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Distribution and organization of a tandemly repeated 352-bp sequence in the oryzae family

A. De Kochko*, M. C. Kiefer, F. Cordesse, A. S. Reddy and M. Delseny*****

Laboratory of Plant Physiology and Molecular Biology URA 565 du CNRS, Avenue de Villeneuve, F-66860 Perpignan-Cédex, France

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Summary. A 352-bp EcoRI fragment from rice DNA was cloned and shown to be a member of a tandem repeat. Sequence determination revealed homologies with human alpha satellite DNA and maize knob heterochromatin specific repeat. This 352-bp sequence is highly specific for the AA genome of rice. However, copy number and sequence organization are variable, depending on the accession analyzed. Several examples of amplification were observed in *O. rufipogon* and *O. longistaminata*. Use of resolutive polyacrylamide gel electrophoresis and 4-bp cutter enzymes allowed one to distinguish between the Indica and Japonica subtypes of *O. sativa*. The same method also discriminates between two groups of *O. rufipogon*, the presumed ancestor of *O. sativa*, suggesting that the present day Indica and Japonica subtypes originated independently from two *O. rufipogon* distinct populations.

Key words: Cultivated rice – Wild rice – RFLP – Satellite DNA – Evolution

Introduction

Tandemly repeated DNA sequences are widely spread in eukaryotic genomes. During the last few years, several such elements have been described in animals and in many plant species (Vedel and Delseny 1987), including rice (Pental and Barnes 1985; Wu and Wu 1987; Zhao et al. 1989). Although these sequences have no known function and are often considered as "selfish" DNA, in situ hybridization, when possible, has revealed that they were located at strategic positions within chromosomes, such as subtelomeric, knob heterochromatin, or centromeric regions (Bedbrook et al. 1980b; Dennis and Peacock 1984; Willard and Waye 1987). Another interesting feature is that these sequences are the substrate for concerted evolution (Dover 1986) and diverge rapidly even among closely related species, so that they can be considered as species-specific sequences (Grellet et al. 1986; Schweizer et al. 1988; Zhao et al. 1989). Accordingly, one might assume that they play a role in defining fertility barriers between species by limiting alien chromosome pairing. So far, very little is known about their organization in the genome, and their origin and evolution in closely related plant species.

The Oryzae family is a rather complex one in which many accessions of wild, as well as cultivated, species are available. Previous studies have focused on the use of isozyme patterns to discriminate between these accessions and to assess their phylogenetic relationships (Second 1984, 1985; Glaszmann 1987, 1988; De Kochko 1987). More recently, variability of the restriction pattern of chloroplast DNA and nuclear ribosomal genes was analyzed, and general trends in the evolution of these genetic entities in cultivated rice species were reported (Dally 1988; Ishii et al. 1988; Cordesse et al. 1990; Dally and Second 1990). In a further effort to discriminate between cultivated and wild rice species at the molecular level, we cloned the ca. 360-bp EcoRI repeated fragment observed by Pental and Barnes (1985). In this paper we extend earlier analysis (Wu and Wu 1987; Zhao et al. 1989) concerning genome specificity, organization, copy number, evolution, and use of this sequence to discriminate between cultivated accessions as well as wild species.

* Present address: Department of Biology, Washington University, St. Louis, MO 63130, USA
** Present address: Department of Biology, Texas A&M University, College Station, TX 77843-3258, USA
*** To whom offprint request should be addressed

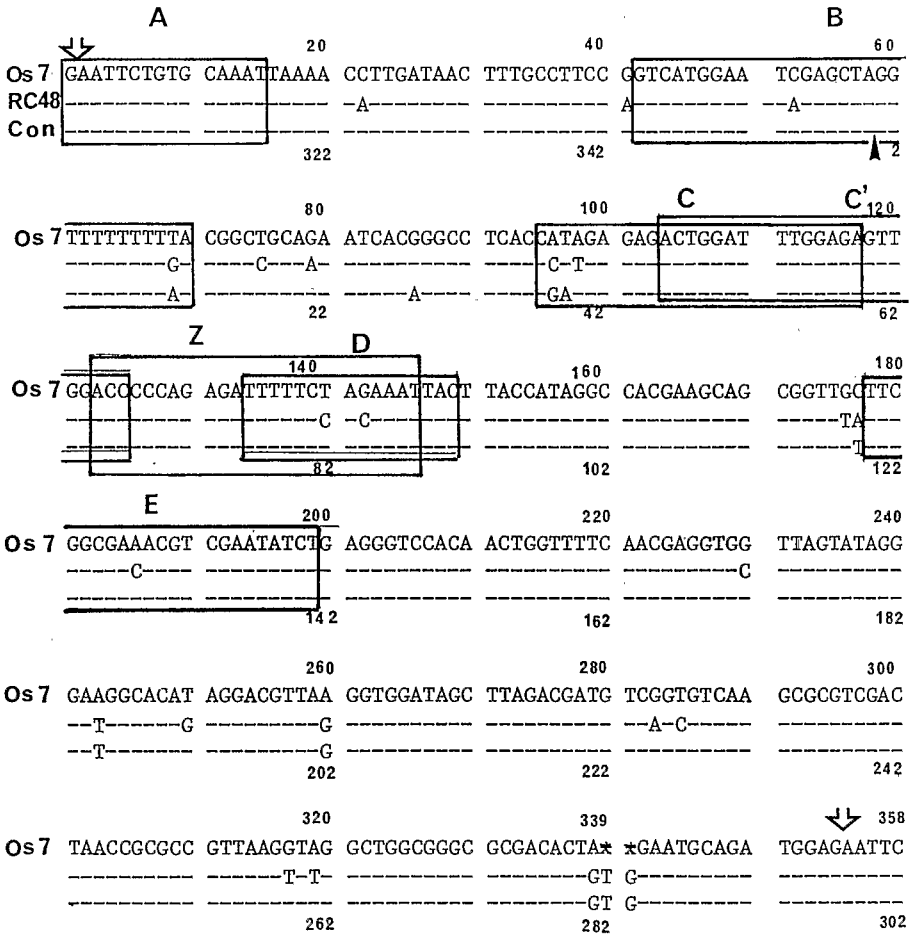

Fig. 1. Nucleotide sequence of clone pOs7. The sequence was aligned with that of clone RC 48 and of consensus sequence (Con), derived from ten clones of cultivar "Labelle" (Wu and Wu 1987). Only nucleotide changes were indicated. A two-nucleotide gap (*asterisks*) has to be introduced to maximize the alignment. Lower numbers correspond to nucleotide positions as described by Wu and Wu (1987). The *black arrowhead* indicates the position of first nucleotide in RC48 and Con. *Boxes,* labelled A, B, C, C', D, E, and Z, correspond to homologous blocks described in Fig. 2. *Open arrowheads* indicate the limits of the satellite unit. Additional nucleotide upstream and downstream of these limits are derived from the polylinker sequence and are present in adjacent units

Materials and methods

A list of the plant materials used in this study is given in Table 1. Cloning work was carried out with the French cultivar "Cigalon," which belongs to the Japonica subtype of *Oryza sativa.* Most of the accessions were part of the collection studied in previous work (Second 1985; Glaszmann 1987; Cordesse et al. 1990). They were grown either in a greenhouse or in a growth chamber at 25°C with a 13-h (day)/11-h (night) photoperiod. Each plant was grown in a pot partially immersed in water.

DNA was extracted as previously described, using a miniprep method (Cordesse et al. 1990) or a CsCl-ethidium bromide centrifugation protocol (Delcasso-Tremousaygue et al. 1988).

DNA digestion, agarose gel electrophoresis, Southern blots, copy number determination, cloning, and sequencing were carried out as already described (Maniatis et al. 1982; Grellet et al. 1986; Delcasso-Tremousaygue et al. 1988). Polyacrylamide gels were prepared according to Gebhardt et al. (1989). Following polyacrylamide gel electrophoresis, DNA was transferred to Hybond-N membrane using 20 × SSPE [3.6 *M* NaCl, 200 m*M* NaH_2PO_4 (pH 7.4), 20 m*M* EDTA] by capillarity (Maniatis et al. 1982).

Results

Isolation and characterization of a 358-bp fragment

As initially reported (Pental and Barnes 1985), a DNA band was clearly visible on ethidium-bromide-stained gels following electrophoresis of EcoRI-restricted DNA from most *O. sativa* accessions, including the Cigalon cultivar. This band was excised from the gel, the DNA was purified and cloned into EcoRI-cut pUC8 plasmid vector. Several white clones were picked up at random and their inserts were characterized by EcoRI digestion. A clone (pOs7) with an insert of the appropriate size was selected and used to probe genomic DNA restricted with the same enzyme. A typical ladder pattern appeared,

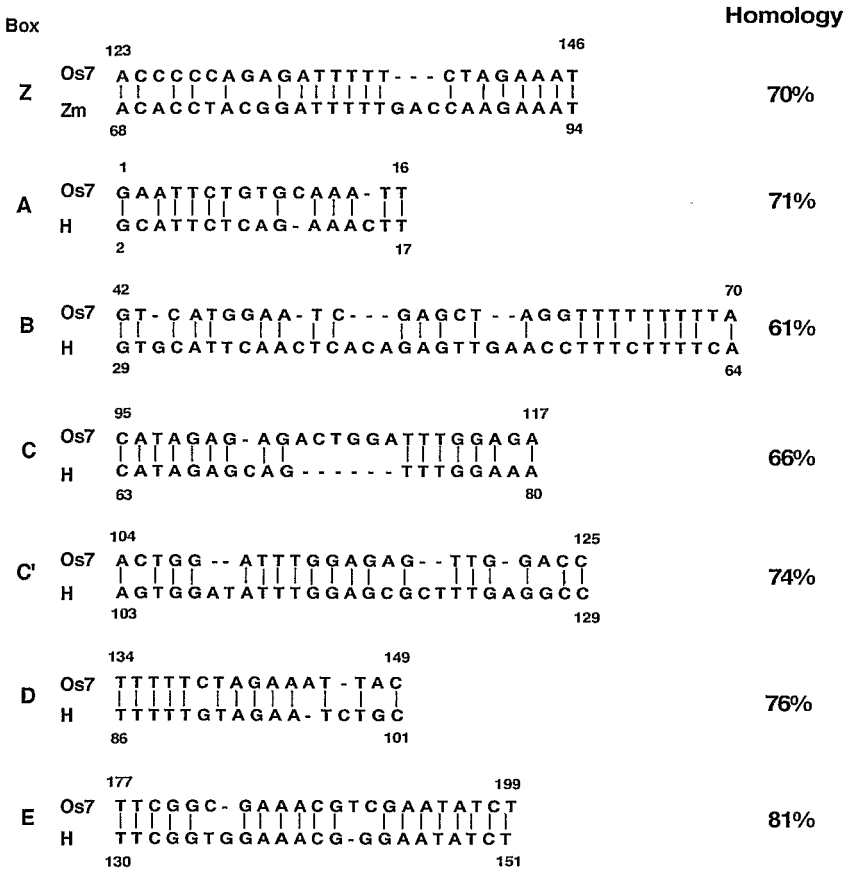

Fig. 2. Nucleotide sequence homologies between pOs7 and other repeated sequences. Os7 is the rice sequence, Zm is the maize repeat, and H corresponds to human alpha satellite DNA consensus sequence. Each homology block is represented by a letter, which is also used in Fig. 1

revealing multimers of ca. 360 bp. Such a pattern is indicative of a tandemly repeated organization and it arises by random loss, during evolution, of restriction sites defining the repeating unit. This clone was further characterized by sequencing and was found to be 352 bp long (Fig. 1). The sequence was entered in the EMBL data bank under accession number X 55642. When compared with the previously described sequence RC 48 isolated from cultivar "Labelle" (Wu and Wu 1987), a strong homology was clearly detected starting at G 59. Indeed, the first 58 nucleotides of pOs7 almost perfectly matched with the last 58 nucleotides of RC 48. The calculated homology with RC 48 is 93.3% and is 97%, with the consensus sequence derived from ten other clones (Wu and Wu 1987). Therefore, the element we have isolated clearly belongs to the same family as the previously described one.

Homology with other repeated sequences

Although no extensive homology could be found with repeated sequences from other plant species, we observed that the 123- to 146-bp region has a 70% homology with a 26-bp region of the 185-bp repeated sequence from maize knob heterochromatin (Dennis and Peacock 1984). We previously showed that this region had some homology with tandemly repeated sequences from radish and broad bean (Grellet et al. 1986). More surprisingly, several blocks of significant homology were also detected when pOs7 sequence was compared with the consensus sequence of human alpha satellite DNA (Willard and Waye 1987). These homologies are listed in Fig. 2 and the corresponding boxes are indicated in Fig. 1.

Distribution of the 352-bp element among the Oryzae

The *Oryza* genus is represented by a large number of species growing in many different habitats and under very different climatic conditions. From cytogenetic studies and the analysis of the fertility of the offspring of interspecific crosses, the existence of several distinct genomes has been deduced (Oka 1974). The A genome is represented by *O. sativa* (cultivated Asian rice Indica and Japonica subtypes), *O. glaberrima* (cultivated African rice), and various wild type species (*O. rufipogon*, *O. longistaminata*, and *O. breviligulata*). The other genomes are all represented by wild species as indicated in Table 1. Information on the isozyme patterns of all these species (Second 1982, 1985; Glaszmann 1987; G. Second, unpublished results) guided the choice of these accessions for molecular analysis. Their DNA was restricted with EcoRI and analyzed on agarose gels. A

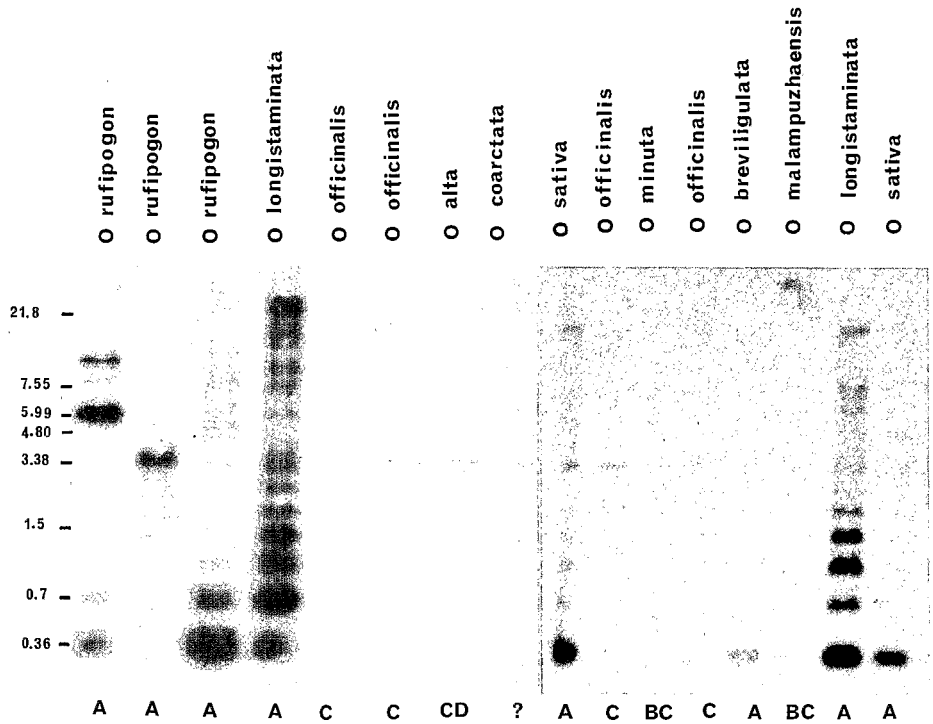

Fig. 3. Satellite DNA pattern in various rice accessions. DNA prepared from various rice accessions was digested with EcoRI, electrophoresed, blot transferred, and hybridized with pOs7. A 3.4-kb faint band (*lanes C, CD,* and *BC*) is a hybridization artifact due to cross hybridization with pUC 8. The lanes, from *left* to *right*, correspond to accessions DN 41, 100968, W 1655, EL 34, W 65, DO 4, W 17, W 551, 58881, 104314, W 1344, W 1306, UB 35, W 1159, EL 15-17, and Cigalon

representative blot is shown in Fig. 3. From these data, it is obvious that probe pOs7 is specific for the A genome when standard hybridization conditions are used. No hybridization at all was observed with B, C, BC, CD, and E genomes, even when hybridizations were carried out at lower stringency.

In all studied A-genome accessions, except for some *O. rufipogon*, a typical regular ladder pattern was observed, with an average increment of ca. 360 bp. In some *O. rufipogon* accessions several much larger fragments were also observed, superimposed on a faint ladder (e.g., DN 41 and 100968). This situation is examined in more detail in the following section.

African rice (*O. glaberrima*, *O. longistaminata*, and *O. breviligulata*) was also extensively surveyed (ten accessions of each), but very little variation was observed. Occasionally, a double repeat was observed in some *O. longistaminata* accessions, indicating the occurrence of at least two subfamilies differing by 10–15 nucleotides (Fig. 4). Since the two ladders remain distinct, one can conclude that members of the two subfamilies are not interspersed and are most likely localized in distinct parts of the genome.

The patterns shown in Figs. 3 and 4 also gave preliminary evidence for different copy numbers in the various accessions. This point was further investigated.

Variation in copy number

Copy number was determined for different accessions of the various species of the A genome by dot blots, using serial dilutions and two hybridization conditions: 65 °C (standard conditions) and 55 °C (less stringent conditions). The results are summarized in Table 2. They demonstrate that the sequence is generally more abundant in the Indica cultivars with a few thousand copies, than in the Japonica ones (ca. 200–1,200 copies), and that the copy number in *O. longistaminata* is also high. The sequence is rather poorly represented in *O. glaberrima*, while the copy number is intermediate in *O. breviligulata*. These data illustrate a general tendency, but there are many fluctuations when more accessions are analyzed, as can be seen from the Southern blots shown in Figs. 3, 5, and 6. For instance, we found one accession of Japonica (563) and one of *O. rufipogon* (DN 9) in which there was almost no detectable sequence homologous to pOs7. *O. rufipogon* accessions indeed fall into two classes: one has a low copy number, similar to the Japonica accessions, the other has a copy number similar to the Indica type and to *O. longistaminata*.

These differences in hybridization intensity may reflect divergence of the sequence rather than that of true copy number. This is unlikely for the Japonica/Indica

Table 1. Plant material used in this study

Genome constitution	Species	Accession	Origin
A	*O. sativa* (Indica)	610	Indonesia
		IR 36	Philippines
		130	Taiwan
	O. sativa (Japonica)	446	Thailand
		563	Japan
		571	Japan
		58881	Japan
		Cigalon	France
	O. glaberrima	GI	Senegal
		G2	Senegal
		CG01-3	Senegal
	O. breviligulata	UB 35	Cameroon
		TB 82	Tchad
	O. rufipogon	100968	Surinam
		DN41	India
		DN34	India
		W 1655	China
		DN9	India
		W 135	India
	O. longistaminata	UL 16-6	Cameroon
		EL 34	Tanzania
		EL 15-17	Tanzania
		AK	Ivory coast
BC	*O. minuta*	W 1344	Philippines
	O. malampuzhaensis	W 1159	India
C	*O. officinalis*	104314	China
		W 65	Thailand
		D04	India
		W 1306	Philippines
CD	*O. alta*	W 17	South America
?	*O. coarctata*	W 551	India

Table 2. Copy number of satellite DNA sequence[a]

Species	Accession	55 °C	65 °C
O. sativa			
– Indica	IR 36	3120	2650
– Japonica	446	1500	1200
– Japonica	563	300	230
O. glaberrima	CG DI-3	190	50
O. breviligulata	TB 82	575	180
O. rufipogon	DN 9	190	100
O. rufipogon	W 135	1400	950
O. longistaminata	ZL 14	1640	1050

[a] The values were determined assuming a haploid genome size of 1.2×10^9 bp

comparison, because the probe is from a Japonica source and hybridization intensity is always lower with Japonica DNA than with Indica. On the other hand, one can consider the fluorescence intensity of the 360-bp band on ethidium-bromide-stained gel. For *O. glaberrima* and *O. breviligulata* a band is clearly present, indicating a copy number of the fragment similar to that in "Cigalon," yet the intensity of hybridization is much lower, presumably reflecting divergence of the sequences. This is confirmed by the fact that a stronger signal is detected when less stringent conditions are used.

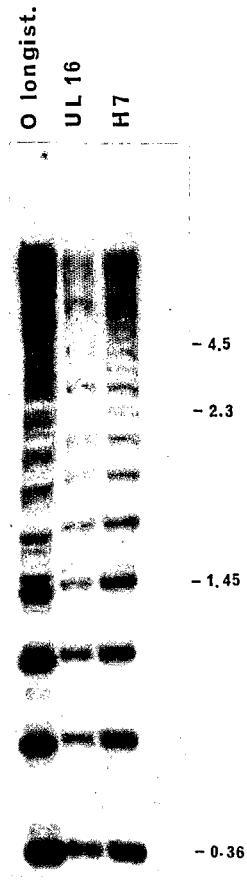

Fig. 4. EcoRI patterns of satellite DNA from *O. longistaminata*. *O. longistaminata* (AK) is from the Ivory Coast, UL 16 has genes introgressed from *O. sativa*, and H7 results from a cross between UL 16 and YS 309, an *O. sativa* having some genes of *O. longistaminata*

Organization of the repeats in O. rufipogon

Three distinct patterns were observed in Fig. 3 for EcoRI digestions of *O. rufipogon* DNA. With DN 41, EcoRI yielded the basic repeat of 360 bp and multiples, as well as an intense band at ca. 6 kbp and its possible dimer, suggesting that a block of 15–16 units had been amplified. In 100968, a single major band at 3.4 kbp was observed suggesting the amplification of ten blocks. On the other hand, W 1655 showed the usual ladder pattern.

In order to identify other evidence of amplification, DNA from three accessions, including DN 41 and W 1655, was digested with three other enzymes (Fig. 5). HinfI yielded a typical ladder pattern, but with BamHI and HindIII a complex pattern was observed. A large

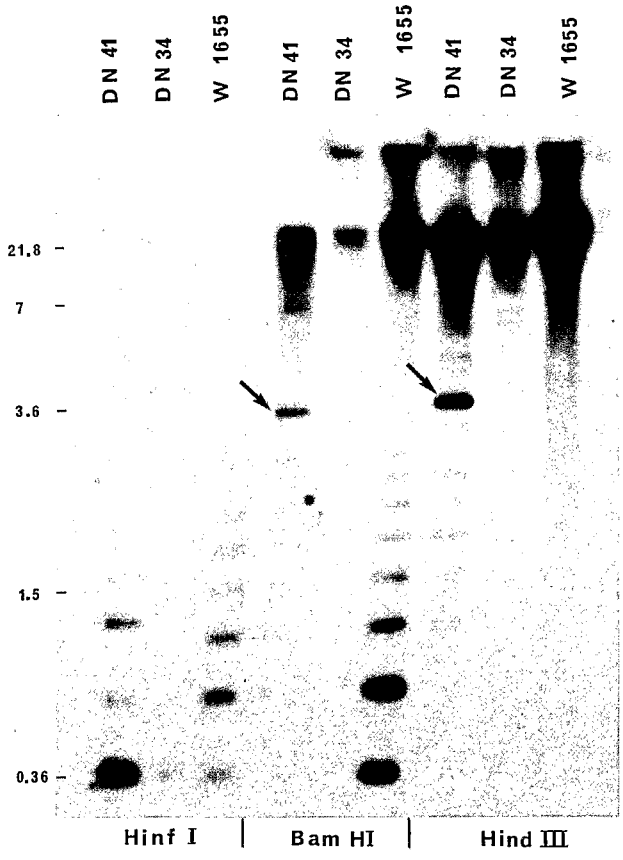

Fig. 5. Restriction patterns of satellite DNA in *O. rufipogon*. Total DNAs of accessions DN 41, DN 34, and W 1655 were digested with HinfI, BamHI, and HindIII. Following electrophoresis, DNA was blot transferred on nylon membrane and hybridized with pOs7. The *arrows* indicate fragments corresponding to amplified blocks

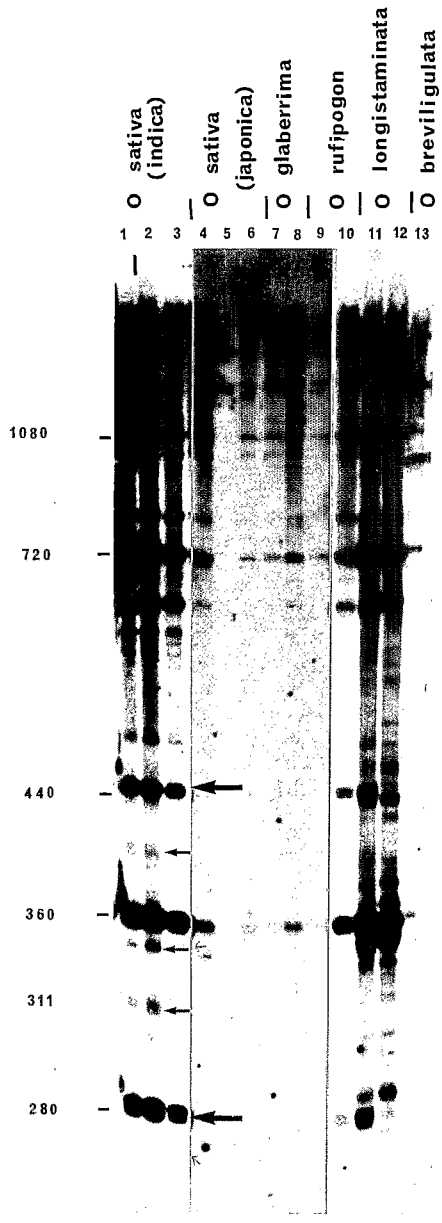

Fig. 6. PAGE of satellite DNA from different accessions of A genomes. DNA was digested with Sau 3A, run on a PAGE gel, transferred by capillarity, and hybridized with pOs7. *Lane 1:* 130; *2:* IR 36; *3:* 610; *4:* 571; *5:* no DNA; *6:* 563; *7:* GI; *8:* G2; *9:* DN 9; *10:* W 135; *11:* UL 16-6; *12:* ZL 14; *13:* UB 35. *Arrows* indicate major and minor additional fragments which discriminate Indica from Japonica

fraction of the DNA remained undigested in all three accessions. BamHI and HindIII revealed a major band in DN 41, indicating that a block of 10 units had been amplified in this species. Another band at 7 kbp possibly corresponds to a dimer of this block. It should be noted that DN 34 satellite DNA has virtually no BamHI or HindIII sites. Few sites for these two enzymes exist in DN 41, yielding mostly multimers larger than dimer or trimer of the 360-bp unit. On the other hand, DNA from W 1655 is readily digested by BamHI. However, with BamHI and HinfI, the dimer band is more intense than the monomer or the trimer, suggesting that blocks of dimers have been amplified somewhere in the genome.

Therefore, in *O. rufipogon* accessions several types of organization of the repeated element coexist. For instance, in DN 41 there are large arrays of satellite DNA sequences without any sites for BamHI and HindIII. Another array is characterized by the presence of BamHI and HindIII sites approximately every 10 units. Finally, another fraction of the satellite DNA can be readily digested by these two enzymes because it contains their recognition sequences in almost all its units. EcoRI also revealed two types of arrays: those where EcoRI defines the basic unit of 360 bp and those which correspond to the amplification of 15–16 blocks. Whether or not each enzyme defines a distinct subfamily remains to be established. In order to confirm that the digestions were not partial, the same filters were probed with an rDNA probe (Cordesse et al. 1990); the expected pattern was observed (not shown).

Polymorphism of the repeat sequence

Agarose gel electrophoresis following digestion with 6-bp recognition enzymes shows very little, if any, polymorphism between the closely related species and subtypes belonging to the A-genome group. Therefore, we used 4-bp cutters and polyacrylamide gels (Gebhardt et al. 1989) as an additional approach. Figure 6 shows an example of digestion with Sau 3A. It very clearly demonstrates differences in the organization of the repeat sequences between Indica and Japonica varieties: several fragments that are present in Indica (e.g., 310 and 400 bp) are absent in most Japonica species, while others are underrepresented (e.g., 280, 440, 640, and 796 bp). The *O. glaberrima* and *O. breviligulata* accessions also show a much simpler pattern, very similar to the Japonica type. The most complex organization is that of the *O. longistaminata* in which there is a doublet at the position of the monomer, as previously observed in Fig. 4b for another accession. Other fragments, such as the 270–280 and 430–440 bp fragments, also correspond to doublets.

A striking feature of the Indica genomes revealed by this analysis is the very regular pattern that is observed with at least five major triplet fragments, corresponding to multimers of 360 ± 80 bp. This indicates that some units have an additional Sau 3A site approximately 80 bp from the one delimiting the repeat. This regular pattern can be explained if not all the Sau 3A sites were cut by the enzyme because of mutation. Following amplification of the 360 bp, the sequences diverged and Sau 3A sites were lost at random, generating the prominent pattern observed for Indica types. Minor fragments, observed at ca. 310 and 400 bp, indicate that there is a subset of the repeats with a similar organization, but in which another Sau 3A site has been fixed ca. 45 bp from the next one. The simpler pattern of the Japonica type is explained by the fact that a single Sau 3A site was present in the amplified unit.

Discussion

In this report we describe a tandemly repeated element from rice about ca. 360 bp long. The clone we have isolated from the cultivar "Cigalon" (Japonica) is very similar to that isolated from cv "Labelle" (Wu and Wu 1987). We suggest that this sequence is a member of the alphoïd satellite DNA family, as illustrated by the homologies with human alpha satellite DNA. Although this sequence is centromeric in human chromosomes, it is certainly premature to extend this conclusion to rice without further experiments. This report provides additional information concerning the occurrence and organization of this repeat in cultivated and wild rice genomes. We have confirmed that this sequence is highly specific for the A genome. Other repeated sequences specific for the other rice genomes (namely B, C, and E) have been isolated (Zhao et al. 1989; A. S. Reddy, M. C. Kiefer, M. Delseny, unpublished results) and shown to have no obvious homology with the 352-bp A-genome element. The copy number is variable depending on the accession. However, the same general trend to a lower copy number in Japonica was observed (Zhao et al. 1989). Our investigation revealed that the evolutionary pathway of this sequence in the different species is rather complex. With most Indica species, following digestion with Sau 3A, we obtained evidence suggesting that several subfamilies exist, differing in the position of an additional Sau 3A site. A slightly different repeat with a single Sau 3A site has been amplified in most Japonica types, as well as in *O. glaberrima* and *O. breviligulata*. Other evidence of rearrangements was observed in a few *O. rufipogon* accessions where decamers and 15- to 16-mers have been amplified. Depending on the restriction enzyme used, at least three subsets of the repeats can be distinguished corresponding to the spreading of different variants. In at least three *O. longistaminata* accessions, two superimposed ladders are observed differing in the increment by 10–15 bp. This indicates that two types of slightly different size units have been amplified and are physically independent in the genome. Such observations support the idea that these repeated sequences evolved by fixation of new restriction sites and small deletions, successive rounds of amplification and divergence, and by concerted evolution (Flavell 1982; Bedbrook et al. 1980a; Walbot and Cullis 1985; Grellet et al. 1986; Vedel and Delseny 1987; Willard and Waye 1987; Dod et al. 1989).

From an evolutionary point of view it is generally assumed that *O. rufipogon* is the ancestor of *O. sativa*. Using polyacrylamide gel analysis, we observed the Indica pattern in one subset of *O. rufipogon* accessions and that of Japonica in the other. We can therefore speculate that the two cultivated subtypes of *O. sativa* arose independently from already divergent *O. rufipogon* populations. Indeed, two groups of species can be distinguished on the basis of the Sau 3A patterns on polyacrylamide gels: one group comprising *O. sativa* Indica subtype, *O. longistaminata*, and some *O. rufipogon*; the other comprising the African rice *O. breviligulata*, *O. glaberrima*, the Japonica subtype of *O. sativa*, and the other *O. rufipogon*. The same grouping is observed when the copy numbers are considered. These patterns allow one to introduce a major dichotomy within the A-genome group and suggest that the separation of the Japonica and Indica subtypes is relatively old and probably occurred before the differentiation of the African species. This finding adds further support to the hypothesis of diphyletic origin of the present day *O. sativa* proposed by Second (1982), as opposed to the monophyletic theory (Oka 1974).

The mechanisms by which these tandemly repeated sequences arose are not yet clear. It has been suggested that these repeats result from amplification of tRNA or 5S rRNA pseudogenes (Benslimane et al. 1986; Wu and Wu 1987). However, in both cases the homology with the presumed ancestral gene is rather low. Tissue culture has been shown recently to induce amplification of specific repeated DNA sequences in rice, but the sequences involved have not yet been characterized (Kikuchi et al. 1987; Zheng et al. 1987). Nevertheless, this demonstrates a relatively large fluidity of the rice genome.

The presence of several subsets of the 360-bp repeat family raises the question of their chromosomal location and whether or not each subfamily is a marker of a specific chromosome, as observed for the human alpha repeat (Jorgensen et al. 1986; Willard and Waye 1987). The identification of polymorphic fragments should facilitate localization of the various subgroups on the rice RFLP map (McCouch et al. 1988).

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