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Chromosomes associate premeiotically and in xylem vessel cells via their telomeres and centromeres in diploid rice (*Oryza sativa*)

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Abstract Studies of the meiosis of diploid plants such as *Arabidopsis*, maize and diploid progenitors of wheat have revealed no premeiotic association of chromosomes. Premeiotic and somatic association of chromosomes has only been previously observed in the anther tissues and xylem vessel cells of developing roots in polyploid plants such as hexaploid and tetraploid wheat, polyploid relatives of wheat and artificial polyploids made from the progenitor diploids of wheat. This suggested that this association was confined specifically to polyploids or was induced by polyploidy. However, we developed procedures for in situ hybridization on structurally well-preserved tissue sections of rice, and analysed two diploid rice species (*Oryza sativa* and *O. punctata*). Contrary to expectation, this has revealed that centromeres and telomeres also associate both in the xylem vessel cells of developing root and in undifferentiated anther cells in these diploids. However, in contrast to wheat and related polyploids, where the initial association in undifferentiated anthers is between either non-homologous or related chromosomes, and not homologous chromosomes, the initial association of rice chromosomes seems to be between homologues. Thus, in contrast to the diploid dicot model *Arabidopsis*, meiotic studies on the diploid model cereal, rice, will now need to take into account the effects of premeiotic chromosome association.

Introduction

The studies of meiosis in the diploid plants *Arabidopsis* and maize as well as the diploid (A and D genome) progenitors of wheat have revealed that chromosomes only associate during meiosis (Bass et al. 1997; Martinez-

Perez et al. 2000; Armstrong et al. 2002; Comai et al. 2003). Premeiotic association of chromosomes has not been reported in these species. In contrast, studies of chromosome pairing in hexaploid (*Triticum aestivum*) and tetraploid (*T. durum*) wheat, both allopolyploids, and their polyploid relatives have shown that chromosomes associate in pairs via their centromeres during anther development prior to meiosis (Martinez-Perez et al. 1999, 2000; Maestra et al. 2002). The initial association of the chromosomes during early anther development is not homologous, being either non-homologous or homoeologous (between related chromosomes from the three wheat genomes). At the onset of meiosis and just prior to the formation of the telomere bouquet, the chromosomes cluster via the centromeres into seven groups (Martinez-Perez et al. 2003). At this stage, the level of association of the homologous chromosomes increases to over 90%, suggesting that the homologous chromosomes locate to the same group. Centromere association also occurs in the xylem vessel cells of developing wheat roots (Martinez-Perez et al. 2001). The *Ph1* locus, which affects both premeiotic and meiotic chromosome pairing, also affects this somatic pairing in the roots. An artificial polyploid made between the A and D progenitors of wheat does exhibit premeiotic centromere pairing (Martinez-Perez et al. 2000). These studies of diploid and polyploid plants suggested that premeiotic association was restricted to polyploids or could be induced following polyploidization.

Rice (*Oryza sativa*) has a relatively small genome (430 Mbp) compared with many cereals and grasses. A draft of its genome sequence is available. It is generally now considered as a model for the cereals. Its overall gene order is quite similar to that of other cereals including wheat and maize. The genes on the rice map can be grouped into sets and these same sets of genes (rice linkage segments) also describe the genetic maps of all the chromosomes of barley, foxtail millet, maize, sorghum, sugar cane and wheat (Moore et al. 1995a,b). Thus the structure of the rice genome provides the solution to representing cereal synteny. Rice is not only a model for

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the cereals, but is one of the major cereal crops. Understanding the factors that affect chromosome pairing and recombination in this species would have important consequences for breeding strategies.

Based on our previous studies of wheat and its diploid and polyploid relatives, and on the studies of maize, we predicted that rice would not exhibit premeiotic association. We therefore undertook a study to test this hypothesis. Studies on wheat and maize have all shown the importance of examining well-preserved material, both to be confident of the three-dimensional (3D) preservation of delicate nuclear structure, and of the cell types that are being analysed (Bennett et al. 1979; Dawe et al. 1994; Aragon-Alcaide et al. 1998). For example, tapetal cells and meiocytes in anthers or xylem vessel cells and other cells in the root can be misclassified, and polarized centromere or telomere clusters can easily be displaced or brought together artefactually in highly squashed preparations. Procedures have not previously been determined for undertaking 3D in situ approaches on either of the monocot or dicot model species, rice and *Arabidopsis*. We therefore initially determined conditions for labelling chromosome sequences in thick rice root and anther sections by in situ hybridization and visualized the resulting specimens by 3D confocal microscopy. Using this approach with probes for centromeres and telomeres, as well as bacterial artificial chromosome (BAC) probes for other chromosomal regions, we found, unexpectedly, that in two diploid and one polyploid rice species, the chromosomes associate as pairs early during floral development in all the cells in the anthers, and in the xylem vessel precursor cells of developing roots. This has led us to reassess the origin and extent of this premeiotic association, which we had previously ascribed to polyploidy in the close wheat relatives.

Results

Centromere and telomere organization in roots

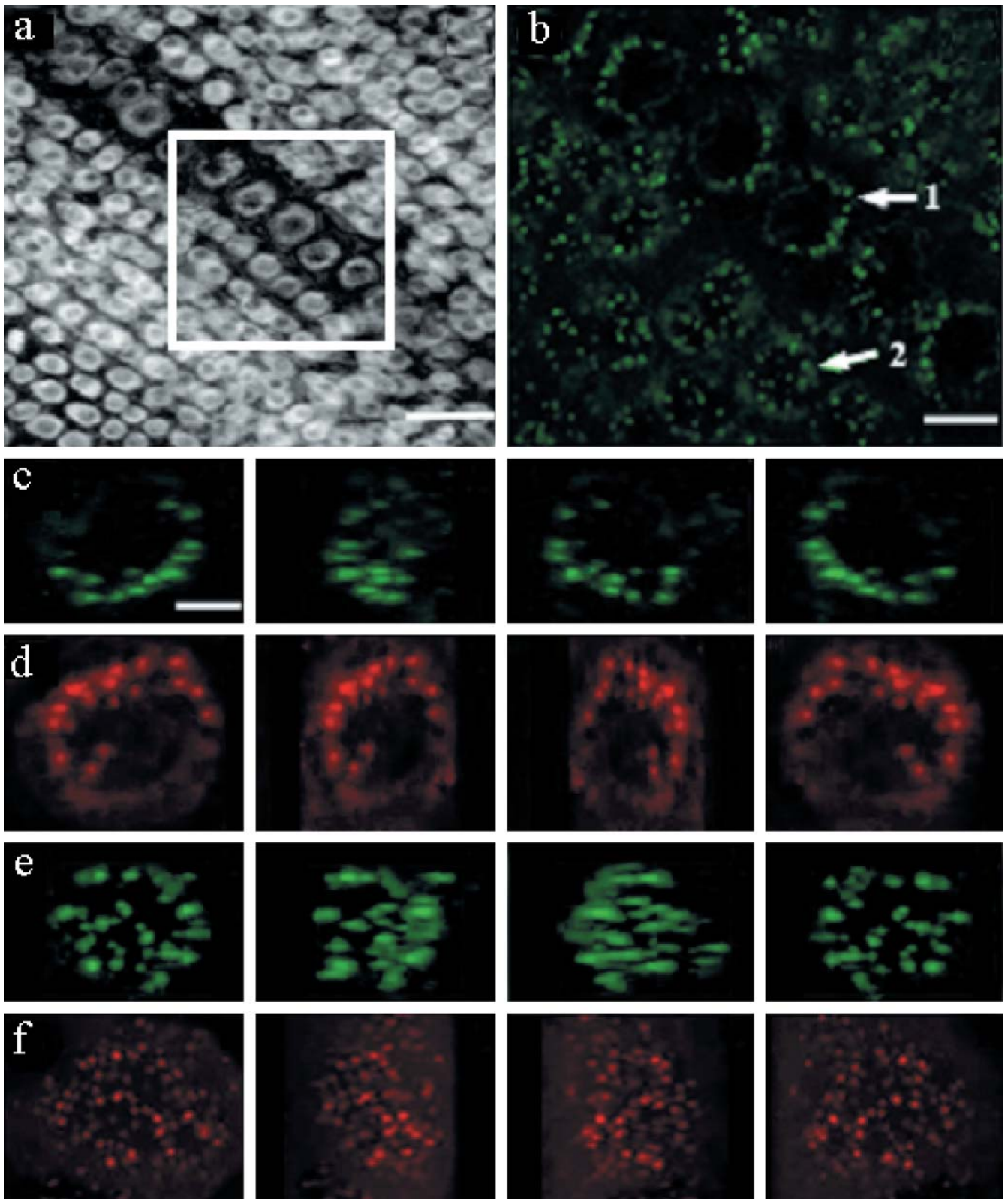
Initially using root tissue, we developed a whole-mount sectioning method for rice using a vibratome. In situ hybridization could be reliably performed on such sections (see Materials and methods). Each root section made is approximately 20 μm thick and contains about 2–3 cell layers. Using longitudinal root sections, we examined the morphology of the developing root tissue of diploid cultivated rice (*O. sativa*) at early stages (Fig. 1). Within such sections, it is possible to identify clearly the developing xylem vessel cells, as the central column of large cells (Fig. 1). There is a substantial increase in size of the nuclei of the cells of the developing xylem vessel column compared with the surrounding tissues. This suggests that endoreduplication may be occurring, as has been described previously for the xylem vessel cells in hexaploid wheat (Martinez-Perez et al. 2001). It is apparent that in most root nuclei there is no preferential organization of the centromeres and telomeres and they

are both dispersed around the periphery of the nuclei. However, in the xylem vessel cells, the centromeres and telomeres do localize to opposite poles of the nuclei, suggesting that there is a Rab1 configuration in these cells (Fig. 1c,d).

Somatic pairing of centromeres and telomeres

In situ hybridization with the RCS2 sequence and (TTTAGGG)_n telomere sequences as probes was used to detect the centromeres and telomeres in the interphase nuclei of the intact root sections in *O. sativa* (Fig. 1). The nuclei within the tissue section are all clearly and specifically labelled with the two probes. We next examined 3D confocal stacks such as those shown in Fig. 1 to determine whether centromere association occurs in diploid cultivated rice (*O. sativa*, A genome). We counted the number of centromeric sites in the developing xylem vessel cells. This showed, surprisingly, approximately half the expected number of centromere sites (about 12 sites seen for the 24 centromeres present; Figs. 1, 2; Table 1) and suggested that centromeres are associated in pairs in the xylem vessel cells. In contrast, the number of centromere sites in the surrounding root cells equated to the number of chromosomes (an average of 22 sites visible) (Figs. 1, 2; Table 1). Therefore the centromeres remain largely unassociated in these cells. The association of centromeres in the xylem vessel cells and not in the surrounding cells of the root is similar to that observed previously in the root tissues of hexaploid wheat (Martinez-Perez et al. 2001). The 48 telomeres of *O. sativa* also reduce to less than half the number of sites (15 sites) in the xylem vessel cells, but not in the surrounding cells (32 sites) (Table 1; Fig. 2). The association of centromeres and telomeres is unexpected in this diploid species as association of chromosomes had previously only been reported in a polyploid situation. The nucleolus is bigger in the xylem cells and could displace some volume of the nucleus, causing a redistribution of centromeres and telomeres and resulting in their apparent “passive” association. Both the in situ hybridization and resulting statistical analysis clearly reveal close to half the number of centromere sites with little deviation from this number between the cells analysed. This is consistent with an active process of association rather than a passive one where both the numbers of centromeres and the size of the associated centromere groups would vary markedly between cells analysed.

Fig. 1a–f Examples of 4',6-diamidino-2-phenylindole (DAPI) and fluorescence in situ confocal images of root sections of *Oryza sativa*. **a** Projection of three adjacent optical sections of the root showing a whole central column of vessel cells. The focal step size between sections was 4 μm . Chromatin was counterstained with DAPI. **b** Fluorescence in situ hybridization in the cells shown in the inset in **a** with the centromere (green) probe. The image is a single confocal optical section to show the different distribution of centromeres between xylem vessel and non-xylem cells in the root. A vessel cell (1) and a non-xylem cell (2) are arrowed. **c** Three-



dimensional (3D) rotation of the vessel cell (*arrow 1* in **b**) exhibiting around 12 centromeres grouped in one side of the cell nucleus. **d** 3D rotation of the same vessel cell in **c**, showing telomeres (*red*) distributed in the opposite side of the centromeres. **e** 3D rotation of a non-xylem root cell (*arrow 2* in **b**) exhibiting around 24 centromeres dispersed in the entire cell nucleus. **f** 3D

rotation of the same non-xylem root cell in **e**, showing that telomeres are distributed around the entire cell. The 3D rotations (0, 45, 135 and 180°) are generated from the projections of 8–10 adjacent optical sections with a 0.6–0.8 μm focal step size between sections. *Bar* represents 20 μm in **a**, 10 μm in **b** and 5 μm in **c–f**

Fig. 2 Centromere and telomere behaviour in diploid rice. All images are projections from confocal sections spaced $0.5 \mu\text{m}$. The projections have been produced to show the centromeres (*green*) and the telomeres (*red*) in root cells and in floral tissue at early developmental stages. The number of centromeres is haploid in vessel cells and in premeiotic anther cells. Not all the centromeres or telomeres are visible in these projections and some of them can be very close or superposed because of the superposition of the images. *Bar* represents $5 \mu\text{m}$

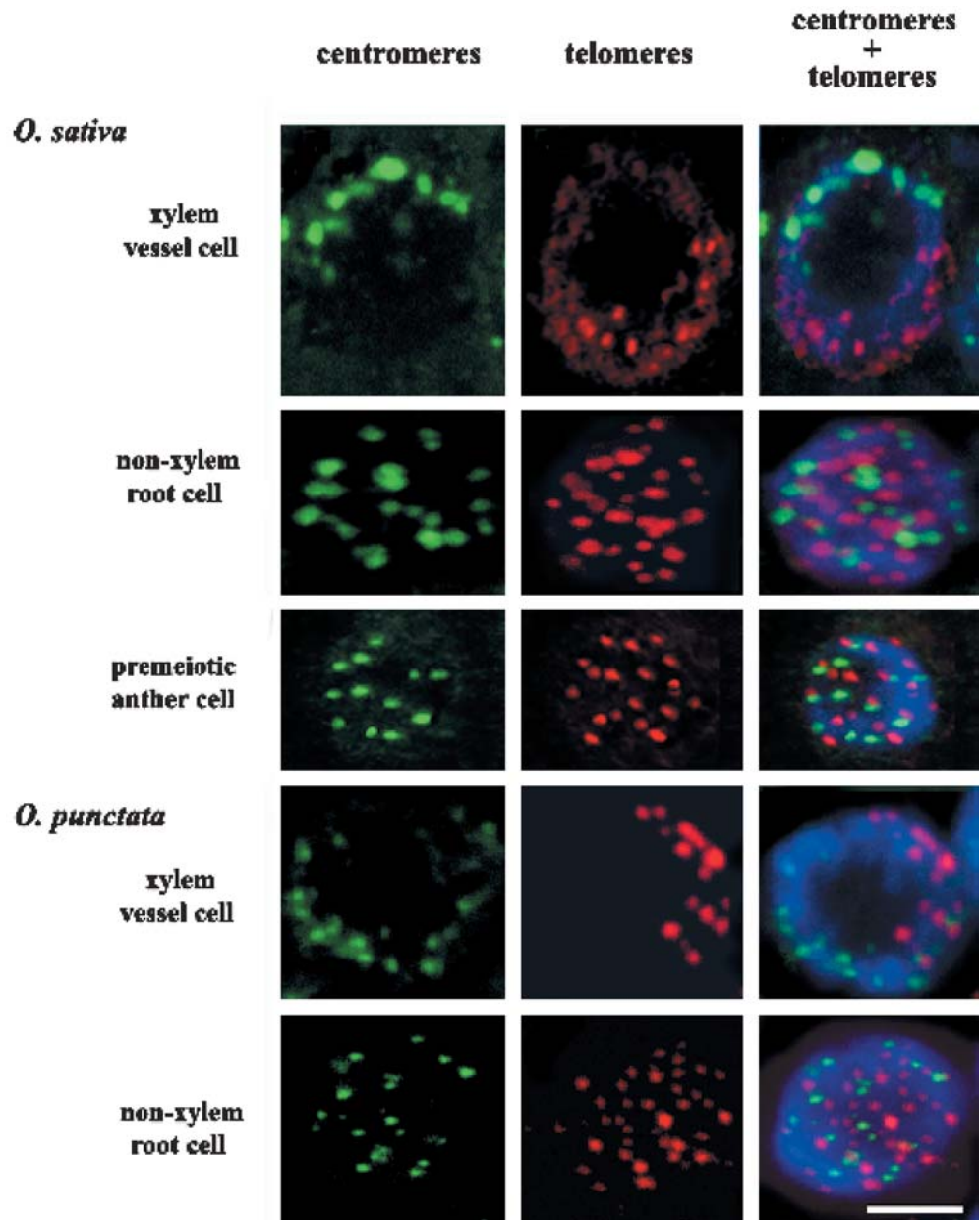


Table 1 Statistics of the number of centromere and telomere sites in root and anthers of diploid and polyploid rice

Species	Tissue	Cells analysed	Centromeres		Telomeres	
			Average (SD)	<i>P</i> Value	Average (SD)	<i>P</i> Value
<i>Oryza sativa</i> (AA) $2n=2x=24$	Non-xylem root cells	80	22.1 (1.7)		32.2 (9.0)	
	Xylem vessel	43	12.5 (0.8)	<0.001	14.7 (2.9)	<0.001
	Premeiotic anther cells	16	12.6 (1.3)	<0.001	16.5 (2.4)	<0.001
<i>O. punctata</i> (BB) $2n=2x=24$	Non-xylem root cells	47	22.9 (1.1)		33.2 (8.2)	
	Xylem vessel	24	12.1 (1.5)	<0.001	15.1 (2.9)	<0.001
<i>O. minuta</i> (BBCC) $2n=4x=48$	Non-xylem root cells	74	23.3 (0.8)		47.2 (2.2)	
	Xylem vessel	24	12.9 (0.8)	<0.001	23.9 (2.2)	<0.001

All centromere and telomere sites were counted on the original three-dimensional confocal stacks. The standard deviation (SD) is given in parentheses. The *P* value indicates the probability of the null hypothesis that the means in the xylem root cells or the premeiotic anther cells and the non-xylem root cells are the same. The telomeres are visualized as much smaller hybridization signals and are therefore more difficult to count accurately; this is reflected in the higher standard deviations for the telomere counts.

In order to determine whether these centromere and telomere associations are specific to *O. sativa*, we also examined the developing root of another diploid rice species, *O. punctata* (B genome). This revealed that the 24 centromeres in this species also reduce to approximately half the number (12 sites) in the xylem vessel cells (Table 1; Fig. 2). Again, the 48 telomeres also reduce to less than half the number of sites (15) (Table 1; Fig. 2). Thus, the chromosomes are associating by their centromeres and their telomeres in the developing column of xylem vessel cells of this diploid rice species. Therefore, centromere association is already present in at least two diploid rice species and does not depend on development of polyploidy.

These observations in the diploid rice species *O. punctata* and *O. sativa* show similarities between the diploid rice species and hexaploid wheat, but contrast with those of the wheat diploid relative *T. monococcum*, which did not exhibit centromere association in either the xylem vessel cells or the surrounding tissue in the roots (Martinez-Perez et al. 2001). Telomere behaviour in the xylem vessel cells of hexaploid wheat was not assessed so a comparison of telomere behaviour cannot be made with the diploid rice species.

Since centromeres and telomeres unexpectedly associate in the diploid rice species, this raises the question of what happens in polyploid rice species. We therefore examined the rice polyploid *O. minuta* (BBCC), which possesses 48 centromeres, to determine centromere behaviour. Unfortunately, the centromere probe used in this study only detected the 24 B genome centromeres and not the C genome centromeres. Moreover, no centromeric signals were detected when in situ hybridization was carried out using our centromere RCS2 probe in the rice diploid *O. officinalis* (C genome). We were therefore unable to examine the nature of pairing in the C genome by in situ hybridization using this probe.

Despite being only able to label half the centromeres, analysis of the xylem vessel cells of a developing root in this polyploid showed that the 24 centromeres of the B genome were visualized as approximately half the expected number of sites (12) (Table 1), again suggesting association of at least the B genome centromeres. Furthermore, the 96 telomeres also associated, reducing to approximately half (47) the number of expected sites (Table 1). The centromeres and telomeres in the surrounding tissue cells did not associate (Table 1). Thus this polyploid rice species showed very similar behaviour to the two diploid rice species described above. However, the diploid species showed significantly less than half the expected number of telomere sites, suggesting that there is either additional non-homologous association or association between chromosomes carrying duplicated regions of the genome.

Premeiotic association of centromeres and telomeres

In hexaploid wheat, the centromeres associate premeiotically in the developing anther tissues, reducing to approximately half the number of sites (Martinez-Perez et al. 1999). Labelling of early, premeiotic anther tissue isolated from *O. sativa* (AA) also showed that the 24 centromeres also reduce to an average of 12 sites during premeiotic floral development (Table 1; Figs. 1, 2). As in the case of the xylem vessel cells in rice, the 48 telomeres also associate at the same time reducing to less than half the number (16). Thus, in common with hexaploid wheat, diploid rice associates its centromeres in both the xylem vessel cells and the anthers. Telomere behaviour has not been assessed in early undifferentiated anthers in hexaploid wheat. Thus a direct comparison of telomere behaviour in hexaploid wheat and rice during anthesis cannot be made.

The chromosome association is likely to be homologous

The chromosomes are associated both via their centromeres and telomeres. This observation raises two questions. Are these associations mostly homologous or non-homologous and are the chromosomes associated along their complete length? There is no centromere or telomere specific probe available to ask whether specific homologous centromeres or telomeres are associating. We therefore tested 30 mappedBACs from chromosome 1, making a probe for each one and testing each individually for hybridization at a single, unique chromosome position. Of the 15 that have a clear, single site, we chose the closest mapped BACs to one of the telomeres and the centromere of the chromosome, and used these as chromosome-unique probes for these chromosomal regions.

In situ hybridization with both these BACs in both the xylem vessel cells and the anther tissue of *O. sativa* clearly shows two sites of hybridization for each BAC (Fig. 3). Thus the homologous chromosomes are separated at these sites, and the chromosomes are not associated along their complete length but only at the centromeres and telomeres. However, the homologous pairs of sites appeared closer together in the xylem vessel and anther nuclei than in most of the other somatic cells. To quantify whether homologous sites are closer together in those tissues in which centromeres and telomeres are associating, the 3D distance between the pairs of hybridization sites was compared in the xylem vessel cells and anther cells with cells in which no centromere/telomere association occurred. The statistical analysis confirms that the hybridising sites are in much closer proximity to each other for both BACs located in centromere and telomere regions than in those tissues in which the telomeres and centromeres are not associating (Table 2). The simplest interpretation of this observation is that the centromere and telomere associations are likely to be between homologous chromosomes.

Fig. 3a, b In situ hybridization with bacterial artificial chromosomes (BACs) in *Oryza sativa*. **a** Confocal images of diploid rice root sections and floral tissue at early developmental stages labelled using two rice chromosome 1-specific BACs as probes. *Bar* represents 5 μm . **b** Location of the a0040G11 clone (*40G11*) and the a0002C21 clone (*2C21*) on chromosome 1 of rice. The centromere of chromosome 1 is shown in *yellow* (adapted from Chen et al. 2002)

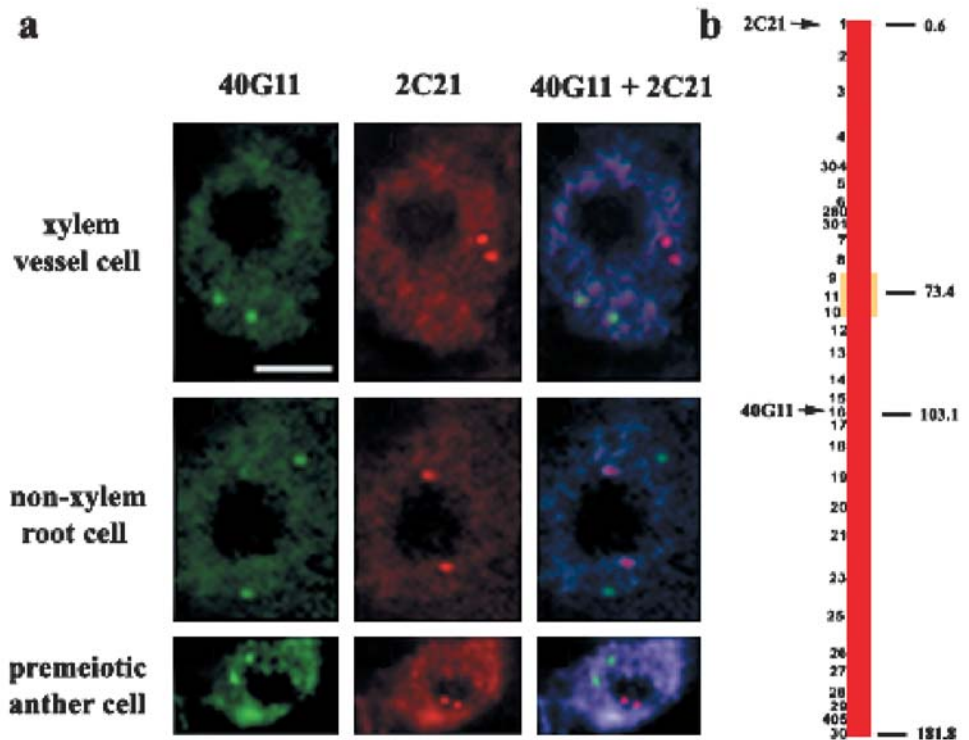


Table 2 Distance between two bacterial artificial chromosome (BAC) signals (μm) after hybridization to root and anther tissues of *Oryza sativa*

	Cells analysed	40G11		2C21	
		Average (SD)	<i>P</i> Value	Average (SD)	<i>P</i> Value
Non-xylem root cells	47	5.1 (0.3)		5.1 (1.1)	
Xylem vessel	22	2.1 (0.5)	<0.001	2.1 (0.3)	0.01
Premeiotic anther cells	62	2.0 (0.5)	<0.001	2.3 (0.4)	<0.001

The standard deviation (SD) is given in parentheses. The *P* value indicates the probability of the null hypothesis that the distances between two BAC signals in the xylem root cells or the premeiotic anther cells and in non-xylem root cells are the same.

Discussion

In hexaploid wheat and its polyploid relatives, centromeres associate premeiotically. On the other hand, there seems to be no premeiotic association of the chromosomes in rye, *Arabidopsis*, the ancient allotetraploid maize and the A and D diploid progenitors of wheat (Bass et al. 1997; Martinez-Perez et al. 2000; Mikhailova et al. 2001; Comai et al. 2003). Moreover there is no general evidence of premeiotic associations of chromosomes reported in the dicots. An artificial polyploid derived from the A and D genome progenitors does exhibit premeiotic centromere association (Martinez-Perez et al. 2000). We therefore previously ascribed the centromere association in the wheat relatives to the ploidy of the genome, with polyploid species undergoing centromere association and diploid species not doing so.

The present study reveals that rice associates both its centromeres and telomeres premeiotically in anther cells and non-meiotically in xylem vessel precursor cells. This is similar to the situation occurring in wheat and related

polyploids. The difference is that, in hexaploid wheat, the initial chromosome associations in premeiotic anthers are mostly not homologous (being either non-homologous or homoeologous) but in the premeiotic rice anthers are homologous. This difference is likely to reflect the presence of related chromosomes derived from the different genomes in the polyploid situation. It is clear that the chromosome pairing in the cereal model rice will not resemble that reported for *Arabidopsis*. Moreover, the presence of premeiotic chromosome pairing will complicate the exploitation of the knowledge of meiotic processes derived from other plant systems to underpin rice breeding strategies. However, the procedures described in the paper will provide tools for developing a framework on which future rice meiotic studies can be based.

These observations suggest that the potential to associate chromosomes via centromeres (and telomeres) both premeiotically and in certain other cell types may be widespread in the cereals. The cereals (family Gramineae) are part of the subclass Commelinidae, which includes the rushes and sedges. The evolution of the cereals within the

Gramineae through breakage and fusion events resembles chromosome evolution within the rushes and sedges (Moore et al. 1997). These species have extensively modified their meiosis so that association between homologous chromosomes is retained through meiosis I (Nordenskiöld 1962). In fact a recent study suggests that the rushes also show premeiotic association of their chromosomes, which may explain their unusual meiotic behaviour (Haizel et al. 2003). The potential to associate the chromosomes during anther development may therefore have arisen in the ancestor of the cereals and rushes and been retained in some but not all cereals. This then raises the issue of what is the consequence of its retention in rice and not, for example, maize? Restriction fragment length polymorphism mapping of the maize genome has revealed an ancient tetraploid structure (Weber and Helentjaris 1989; Whitkus et al. 1992; Ahn and Tanksley 1993; Moore et al. 1995a,b). However, recent sequencing of regions of the maize genome indicates that it has extensively diploidized itself, deleting duplicated copies (Ilic et al. 2003). Thus the maize genome is relatively unstable.

In contrast, when the first synteny analyses were undertaken, it was suggested that the genome structure of rice is closer to the genome structure of the ancestor of the cereals than that of the other cereals. Over the last 5 years, more detailed synteny comparisons at the sequence level have been undertaken. They reveal that most rearrangements detected in comparisons between other cereals and rice are found in the other cereal genome rather than in rice (Bennetzen and Ma 2003). This supports the theory that the present day rice genome is more similar to the ancestral cereal genome than those of the other modern cereals. It also raises the question of why the chromosomes of rice are relatively more stable than those of other cereals. The only other cereal genome that has been reported to be relatively stable is hexaploid wheat, which has also not undergone major rearrangements of its genome following polyploidization (Moore 2000). When the rice genome was first organized into linkage segments in the syntenic map with the other cereals, it was known that telomeres defined the ends of some segments (Moore et al. 1995a,b). However, it was not known what genomic structures defined the remaining ends (Moore et al. 1997). The mapping of rice centromeres revealed that the linkage segments were in fact defined both by centromere and telomere locations. This suggests that telomeres and centromeres are important in defining the chromosome rearrangements that have taken place during cereal evolution.

The observations we report here also mean that we need to reconsider premeiotic chromosome association as being polyploid specific. Previous results showed that such premeiotic association did not occur in a variety of diploid species related to wheat. This suggested that premeiotic association was only induced on polyploidization. However, none of the diploid species surveyed contained the B genome progenitor of wheat, since there is currently no known diploid relative extant, and it is

possible that the B genome carries the genes controlling centromere and telomere association, as it does for genes controlling homologue pairing. The presence of premeiotic pairing in diploid rice suggests that this model diploid cereal may possess similar mechanisms for the regulation of homologue pairing to those previously thought to be restricted to wheat.

Materials and methods

Plant material

Rice lines used in the present study were a cultivated rice (*O. sativa*, AA, $2n=24$, cv. Bengal) and three wild *Oryza* species: *O. punctata* acc. 105980 (BB), *O. minuta* acc. 101141 (BBCC) and *O. officinalis* acc. 100896 (CC). The *O. punctata*, *O. minuta* and *O. officinalis* lines were kindly provided by Dr. Darshan S. Brar from the Plant Breeding, Genetics and Biochemistry Division in the International Rice Research Institute (IRRI), Metro Manila, Philippines.

Seeds were pretreated at 37°C for 24 h and then germinated for 3 days at 24°C on moist filter paper. The root tips were excised and fixed in 4% (w/v) formaldehyde freshly prepared from paraformaldehyde in PEM buffer (50 mM PIPES/KOH, pH 6.9, 5 mM EGTA, 5 mM MgSO₄) for 1 h at room temperature (RT) and then washed in TBS (10 mM TRIS-HCl, pH 7.4, 140 mM NaCl) for 10 min. Sections (20–50 µm) were cut from the root tips using a Vibratome Series 1000 (TAAB Laboratories Equipment, Aldermaston, UK).

Rice plants were grown to flowering in a controlled environment room to rice spikes at different developmental stages and were fixed for 1 h at room temperature in 4% (w/v) paraformaldehyde in PEM buffer. To facilitate penetration of the fixative, vacuum infiltration was used. Single spikelets were detached from the spike and sectioned (50–100 µm thick sections) under water using a Vibratome series 1000.

Sectioning and DNA probes

Vibratome sections from roots or spikes were allowed to dry on multi-well slides (ICN Biomedicals). The slides were pretreated by washing in 3% Decon for at least 1 h, rinsing thoroughly with distilled water and then coated with a freshly prepared solution of 2% (v/v) 3-aminopropyl triethoxy silane (APTES, Sigma) in acetone for 10 s and activated with 2.5% (v/v) glutaraldehyde in phosphate buffer for 30 min, rinsed in distilled water and air dried.

The RCS2 centromeric probe (GenBank Accession number AF058902) was obtained by polymerase chain reaction amplification of total genomic *O. sativa* DNA with a pair of primers (5'-TACTGGAATCAAAAAGTTCAAAAAGAGCCA-3' and 5'-TCG-TGTATGCACTTGGCATTAAATGAC-3') based on sequences that flank the conserved centromeric region of rice (Dong et al. 1998). Two BACs for rice chromosome 1 were also used as probes (Chen et al. 2002). The a0002C21 clone is located in contig 1 of chromosome 1 and mapped approximately between 1 and 7 cM. The a0040G11 clone is located in contig 16 in rice chromosome 1 and mapped approximately at 100 cM (Chen et al. 2002). The centromere of chromosome 1 is mapped to a single genetic position at 73.4 cM.

Fluorescence in situ hybridization on rice root sections

To improve the permeability step, the tissue sections were first dehydrated in a series of 30, 50, 70 and 100% methanol. Immediately afterwards, the tissue sections were treated with the enzyme mix, 1% (w/v) cellulase (Onozuka R-10), 0.5% pectolyase (Kikkoman, Japan) in TBS for 30 min at RT, washed in TBS for

10 min, and dehydrated again in a series of 30, 50, 70 and 100% methanol. The hybridization mixture contained 50% deionized formamide, 20% dextran sulphate, 0.1% sodium dodecyl sulphate, 10% 20×SSC, 200 ng of centromeric, telomeric or BAC probe, 1 µg of sonicated salmon sperm as blocking DNA. Probes were labelled with digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, Ind.) or biotin-16-dUTP (Boehringer Mannheim) by nick translation. The hybridization mixture was denatured at 95°C for 5 min, cooled in ice for another 5 min and immediately applied to the sections. Target DNA denaturation was carried out in a modified thermocycler (Omnislide, Hybaid, Long Island, N.Y.) at 78°C for subsequent hybridization at 37°C overnight. Post-hybridization washes were carried out using 20% formamide in 0.1×SSC at 42°C. Probes labelled with digoxigenin were detected by an anti-digoxigenin antibody conjugated to fluorescein isothiocyanate (Boehringer Mannheim, Indianapolis, Ind.) and biotin-labelled probes were detected with extravidin-cy3 (Sigma).

Confocal fluorescence microscopy, image acquisition and data analysis

Confocal optical section stacks were collected using a Leica TCS SP confocal microscope (Leica Microsystems, Heidelberg, Germany) equipped with a Krypton and an Argon laser. The microscopy data were recorded and then transferred to NIH image (a public domain program for the Macintosh by W. Rasband available via ftp from ftp://Zippy.nimh.nih.gov) and composited using Adobe Photoshop 5.0 (Adobe Systems, Mountain View, Calif.). Final images were printed on a Pictography P3000 printer.

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