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Genome specificity of rDNA spacer fragments from *Oryza sativa* L.

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Summary. The intergenic spacer derived from a cloned rDNA unit from a cultivated rice was dissected into several subclones, which were used as probes to analyze sequence homologies between rDNA spacers from wild rice belonging to genome types AA, BB, CC, EE, FF, BBCC, and CCDD. This analysis allowed us to detect several regions with different degrees of homology. A series of 250–260 bp repeats is located in the central part of the AA spacer. This sequence cross-hybridizes with the BB, CC, BBCC, and FF genomes, but is absent in the EE and CCDD genomes. Regions proximal to 25S and 18S sequences are well conserved in all genomes. Finally, two adjacent sequences of 61 bp and 94 bp, located downstream from the repeats, have been found to have a narrow genomic specificity, restricted respectively to AA and FF genomes for the first one and to AA for the second. These data provide new information on the evolution of the ribosomal RNA gene spacer within a complex of related species, and add to our knowledge on the relationship between the various rice genomes.

Key words: Wild rice – rDNA spacer – Genome-specific DNA sequence – Evolution

Introduction

Cytogenetic studies have revealed the occurrence of several genome types within the *Oryza* genus (Tsunoda and Takahashi 1984). Both cultivated species (*O. sativa* and *O. glaberrima*) belong to the AA genome, as well as a few wild species, which are presumed to be their ancestors (*O. rufipogon*, *O. longistaminata*, and *O. breviligulata*). Species with the AA genome constitute the *Sativa* complex, whereas the remaining species constitute the *Latifolia* complex. This complex is composed of species having the BB genome (*O. punctata*), the CC genome (*O. officinalis*, *O. eichingeri*, and *O. collina*), the EE genome (*O. australiensis*), and the FF genome (*O. brachyantha*). In addition to these diploid species, the *Latifolia* complex contains allotetraploids that have a combination of these genomes such as BBCC (*O. minuta* and *O. punctata*) and CCDD (*O. latifolia*, *O. alta*, and *O. grandiglumis*). The D genome donor to the latter is still unknown (Vaughan 1989; Jena and Kochert 1991).

A major trend in rice breeding is to introgress valuable traits, which occur naturally in wild species, such as resistance to drought or to a large variety of insect, fungus, or bacteria pests, into cultivated varieties (Jena and Khush 1990). The general strategy in this approach is wide crossing between *O. sativa* and a wild species, followed by repeated backcrosses with *O. sativa* and selection for the desirable character. However, this approach is time-consuming and could be accelerated if genome-specific markers were available to determine whether or not fragments of the wild genome have introgressed into the cultivated one. A major effort towards this goal has been to select anonymous RFLP markers and to establish saturated RFLP maps for both *O. sativa* and *O. officinalis* (McCouch et al. 1988; Jena and Kochert 1991). Another strategy is to isolate genome-specific repeated sequences, since they should allow one to very rapidly discriminate between two genomes (Vedel and Delseny 1987). The most interesting type of repeated sequences are those that are dispersed throughout the genome, but those restricted to specific sites might still be of interest if they can be linked to a useful character. In rice,

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tandemly repeated sequences specific for the AA genome (Wu and Wu 1987; De Kochko et al. 1991) and the CC, EE, and FF genomes (Zhao et al. 1989; Cordesse et al. 1991) have already been isolated, but their specific chromosomal location is not yet determined. As part of this search for genome-specific sequences, we have analyzed the nuclear ribosomal gene intergenic spacer, since previous work on crucifers in our laboratory has demonstrated that this region contains sequences with very narrow specificity (Delcasso-Tremousaygue et al. 1988; Tremousaygue al. 1988; Grellet et al. 1989). In addition, we and others have demonstrated extensive variability in the rice rDNA spacer (Cordesse et al. 1990; Sano and Sano 1990). In the present study we have dissected a previously cloned ribosomal RNA gene unit (Takaiwa et al. 1984) and used various subclones to probe for specificity towards the different rice genomes. We observed that the various spacer regions did not diverged at the same rate and that a short sequence was absolutely specific for the AA genome.

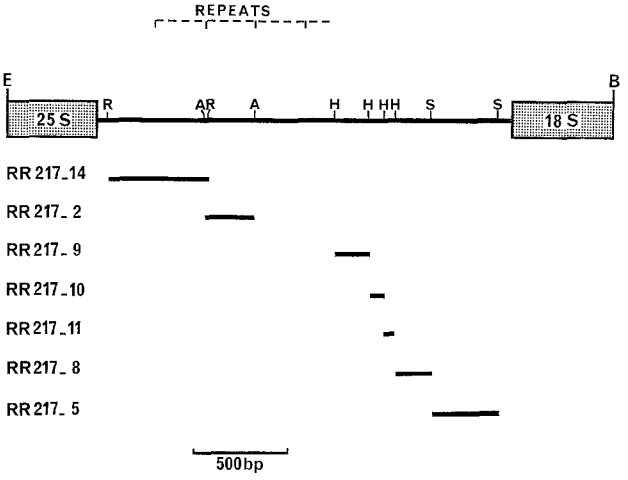

Fig. 1. Restriction map and subclones in the rDNA intergenic spacer region (pRR 217-3000). *Stippled boxes* represent sequences coding for 25S 3′-end and 18S 5′-regions. Position of the various subclones used in this study is given in the lower part of the drawing with a scale. *Dotted lines* above the restriction map indicate the position of three direct tandem repeats and of a fourth incomplete one. Symbols for restriction sites are as follows: R, *Rsr*I; A, *Acc*I; H, *Hin*fI; S, *Sma*I; E, *Eco*RI; *B*, *Bam*HI. Only those sites that have been used for subcloning are indicated

Materials and methods

Samples used in this study are fom the rice germ plasm collection that was previously analyzed for rDNA variability (Cordesse et al. 1990). These accessions have either been collected by G. Second or originate from IRRI (Manila) or NIG (Mishima) germ plasm collections. Plants were maintained in a growth chamber, and young or adult healthy leaves were periodically collected. DNA was prepared according to previously published protocols (Grellet et al. 1986). Rice DNA was digested in the presence of a ten fold excess of restriction enzyme with the appropriate buffer. Restriction fragments were separated by 0.8% agarose gel electrophoresis, transferred to nylon Hybond N membranes, and hybridized as previously described with the selected probes (Cordesse et al. 1990). Probes were usually labelled using random priming (Feinberg and Vogelstein 1983) and ^{32}P-dCTP from Amersham Ltd. (UK). Short fragments were end-labelled using the Klenow fragment of *E. coli* DNA polymerase (Sambrook et al. 1989).

A cloned rDNA unit, pRR 217, was kindly made available for this study by Dr. F. Takaiwa (NIAR, Tsukuba, Japan). All probes used in this work were derived from this initial plasmid by subcloning various restriction fragments into the pUC18 vector (Yanisch-Perron et al. 1985). Sequencing was made by the Sanger method adapted to double-stranded plasmids (Chen and Seeburg 1985).

Results

General organization of the rice rDNA spacer

The position of rice *Eco*RI and *Bam*HI sites in rDNA was known from previous studies (Olmedilla et al. 1984; Takaiwa et al. 1984). The large *Eco*RI-*Bam*HI fragment overlapping the 3′ end of the 25S rRNA sequence, the complete spacer, and the 5′ end of the 18S rRNA sequence was sub-cloned from pRR 217 as pRR 217-3000 in pUC 18. This subclone was digested with *Bgl*I, *Sma*I, *Acc*I, and in double digestions with these enzymes plus either *Eco*RI or *Bam*HI. This large fragment was also end-labelled at the *Hin*dIII polylinker site and partially digested with *Hin*fI. The derived restriction map (Fig. 1) was used for subcloning the the various regions of the spacer. The various subclones used in this study are indicated in this figure. The complete sequence of this region has been determined (EMBL accession number: X58275) and revealed that the spacer is 2,141 bp long and contains, as anticipated (Cordesse et al. 1990), a central region made of three imperfect repeats of 253, 254, and 254 bp. Downstream from the third repeat is a 100-bp sequence probably corresponding to a fourth repeat which has undergone a deletion. The position of these repeats is also indicated in Fig. 1. The sequence of this region has recently been reported independently (EMBL accession number X54194, Takaiwa et al. 1990). Although there are 30 differences between the two sequences, most likely due to reading errors, the overall organization deduced for the spacer is the same.

Strategy for determining genome-specific regions

Initial mapping of rice rDNA (Olmedilla et al. 1984; Takaiwa et al. 1984, 1985a, b; Cordesse et al. 1990) indicated that it contains two *Bam*HI sites located, respectively, in the 18S and 25S coding sequences. Accordingly, digestion of DNA samples with *Bam*HI yields a constant fragment of 3.8 kbp and one (or several) larger frag-

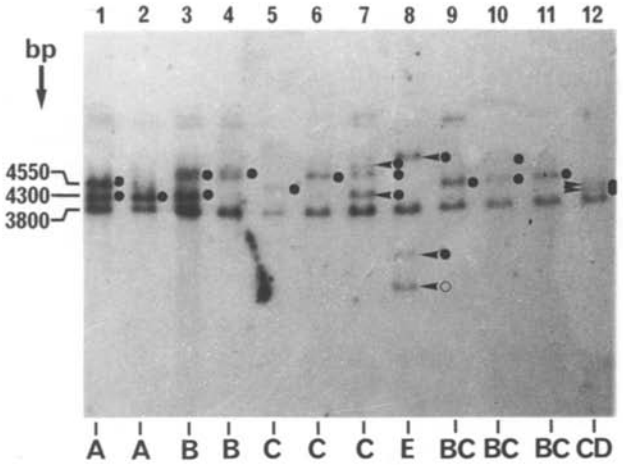

Fig. 2. *Bam*HI restriction patterns of various cultivated and wild rice following hybridization with pRR 217. DNA are originating from the following accessions: *1*, *O. sativa* S35; *2*, *O. sativa* cv Cigalon; *3*, *O. punctata* W 1515; *4*, *O. punctata* W 1586; *5*, *O. officinalis* W 65; *6*, *O. officinalis* W 1306; *7*, hybrid between *O. officinalis* W 553 and *O. officinalis* W 1199; *8*, *O. australiensis* OA 27; *9*, *O. malanpuzahensis* W 1159; *10*, *O. minuta* W 1331; *11*, *O. punctata* W 1408; *12*, *O. latifolia* W 1168. The genome type is indicated in the lower part of the picture. *Dots* indicate the spacer fragments as detected by probe pRR217-3000; the *closed dots* point to the fragments that hybridize with probe pRR 217-14 and the *open one* marks a fragment that does not hybridize with this probe but with the 18S proximal region (compare with Fig. 3). *Arrows* indicate spacer fragments that no longer hybridize with probe pRR 217-5 and pRR 217-2 in subsequent hybridizations (compare with Figs. 3 and 4)

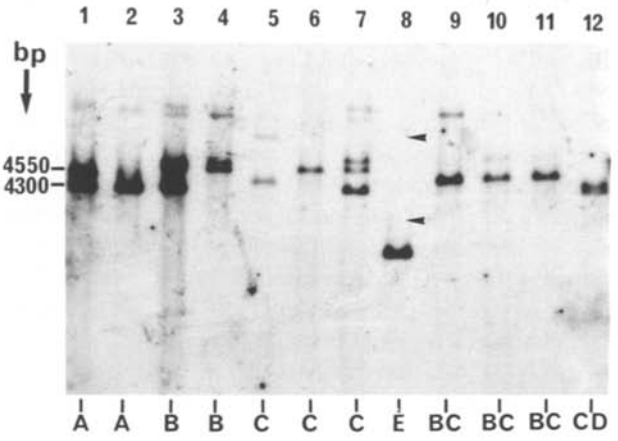

Fig. 3. *Bam*HI spacer fragments revealed by probe pRR 217-5 (proximal to 18S RNA sequence). After the experiment described in Fig. 2, the probe was stripped off and the membrane hybridized again with pRR 217-5 and autoradiographed. *Arrows* point to the two EE fragments that no longer hybridize with this probe

ment(s) containing the variable spacer region. Samples representative of AA, BB, CC, EE, BBCC, and CCDD genomes were digested with *Bam*HI and run on the same agarose gel. In other experiments, samples corresponding to the FF genome were also included. These digests were first probed with pRR 217 and pRR 217-3000, in order to unambiguously identify the spacer fragments. The results of the hybridization with pRR 217 are shown in Fig. 2. On this figure we have also indicated by dots the fragments that hybridize to pRR 217-3000 and that contain the intergenic spacer region. There are between one and three spacer fragments, depending on the samples. The unmarked fragment at 3,800 bp is a constant one and corresponds to part of the gene expressed as ribosomal RNA. Bands larger than 7,500 bp most likely correspond to monomer or multimer rDNA units resulting from the unavailability of some *Bam*HI sites for digestion. After this initial hybridization, the probe was stripped off the filter, which was then hybridized serially with each of the subclones corresponding to the different regions of the spacer. Doing these experiments we observed that some of these shorter probes no longer hybridized to the spacer fragment(s) of a particular genome and therefore have a restricted specificity

Probes located downstream of the 25S and upstream of the 18S rRNA sequences are not genome specific

The region located downstream of the 25S RNA sequence corresponds to probe p RR 217-14. As indicated by black dots in Fig. 2, it hybridizes to all spacer fragments except to the smallest one of the EE genome (open dot). The region proximal to the 18S rRNA sequence was assayed with probe RR 217-5 (a 347-bp *Sma*I fragment). As shown in Fig. 3, most of the spacer fragments are still detected with this probe, except two from the EE genome. The EE genome rDNA displays three *Bam*HI fragments containing portions of the spacer region. One hybridizes with the probe proximal to the 18S sequence and the two others to the probe close to 25S sequence. The simplest explanation for this pattern is that the spacer region of the EE genome rDNA is interrupted by an additional *Bam*HI site. The largest fragment corresponds either to a larger unit type, or to units in which the *Bam*HI site in the 25S sequence is not available. Additional experiments were carried out with the *Hin*fI-*Sma*I upstream adjacent fragment, pRR 217-8, but since the patterns were identical to the one in Fig. 3, they are not presented.

Specificity of the probes located in the central repeated region

Figure 4 shows the results of a hybridization with probe pRR 217-2, corresponding to the 254-bp *Acc*I fragment which contains the complete second repeat. Identical results (not shown) were obtained with probe pRR 217-9, which contains the fourth deleted repeat. Probes corresponding to the region between these two fragments were

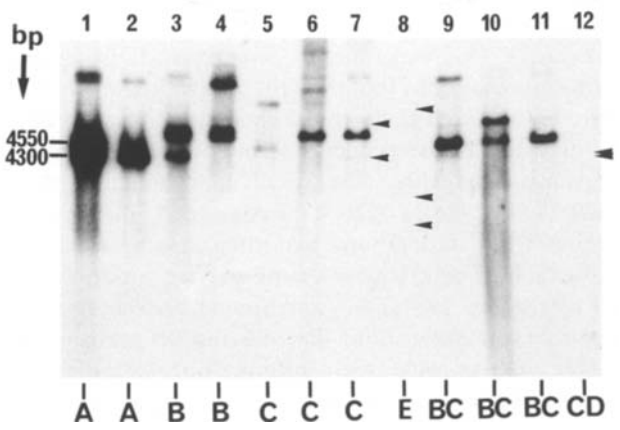

Fig. 4. *Bam*HI spacer fragments revealed by probe pRR 217-2 (repeat region). Following hybridization with pRR 217-5, this probe was stripped off and the same membrane was hybridized again with pRR 217-2, as a new probe. *Arrows* point to fragments that are no longer recognized by this probe

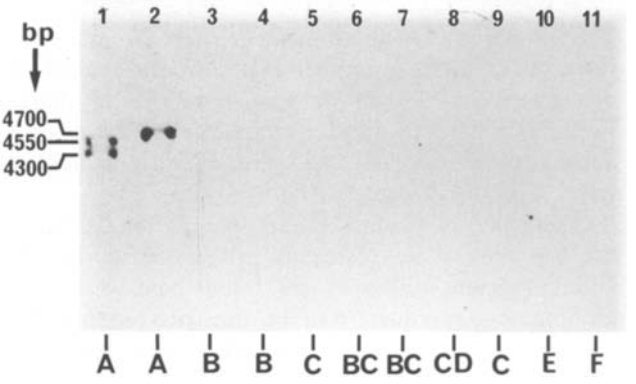

Fig. 5. Detection of sequences specific for the AA genome. DNA from various accessions was digested with *Bam*HI and, following electrophoresis and transfer, was hybridized with probe pRR 217-10. Probe was end-labelled. Samples are the following: *1*, *O. sativa* S35; *2*, S05; *3*, W 1515; *4*, W 1586; *5*, W 65; *6*, W 1159; *7*, W 1331; *8*, W 1168; *9*, W 553 × W 1199; *10*, OA 27; *11*, *O. brachyantha* W 1507. Position of all spacer fragments was revealed afterwards by hybridization with pRR 217 (not shown)

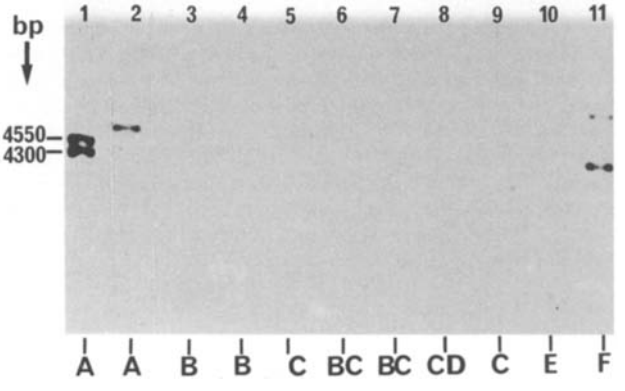

Fig. 6. Detection of sequences restricted to the AA and FF genomes. Following removal of the probe, the membrane used in the experiment described in Fig. 5 was hybridized again with probe pRR 217-11. This probe was also end-labelled

not tested because they are repeated sequences 95% homologous to pRR 217-2.

From this experiment, it is obvious that all the spacer fragments from the EE genome have now disappeared from the autoradiogram. Fragments corresponding to the AA and BB genomes hybridize very strongly to the probe. Fragments from the CC genome also hybridize, but with a somewhat lower intensity, suggesting that repeats are also present in this genome but have already slightly diverged.

A surprising result from this experiment is that although a sequence homologous to the repeated region is clearly detected in the CC genome, it no longer shows up in the allotetraploid CCDD genome (lane 12). We have also analyzed a hybrid between two CC-type *O. officinalis* accessions (W 1199 × W 553) because, following RFLP experiments on chloroplast DNA (Dally 1988), it has been suggested that W553 was indeed of CCDD type. We observed that two of the three *Bam*HI fragments spanning the rDNA spacer did not hybridize with pRR217-2 (lane 7). This observation suggests that these two fragments probably did not derive from a CC parent, and this is consistent with the suggestion made from analysis of chloroplast DNA that W553 might be of the CCDD type. On the other hand, a fragment clearly hybridizes in all the BBCC genomes; however, we do not know whether it is contributed by the CC, the BB, or both genomes.

Restricted specificity of the region immediately downstream from the repeats

The homology in the region downstream from the repeats was assayed using two cloned *Hin*fI fragments adjacent to pRR 217-9. Probe pRR 217-10 is 93 bp long, and probe pRR 217-11, located further downstream, is 61 bp long. Figure 5 shows that probe pRR 217-10 is strictly specific for the AA genome and does not recognize any of the spacer fragments from the other genomes. Probe pRR 217-11 shows a similar specificity but unexpectedly also recognizes the spacer fragment from the FF genome (Fig. 6).

In order to investigate the possibility of an even greater specificity of probe pRR 217-10, representatives of three species with the AA genome (*O. sativa*, *O. glaberrima*, and *O. rufipogon*) were analyzed. As illustrated in Fig. 7 the probe recognizes spacer fragments of all these species. Sequence of this probe is given in Fig. 8.

Discussion

This dissection of a ribosomal gene spacer from a cultivated rice confirms our hypothesis (Cordesse et al. 1990) that spacer variability in wild species was not only due to variation in the copy number of the 250–260 bp repeats, but that other regions of the spacer are also involved in variability.

Dissection of a cloned rDNA spacer from cultivated rice has allowed us to prepare probes specific for the different regions of this element. Using them, we assayed for homologous sequences in the other rice genomes. We observed varying degrees of homology throughout the spacer as already mentioned in wheat (Appels and Dvorak 1982). Our experiments were repeated using less stringent conditions (not shown), but the fragments that were not detected in the initial experiment light up only faintly under the new conditions, indicating considerable sequence divergence. The results are summarized in Table 1. As far as rDNA is concerned, the various genomes fall into groups that roughly correspond to those defined by extensive use of isozyme variation and RFLP (Second 1984; Dally and Second 1990; G. Second, personal communication). The AA and BB genomes are rather close to each other, differing only in the region spanned by pRR 217-10 and 11. The CC genome seems to be more divergent because, although CC spacer fragments hybridized to the same probes as the BB fragments, the intensity of the hybridizing bands was always lower. Finally, the EE genome shows extensive divergence. The rDNA spacer from the FF genome shows the closest similarity to that of AA, with only the pRR 217-10 probe discriminating between them, although the two genomes, as a whole, are clearly different by most other criteria. Since the distribution area of *O. brachyantha* largely overlaps that of other species of the AA genome in Africa, the possibility exists for a coevolution of ribosomal genes between the two genomes, or for an early introgression of AA genome ribosomal genes into the FF genome.

Another surprising feature revealed by this analysis is the discrepancy between observations on CC and CCDD genomes. Our results indicate that the genes coming from the CC genome have been eliminated in the allotetraploid and that the remaining genes probably originated from

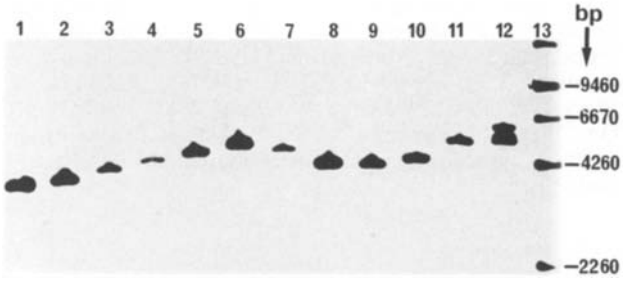

Fig. 7. Distribution of the AA specific sequence in representative accessions of three species of the AA genome. DNA was digested with *Bam*HI and the membrane was probed with pRR 217-10. Samples are the following: *1*, *O. glaberrima* GO1; *2*, *O. sativa* (*Japonica*) S27; *3*, *O. sativa* (*Japonica*) S41; *4*, *O. sativa* (*Japonica*) S16; *5*, *O. sativa* (*Indica*) S38; *6*, *O. sativa* (*Indica*) S31; *7*, *O. sativa* (*Indica*) 160; *8*, *O. rufipogon* DN 9; *9*, *O. rufipogon* W 1191; *10*, *O. rufipogon* W 1627; *11*, *O. rufipogon* W 555; *12*, *O. rufipogon* W 162; *13*, end-labelled *Hin*dIII fragments of lambda phage DNA (marker)

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1401
  ↓
  gACTCACGGCACTAGCCGAACCCCGGCCGCTGCGGGGTGTCGCACGTGA
  ctgaGTGCCGTGATCGGCTTGGGGCCGGCGACGCCCCACAGCGTGCTCT

                                              1493
                                               ↓
  GATCCTTCCCACCGCCTCCTAGCCTGCTGGCACGGCGCCCTGGCGagtc
  CTAGGAAGGGTGGCGGAGGATCGGACGACCGTGCCGCGGGACCGCTCAg
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Fig. 8. Nucleotide sequence of pRR 217-10 probe specific for the AA genome. Numbers refer to the position within the complete spacer sequence as deposed in the EMBL data bank (X 58275). Lowercase letters correspond to the protruding ends of the restriction site

Table 1. Summary of the genome specificity of the various probes. Symbol (+) indicates that the probe hybridizes to the spacer(s) fragment(s), symbol (−) that there is no hybridization at all, and (−/+) that only some of the fragments hybridize

Probes / Genomes	RR 217 14	RR 217 2	RR 217 9	RR 217 10	RR 217 11	RR 217 8	RR 217 5
A	+	+	+	+	+	+	+
B	+	+	+	−	−	+	+
BC	+	+	+	−	−	+	+
C	+	+	+	−	−	+	+
CD	+	−	−	−	−	+	+
E	−/+	−	−	−	−	−/+	−/+
F	+	+	+	−	+	+	+

the DD genome which, so far, has not yet been identified at the diploid level in present-day species (Jena and Kochert 1991). Cloning of these genes should provide probes to trace this genome in wild relatives of rice in America, since CCDD species are found mostly on this continent. Although we have no definitive proof, results for the BBCC genome also suggest that rDNA from one of the two parental genomes has been eliminated, since in most of the analyzed accessions we found a single spacer fragment. Again, cloning of rDNA from the BB and CC genomes would be very useful to isolate specific probes and determine in which genome rDNA has been eliminated. This situation is different from the one met in amphidiploids *Brassica*, which have fragments originating from both parents (Delseny et al. 1990). Loss or inactivation of genes are not infrequent in allotetraploids and a well-documented example is the case of the genes coding for the small subunit of rubisco in tobacco (Jamet et al. 1987). However, to our knowledge, there is not yet any convincing data for ribosomal genes.

A major outcome of this study is the discovery of a short sequence that has a narrow specificity restricted to the AA genome, thus demonstrating that short sequences from the intergenic rDNA spacer can be used as highly specific genome markers. In addition, this sequence is site specific because it is present only on the chromosome(s) that carries the nucleolar organizer. Practical use of this finding has still to await the isolation of a specific sequence from genome BB or CC rDNA and linkage of rDNA with a useful agronomic trait originating from these genomes. However, it should be emphasized that such a favorable situation has recently been reported for tomato (Levesque et al. 1990). Although we looked for it, we were not able to find a rDNA spacer sequence that would be specific for either the *Japonica* or *Indica* subtype of *O. sativa*. This does not mean that such a sequence does not exist because, as observed earlier (Cordesse et al. 1990; Sano and Sano 1990), the rDNA spacer from the *Japonica* subtype is usually shorter than that of *Indica*. Since the gene we dissected originated from a *Japonica* cultivar, the possibility still remains of finding such a sequence in an *Indica* variety.

Finally, these results confirm that the various regions of the rDNA spacer are not evolving at the same rate and contribute to some extent to species differentiation. An interesting question is to understand how these various spacer regions have been assembled and how they evolved. Sequence analysis of corresponding regions from wild rice rDNA spacers should provide us with some answers.

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