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Characterization of a *DMC1* homologue, *RiLIM15*, in meiotic panicles, mitotic cultured cells and mature leaves of rice (*Oryza sativa* L.)

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Abstract *DMC1* is one of the most important genes involved in meiotic homologous recombination in *Saccharomyces cerevisiae*. Homologues of *DMC1* have been isolated recently from some plant species, and in this study, we characterized the structure and expression of a *DMC1* homologue, *RiLIM15*, in a Japonica rice, strain A58. *RiLIM15* was found to be a gene family consisting of two genes, *RiLIM15A* and *RiLIM15B*, in the rice genome. The DNA sequence of *RiLIM15A* was highly homologous with that of *RiLIM15B* in the exon regions, although it was less homologous with that of *RiLIM15B* in the intron regions. Analysis for the expression of *RiLIM15* by a combination of Southern blot hybridization and reverse transcription-polymerase chain reaction (RT-PCR) showed that *RiLIM15* was expressed not only in meiotic young panicles, but also in mitotic cultured cells, although not in the mature leaves. Analysis of the sequences of these *RiLIM15* cDNAs amplified by RT-PCR showed that the sequences of exon 5 were deleted from the cDNAs derived from the meiotic young panicles. Also, exons 5, 10 and 11, as well as 29 bp of exon 8, were deleted from some types of cDNA from the mitotic cultured cells. These results suggest that these deletions may be caused by alternative splicing.

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Introduction

The homologous recombination of DNA strands in meiosis is an important mechanism for inducing genetic variation in animals, plants and fungi. In *Escherichia coli*, the RecA protein has a function in homologous recombination (DasGupta et al. 1981), and *recA* homologues have been cloned from many other organisms. All *recA* homologues found so far have a specific domain, a homologous core. The amino acid sequences of the homologous cores are highly conserved in bacteria, fungi, animals and plants. As the homologous core has the ATP-binding consensus sequences, motifs A and B, it has been suggested that the domain dealing with ATP and Mg^{++} is important for the recombination of DNA strands (Sung 1994).

DMC1, a *recA* homologue, has been isolated in *Saccharomyces cerevisiae* (Shinohara et al. 1992). It has been predicted that *DMC1* is required for homologous recombinations only at meiosis in *S. cerevisiae* (Bishop et al. 1992). Recently, *DMC1* homologues have also been isolated in some plant species, and it has been found that those of lily (*LIM15*) and *Arabidopsis thaliana* (*AtDMC1*) are specifically expressed in the early and middle stages of prophase I in the meiotic germ cells (Kobayashi et al. 1994; Klimyuk and Jones 1997). Analysis of a mutant of *AtDMC1* suggests that *DMC1* homologues of plant species must play an essential role in the pairing of homologous chromosomes during meiosis (Couteau et al. 1999).

A rice *DMC1* homologue, *RiLIM15*, was first cloned from an indica type strain IR36 by Sato et al. (personal communication). This gene consists of 14 introns and 13 exons. The size of the amino acid sequence predicted from the DNA sequence is 344 amino acid residues. The aim of the study reported here was to investigate further the structure and expression of *RiLIM15* in Japonica rice.

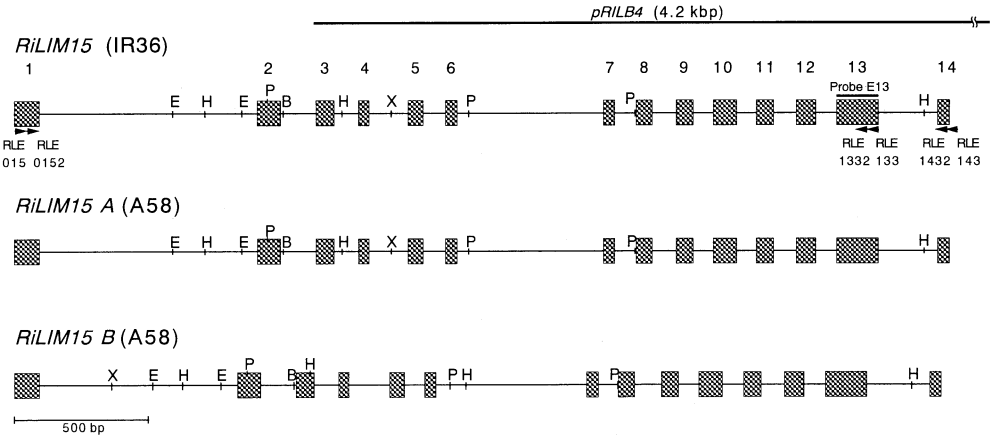

Fig. 1 Structures of the *RiLIM15* genes, *RiLIM15A* and *RiLIM15B*, isolated from strain A58 (Japonica rice) and the original *RiLIM15* from strain IR36 (Indica rice). The genes are composed of 14 exons (*hatched boxes*) and 13 introns (*thin lines*), respectively. *pRILB4* (*thick line* at the *top* of the figure) is a genomic subclone encoding *RiLIM15* isolated from the genomic library of IR36. Probe E13 (*thick line* above exon 13 of the IR36 *RiLIM15* restriction map), which covers the whole sequence of exon 13 derived from *pRILB4*, was used for Southern blot hybridization of RT-PCR. Primers RLE015, RLE0152, RLE133, RLE1332, RLE143 and RLE1432, used for the amplification of the *RiLIM15* genes and their cDNA in strain A58 are indicated by *arrowheads*. *B Bam*HI, *E Eco*RI, *H Hin*dIII, *P Pst*I, *X Xba*I

Materials and methods

Plant materials

Japonica rice strain A58 was used for the experiments. Mature leaves were sampled from the plants before the heading date. Young panicles at meiosis were obtained from leaf sheaths before the heading date and were sorted by length into early-stage (4–8 cm), middle-stage (8–15 cm) and late-stage (longer than 15 cm) meiosis. Calli induced from the scutella were subcultured in suspension medium for about 5 months.

Cloning and sequencing of the genomic clones of *RiLIM15*

Total DNA of mature leaves was isolated and purified by equilibrium centrifugation in CsCl-ethidium bromide gradients. Polymerase chain reaction (PCR) was carried out in a 100-ng aliquot of total DNA and the primers RLE015 (5′-ATG GCG CCG TCC AAG CAG-3′) and RLE143 (5′-TCA GTC TTT CGC ATC CAT TAT T-3′) (Fig. 1). Nested PCR was subsequently carried out with the first PCR product as a template and primers RLE0152 (5′-AGG CGG GCA GCT CCA GCT-3′) and RLE1432 (5′-CCA CCT GAT GTT ACC TGG AA-3′) (Fig. 1). The DNA fragment of *RiLIM15* amplified by PCR was cloned into pBluescript II KS(+), and the DNA sequence of the *RiLIM15* genomic clone was subsequently analysed with an automated sequencer (Li-Cor model 4000L).

Southern blot hybridization of reverse transcription (RT)-PCR product

Total RNAs of mature leaves, young panicles and calli were first isolated with guanidine isothiocyanate followed by centrifugation in CsCl solutions and then purified in a RQ RNase-free DNase (Promega). RT-PCR was carried out with 20-μg aliquot of total RNA as a template and primers RLE015 and RLE133 (5′-AGC TTC TCC CTC AGG CAG-3′) (Fig. 1). The RT-PCR product was separated on 2.0% agarose gel and then transferred and bound to a nylon membrane (Boehringer Mannheim) by baking. The DNA fragment of exon 13 of *RiLIM15*, probe E13 (Fig. 1), was used as a probe. The labeling of the probe and hybridization to the blot were carried out with a DIG DNA Labeling and Detection kit (Boehringer Mannheim). The blot was exposed to Kodak XJB-1 X-ray film.

Cloning and sequencing of the RT-PCR product

Specific amplification of the cDNA of *RiLIM15* from the RT-PCR product obtained above was performed using nested PCR with the RT-PCR products as templates and primers RLE0152 and RLE1332 (5′-AGG GGC ATC AAA GAT CTT GCA-3′) (Fig. 1). After the reaction, amplified cDNA was analysed by electrophoresis on a 2.0% agarose gel. Cloning and sequence analysis of the amplified *RiLIM15* cDNA were carried out by the method described above.

Results

The *RiLIM15* family consists of two genes

The rice *RiLIM15* gene was first cloned from a commercial genomic library of indica rice strain IR36. Additional Southern blot analyses of total DNA of another 146 rice varieties and strains using the IR36 *RiLIM15* genomic clone *pRILB4* as a probe suggested that the rice genome has more than two homologues of *RiLIM15*.

To obtain genomic clones of *RiLIM15* from Japonica rice strain A58, we amplified most of the coding regions from exon 1 to exon 14 by PCR using total DNA of mature leaves as a template and primers RLE015, RLE 0152, RLE143 and RLE1432 (Fig. 1). Based on the DNA sequences of the PCR products, we classified the clones into two types, indicating the presence of two copies of the gene, hereafter designated *RiLIM15A* and *RiLIM15B*. The sizes of the *RiLIM15A* and *RiLIM15B* sequences were 3,615 and 3,576 bp, respectively (Fig. 1), excluding exons 1 and 14 (whose sequences were not determined completely). The homology between the DNA sequences of *RiLIM15A* and *RiLIM15B* in the exon regions was very high (95.3%), while their homology in the intron regions was comparatively low

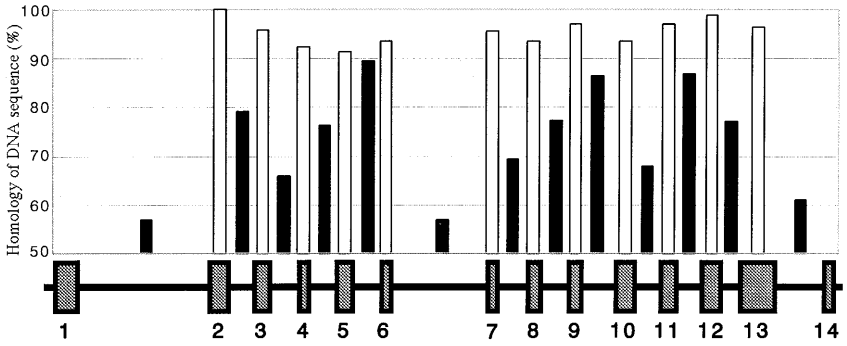

Fig. 2 Homologies between *RiLIM15A* and *RiLIM15B* DNA sequences in the region of exon and intron. The *white bars* show the homologies of the sequences of exons between *RiLIM15A* and *RiLIM15B*. The *black bars* show homologies of introns between *RiLIM15A* and *RiLIM15B*. The *number under* the bar chart shows the structure of *RiLIM15*. Exons and introns are indicated by *hatched boxes* and *thick lines*, respectively. Because the sequences of exon 1 and 14 have not been determined completely, it was not possible to calculate the homologies of these sequences

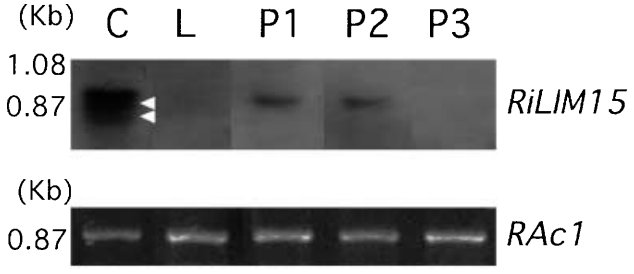

Fig. 3 Analysis of expression of *RiLIM15* in cultured cells, meiotic young panicles and mature leaves of strain A58. RT-PCR was carried out using total RNA extracted from calli subcultured for 5 months (*C*), mature leaves (*L*), and young panicles at early (*P1*), middle (*P2*), and late (*P3*) meiotic stages with primers RLE015 and RLE133 (**Fig. 1**). The *RiLIM15* cDNA amplified by RT-PCR was analysed by Southern blot hybridization with probe E13 (**Fig. 1**). Two types of amplified cDNA molecules shorter than the 0.94 kb of full-length cDNA are indicated by *open arrowheads*. RT-PCR analysis for the expression of a rice actin gene, *RAc1*, in these tissues was carried out as a control

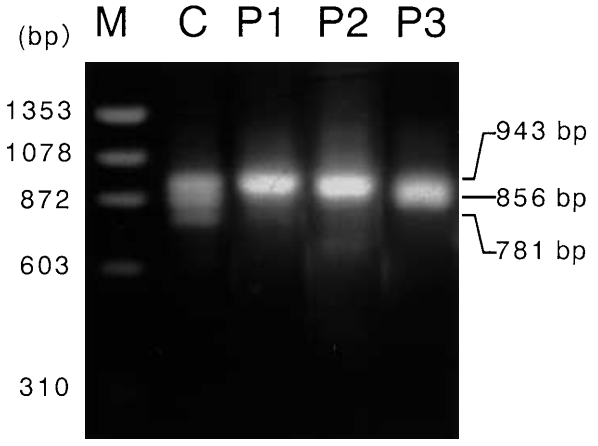

Fig. 4 Electrophoretic analysis of the amplified *RiLIM15* cDNAs derived from cultured cells and meiotic young panicles. *RiLIM15* cDNA was amplified by nested PCR using RT-PCR products derived from cultured cells (*C*), and from young panicles at early (*P1*), middle (*P2*), and late (*P3*) stages of meiosis. Primers RLE 0152 and RLE1332 (**Fig. 1**) were used in the PCR. The amplified cDNA was separated on 2.0% agarose gel. The size marker (*M*) was ϕX174 DNA digested with *Hae*III

(76.1%) (Fig. 2). These results indicate that *RiLIM15* in the rice genome may be composed of a multi-gene family with two types of genes. Comparison between the exon sequences for *RiLIM15A* and *RiLIM15B* revealed 28 transitions and four transversions. Three of the base substitutions gave rise to the substitutions of three amino acid residues. Thus, the amino acid sequences predicted from *RiLIM15A* and *RiLIM15B* are highly homologous (98.2%), suggesting that *RiLIM15A* and *RiLIM15B* may be functionally equivalent.

Expression of *RiLIM15* in young panicles at meiosis and in cultured cells

The expression of the *RiLIM15* genes was investigated in cultured cells, young panicles at early, middle and late stages of meiosis and mature leaves of A58. The cDNA was amplified from the total RNA samples by RT-PCR with primers RLE015 and RLE133 and then analysed by Southern blot hybridization with probe E13 (Fig. 1). We observed a single 0.98-kb band of amplified cDNA, just as predicted from the genomic DNA of *RiLIM15* (Fig. 3). This band was present not only in the young panicles at the early and middle stages of meiosis but also in the cultured cells. On the other hand, it was not detected in young panicles at late meiosis or in mature leaves. In addition to the 0.98 kb of cDNA found in the cultured cells, at least two unexpected smaller cDNAs were also amplified.

To analyze the sequences of *RiLIM15* cDNA amplified by RT-PCR, we amplified these cDNA fragments further by nested PCR using primers RLE0152 and RLE1332 (Figs. 1, 4). Ultimately, four types of *RiLIM15* cDNA clones were isolated. Type-1 cDNA, at 943 bp, had no deleted regions and was isolated from both cultured cells and young panicles. Type 2, at 885 bp, was characterized by the deletion of exon 5 of *RiLIM15* and was isolated from only the meiotic young panicles. It could not be separated from type 1 by agarose gel elec-

trophoresis analysis. Type 3, at 856 bp, was characterized by the deletions of exon 5 and part of exon 8 (29 bp) and could be isolated only from cultured cells. Type 4, at 781 bp, was characterized by the deletion of exons 10 and 11 and was isolated only from cultured cells. These deletions of the exons in the cDNA sequences may have been caused by alternative splicing.

Discussion

Expression of *RiLIM15*, which is believed to be meiosis-specific in rice, was detected in cultured cells and in meiotic young panicles, but not in mature leaves. These results give rise to two questions. The first question concerns the high expression of *RiLIM15* in cultured cells of rice. Doutriaux et al. (1998) demonstrated a high expression of *AtDMC1* in cultured cells with higher mitotic activity and a low expression in mature leaves with weaker mitotic activity. In the case of the rice plant, *RiLIM15* is strictly regulated so that it is expressed only in meiotic germ cells, but this expression may be deregulated in mitotic cultured cells that are dividing actively. The second question revolves around the function of the transcripts from *RiLIM15* in cultured cells of rice. Using *A. thaliana* and rice, Doutriaux et al. (1998) and Akama et al. (1999), respectively, reported that a *recA* homologue, *RAD51* was also expressed in suspension-cultured cells and in meiotic flower buds. *RAD51* functions not only in meiotic homologous recombination by interacting with *DMC1* (Bishop 1994) but also in the repair of broken DNA strands in *S. cerevisiae* (Game 1993). Moreover, Loh et al. (1987) and Giorgetti et al. (1995) obtained some proof of genetic segregation in somaclonal regenerants of tomato and carrot. It may be assumed that the high and simultaneous expression of *DMC1* and *RAD51* homologues in mitotic cultured cells of plant species induce a certain homologous recombination in mitosis.

Large differences in the DNA sequences of the intron regions of some genes may have effects on splicing. McCullough et al. (1996) constructed five types of artificial genes and investigated the splicing patterns of these genes in the nuclei of leaf cells of *Nicotiana benthamiana*. The 5' and 3' regions of the introns of these genes had different sequences originating from the substitution of nucleotides. One mRNA that was transcribed from these genes had a deletion of one exon. This was similar to the deletion that might arise from alternative splicing, thereby indicating that the sequences of introns may influence the regulation of splicing. In this study also, our analysis of cDNA sequences of *RiLIM15* showed that the extensive difference in intron sequences between *RiLIM15A* and *RiLIM15B* appears to have an effect on the splicing patterns, since it was found that a certain cDNA with a deletion of some exons arose from only one type of *RiLIM15*, namely from *RiLIM15A* or *RiLIM15B*.

mRNA with some deleted regions was produced in both cultured cells and meiotic young panicles of rice. The deletions of exons 5, 10, 11 and part of exon 8 in type-2, -3 and -4 cDNAs might result in the deletion of the amino acid sequences located in the homologous core of putative RiLIM15 proteins. In addition, the deletion of exon 5, and exon 5 and part of exon 8 in type-2 and -3 cDNAs, respectively, might result in the substitutions of many amino acid residues by a frameshift in the homologous core. The deletion of part of the homologous core in the cDNA sequence of the *DMC1* homologue isolated from mouse testis has been reported by Habu et al. (1996). These deletions should markedly affect the functions of DMC1-like proteins. Nevertheless, they indicated that a DMC1-like protein with deleted regions might have a function in the regulation of meiotic homologous recombination. However, the real function of a mouse DMC1 protein with deletions and also putative RiLIM15 proteins of type-2, -3, and -4 cDNAs in this study has not been clarified.

Analysis of the cDNA sequences of *RiLIM15* indicated that some of these sequences appear to be combinations of sequences derived from *RiLIM15A* and *RiLIM15B*. This suggests two possible reasons for the formation of these chimeric cDNA sequences: one is *in vivo* intermolecular splicing (*trans*-splicing); the other is artificial recombination between cDNAs from *RiLIM15A* and *RiLIM15B* by PCR. The actual cause of the formation of these chimeric cDNAs was not established in this experiment.

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