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ACKNOWLEDGEMENTS. Financial assistance from CSIR to V.S.S.P. as SRF is acknowledged.

Received 30 December 2003; revised accepted 10 March 2004

Cloning and characterization of the *DMC1* genes in *Oryza sativa*

Shalaka S. Metkar, Jayashree K. Sainis* and Suresh K. Mahajan

Molecular Biology Division, Bhabha Atomic Research Centre, Mumbai 400 085, India

The *DMC1* gene is a major homologous recombination gene, expressed during prophase I of meiosis. We have isolated and analysed two *DMC1* genes, viz. type A and type B from rice. It was observed that *DMC1* type A is located on chromosome 12 whereas *DMC1* type B is on chromosome 11. The location of *DMC1* type A in the region on chromosome 12 that is duplicated on rice chromosome 11 is a new finding in this report. Earlier *DMC1* orthologues have been reported on chromosomes 9 and 11. Partially overlapping 5′ and 3′ cDNAs of one of the *DMC1* genes were obtained and used to generate the full-length *DMC1* gene, which was cloned and over-expressed in *E. coli*.

GENETIC recombination is a fundamental process in living cells. Studies on homologous recombination have potential applications in gene targeting[1,2] and in the development of apomictic varieties in plants like rice[3]. A number of plant recombination genes have been reported recently[4]. One of these is *DMC1*, which was identified in *S. cerevisiae* as a meiosis-specific homologue of the *E. coli recA* gene[5], required for recombination, synaptonemal complex formation and cell cycle progression[6] and later shown to be present in several mammalian, fungal and plant species[7]. The protein products [Dmc1] of the yeast *DMC1* and its human and basidiomycetes orthologues have been shown to possess biochemical properties similar to the bacterial RecA[8–10]. Plant *DMC1* orthologues have been identified in *Lilium longiflorum*[11,12], *Arabidopsis thaliana*[13,14], *Hordeum vulgare* (GenBank Accession Number AF234170), *Glycine max* (GenBank Accession Number U66836) and *Oryza sativa*[3,15,16], but so far there is no report on the characterization of any plant Dmc1 protein.

The important role of the *DMC1* genes during meiosis was demonstrated by gene expression analysis in meiotic tissue using RT–PCR, Northern blot and *in situ* hybridization in lily, *A. thaliana* and rice[3,12,14,17]. Immunofluorescence localization of the DMC1 (LIM15) protein in the leptotene and zygotene stages of meiosis prophase I was observed in lily[18,19]. The important role of this gene during meiosis was confirmed with the characterization of the *DMC1* mutant of *A. thaliana*[20]. This mutant showed drastically aberrant chromosome behaviour in prophase I; bivalent formation by pairing of homologous chromosomes was impaired and the ten univalent chromosomes

*For correspondence. (e-mail: jksainis@magnum.barc.ernet.in)

segregated randomly to the two poles, which resulted in abnormal pollen grain formation and drastically reduced fertility[20].

All the above results are consistent with a role for the Dmc1 protein in the homology search stage during chromosome pairing and synapsis. At the same time, it is important to note that the *DMC1* genes are also expressed in actively dividing tissues like cell suspension cultures, callus and root apices[3,14].

The present work involves isolation and characterization of the *DMC1* gene in rice and over-expression of one of these in *E. coli* to produce the Dmc1 protein. Initially, sequences of the *DMC1* gene from *A. thaliana* (*ArLIM15*) and lily (*LIM15*) were compared and degenerate primers containing inosine and wobbles at certain positions were designed on the basis of conserved regions. Figure 1 shows the positions and sequences of various degenerate and gene-specific primers used in this study with respect to introns and exons of the *DMC1* gene. PCR with degenerate primers P4 and P6 using genomic DNA of indica rice resulted in amplification of a 0.8 kb DNA fragment that on sequencing was confirmed to be a part of the rice *DMC1* gene corresponding to the region between intron 9 and exon 14 of *ArLIM15* (GenBank Accession Number U85613). There was 64% and 92% homology respectively in the nucleic acid sequence and the amino acid sequence levels between the rice *DMC1* gene and the corresponding region of the *ArLIM15*. This indicated the presence of a *DMC1* homologue in indica rice. This was further supported by Southern blotting experiments in which indica rice genomic DNA digested with various restriction nucleases was probed with wheat *DMC1* probe prepared by RT–PCR of the total mRNA isolated from a wheat callus. The primer P6 was used for the first strand cDNA synthesis and the P6–P15 pair was used for the PCR amplification to generate probe. This probe was 280 bp long and had sequence homology with the exons in the corresponding region (2069 bp to 2616 bp) of genomic DNA of *ArLIM15*. Single bands were seen with *Apa*I, *Eco*RI and *Eco*RV digests. However, three very closely spaced bands were obtained with *Bam*HI and *Sac*I digests (Figure 2), suggesting multiple copies of *DMC1* gene in rice.

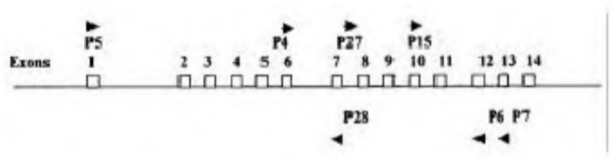

Figure 1. Primers used in this study. The positions of various primers used in this study and their directions are indicated. Primers P4 and P6 are degenerate and contain wobbles or inosine at certain positions. The remaining primers are gene-specific primers for the rice *DMC1* gene. The open boxes numbered 1–14 denote exons and the intervening lines denote introns in figure. The sequences of the primer [5′ to 3′] are as follows: P4, [tacatcgacacaga[g/a]ggiacitt]; P5, [tgtgtgagcatatggcgcggtccaa]–5′ upstream primer; P6, [tgicciccctgctggctt[c/t]ttigg]; P7, [gttcggatccttagtctttcgcatccattatt]–3′ downstream primer; P15, [accagtacaacttgctccttggcctcgctg]; P27, [ggaagacccagttggctcacact]; P28, [gacacatagagtgtgagccaact].

In order to isolate these *DMC1* genes, a *Hin*dIII genomic BAC library of *O. sativa* ssp *japonica* var *nipponbare* available with Dr K. S. Gill, University of Nebraska, Lincoln, USA was screened with the *DMC1* cDNA probe and 14 positive BAC clones were obtained.

Using the site [http://www.genome.clemson.edu/projects/rice/fpc], ten BAC clones were located on contig 224, on the rice chromosome 11, while four BAC clones were placed on contig 250, which lies on rice chromosome 12, indicating that they fall into two groups and were therefore classified as A and B types (Table 1). Contigs 224

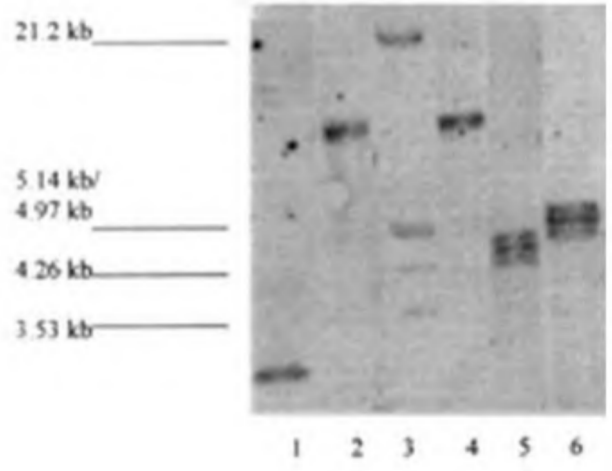

Figure 2. Southern hybridization of rice DNA with *DMC1* probe. Genomic DNA of rice was digested with different number of restriction enzymes, the restriction digests were run on a 1% agarose gel, transferred to nylon membrane and hybridized with the *DMC1* cDNA probe. Lane 1, *Apa*I; Lane 2, *Eco*RI; Lane 3, λ *Hin*dIII mol. wt marker, ^{32}P labeled; Lane 4, *Eco*RV; Lane 5, *Bam*HI; Lane 6, *Sac*I.

Table 1. Two groups of BAC clones from genomic library of japonica rice hybridizing to *DMC1* probe

Group I – *DMC1* type B Contig 224 – Chromosome 11		Group II – *DMC1* type A Contig 250 – Chromosome 12	
Sr. No.	BAC clone	Sr. No.	BAC No.
1	OSJNBa0027L04	2	OSJNBa0029J04
5	OSJNBa0030E22	3	OSJNBa0029O07
6	OSJNBa0030B22	4	OSJNBa0042O07
7	OSJNBa0017O22	14	OSJNBa0015L17
8	OSJNBa0004K03		
9	OSJNBa0039D03		
10	OSJNBa0029P24		
11	OSJNBa0047G16		
12	OSJNBa0011K13		
13	OSJNBa0010J24		

BAC clones 1 and 5 to 13 lie on contig 224, located on chromosome 11, while BAC clones 2, 3, 4 and 14 are located on contig 250, which is on rice chromosome 12. The group I BAC clones contain the type B *DMC1* gene, while the group II BAC clones contain the type A *DMC1* gene of rice.

and 250 are located within duplicated region of the rice genome present on chromosomes 11 and 12, close to the markers S10637A and S10637B respectively[21], which may explain the presence of two similar *DMC1* genes in rice[16]. To date, all other plants have shown only one type of *DMC1* gene but in rice the presence of *DMC1* orthologues on chromosomes 9 and 11 has been reported[3].

One BAC clone was taken from each of the two groups and the *DMC1* gene in it was sequenced (BAC clone OSJNBa0029O7 – type A, *OsDMC1*, GenBank accession number AY123338; and BAC clone OS–JNBa0027L04 – type B, *OsDMC1B*, GenBank accession number AF375982). Using the N- and C-terminal gene-specific primers P5 and P7 (Figure 1), we also amplified and sequenced the two types of *DMC1* genes from the genomic DNA of *O. sativa* ssp *indica* (GenBank accession numbers AY123340 and AY123339 – types A and B respectively).

Analysis of the four rice *DMC1* genes sequenced showed that there are 14 exons and 13 introns in each, consistent with the *DMC1* genes reported by others[3,15,16]. The organization of the rice *DMC1* gene was similar to that of the barley *DMC1* gene (GenBank Accession Number AF234170). They differed from the *AtDMC1* and *ArLIM15* genes of dicot *A. thaliana*, which have 15 exons. The exons 1 and 2 of *AtDMC1* are fused in the monocots, rice and barley, to constitute exon 1. The DNA sequence homology between the type A and type B genes is 90%, whereas within each type, the homology between japonica and indica cultivars is 99%. The deduced amino acid sequences of the Dmc1 proteins of both types obtained from this study as well as previous studies with other rice strains revealed variations at certain amino acid positions (Table 2). A careful analysis of these variations allowed us to cluster them into types A and B. The amino acids at positions 8, 93 and 288 can be used to determine the type of rice Dmc1 protein. Type A proteins have serine at amino acid position 8, isoleucine at position 93 and leucine at position 288, while type B proteins have aspartic acid, methionine and proline respectively at these positions. The Dmc1 proteins of lily, *Arabidopsis*, soybean and barley are similar to type B Dmc1 proteins from rice with respect to the amino acids at positions 8 and 288. The amino acid at position 93 shows either I or M in these plants.

As mentioned earlier, these two types may have arisen due to a chromosomal duplication event in rice. In addition, differential expression of types A and B *DMC1* genes has been reported[3]. Though both types of transcripts were observed during meiosis, the type B *DMC1* transcript was also expressed post-meiotically, during two haploid mitoses at the time of pollen maturation[3]. Thus, the two genes may have functionally diverged.

None of the plant *DMC1* genes have been cloned and over-expressed in bacterial systems. In the present work, *DMC1* type A gene was cloned and over-expressed in *E. coli*. Total RNA was isolated from the anthers of 2–3 selected spikelets in early meiosis prior to dyad stage of rice pollen mother cells (Figure 3) and used for RT–PCR. N- and C-terminal

Table 2. Variant amino acid positions in rice Dmc1 proteins in different strains of rice

Rice strain/GenBank Acc. No.	Gene	Type	Positions of amino acid variations 8	93	117	150	246	288
Japonica Nipponbare/AY123338*	*OsDMC1A*	A	S	I	E	A	E	L
Indica TSSR/AY123340*	*OsDMC1A-In*	A	S	I	K	A	K	L
Japonica A58/AB065111	*RiLIM15A*	A	S	I	K	A	E	L
Indica IR64/AF265548	*DMC1A*	A	S	I	K	T	E	L
Japonica Nipponbare/AB079873	*OsDMC1A*-RNA	A	S	I	K	A	E	L
Japonica Nipponbare/AF375982*	*OsDMC1*	B	D	M	E	T	E	P
Japonica A58/AB065112	*RiLIM15B*	B	D	M	E	T	K	P
Indica TSSR/AY123339*	*OsDMC1-In*	B	D	M	E	T	E	P
Indica IR64/AF265549	*DMC1B*	B	D	M	E	T	E	P
Japonica A58/AB064544	*RiLIM15B*	B	D	M	E	T	E	P
Japonica Nipponbare/AB079874	*OsDMC1B*-RNA	B	D	M	E	T	E	P

*This work.

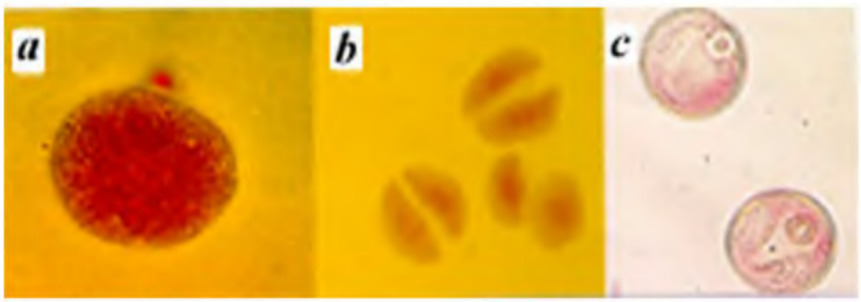

Figure 3. Rice anthers were isolated from the spikelets, placed in a drop of acetocarmine stain, covered with a cover slip and tapped lightly. They were observed under the light microscope to identify the stages of meiosis. ***a***, Early prophase in meiosis I; ***b***, Dyads; ***c***, Mature pollen grains.

primers P5 and P7 of the rice *DMC1* genes were designed on the basis of the genomic sequence. However, full-length *DMC1* cDNA could not be amplified probably due to the inability of reverse transcriptase to reach the end of the *DMC1* RNA. Therefore, reverse transcription was done to obtain partial 5′ and 3′ fragments of the type A *DMC1* cDNA and the full-length gene was generated by fusion of the 5′ and 3′ cDNA fragments by PCR (Figure 4 *a* and *b*). This cDNA was cloned in pET28a vector in *E. coli* BLDE3 host and over-expression of a protein around ~ 38 kDa with histidine tag was obtained (Figure 5). The identity of the protein was checked by performing a Western blot with anti His-tag antibodies as well as with anti Yeast Dmc1 antibodies. These antibodies showed a positive reaction with the ~ 38 kDa over-expressed protein (Figure 6 *a* and *b*, lanes 3 and 4), while the controls (cell extracts from host cells, lane 1; host cells containing vector alone, lane 2) did not show any reaction with antibodies. Leaky expression of OsDmc1 without IPTG was observed in the clone, which was used for Western blotting. Further work is aimed at the biochemical characterization of the over-expressed Dmc1 protein.

Based on the analysis of the chromosomal locations of the BAC clones hybridizing to the *DMC1* probe in the present work, we have observed the presence of two *DMC1* genes type A and B on rice chromosomes 12 and 11 respectively. Earlier, Kathiresan *et al.*[3] reported that *DMC1A* is located on chromosome 9 of rice and *DMC1B* is located on the short arm of chromosome 11. Interestingly, based on Southern hybridization, Shimazu *et al.*[16] reported the presence of more than two copies of the *DMC1* genes in 146 rice varieties. In Southern blot analysis we also observed three hybridizing fragments (Figure 2). Taken together, these observations indicate the presence of multiple copies (possibly three) of the *DMC1*-like genes in rice. In addition, the report[3] regarding the location of *DMC1A* gene close to the STS marker G123, which was earlier located[22] at position ~ 65.4 CM on chromosome 9L has an exciting possibility. This region shows synteny

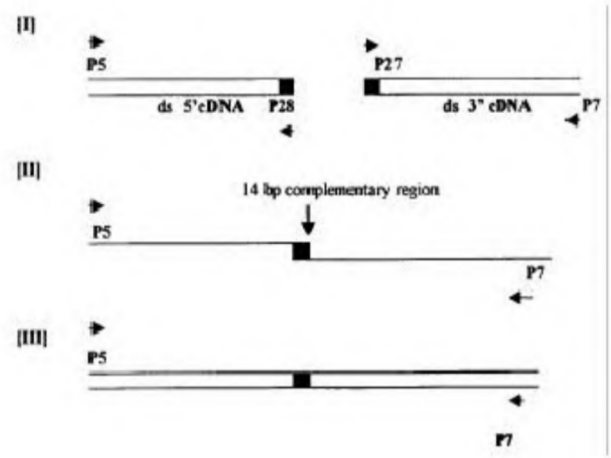

Figure 4 *a*. Generation of full-length rice *DMC1* cDNA. **I**, Primers P5 and P28 were used to amplify the 5′ fragment of the rice *DMC1* cDNA by PCR, while primers P27 and P7 were used to amplify the 3′ fragment. Primers P27 and P28 show a 14 bp overlap (see Figure 1 for sequence). **II**, Denaturation of the 5′ and 3′ cDNA fragments followed by annealing of the complementary 5′ and 3′ strands due to 14 bp overlap and extension to full length by Pwo polymerase. **III**, Subsequent PCR (with full-length cDNA as template and primers P5 and P7) amplified for 5 cycles, using an annealing temperature of 42°C and a further 10 cycles with annealing at 55°C, resulted in the amplification of full-length *DMC1* cDNA.

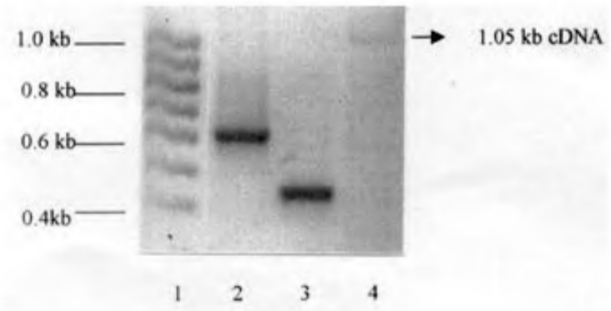

Figure 4 *b*. Agarose gel picture showing constructions of full-length rice *DMC1* gene. 1st strand was synthesized using oligo d[T] or primer P6 with the 1st strand cDNA synthesis kit from Amersham. The product of this reaction was used as template for PCR with Pwo polymerase, which gives blunt-ended PCR products. Lane 1, 100 bp ladder (up to 1 kb). Lane 2, partial 5′ ds cDNA, amplified with N-terminal primer P5 and internal primer P28; Lane 3, Partial 3′ ds cDNA, amplified with internal primer P27 and C-terminal primer P7; Lane 4, Full-length *DMC1* cDNA amplified with P5 and P7.

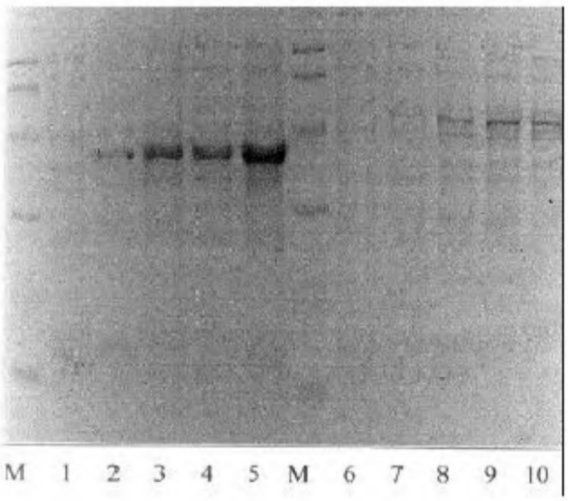

Figure 5. Over-expression of DMC1 in *E. coli*. The *E. coli BLDE3* host strain with the recombinant plasmid was grown overnight in LB containing kanamycin (25 µg/ml) and diluted 1 : 50 in fresh medium with kanamycin. After ~ 3 hours of growth at 37°C on shaker water-bath, IPTG was added at a concentration of 1 mM for the induction of the DMC1 protein. Samples (1 ml) were taken at 0, 1, 2, 3 and 4 h after induction. The cell pellets were boiled in 2X SDS–PAGE buffer for 3 min and centrifuged. The supernatants were run on SDS–PAGE gel and stained with Coomassie Blue R-250. Lanes 1–5 show the samples with *DMC1* insert (Lane 1, uninduced; lanes 2 to 5, induced for 1, 2, 3 and 4 h respectively). Lanes 6–10 show the corresponding controls for vector without the insert (Lane 6, uninduced; Lanes 7 to 10, induced for 1, 2, 3 and 4 h respectively). Lane M shows the molecular weight markers, 94 kDa, 67 kDa, 43 kDa, 30 kDa, 20.1 kDa and 14.4 kDa respectively from top.

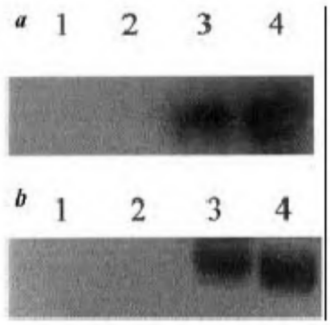

Figure 6. Western blot of over-expressed protein. Extracts of *E. coli BLDE3* (lane 1), *E. coli BLDE3* with pET28a vector (lane 2), *E. coli BLDE3* with pET28a vector with *DMC1* insert (uninduced) (lane 3), *E. coli BLDE3* with pET28a vector with *DMC1* insert (induced with IPTG) (lane 4) were subjected to SDS–PAGE, proteins were transferred on nitrocellulose and detected with anti-His tag antibody (***a***) and anti-yeast Dmc1 antibody (***b***).

with the group 5B chromosome of wheat, in the region between markers C1227 and R2790 (see Figure 1 in ref. 23), the deletion of which results in the Ph1 mutant phenotype. The Ph1 locus allows pairing of only homologous chromosomes and prevents pairing of homeologous chromosomes and the identity of the genes responsible for Ph1 phenotype is an active area of research[24]. The Ph1 locus has recently been shown to play a role in specific somatic and meiotic centromere association to prevent pairing of homeologous chromosomes. Deletion of this region in conjunction with the disruption of pairing of homologous chromosomes, possibly under the control of another gene, has been postulated to confer the Ph1 mutant phenotype[25]. The possible map location of the *DMC1A* gene within the Ph1 region derived from synteny between rice and wheat genomes and its role in homology search indicate a possible function for this gene in the Ph1 phenotype.

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ACKNOWLEDGEMENTS. We thank Dr K. S. Gill, University of Nebraska, Lincoln, USA for screening and sending the positive BAC clones and Dr D. Bishop, University of Chicago, Illinois for antibodies to the yeast DMC1 protein. We also thank Dr S. J. Jambhulkar for providing rice seeds and tissue samples; Dr D. C. Joshua for help with identification of meiotic stages in rice; Dr R. Mukhopadhyaya for help with automated DNA sequencing and C. Rajanikant for Western blot of the over-expressed Dmc1 protein. We are grateful to Dr B. J. Rao, TIFR for useful discussions. S. S. Metkar was a recipient of the UGC fellowship under the Joint UGC–CSIR Scheme, India.

Received 17 January 2003; revised accepted 31 March 2004