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Mapping of centromeric regions on the molecular linkage map of rice (*Oryza sativa* L.) using centromere-associated sequences

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Abstract Genetic mapping of centromeres has been a challenge for plant geneticists. The objective of this study was to develop a new strategy for determining the locations of centromeric regions on genetic maps by mapping centromere-associated sequences, to make it possible to define the centromeric region of each chromosome as a single Mendelian locus on the molecular linkage map. Two DNA probes containing sequences specifically associated with the centromeres of grass species were used for genetic mapping. The centromere-associated sequences for all 12 rice chromosomes were mapped on the molecular map with either or both of the probes, and flanking molecular markers on one or both sides were localized 0 to 8 cM away. The map locations of the centromere-associated markers corresponded very well with the positions of centromeric regions determined previously using trisomic analyses for 11 of the 12 chromosomes. The precise mapping of the centromeric regions using these probes makes the molecular map a more complete and informative tool for genomic studies, which will facilitate studies of the structure and function of the rice centromeres. The simplicity of this technique, together with the fact that these probes are also associated with the centromeric regions in other grass species, may provide a general approach to the mapping of centromeric regions in the genomes of other cereal crops.

Key words Centromere · Genetic mapping · Genome analysis

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Introduction

The centromere is one of the most important functional elements of eukaryotic chromosomes, and ensures proper cell division and stable transmission of the genetic material. The DNA composition and sizes of centromeres vary significantly in different organisms. For example, the centromeres of budding yeast (*Saccharomyces cerevisiae*) chromosomes consist of only about 125 bp of DNA and are free of repetitive DNA sequences (Clarke and Carbon 1980, 1985; Clarke 1990). Human centromeres, on the other hand, are composed mainly of α-satellite DNA which is the essential element for centromere function (Harrington et al. 1997; Ikeno et al. 1998). The centromeres of fission yeast (*Schizosaccharomyces pombe*) and *Drosophila melanogaster* chromosomes include complex DNA, including repetitive DNA and transposon sequences (Clark 1990; Murphy and Karpen 1995; Sun et al. 1997). The centromeres of higher plants also consist of different types of repetitive DNA sequences (Alfenito and Birchler 1993; Aragon-Alcaide et al. 1996; Jiang et al. 1996; Round et al. 1997; Ananiev et al. 1998; Dong et al. 1998; Miller et al. 1998a, b). Therefore, two types of centromeres can be distinguished, based on the length of the DNA sequences involved: regional centromeres with lengths ranging from several kilobases to several megabases, and point centromeres consisting of only a few hundred bases (Clarke 1990).

Rice has now become a model system for grass species in genome research because of its small genome size, and the availability of comprehensive molecular linkage maps and a highly efficient transformation system. The genetic linkage maps of rice are among the most saturated molecular marker-based maps constructed for the major crop plants (Causse et al. 1994; Harushima et al. 1998). Singh et al. (1996) determined the position of the centromeric region for each of the 12 chromosomes by dosage analysis of secondary trisomics and telotrisomics using DNA markers on the linkage map constructed by

the Cornell University group (Causse et al. 1994). Using a similar strategy, Harushima et al. (1998) also determined the locations of the centromeric regions on the molecular map constructed by the Japanese Rice Genome Research Program. Although the resolution of the centromeric locations in the two maps were better for some of the chromosomes than for others, the deduced centromeric regions identified for various chromosomes still involved stretches of chromosomes that varied in length from less than one centi-Morgan (cM) to more than 20 cM. Thus, it is still not known precisely where the centromere is located on each of the chromosomes.

In the study reported in this paper, we mapped genetically two centromere-associated DNA sequences to the 12 chromosomes with reference to the molecular marker linkage map of rice, using Southern hybridization and linkage analysis. The objective of the study was to develop a new strategy for determining the locations of centromeric regions on genetic maps by mapping centromere-associated probes, which should make it possible to define the centromeric region of each chromosome as a single Mendelian locus on the molecular marker linkage map.

Materials and methods

Centromere-associated probes

Two clones were used as hybridization probes to map centromere-associated sequences to the chromosomes. The first clone, pSau3A9, was isolated from sorghum (*Sorghum bicolor* L.) and contains sequences specific to the centromeres of grass chromosomes (Jiang et al. 1996). The second clone, N.36A (GenBank Accession No. AF091233), was obtained in our previous study (S. Wang et al. 1998) by PCR amplification of total DNA from a rice cultivar, Aijiao Nante (*Oryza sativa* ssp. *indica*) with a pair of primers (5′-ATGGTGTTCGGATTATGGGG-3′ and 5′-TTGTGTCGGAGTTGGAGGTG-3′) based on sequences that flank the conserved region of tobacco mosaic virus resistance gene *N* (Whitham et al. 1994).

Mapping populations

Three populations were used for mapping of the centromere-associated sequences. The first population consisted of 235 individuals and was derived from a three-way cross, Balilla (*O. sativa* ssp. *japonica*)/Dular (*O. sativa* ssp. *indica*)//Nanjing 11 (*O. sativa* ssp. *indica*). A molecular linkage map containing 158 RFLP loci was developed using this population (J. Wang et al. 1998). The second population, consisting of 172 F_2 individuals, was derived from a cross between the cultivar Aijiao Nante and P16, an accession of the common wild rice (*O. rufipogon*). A molecular linkage map based on this F_2 population contained a total of 612 loci, including 412 RFLPs, 28 SSRs and 172 AFLPs (Xiong et al. 1998). The third population of 230 individuals was also from a three-way cross, 02428 (*O. sativa* ssp. *japonica*)/Nanjing 11//Balilla. A RFLP linkage map containing 191 loci was also constructed using this population (Liu et al. 1997).

DNA hybridization and data analysis

The DNA samples were digested with one of seven restriction enzymes (*Apa*I, *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV or *Hin*dIII).

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RCS1     18  TTCGATTGCTTGCAG-TTCAAG-TTGTTCTTGGCATGGCA-GAACAGCAA  64
N.36A   507  TTCGATTGCTTGCAGGTTCAAGGTTGTTCTTGGCACGGCAAGAACAGCAA 556
MCS1a    56            GCAGGTTCAGTGCTGTTCGTGGCACGGCAAG          86

RCS1     65  CAACGTGGAGTCAGTGTACCGGTGCTAAGGCGCAGCACCCAA-TAGTAGT 113
N.36A   557  CAACGTGGAGTCGGTGTACTGGTTGTAAGGCGCAGCACCCCTGTGGTAGT 606
MCS1a   214                          AAGGCGCCACACCATGTTGGTTGT 237

RCS1    114  TGTAGTTGGACCGCCAACGTCGTGTCCTCCCTCAAATCGAAGTTTATCCT 163
N.36A   607  TGTAGTCGGACCGCCAACGTCGTATCCTTCCCCAAATCGAAGTTTATCCT 656
MCS1a   238  GGTAGTCGGGCAGCCAACGTCGTTCTCCAACAAGTTATCCACCTCCATCA 287

RCS1    164  CCTCTCATCGAAAGATTGGGAACCCCCGTTGCTGCATCA            202
N.36A   657  CCTCTCATCGAAAGATCGGGAACGCCCGTTGCTGCATCA            695
MCS1a   288  TCTCTCATCGAAAGATCGGGCAC                            310
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Fig. 1 Comparison of the nucleotide sequence of N.36A with known rice (RCS1) and maize (MCS1a) centromeric sequences, aligned using BLASTN. The *vertical bars* indicate sequence identities

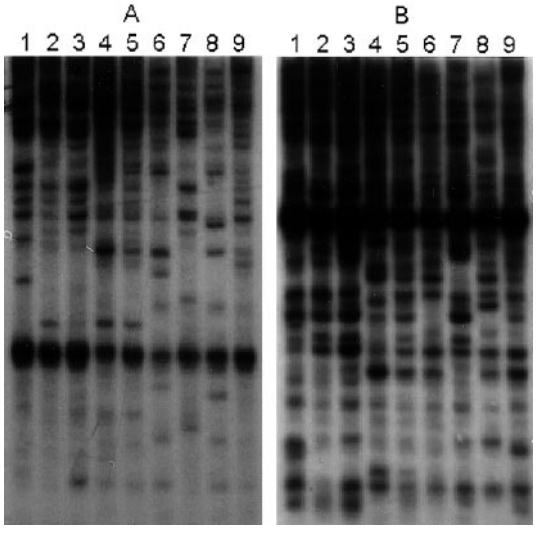

Fig. 2A, B Southern analysis of rice varieties using N.36A (**A**) and pSau3A9 (**B**) as probes. The DNA was digested with *Apa*I in both cases. Lane 1, Zhenshan 97 (*indica*); 2, Minghui 63 (*indica*); 3, Aijiao Nante; 4, P16; 5, F_1 of Aijiao Nante/P16; 6, Balilla; 7, Nanjing 11; 8, 02428; 9, Dular

The hybridization was conducted according to the procedures described previously (Wang et al. 1997). After hybridization, the filters were washed in 0.5× SSC and 0.1% SDS once for 5 min at room temperature and twice for 15 min at 65 °C. The locations of centromere-associated markers on the molecular linkage maps were determined using Mapmaker/Exp 3.0 (Lincoln et al. 1992) at a LOD threshold of 3.0.

Results

Clones containing centromere-associated sequences

Clone N.36A contained an insert of 1026 bp in length. A BLAST search (Altschul et al. 1997) showed that about

190 bp of the clone was highly homologous [expect (E) = 3e-49] to a portion of a 306-bp rice centromeric sequence, RCS1, with 91% nucleotide identity between them (Fig. 1) (Aragon-Alcaide et al. 1996). The same region of N.36A was also partially homologous (E = 2.2e-08) to a maize centromeric sequence, MCS1a (Fig. 1) (Aragon-Alcaide et al. 1996).

The sequence of the second clone, pSau3A9, was found to be present in the centromeric regions of all rice chromosomes (Jiang et al. 1996). A part of this fragment was highly homologous to Ty3/*gypsy* retrotransposons of *Zea mays*, *Drosophila buzzatii*, *Tribolium castaneum* and *Magnaporthe grisea* (Miller et al. 1998a).

Both clones detected multiple hybridization signals on Southern blots of genomic DNA from both *indica* and *japonica* varieties, with no distinct difference being detectable between the two subspecies (Fig. 2). The banding patterns detected by N.36A and pSau3A9 were clearly different (Fig. 2).

Mapping of centromere-associated clones

The locations of the centromere-associated markers on all 12 chromosomes in the rice molecular linkage map are presented in Fig. 3. Owing to insufficient polymorphism between the parents of the mapping populations, only one of the two probes could be mapped for nine of the 12 chromosomes (see Fig. 4 for the exceptional

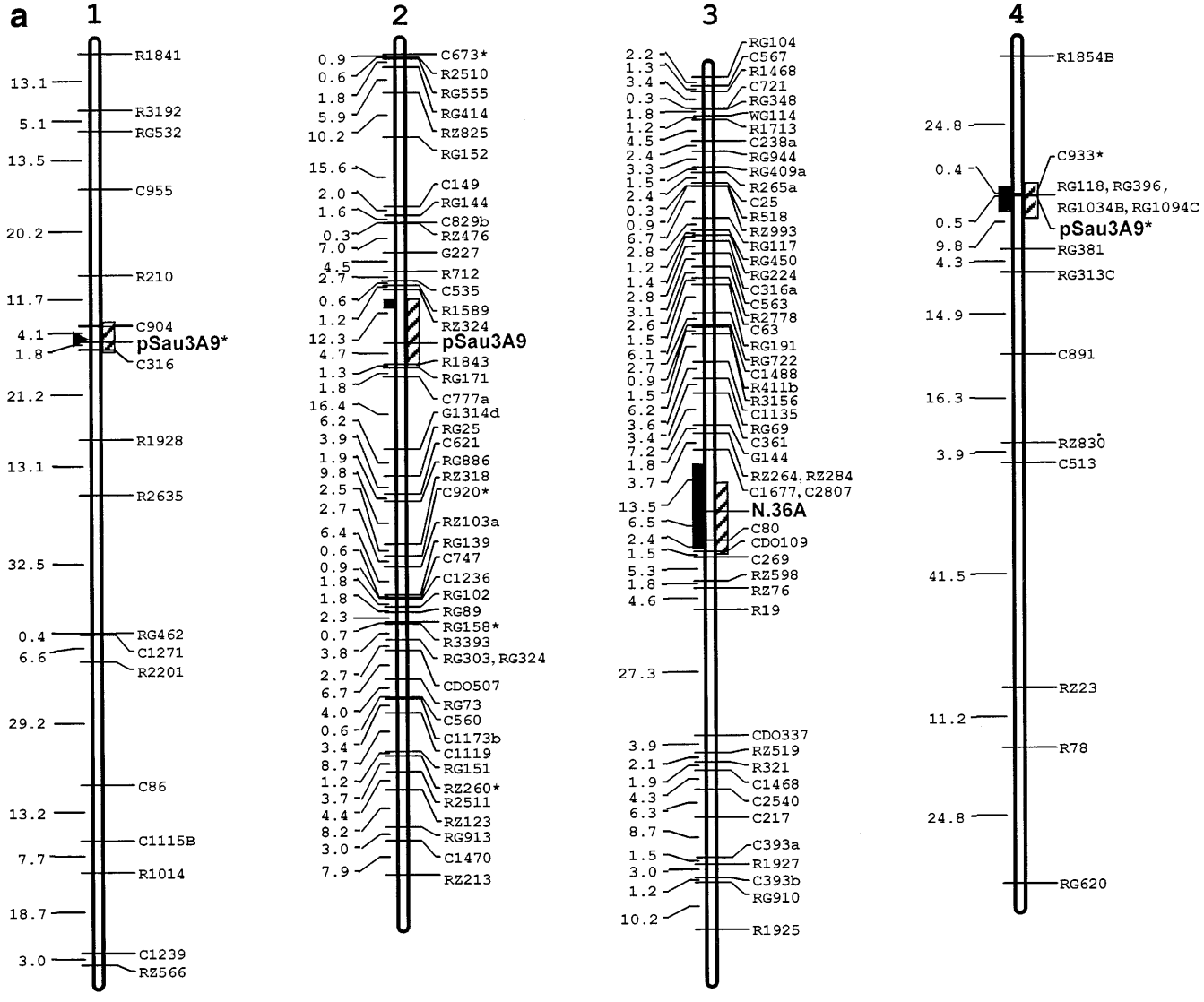

Fig. 3a–c Locations of the centromere-associated markers on the molecular linkage map, determined using N.36A and pSau3A9 as hybridization probes. The maps of chromosomes 1, 4, 5, 6, 10, and 12 were based on the three-way cross Balilla/Dular//Nanjing 11, and the maps of chromosomes 2, 3, 7, 8, 9, and 11 were based on the F_2 population from Aijiao Nante/P16. The orientation of the chromosomes follows Singh et al. (1996), with the short arm placed on top. The *bar or triangle* on the left-hand side of each chromosome indicates the centromeric region deduced on the basis of trisomic analysis by Harushima et al. (1998) and the *bar* on the right hand side of each chromosome is the centromeric region by Singh et al. (1996). The open bar on chromosome 10 in the map of Singh et al. (1996) indicates that the centromeric region is not precisely defined. Map distances are given in cM to the left of each chromosome. *Asterisks* indicate assignment at LOD 2.0 and the others are at LOD 3.0

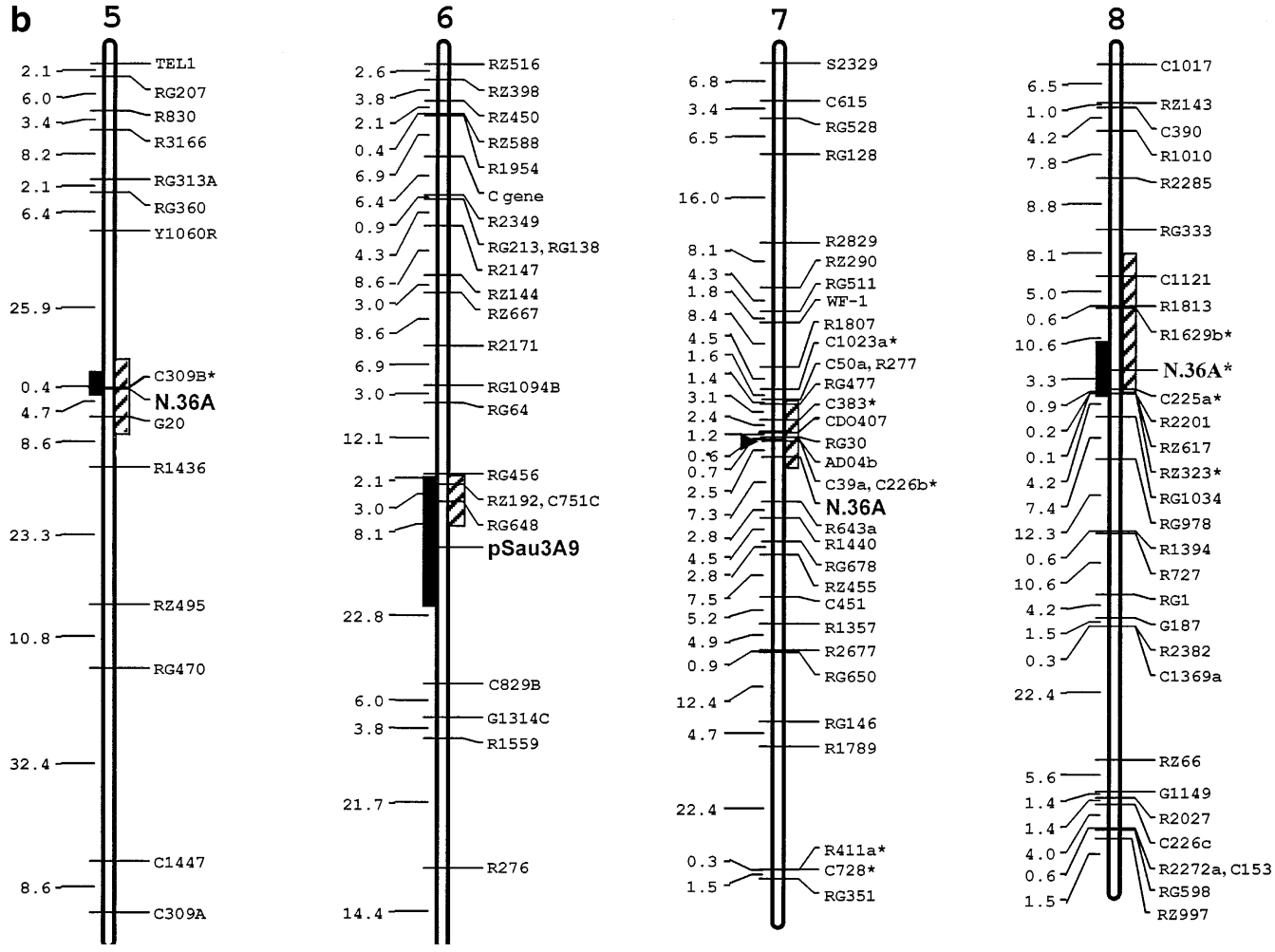

Fig. 3b (Contd.)

cases). Also, the probes seemed to be preferentially associated with particular chromosomes. For example, the centromere-associated sequence on chromosome 3 was mapped with N.36A in all three mapping populations (data not shown). Similarly, the centromere-associated sequence on chromosome 11 was mapped with pSau3A9 in all the populations. In cases where a probe could be mapped to the same chromosome in different mapping populations, the locations were very consistent across the populations.

Each of the centromere-associated markers was mapped within a few cM of at least one flanking molecular marker locus (Fig. 3). The centromere-associated marker on chromosome 9 cosegregates with two RFLP loci. On chromosomes 1, 5 and 11, the centromere-associated markers were flanked by RFLP markers within 5 cM on both sides. Closely linked RFLP markers (less than 5 cM away) were also found for the centromere-associated markers on chromosomes 2, 4, 7, 8 and 12. The linkages between the centromere-associated marker loci and flanking molecular markers are relatively loose (6–8 cM) for the remaining three chromosomes (3, 6 and 10).

Correspondence between the map positions of the centromere-associated markers and centromeric regions determined by trisomic analyses

In general, the locations to which the centromere-associated markers were mapped on the chromosomes corresponded well with the results obtained by trisomic analyses (Singh et al. 1996; Harushima et al. 1998). We now compare our mapping results with those of the two previous studies.

Harushima et al. (1998) resolved the centromeric region to a specific point between two cosegregating marker loci for each of the chromosomes 1, 7, 9 and 11. The map positions of the centromere-associated markers on chromosomes 1, 9 and 11 corresponded exactly to those on their map (Fig. 3). The centromere-associated probes were mapped in all three populations for chromosome 7 in the present study. In two of the populations the mapped probe was N.36A, which was linked to the RFLP marker C39 at 7.2 cM (using the 02428/Nanjing 11//Balilla population) and 2.5 cM (using the Aijiao Nante/P16 population), respectively (Figs. 3 and 4). In the other population the mapped probe was pSau3A9, which was linked to C39 at 0.9 cM (Fig. 4).

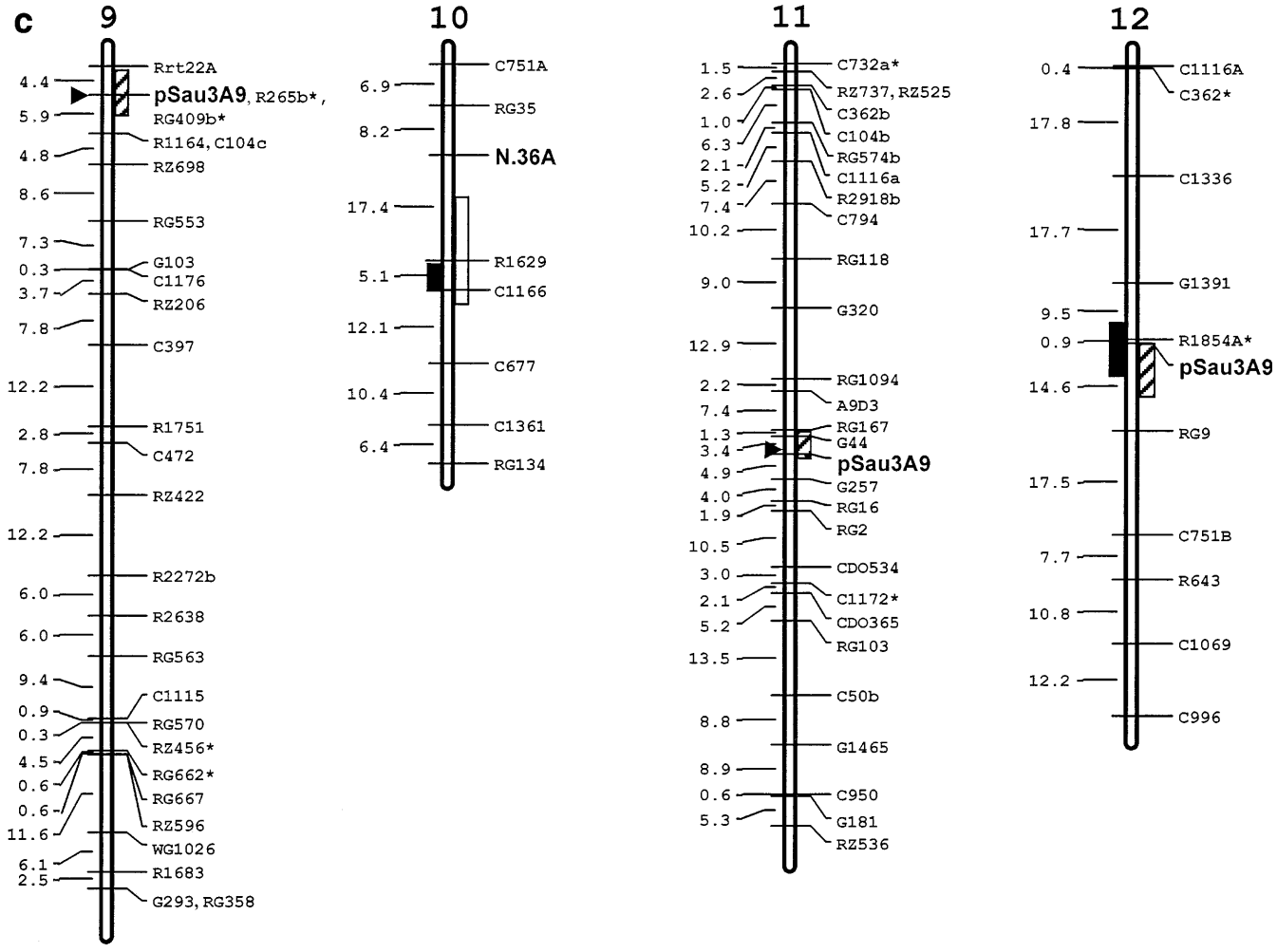

Fig. 3c (Contd.)

The orders of the RFLP markers relative to the centromere-associated probes were slightly different in these four cases. C39 and several other RFLP markers cosegregated with the centromeric region on the map of Harushima et al. (1998). With respect to the centromere-associated probes, C39 would be placed on the long arm as mapped using the two three-way crosses (Fig. 4), whereas this marker would be placed on the short arm based on the Aijiao Nante/P16 cross. Such a difference is most likely to be due to a statistical error in the computation rather than an inversion in Aijiao Nante or P16, because the relative order of markers on the map of the Aijiao Nante/P16 cross (Xiong et al. 1998) was the same as on the maps constructed with the other two crosses if the centromere-associated probe N.36A was omitted from the map.

The centromeric regions deduced for the remaining eight chromosomes (2, 3, 4, 5, 6, 8, 10 and 12) on the map of Harushima et al. (1998) each involved a linkage block that varied in length from 0.3 to 6.8 cM. All of them showed good correspondence with those on the map of Singh et al. (1996), although the lengths of the linkage blocks involved were very different on the two maps. For seven of the eight chromosomes, the mapped positions of the centromere-associated markers lay within the deduced centromeric regions in one or both maps provided by Singh et al. (1996) and Harushima et al. (1998). However, the present map locations of the centromere-associated markers can be used unambiguously to place all the markers on the long or the short arm of each chromosome. Thus such mapping can not only provide precise genetic locations for the centromeric regions, but also resolve the arm locations of all the markers on these chromosomes.

The only exception is chromosome 10. The centromeric region of chromosome 10 was not clearly defined on the map of Singh et al. (1996). On the map reported by Harushima et al. (1998) the RFLP marker R1629 was on the short arm, whereas this marker was placed on the long arm based on the position of the centromere-associated probe N.36A. Thus the position of the centromere-associated probe does not seem to agree with the centromeric region proposed by Harushima et al. (1998).

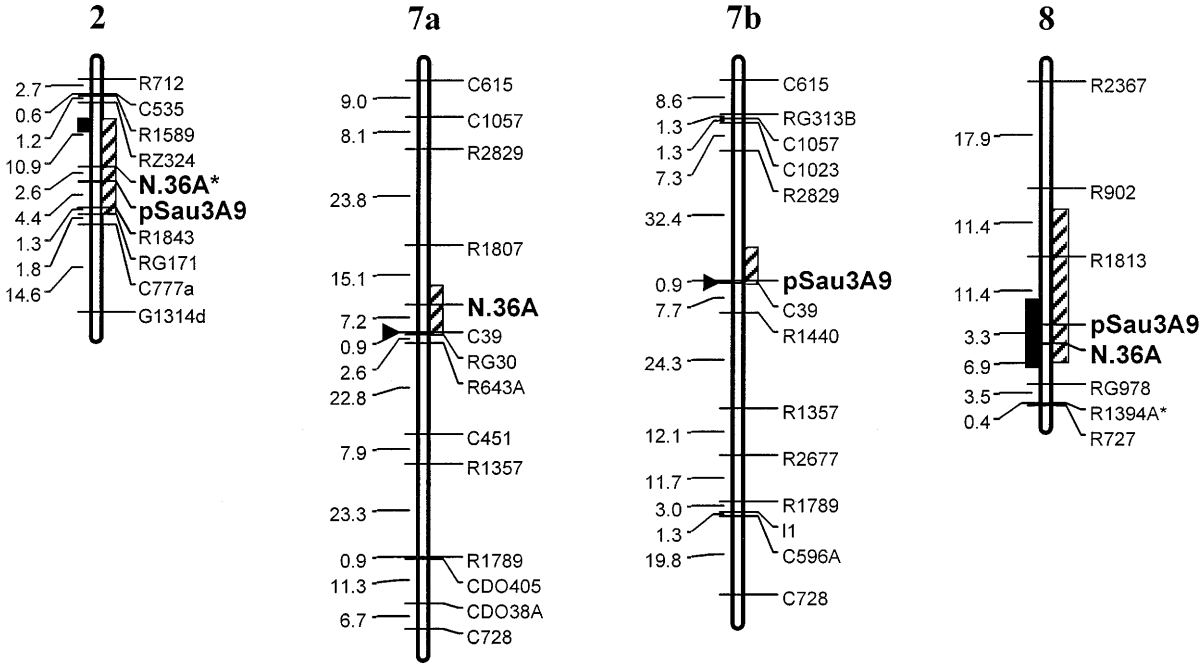

Fig. 4 The locations of the two centromere-associated probes N.36A and pSau3A9 on the same chromosomes (2 and 8) or on the same chromosome but presented on different maps (7a and 7b). The map of chromosome 2 was based on the F_2 population from Aijiao Nante/P16. The maps of chromosomes 7a and 8 were based on the three-way cross 02428/Nanjing 11//Balilla. The map of chromosome 7b was based on the three-way cross Balilla/Dular//Nanjing 11. The *bar or triangle* on the left-hand side of each chromosome indicates the centromeric region deduced on the basis of trisomic analysis by Harushima et al. (1998) and the *bar* on the right-hand side of each chromosome is the centromeric region assigned by Singh et al. (1996). Map distances are presented in cM to the left of the chromosomes. *Asterisks* indicate assignment at LOD 2.0 and the others are at LOD 3

The relative map positions of the two probes

In two cases, N.36A and pSau3A9 were simultaneously mapped to the same chromosomes. The first case occurred in the Aijiao Nante/P16 F_2 population, where the two probes were mapped to chromosome 2, a distance of 2.6 cM apart (Fig. 4). The second case was in the 02428/Nanjing 11//Balilla population, in which the two probes were mapped to chromosome 8, some 3.3 cM apart (Fig. 4). Comparison of the mapping results for the two probes on chromosome 7 (Figs. 3 and 4) also indicated that the positions of the two probes were separated by only a few cM on the molecular linkage map.

Discussion

Although knowledge of the precise genetic locations of the centromeres is very important for studying the structure and function of the genome and also for molecular cloning of genes, genetic mapping of centromeres has been a challenge for plant geneticists. Traditionally, the centromere positions on genetic maps are determined by an aneuploid analysis that places the centromere between morphological markers, if such cytogenetic stocks are available for a particular species. Such traditional methods have been used in several plant species, including maize (Rhoades 1940), oat (McGinnis et al. 1963), cotton (Endrizzi and Kohel 1966), tomato (Khush and Rick 1968), and barley (Tsuchiya 1972; Fedak et al. 1974). Southern analysis of aneuploids can also be used to place individual DNA markers on specific chromosomal arms. The positions of the centromeres can then be inferred, making use of marker information on the linkage maps. Several different types of aneuploids have been utilized in such analyses, including the B-A translocations of maize (Weber and Helentjaris 1989), ditelocentric lines of wheat (Anderson et al. 1992), and trisomics in rice (Singh et al. 1996; Harushima et al. 1998), tomato (Frary et al. 1996), and maize (Schneerman et al. 1998). However, such aneuploid analyses can only assign the centromeric region to an approximate interval in the genetic map, although the length of this interval can be greatly reduced using molecular markers from high-density maps (see, for example, Harushima et al. 1998).

Two different approaches were developed recently to map the centromeres of all five chromosomes of *Arabidopsis thaliana*. The first approach involves tetrad analysis, and makes use of pollen mutants in which the four products of pollen meiosis remain attached (Copenhaver et al. 1998). The second approach is genetic mapping of a centromeric repeat using pulsed field gel electrophoresis and Southern analysis (Round et al. 1997).

The present study mapped centromere-associated markers to each of the 12 rice chromosomes using two cloned fragments as probes and the results showed a very good correspondence between the mapped probe loci and the centromeric regions determined in previous studies

using trisomic analyses. Such results confirm the locations of the centromeric regions on the molecular linkage maps determined in the previous studies, although the discrepancy in the centromeric location of chromosome 10 between the earlier results derived from trisomic analysis and the present study needs to be resolved.

The significance of this work lies partly in the technical approach, conventional Southern hybridization of the centromere-associated probes and linkage analysis of the resulting hybridization bands, which we used to resolve the locations of the centromeric regions as Mendelian loci on the molecular maps of the 12 rice chromosomes. This approach is very similar to the one adopted by Round et al. (1997) for mapping *A. thaliana* centromeres. The simplicity of this technique, together with the fact that these probes are also associated with the centromeres in other grass species, may provide a general approach to the mapping of centromeric regions in other cereal crops.

As in humans and other mammals, centromeric regions in plants are also full of repetitive DNA sequences (Aragon-Alcaide et al. 1996; Jiang et al. 1996; Round et al. 1997; Ananiev et al. 1998; Dong et al. 1998; Miller et al. 1998a, b; Presting et al. 1998). Retroelements are the major class of repetitive DNA in plants. The sequence similarity of pSau3A9 to retroelements suggests that rice centromeric regions consist, at least in part, of retroelements. Supporting evidence can also be found in the results of a study of retrotransposon mapping in rice, which showed that clusters of retrotransposons occur in the centromeric regions of several rice chromosomes (Wang et al. 1999). Retroelements may also be a constituent element of the centromeric regions in other cereal species, since sequences homologous to the pSau3A9 fragment have also been detected in a number of other cereal species including maize, wheat, barley, rye and oat (Jiang et al. 1996; Ananiev et al. 1998; Miller et al. 1998a; Presting et al. 1998). In addition to retroelements, rice centromeric regions may contain other types of repetitive sequences, as indicated by the fact that a BLAST search did not find any similarity between N.36A and either retroelements or other genes.

The centromere is one of the most important landmarks on the chromosome. The molecular linkage maps used in the present study combined the RFLP markers in two high-density maps, constructed by Cornell University group and the Japanese Rice Genome Research Program, respectively (Causse et al. 1994; Harushima et al. 1998). Localization of centromeric regions on the molecular linkage map makes the map a more complete and informative tool for genomic studies. The precise mapping of the centromeric regions will also facilitate the study of the structure and function of the rice centromeres.

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