The existence of 254-bp subrepeats in the FF genome was unknown at that time. However, a fragment of 61 bp downstream of those subrepeats in IGS of AA genome was found also to be also specific to the FF genome [15].
In this study, PCR profiles and genomic southern analyses show that the organizations of IGS of *O. australiensis* and *O. brachyantha* differ from those of other *Oryza* genomes. We cloned and characterized the IGS fragments amplified from *O. australiensis* and *O. brachyantha*, respectively. The results show that both *O. australiensis* and *O. brachyantha* have IGS that are extremely genome specific, although their rDNA loci are at a conserved chromosomal position in the genus *Oryza*. Together with the results presented in the present paper and the variations in the number and position of rDNA loci reported previously [12], the genome-specific IGS in *O. brachyantha* and in *O. australiensis* suggest different evolution processes of rDNAs from other *Oryza* species.

### Table 1

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</table>

* Each accession in the figures is represented by the number denoted here.

### Materials and methods

#### Plant materials

Sixteen accessions representing 11 *Oryza* species were used in this study (Table 1). In the following figures, each accession is represented with a number identical to that in Table 1. The original seeds of wild species of rice, except for those of *O. brachyantha* (FF), were kindly provided by the National Institute of Genetics, Japan (http://www.shigen.nig.ac.jp/rice/oryzabase/wild/coreCollection.jsp). The International Rice Genebank at the International Rice Research Institute (IRRI) in the Philippines kindly provided the seeds of *O. brachyantha*. Plants were grown at the experimental field of the Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan. Genomic DNA samples were extracted from young leaves using DNeasy Plant Mini Kit (Qiagen, Oslo, Norway).

#### PCR analysis

Appropriate primers were used in the PCR to amplify fragments of 18S rDNA, IGS, and subrepeats in the IGS from genomic DNA of accessions (Fig. 1A). The primers for 18S rDNA are P1 (5′-CGAACGTGAAACTGCGAATGGC-3′) and P2 (5′-TAGGAGCGACGGGCGGTGTG-3′) [18]. The primers for the IGS region are P3 (5′-TTGCTGCCACGATCCACTGAG-3′) and P4 (5′-CTACTGCGAGATCAACACCG-3′), which are designed according to the sequences at the 3′ end of 18S rDNA and the complementary sequence at the 5′ end of 25S rDNA [20]. The primers for the IGS subrepeat are P5 (5′-CTCGCCCCAGCTCCCGAG-3′) and P6 (5′-GGCTACGTGCCCGAACAC-3′) [18].

Each 25-μL reaction mixture contained 25 ng of total genomic DNA, 200 μM of each dNTP, 0.1 μM of each primer, 1× PCR buffer...
(10 mM Tris–HCl, pH 8.8, 1.5 mM MgCl₂, 50 mM KCl, and 0.1% Triton X-100), and 1 U HotstarTaq DNA polymerase (Qiagen, Oslo, Norway). The amplification conditions for the 18 S rDNA fragment were 95 °C for 15 min; 45 cycles of 95 °C for 1 min, 68 °C for 1 min, and 72 °C for 1.5 min; and 72 °C for 10 min (Thermal cycler 2720, Applied Biosystems, Foster City, CA, USA). Except for the annealing temperature, similar conditions were used to amplify the IGS region and IGS subrepeats. The annealing temperature for amplification of the IGS region or IGS subrepeats was 55 °C and 59.2 °C, respectively. PCR products were separated by electrophoresis in a 1.0% agarose gel with a restrictive enzyme (New England Biolabs, Hertfordshire, UK), Southern blot hybridization kit and an autosequencer (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

Southern blot hybridization

Genomic DNA of each genome (20 μg) were completely digested with a restrictive enzyme (New England Biolabs, Hertfordshire, UK), separated by electrophoresis at 40 kV (1 kV/cm) overnight on a 0.8% agarose gel in 0.5× TBE buffer, then blotted onto Hybond-N+nylon membrane by using ethidium bromide staining under UV illumination. Selected PCR products were eluted and cloned into a pCRTMII vector by use of a TA cloning kit (Invitrogen, Carlsbad, CA, USA). Inserts were sequenced by use of an Autoread sequencing kit and an autosequencer (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

FISH on chromosomes

The protocol for FISH was as previously described[12]. Probes used in FISH were labeled with digoxigenin-11-dUTP or biotin-14-dATP by nick translation. The construct pRY18 with partial coding region of 45 S rRNA gene (3.8 kb) of rice[17] was labeled and used as a probe for indicating the chromosomal position of rDNA locus. Hybridization signals for digoxigenin-labeled probe were immunodetected by using an antibody with rhodamine conjugated (Roche Diagnostics GmbH, Penzberg, Germany). Hybridization signals for biotin-labeled probes were detected by using fluorescein isothiocyanate (FITC)-conjugated avidin (Vector Laboratories, Burlingame, CA, USA). Chromosomes were counterstained with 4’, 6-diamidino-2-phenylindole (DAPI).

FISH on DNA fibers

Stretched DNA fibers were prepared following the protocol of Cheng et al., [21]. FISH and post-hybridization washing stringency were the same as those for FISH on chromosomes. Digoxigenin-labeled probes were immunologically detected with an antibody of sheep anti-digoxigenin-rhodamine (Roche) and then amplified with an antibody of rabbit anti-sheep-rhodamine (Upstate, NY, USA). The three-layer detection scheme for biotin-labeled probes included the use of FITC-conjugated avidin (Vector Laboratories), biotinylated goat anti-avidin (Vector Laboratories), and another FITC-conjugated avidin. After the last detection and washing step, the slides were mounted in Vectashield (Vector Laboratories).

Results

Variations in the IGS region of rDNA units in the genus Oryza

A noticeable 1600-bp band corresponding to 18 S rDNA was consistently amplified in each accession by using the primer pair P1–P2 (Fig. 2A). In addition, one faint band around 3000–4000 bp was amplified in each accession, which suggests the existence of DNA variants with different structural conformation in these Oryza genomes (Fig. 2A). The PCR profiles of the primer pair P3–P4 showed both similarity and polymorphism of the IGS in the genus Oryza. Two conspicuous bands, 500–600 bp and 1500–2000 bp, were amplified in four accessions of O. sativa (AA, lanes 1–4, Fig. 2B) and in O. brachyantha (FF) (lane 16, Fig. 2B). In the same reaction, one additional band of 300 bp was amplified in cultivar TNG 67 (lane 4, Fig. 2B). Two conspicuous bands, 1000–1500 bp and 1500–2000 bp, were amplified in Oryza glaberrima (AA, lane 5, Fig. 2B). One conspicuous band was observed in O. australiensis (EE, lane 8, Fig. 2B) and in Oryza latifolia (CCDD, lane 15, Fig. 2B), respectively. PCR products in other accessions appeared as multiple faint bands. The bands around 2000 bp in O. australiensis (EE, lane 8, Fig. 2B) and in

![Fig. 2. The conservation and polymorphism in PCR profiles of rDNAs among Oryza species.](image)

![Fig. 3. EcoRV-digested length polymorphism of rDNA in the Oryza genomes. Southern blot analysis of EcoRV-digested genomic DNA hybridized to the 18 S rDNA which was amplified from Oryza sativa ssp. japonica cv. Nipponbare by primers P1–P2 and used as a probe.)](image)
O. brachyantha (FF, lane 16, Fig. 2B) were separately eluted and cloned for further characterization. The fragment from O. brachyantha was denoted as Obra2.0 and the fragment from O. australiensis was denoted as Oaus2.0.

The subrepeats in the IGS of the Oryza genomes were amplified by use of the primer pair P5–P6 (Fig. 2C). One or two conspicuous bands accompanied by several faint bands were observed in most of the accessions, but no bands in excess of 100 bp were amplified from O. australiensis (EE, lane 8) nor from O. brachyantha (FF, lane 16). A band around 600 bp was predominantly amplified in the accessions of O. sativa (AA, lanes 1–4 in Fig. 2C), O. glaberrima (AA, lane 5 in Fig. 2C), and O. rufipogon (AA, lane 7 in Fig. 2C). A band about 900 bp was amplified in accession with B, BC, C, and CD genomes (lanes 9–14 in Fig. 2C). Several faint bands were amplified from O. latifolia (CCDD, lane 15 in Fig. 2C).

Each rDNA unit of O. sativa has one EcoRV site in its 5.8 S rDNA region (Fig. 1A; [18]). Southern hybridization EcoRV-digested genomic DNA to 18 S rDNA showed a band around 8 kb in AA, BB, and BBCC genome accessions (lanes 1–12 in Fig. 3) and a band around 3 kb in other accessions (lanes 11–16 in Fig. 3). These results suggest that the full length of an rDNA unit is about 8 kb in Oryza species with A and B genomes, while one additional EcoRV site may exist in the IGS region of the rDNA unit in C, CD, and F genomes. We later confirmed the existence of this additional EcoRV site in the IGS of the FF genome by sequence analysis (Figs. 1C and 5).

There are two BamHI sites in the coding regions of both 18 S and 25 S rDNAs of Oryza (Fig. 1A; [18]). Genomic DNA digested with BamHI and southern hybridized to the 18 S rDNA showed a 3.8-kb band corresponding to the coding region of each rDNA unit in each accession (Figs. 1C and 4A). At the same time, one or more bands of different lengths corresponding to the IGS regions were detected in each accession, representing the intraspecific or interspecific polymorphism in the IGS lengths (Figs. 1C and 4A). These bands were further identified as IGS fragments by hybridizing the same blots to IGS subrepeats (Fig. 4B). Such IGS subrepeats were absent in O. australiensis (EE, lane 8 in Fig. 4B), O. latifolia (CCDD, lane 15 in Fig. 4B), and O. brachyantha (FF, lane 16 in Fig. 4B). A particular band approximate 2.8 kb was detected by using 18 S rDNA as a probe in O. australiensis (EE, lane 8 in Fig. 4B), suggesting the existence of this additional BamHI site in its IGS region. Later, this additional site was confirmed by sequence analysis (Fig. 6A). Up to three bands in excess of 8 kb were also detected in the same hybridization, which suggests the existence of rDNA variants in these Oryza genomes (Fig. 4A). One of the BamHI sites may have been lost in these rDNA variants.
Characterization of specific IGS in O. brachyantha

The PCR product of 2.0 kb amplified from O. brachyantha (FF) with the use of the primer pair P3–P4 (lane 16 in Fig. 2B) was cloned and assigned as Obra2.0. Its sequence appears in the GenBank/EMBL database under the accession number FJ411058. Sequence analysis revealed that Obra2.0 is 2047 bp (Fig. 5A). BLASTN search results showed that segments at two ends of Obra 2.0 were homologous with several published sequences of O. sativa (Fig. 5A). The first 57 nucleotides (1–57 nt) at the 5′ end of Obra2.0 are identical to that at the 3′ end of the 25 S rDNA on chromosome 9 of O. sativa ssp. japonica cv. Nipponbare [18]. Another segment of 577 bp (1470–2047 nt) at the 3′ end is complementary with 83% homology to the segment involving the partial IGS and the 5′ end of 17 S rDNA on chromosome 9 of O. sativa ssp. japonica cv. Nipponbare [18]. An EcoRV site at position 1007 nt (Fig. 5A) explains the existence of the 3-kb band in southern hybridization of EcoRV-digested FF genomic DNA to 18 S rDNA (Fig. 1B–C, lane 16 in Fig. 3). This fragment, between those two EcoRV sites is approximately 3000 bp long and consists of about 1000 bp of the IGS region and 2000 bp of the coding region (Figs. 1C and 5A).

Instead of the 254-bp subrepeats, four tandem repeat elements were found in the Obra2.0 sequence. From the 5′ to the 3′ end, the first repeat element (R1) is a full length of 200 bp, the next is of 193 bp (R2) with several deletions and insertions, and the following two are truncated elements of 123 bp (R3) and 35 bp (R4). These four repeat elements shared more than 90% homology with the corresponding regions with several substitutions and deletions (Fig. 4A–B). The Obra2.0 sequence contains three transcriptional initiation sites (5′-TATAGTAGGGC-3′, +1 is underlined; [20]): two within repeat elements R1 (515–526 nt) and R3 (858–869 nt) and one at position 1153–1164 nt, downstream of R4 closest to the 18 S rDNA coding region (Fig. 4–C). Within repeat element R2, several nucleotides were deleted, including TATA at the position corresponding to the putative transcription initiation sites in R1 and R3 (Fig. 4B).

It is worth noting that the nucleotides (58–1469 nt) between the above two conserved segments lack homology to any published sequences in GenBank database. We designed a pair of primers, P7 (5′-AACCATCAGTGTCAGGAGC-3′) and P8 (5′-ACCACCATCAGTGGTCAC-3′), to amplify this specific fragment from O. brachyantha (Figs. 1B and 5A). A fragment of 1145 bp, named Obra1.0, was amplified as expected. Obra1.0 which was cloned and confirmed by sequencing analysis was later used as a probe in southern hybridization and FISH analyses. Southern hybridization with Obra1.0 to BamHI digestion of Oryza genomic DNA showed that Obra1.0 existed specifically in the IGS region of O. brachyantha (FF, lane 16 in Fig. 4C) compared to the constant presence of those fragments homologous with 18 S rDNAs in every Oryza genome (Fig. 4A).

Characterization of specific IGS in O. australiensis

The PCR product of 2.0 kb of O. australiensis (EE) by using the primer pair P3–P4 (lane 8 in Fig. 1B) was cloned and denoted as Oaus2.0. Its sequence appears in the GenBank/EMBL database under the accession number GU111553. Sequence analysis revealed that Oaus2.0 is 2427 bp (Fig. 6A). Similar to those of Obra2.0, segments at two ends of Oaus2.0 (1–58 nt at 5′ end and 1842–2427 nt at 3′ end) are separately homologous with the 3′ end of the 25 S rDNA and with the 5′ end of 17 S rDNA of O. sativa ssp. japonica cv. Nipponbare [18]. In addition to those two BamHI sites in the coding region (Fig. 1A), the third BamHI site in rDNA unit of O. australiensis (EE) presents at the position 614 nt of Oaus2.0 (Figs. 1B and 6A) which interprets the existence of a shorter IGS fragment of approximately 2.8 kb of O. australiensis (EE) (Fig. 1C and lane 8 in Fig. 4A).

In total, 13 tandem repeats were found in Oaus 2.0 by using the tandem repeat finder program [22]. These repeats are around 50 bp long and share more than 90% homology to the consensus pattern with several indels (Fig. 6A–B). Oaus2.0 contains only one transcriptional initiation site at position 1671–1680 nt, out of the region of tandem repeats (Fig. 6A). The nucleotides (59–1841 nt) between those two conserved segments lack homology to any published sequences in the GenBank database. We designed a pair of primers, P9 (5′-CAGCCAGCGAGACTTAC-3′) and P10 (5′-ATAGTAGGGGAAGGAGCCA-3′) to amplify this specific fragment from O. australiensis (Figs. 1B and 6A). A fragment of 1553 bp long was amplified as expected, which was cloned, denoted as Oaus1.5, and confirmed by sequencing analysis. Southern hybridization with Oaus1.5 to BamHI digestion of Oryza genomic DNA showed two homologous bands that were specific in the IGS region of O. australiensis (EE, lane 8 in Fig. 4D): one band around 2.8 kb and one band of 1.2 kb (Figs. 1C and 4D). The band around 2.8 kb was also homologous to the 18 S rDNA fragment (Fig. 4A). The summation of these two bands (~4 kb) was evident over the length of Oaus2.0 (2427 bp). It is worth noting that two homologous bands of 4.0–5.0 kb, corresponding the IGS fragments in Fig 4A, were also detected in Oryza punctata (BB, W1577, lane 10 in Fig 4D) and in O. latifolia (CCDD, lane 15 in Fig 4D) by Oaus1.5.

Cytological visualizing the colocalization of specific IGS and 45 S rDNA

The results of FISH on mitotic chromosomes showed that Obra1.0 was colocalized with the coding region of 45 S rDNAs at the end of chromosome 9 S of O. brachyantha (Fig. 7A–C). Fiber FISH images showed that Obra1.0 sequences appeared in the intervals of the tandem-arranged rDNAs array (Fig. 7D). FISH results also showed the colocalization of Oaus1.5 and the coding region of 45 S rDNA at the end of chromosome 9 S of O. australiensis (Figs. 7E–G).

Discussion

In this study, we showed two distinct and genome-specific IGS of 45 S rDNAs which are located at a conserved position on the chromosomes of Oryza species. The Oryza genomes are known relatively stable compared with other grass genomes [23] and the morphologies of their chromosome complements are highly similar [12,24] although there is more than a twofold difference in their genome sizes [25]. Among diploid Oryza species, O. australiensis (EE) has the largest genome size (1054 Mbp/1C) while O. brachyantha (FF) has the smallest one (343 Mbp/1C) [26]. As shown in a previous study [25], the chromosomes of O. australiensis (Figs. 7E–G) are on average longer than those of O. brachyantha (Figs. 7A–C). Sequencing analyses suggested that the O. australiensis genome has been doubled by massive bursts of three LTR-retrotransposon families after its speciation in the last three million years [27]. Phylogenetic analyses have indicated that O. australiensis is closely related to several diploid species, including O. sativa (AA), O. glaberrima (AA), O. officinalis (CC), and O. punctata (BB) [27], even the undiscovered DD genome [28]. An inversion/deletion spanning ~350 kb was identified in O. australiensis (EE) by a comparative physical map analysis of O. australiensis and O. sativa ssp. japonica [29]. O. brachyantha is phylogenetically separate from other Oryza genomes [28] and is the most distinct and the most

![Fig. 6. Sequence organization of E genome (Oryza australiensis)–specific IGS Oaus2.0. (A) Nucleotide sequence of Oaus2.0. At both ends, nucleotides indicated by gray backgrounds are completely or partially homologous to published rDNA sequences of Oryza sativa ssp. japonica cv. Nipponbare [18]. The positions and orientations of primers used for PCR are indicated with arrows and primer names (P3, P4, P9, and P10). The restriction site for BamHI is labeled at position 614–619 nt. Each of the 13 subrepeat elements (R1–R13) is bordered by two arrows. The putative transcription initiation sites are surrounded by a box. (B) Alignment of consensus sequences and 13 subrepeat elements (R1–R13) in Oaus2.0. Nucleotides indicated by gray backgrounds are consensual.](image-url)
divergent species in the *Oryza* genus based on many taxonomic and molecular studies [25,30–36]. *O. brachyantha* has a genome-specific repetitive sequence, TrsB, which was mapped to the interstitial regions of one pair of chromosomes by FISH [25]. The results of comparative sequence analyses in the coding regions suggest that the AA and FF genomes diverged around 10 million years ago [37]. A genus-wide comparative genomic analysis within the genus *Oryza* suggested that the FF genome and the AA-BB-CC-EE clades shared a common ancestor about 13 to 14.6 million years ago [29], although their gene contents and order are highly conserved [37]. Mutations in this consensus region may reduce transcriptional activity and sometimes alter the site of transcription initiation sequences [20,43]. However, there is little sequence similarity among the IGS subrepeats of different species [47]. In plants, the sequences flanking transcription start sites are highly conserved [11]. Mutations in this consensus region may reduce transcriptional activity and sometimes alter the site of transcription initiation [48]. In rice, the putative transcription

As shown in this study, PCR with universal primers enables chromosome-specific IGS fragments of *Oryza* genomes to be amplified. Our results of PCR (Fig. 2B–C) and southern hybridization (Fig. 3) showed both the conservation and the diversity in IGS regions among *Oryza* genomes. However, the IGS variants that appeared in the PCR profiles and southern hybridization (Figs. 2B–C and 3) were inconsistent with the variations in the number of rDNA loci revealed by FISH [12]. In those genomes with only one rDNA locus at the end of chromosome 9 S [12], i.e., *O. sativa* ssp. *japonica* cv. Nipponbare (lane 1), *O. glaberrima* (lane 5), *O. australiensis* (lane 8), *O. latifolia* (lane 15), and *O. brachyantha* (lane 16), their PCR profiles (Fig. 2B–C) or southern patterns (Fig. 3A–B) were similar but not identical. In the present study, we show that the IGS sequences of both *O. australiensis* (EE) and *O. brachyantha* (FF) are distinct from other *Oryza* species. The results of PCR and southern hybridization showed that the typical 254-bp repeat which commonly exists in the IGS of most *Oryza* species is absent from both *O. australiensis* and *O. brachyantha* ([15]; lanes 8 and 16 in Figs. 2C and 4B in this study). As shown in Fig. 4D, two bands in *O. punctata* (BB, lane 10) and in *O. latifolia* (CCDD, lane 15) were detected homologous with *Oaus2.0*, suggesting a close phylogenetic relationship among these two species and *O. australiensis*. PCR profiles and genomic southern analyses revealed an intragenome variation of rDNAs in *O. punctata* (lanes 9 and 10 in Figs. 2B–C, 3, and 4A–B, D). These two accessions of *O. punctata* (W1577 and W1593) show an identical morphology and share the most common of random amplified polymorphic DNA (RAPD) [41]. In addition, both accessions of *O. punctata* have three 45 S rDNAs loci at identical positions [12]. Among the *Oryza* genomes, it seems that the evolutionary changes in specific parts, even with conserved functions, may be influenced by different factors.

In higher eukaryotes, 45 S ribosomal RNA is transcribed by RNA polymerase I, which is controlled by DNA elements within the IGS [42–44]. Subrepeats in IGS regions may vary in length, and number [42–46] and some of these subrepeats may carry promoter or potential transcription initiation sequences [20,43]. However, there is little sequence similarity among the IGS subrepeats of different species [47]. In plants, the sequences flanking transcription start sites are highly conserved [11]. Mutations in this consensus region may reduce transcriptional activity and sometimes alter the site of transcription initiation [48]. In rice, the putative transcription
initiation site (5′-TATAGTGGGG-3′) was found within the subrepeats of IGS [20]; the variation in the number of this motif is coincident with the change in the number of subrepeats [18]. Similar to the IGS of A. thaliana [49,50], the putative transcriptional initiation site of Obra2.0 (1153–1164 nt), which is downstream of R4, closest to the coding region of 18 S rDNA, is a gene promoter, whereas the other two motifs within subrepeats R1 and R3 may be spacer promoters (Figs. 5A–C). However, Oaus2.0 shows a different organization with 13 tandem repeats and one putative transcription initiation site out of that repeated region (Fig 6). The sizes of the major fragments which were amplified in PCR (800 bp, Fig 2B) or were detected by southern hybridization (~4.0 kb in Figs. 1C and 4D and ~2.8 kb in Fig 4A) are inconsistent with the size of Oaus2.0 (2427 bp), suggesting the existence of IGS variants other than Oaus2.0 in the O. australiensis genome. These IGS variants may be similar in organization but are different in length due to the fact that they contain different numbers of subrepeats. Among those rDNA variants of O. australiensis, those variants of 800 bp were the major product amplified by PCR, while the IGS variants of about 3 kb detected by southern hybridization should be the major class in the O. australiensis genome. Together with the variations in the number and chromosomal positions of rDNA loci detected by FISH [12] and the polymorphism in PCR profiles and southern hybridization we have shown here, the unique IGSs in O. brachyantha and in O. australiensis clearly explain both the conservation and the variation in the IGS regions of rDNAs among Oryza species. The evolution processes of IGS may include successive duplication, deletion, and divergence, thus dramatically changing the composition of subrepeats of an ancestral IGS sequence. Meanwhile, the IGS sequences undergo conversion and concerted evolution to reach a homogenization within an array of repeats [47]. The results presented in this study collectively indicate that both O. brachyantha and O. australiensis have distinct IGS compositions from other Oryza species. The mutation may have been formed in an ancestral genome before both species separated from the other Oryza genomes. Under concerted evolution, homogenization would have continuously swept this mutation to fixation within the array and led to the formation of such unique IGS sequence Obra2.0 in O. brachyantha and Oaus2.0 in O. australiensis. An investigation of the organization of orthologous and paralogous rDNA loci in the genus Oryza is expected to clarify the evolutionary processes of the genus Oryza.

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