RIRE1*, a retrotransposon from wild rice *Oryza australiensis

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RIRE1 is a retrotransposon present in wild rice *Oryza australiensis* in an extraordinary number of copies, and only a portion of the LTR sequence has been determined previously. Here, we isolated and sequenced DNA segments of various portions of *RIRE1*, revealing that the sequences of LTR and the internal region were 1523 and 5277 bp in length, respectively. The internal region shows homology with the *pol* region in *copia*, a *Drosophila* retrotransposon, indicating that *RIRE1* is a *copia*-like retrotransposon. The internal region of *RIRE1* contained an open reading frame coding for genes, *gag*, *pro*, *int*, *rt* and *rh*, like *copia* and retroelements related to it. A clone screened from a library of the *O. australiensis* genomic DNA contained solo LTR, which was flanked by direct repeats of a 5-bp sequence. This suggests that *RIRE1* generates a duplication of the target sequence of 5 bp upon retroposition. We observed that many *RIRE1* members were nested by another *RIRE1* member. This indicates that these *RIRE1* members have received another *RIRE1* to make an extraordinary number of copies in the *O. australiensis* genome without giving a deleterious effect on the growth of rice cells.

INTRODUCTION

Retrotransposons are mobile genetic elements, which make their copies by retroposition (for a review, see Bingham and Zachar, 1989). Structural and functional analyses of retrotransposons in *Drosophila* indicate that they are similar to proviruses, the integrated forms of retroviruses (for a review, see Varmus and Brown, 1989). Many retrotransposons in plants have been identified and characterized. Examples are *Ta1-3* in *Arabidopsis* (Voytas and Ausubel, 1988), *Tnt1* in tobacco (Grandbastien et al., 1989), *del* in lily (Smyth et al., 1989), *PDR1* in pea (Lee et al., 1990), *Wis-2-1A* in wheat (Lucas et al., 1992; Murphy et al., 1992), *Tos3-1* in rice (Hirochika et al., 1992), and *BARE-1* in barley (Manninen and Schulman, 1993). Homologues of some of these elements seem to be present ubiquitously in many kinds of plants (Flavell et al., 1992; Moore et al., 1991; Voytas et al., 1992; Hirochika and Hirochika, 1993).

Retrotransposons have long terminal repeats (LTRs), which are present as direct repeat sequences at their 5' and 3' ends. The LTR sequence is bound by 5'-TG·· and ··CA-3' and contains functional sites, such as enhancer, promoter, polyA signal, termination signal, etc. The internal region flanked by two LTRs contains an open reading frame(s), which is divided into two regions, *gag* and *pol*.

gag is the gene encoding an RNA-binding protein, Gag, which encloses RNA transcribed from retrotransposons in the chromosome and forms virus-like particles. *pol* is the region which is highly homologous to that in some retroviruses and encodes several kinds of proteins, such as protease, integrase, reverse transcriptase and RNase H, which are required for retroposition. A primer-binding site (PBS) exists in the region immediately downstream of one LTR at the 5' end, which has a complementary sequence to the 3' portion of tRNA encoded by the host. tRNA annealing to PBS works as a primer to initiate reverse transcription of RNA to synthesize (–) DNA strand. A polypurine tract (PPT) exists in the region immediately upstream of the other LTR at the 3' end, which is a site used to initiate synthesis of (+) DNA strand.

We have previously used a PCR method to analyze tandem repeat sequences in rice genomes, and found that the pOa4 sequence (511 bp in length), which has been reported as a tandem repeat sequence in wild rice *Oryza australiensis* (Zhao et al., 1989), is actually a segment of an interspersed DNA sequence (Nakajima et al., 1996). The sequences expanded from pOa4 show partial homology with the LTR sequences of *BARE-1* and *Wis-2-1A* (Nakajima et al., 1996). This indicates that pOa4 and the sequences expanded from it are derived from a retrotransposon, which is named *RIRE1* (for Rice RetroElement 1) (Nakajima et al., 1996). Copy numbers of LTR of *RIRE1* have been determined to

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be about 7500 and 180 per haploid genome of *O. australiensis* and *O. sativa* cv. Nipponbare, respectively (Nakajima et al., 1996). In this paper, we report the entire nucleotide sequence of *RIRE1* in *O. australiensis*, and show that *RIRE1* is a *copia*-like retrotransposon with a long LTR. Many *RIRE1* members with an insertion of another *RIRE1* member existed in the *O. australiensis* genome with an extraordinary number of copies of *RIRE1*. Significance of this result will be discussed.

MATERIALS AND METHODS

Plant DNA preparation. Total DNA was isolated from the leaves of 2 weeks-old seedlings of *O. australiensis* W1538 according to the method described previously (Ohtsubo et al., 1991).

PCR. PCR (polymerase chain reaction) was carried out as described (Tenzen et al., 1994). Primers used are listed in Table 1. They were chemically synthesized using DNA synthesizer oligo 1000 M (Beckman). For cloning of the PCR products, they were ligated to pCRTMII vector by using the Cloning Kit (Invitrogen) and the ligation product was transformed into *Escherichia coli* XL1-Blue MRF⁺ cells

(STRATAGENE). The ampicillin-resistant transformants were selected on plates containing 100 µg/ml of ampicillin.

The frequency of mutations induced by PCR under the condition described above is 1 per 642 bp (Tenzen et al., 1994).

Construction of genomic libraries and screening clones from libraries. A genomic library of rice were constructed as described by Sambrook et al. (1989), using Lambda EMBL4/*Eco*RI Vector Kit and Gigapack Gold Packaging Extract (STRATAGENE).

To construct a plasmid genomic library, total DNA from the *O. australiensis* was digested with *Bam*HI, and the digested fragments were ligated with plasmid pUC118 (Vieira and Messing, 1987) which was also digested with *Bam*HI. The ligated molecules were transformed into *E. coli* XL1-Blue MRF⁺ cells, and the ampicillin-resistant transformants were selected on plates containing 100 µg/ml of ampicillin.

Hybridization. The recombinant phage plaques or transformants were subjected to blotting and hybridization with the *RIRE1* probe, pRN28, as follows: The recombinant phage plaques or transformants were transferred to a nylon membrane filter Hybond-N⁺ (Amersham), as described by Sambrook et al. (1989). The filter was incubated at 65°C for 20 min in 4 ml/100 cm² of a hybridization solution, consisting of 6× SSC (0.9 M NaCl, 0.09 M trisodium citrate), 5× Denhardt's solution [0.1 % Ficoll (Pharmacia), 0.1% polyvinylpyrrolidone (Nakarai), 0.1% (w/v) bovine serum albumin (Seikagaku Kogyo)], 0.5% (w/v) SDS, and 100 µg/ml of sonicated salmon sperm DNA (Sigma). The filter was soaked in 2 ml/100 cm² of the hybridization solution containing ³²P-labeled probe DNA, and incubated at 55°C for 12 to 18 h. The filter so treated was washed two times in 2× SSC containing 0.1% SDS for 5 min at 55°C and 0.1× SSC containing 0.1% SDS for 10 min at 55°C.

The DNA fragment used as probe was cut out of plasmid pRN28 and labeled with [³²P]dCTP (Amersham, 185 TBq/nmol) using Random Primer DNA Labeling kit Ver. 2 (Takara).

Nucleotide sequencing and computer analysis of the sequences obtained. DNA sequencing was carried out by the dideoxynucleotide chain termination method (Messing, 1983; Sanger et al., 1977), using dye-labeled primers (-21M13, PR1) and ABI PRISM Dye Primer Cycle Sequencing Ready Reaction kit (Perkin Elmer) with AmpliTaq DNA Polymerase, FS, or using dye-labeled terminator DyeDeoxy Terminator Cycle Sequencing kit with AmpliTaq (Perkin Elmer) and using relevant oligodeoxyribonucleotides primers (see Table 1). Heat-denatured plasmid DNA were used as templates. Sequencing reaction was carried out using Catalyst A800 (Perkin Elmer) and the reaction products were run using ABI 373S-36 DNA Sequencer.

Table 1. Synthetic oligodeoxyribonucleotide primers used

Primer ^a	Sequence ^b (5'→3')	Position ^c
E1	ATCCAACACAATGTGTCTCT	316- 298
E5'	GTGCCATTAGTTGGTCTCA	347- 366
E6'	TGTTAGTGATATGCCCT	1- 17
E6	ATCCCTTTGCCCTCACGAT	726- 709
E7	ggatccGGCTCTGATACCA	1543-1525
E8'	TACGACGCTTAAGTG	891- 906
E10	CCAGGAACACTTGCAAGTA	1467-1449
E12'	CTGTTGACGGCTCGGCTGAGTT	804- 825
E13	CGTGATATCCAACATCTCC	7- 29
E14'	AACGATCTATCGGCCCTT	1432-1449
KN5'	AGGGACTGCGTGGATATT	3132-3150
KN6	AGCGTACGAGACATCTGG	6178-6161
KN10	CTGTTGGTGTGGCATCTC	2597-2580
KN11'	CAGTAGCTCATAGTTGCAAG	3750-3769
KN15	CGAATATTCCAACTCCGAGA	5702-5683
KN16'	GGTTTACTTAACAAGGCTATC	2515-2535
KN17	CCTTCTCAATCTCTACTTC	3808-3789
KN18	CCATATCTACTAAAGTCATCG	4414-4403
KN19'	GACCAATGAGCTCAACG	4432-4358
KN20'	TCGATCCAGAATCGCCT	5618-5634

^a Primers with non-primed numbers hybridize to the strand corresponding to the *RIRE1* transcript. Primers with primed numbers hybridize to the complementary strand. A pair of primers E7/E8' were used to obtain clones pRN116 and 118 (see Fig. 2). Similarly, five pairs of primers, E14'/KN10, KN16'/KN17, KN11'/KN18, KN19'/KN15, and KN20'/E13, were used to obtain clones pKN20 ~ pKN29 (see Fig. 2).

^b Lowercase letters indicate nucleotides of a linker.

^c Numbers indicate coordinates to the *RIRE1* sequence (see Fig. 4).

Alignments of sequence data were carried out using HarrPlot 1.2.2 and GENETYX-MAC 7.3 programs. A search for homology of nucleotide and amino acid sequences was performed by running the programs FASTA and BLAST against the sequences in GenBank and EMBL.

Accession number. The nucleotide sequence data reported will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession number D85597.

RESULTS

Determination of the entire nucleotide sequence of *RIRE1*. We have previously shown that pOa4 is the sequence not repeated in tandem, but is a portion of LTR of a retrotransposon (named *RIRE1*) interspersed in the *O. australiensis* genome. In the previous experiments to show this, we carried out PCR using a pair of primers, Oa4b and Oa4f, which hybridize to the pOa4 sequence and prime DNA synthesis in one and the other directions, respectively (see Fig. 1), and obtained the fragments containing the 5' portions of the LTR sequence (see representative clones, pRN11, pRN17 and pRN28 shown in Fig. 2). Note that these fragments are derived from the *RIRE1* members with an insertion of another *RIRE1* member at different positions in the LTR sequence of *RIRE1*, as shown in Figure 1.

To determine the 3' portion of the LTR sequence of *RIRE1*, we obtained a clone, pRN134, by screening from a phage library containing *Bam*HI fragments of the *O. australiensis* genomic DNA using pRN28 containing a 5' portion of LTR

Fig. 1. Schematic representation of the structure of *RIRE1* and a model for amplification of an *RIRE1* segment by PCR. Positions of LTRs and internal regions containing *gag* and *pol* of *RIRE1* are indicated at top. An *RIRE1* element with an insertion of another *RIRE1* element at a site within an LTR sequence is shown. Note that the *RIRE1* complex has region *a* in an LTR which is connected with region *b* in another LTR, as shown. PCR using a pair of primers which hybridize to the sequences in the pOa4 sequence (shaded boxes) in LTRs can amplify a fragment shown at the bottom. Arrowheads indicate primers (Oa4f and Oa4b) used to amplify the fragment from total DNA of *O. australiensis* (Nakajima et al., 1996). Numbers are coordinates given to the consensus sequence of *RIRE1* (see Fig. 4).

Fig. 2. Schematic representation of the structure of *RIRE1* and clones containing various portions of *RIRE1*. Thick lines indicate portions of *RIRE1* carried by the clones. pRN134 carries an LTR sequence, which cannot be assigned to the sequence at the left or right side of *RIRE1*; therefore, the LTR sequence in pRN134 is indicated by two thick lines, which are separated by a dotted line, but are actually connected at sites indicated by asterisks in this figure. Numbers along the structure of *RIRE1* are coordinates to the *RIRE1* sequence (see Fig. 4). TSD (open triangles), target site duplication of the sequence 5'-GGTCG-3'; LTR (open boxes), long terminal repeat; PBS, primer-binding site; PPT, polypurine tract; *gag*, *gag* region; *pol*, *pol* region; *pro*, aspartic protease gene; *int*, integrase gene; *rt*, reverse transcriptase gene; *rh*, RNase H gene.

Element	PBS	PPT
<i>RIRE1</i>	5' - ca G-TGGTATCA-GAGCTAATCT...//...AGTGCAAGTGGGAGAT tg - 3'	
<i>BARE-1</i>	ca-TGGCATCGT GAGCTAGGTT ...//...AGTGCAAGTGGGAGAC tg	
<i>Wis-2-1A</i>	caACTGGCATCAT GAGCCAGGTC ...//...AGTGCAAGTGGGAGAC tg	
<i>Tnt1</i>	caACTGGTATCA-GAGCACAGGT...//...GACTGGAGGGGGAGAT tg	
<i>PDR1</i>	ca--TGGTATCAAGAACTCCCGA...//...CCAGCTTGAGGGGAG-tg	
<i>Tos3-1</i>	caAGTGGTATCA-GAGCCTCGTT...//...GAAGGGGGAGAT tg	
tRNA _{Met}	TGGTATCA-GAGCCAGGTT	

Fig. 3. Primer-binding sites (PBS) and polypurine tracts (PPT) in the internal regions of retrotransposons. The complementary sequence to the 3' region of methionyl tRNA (tRNA_{Met}) which initiates (–) strand DNA synthesis is also shown. Two di-nucleotide sequences of *RIRE1* that are boxed and indicated by lowercase letters are of the LTR sequences, which are immediately adjacent to the internal sequence (see Fig. 2). These sequences are also present in other retrotransposons as shown. Boldface letters indicate PBS and PPT sequences in each retrotransposon.

as probe (see Fig. 2). Comparison of the sequence of pRN134 with that in pRN28 and with those of the other clones to be described below revealed that pRN134 contained solo LTR bound by 5'-TG- and -CA-3' without the internal region of *RIRE1* (Fig. 2). The solo-LTR sequence was flanked by direct repeats of a 5-bp sequence, 5'-GGTCG-3' (see TSD in Fig. 2).

We also obtained another clone pRN121 by screening from a plasmid library containing *Bam*HI fragments of the *O. australiensis* genomic DNA using pRN28 as probe. Nucleotide sequencing revealed that pRN121 contained not only the 5' portion of LTR, which may correspond to that present at the right side of *RIRE1* (see Fig. 2), but also PPT (Fig. 3). As described below, this clone also contained the 3' portion of the *rh* gene in the *pol* region (see Fig. 2).

To determine the entire internal region of *RIRE1*, we carried out PCR to clone the fragments containing the PBS region adjacent to LTR at the 5' end of *RIRE1* using a pair of primers (E13, E14'; Table 1), which hybridize to the end regions of LTR and prime DNA synthesis towards outside of the sequence. This is based on the assumption that if *RIRE1* members with an insertion of another *RIRE1* member exist in the internal region as depicted in Figure 1, the fragments containing the PBS region could be amplified by PCR using the primers described above. Nucleotide sequencing of several clones obtained revealed that two clones (pRN5 and pR N7; Fig. 2) contained PBS, which showed homology with the PBS sequences in other plant retrotransposons and contained a complementary sequence to the 3'-end region of tRNA_{Met} (Fig. 3). There existed the putative *gag* region downstream of PBS (Fig. 2). We then carried out PCR using a pair of primers (KN5' and KN6'; Table 1), which hybridize to the sequences at the 3' end of pRN7 and at the 5' end of pRN121. Sequencing of a clone pRN19 (Fig. 2) obtained revealed that it contained the *pol* region of *RIRE1*, leading to determination of the entire nucleotide sequence of *RIRE1*.

To confirm the entire sequence of *RIRE1* determined above, we carried out PCR using relevant pairs of primers, which hybridize to appropriate sites in the *RIRE1* sequence (Table 1), and obtained clones containing DNA segments of

RIRE1 (pRN116, pRN118, and pKN20 ~ 29; Fig. 2). Nucleotide sequencing of these clones revealed that they contained *RIRE1* sequences with a sequence difference, almost by base substitutions. From the sequences obtained, we could derive a consensus sequence of LTR and the internal region of *RIRE1*, 1523 and 5277 bp in lengths, respectively (Fig. 4). Comparison of the nucleotide sequences of the clones with the consensus sequence revealed that a few base changes, mostly by substitutions, occurred at a frequency of 1.1 ~ 3.4%, which is higher than the frequency (about 0.2%) of mutations induced by PCR under the condition used in the present experiment. This suggests that the clones obtained are derived from several copies of *RIRE1* interspersed in different loci. Note here, however, that in the *pol* region, which is conserved well among retrotransposons, *RIRE1* showed sequence homology of about 48.2% with that in *copia*, a *Drosophila* retrotransposon (Fig. 5A), and of about 66.5% with that in *BARE1*, a *copia*-like retrotransposon in barley (Fig. 5B). These numbers are much lower than that (about 98.5%) of any one of the clones containing the *pol* region. Note also that more than 72% of the base substitutions observed in the internal coding region did not change amino acids or changed to conservative amino acids. We, therefore, believe that the consensus sequence obtained is the master sequence of *RIRE1*, from which many copies of *RIRE1* have been originated.

Coding region. The internal region (5277 bp) contained one large open reading frame, which could code for genes, *gag*, *pro*, *int*, *rt*, and *rh*, encoding Gag protein, protease, integrase, reverse transcriptase, and RNase H, respectively, in this order (Figs. 2 and 4). This confirms that *RIRE1* is related to *copia* and the *copia*-like retrotransposons, which encode such genes in the same order and produce their proteins as a polyprotein.

The *gag* gene encodes an RNA-binding protein, Gag, which has been demonstrated to mediate positioning of the tRNA on (–) strand of retroviral RNA and initiating reverse transcription (Prats et al., 1988). There exists amino acid motif C (2) C (4) H (4) C which is conserved in a large family of nucleic acid-binding proteins (Berg, 1986). In

1 LTR100
 TGTAGTGATATGCCCTAGAGGCAACATGGAGATGTTGGATATCACGTCAATGTTTATGTATAAATGAATAAAGTGTATTTCAGTTCATGAAATAGACATCTTGTATTGATCAATAAGT
 .200
 ACGTGACTTATTTATGAGACTCTATATGTATGAATCTATTTCATAACGATCCCTGATCATATGTCTATGTTGGAAACAAATATGATTCTAAGATCGGCATGTTTATTGATTGATGATCA
 .300
 TGTCTCATAGATCATGGGTATGGAGATACCAATCAATAAACATGGACATATGTGTTAGAGAACACATTGTTGGATAGACCCACCATGAGACACTACAGGAATTAATGTGCCATTAGTTG
 .400
 GTCTCAGGTAGTGTGGTACAAAGTCTTAGACCTGAGATCACCATGGATTCCAACATGTGTAGTAGCCTACTTTGGGACTACCAAACGCTATTCCGTAAGTGGGTAGTTATAAAGGTAG
 .500
 TTTTCGGGTTTGCTATGAACATGGAGTGGGATGTGAGTGATCAAGATGGAATTTGCCCTCCTTTGGAGAGATATCTCTGGGCCCCCTCGAGGTAATGGATTATGGAAATGCATGGCCAT
 .600
 GCTAAGTTGATTGAGGAGTCAATCAACAGACAGATAATCCAATACGGATCGAGTGAATGGTTAAGCTATTGAAGGGATGGCACACATCTTGCCTATAGCTTAACTGGTATCGTGAGGCAA
 .700
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 .1400
 GATTGATCTGGAACAAGTTTTGCGCACGGGTTGATTAGATCAATCGGTTTTACGGGATTTGTTGAGGAGAGTTGATGTAATTCGCTTCCTTGATAGTGTATCGGAACGACGTAATGTG
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 .1523
 TCGGCCCTTACTTGCAAGTGTTCCTGGTTTTCGCGGTAAAAAGTTTTTGTTCGCGGTAGCGTAGCTTCCGCGTAACCCCTTCASTGGTATCAGAGCTAATCTTGCTTAGTTCCGGTTTTA
 .1600
 GATTGATCTGGAACAAGTTTTGCGCACGGGTTGATTAGATCAATCGGTTTTACGGGATTTGTTGAGGAGAGTTGATGTAATTCGCTTCCTTGATAGTGTATCGGAACGACGTAATGTG
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 .2800
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 .3200
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 .3600
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 .4200
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 .4600
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 .4700
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 .4800
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.4300
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G L L H S F D F E S F E T C E S C L L G K M T K A P F T G H S E R A S D L L A L
.4400
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V H T D V C G P M S S T A R G G Y Q Y F I T F T D D F S R Y G Y I Y L M R H K S
.4500
GAATCCTTTGAAAAGTTCAAAGAATTCAGAAATGAAGTACAGAATCATTAGGGAAAACAATCAAGTTTCTACGATCAGACCGTGGAGGGGAATACGTGAGCCAAGAGTTTGGTAATCAT
E S F E K F K E F Q N E V Q N H L G K T I K F L R S D R G G E Y V S Q E F G N H
.4600
CTGAAAGATGTGGAATTTGTCCACAGTTGACTCCGCCAGGAATCCACAATGGAACGGAGTGTCCGAACGGAGAAATCGCACCTTGTGGACATGGTGGCGTGCATGATGAGCCAAAGT
L K D C G I V P Q L T P P G T P Q W N G V S E R R N R T L L D M V R S M M S Q S
.4700
GATCTTCCGTATCCTTCTGGGGATATGCTCTTGAACAGCTGCGCTCACAATAAGAGTTCCATCTAAGTCAGTTGAAAAGACACCATATGAGATATGGACAGGGCAACCCCTAGT
D L P L S F W G Y A L E T A A L T L N R V P S K S V E K T P Y E I W T G Q P P S
.4800
TTGTCTTTTCTCAAAATTTGGGGATGTGAGGCTTATGTAAACGTTTACAATCTGACAAGCTCACACCCAAATCTGACAAGTGCTTCGTTGTGGGATATCCTAAGGAACTAAGGGATAT
L S F L K I W G C E A Y V K R L Q S D K L T P K S D K C F V V G Y P K E T K G Y
.4900
TACTTTTATAATCGGGAACAGGCAAGTGTGTGCGCCGACATGGTCTCTTCTGGAGAAAGAGTTTCTTTCAAGAAGGGTTAGTGGGAGCAGGGTGCATCTTGAAGAAGTTCAAGAA
Y F Y N R E Q G K V F V A R H G V F L E K E F L S R R V S G S R V H L E E V Q E
.5000
ACACCAGAAAATGTTTCAGGACCACAGAACCAACAGGAGGACCAAGTGTTCGCCGCCAGTGTAGATACACAGCCCGACGGAGTCTGAAAGATCAGCTCGTGCCTGACAGG
T P E N V S A T T E P Q Q E D Q S V A P P V V D T P A P R R S E R S R R A P D R
.5100
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Y T G A E Q R D I L L L D N D E P K T Y E E A M V G P D S N K W L G A M K S E I
.5200
GAATCCATGTACGACAATCAAGTTTGAAGCTTGGTTGATCCACCTGATGGTGTCAAAACCATGAGTGTAAATGGGTTTAAAGAAAAGGCCGATATGGATGGAAATGTTACATCTAT
E S M Y D N Q V W N L V D P P D G V K T I E C K W V F K K K A D M D G N V H I Y
.5300
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K A R L V A K G F K Q I Q G V D Y D E T F S P V A M L K S I R I I L A I A A Y F
.5400
GATTATGAGATATGGCAGATGGATGTCAAAACGGCTTCTCTAAATGGAAACCTAAGCGAGGATGTATACATGATACACCTCAGGGTTTGTGCGATCCAGAATCGCCTGGAAAGATATGC
D Y A E I W Q F L N G N L S E D V Y M I Q P Q G F V D P E S P G K I C
.5500
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K L Q K S I Y G L K Q A S R S W N I R F D E V I K G F G F I K N E E E A C V Y K
.5600
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.5700
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.5800
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H D S K K G F L P M S H G I N L S K N Q C P Q T H D E R N K M G M V P Y A S A I
.5900
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G S I M Y A M L C T R P D V S Y A L S A T S R Y Q S D P G E G H W T A V K N I L
.6000
AAGTACTTGAGAAGAACTAAGGATATGTTCTAGTCTATGGAGGTGAAGAGCTCGTTGTAAGTGGTTACACCGATGCTAGCTTCCAAACAGACAAGGATGATTATAGATCGCAATCT
K Y L R R T K D M F L V Y G G E E E L V V S G Y T D A S F Q T D K D D Y R S Q S
.6100
GGGTCGTGTTCTGCTGAAACGGAGGCGAGTCACTGGAAGAGTTCCAAGCAGGATGTTGCTGATTCTACAACGGAGGCGAGTACATTGCTGCTTCGGAAGCTGCAAAGGAGGCT
G F V F C L N G G A V S W K S S K Q D T V A D S T T E A E Y I A A S E A A K E A
.6200
GTTTGGATCAAGAAATTCGTTCTGAGCTTGGTGTGATGACTAGTACGACTGGTCCAATGTCTCTCTATTGTGATAATAGTGGAGCCATTGCGCAAGCCAAGGAGCGGAGGTACATCAG
V W I K K F V S E L G V M T S T T G P M S L Y C D N S G A I A Q A K E P R S H Q
.6300
AAGTCCAAACACATACTTCGCGGATATCATCTCATCCGCGAGATAGTGGACAGAGTGTGTAAGATGCAAAAGTGCACACGGATCTCAACATAGCCGATCCGCTGACAAAACCTCTC
K S K H I L R R Y H L I R E I V D R G D V K I C K V H T D L N I A D P L T K P L
.6400
CCTCAGCCGAGCATGAGGCGCACACAAGAGCAATGGGTATTAGATACTTACATGATTGACTCTAGTGAAGTGGGAGATTCCTAGTATATGCC (1492 bp) TCCGCGTAACCTTCA
P Q P K H E A H T R A M G I R Y L H D *

```

Fig. 4. The nucleotide sequence of *RIRE1*. This sequence is a consensus sequence, which was derived from the sequences of the fragments in the clones shown in Fig. 2. Numbers are coordinates given to the *RIRE1* sequence. Boldface letters indicate the LTR sequences, in which the LTR sequence at the 3' end of *RIRE1* is supposed to be the same or almost identical to the LTR sequence at the 5' end, and the middle region of LTR at the 3' end is thus abbreviated in this Figure. Two di-nucleotide sequences, which are commonly present at the ends of LTR of retrotransposons, are boxed. PBS and PPT sequences are underlined. The other sequences underlined in LTR are explained in the text. Vertical arrows indicate start sites of genes, *gag*, *pro*, *int*, *rt* and *rh*. See a schematic representation of the structure of *RIRE1* shown in Fig. 2.

RIRE1, the coding region corresponding to *gag* existed at positions from 2827 to 3675 (Fig. 4) and contained this motif (Fig. 6).

In the *pol* region, the *pro* gene encodes an aspartic protease which cleaves the polypeptide. An amino acid sequence D-T-G in proteases is suggested to be an active site

with a zinc-finger motif, which is supposed to be involved in DNA binding (Johnson et al., 1986). The coding region corresponding to *int* existed in *RIRE1* at positions 4123–5022 (Fig. 4) and contained this zinc-finger motif (Fig. 6). Besides the zinc-finger motif, another motif D, D (35) E involving in processes of integration is conserved in integrases encoded by retroelements (Khan et al., 1991; Kulkosky et al., 1992; Doak et al., 1994). This motif was also present in *int* in *RIRE1* (Fig. 6). The *rt* gene encodes a reverse transcriptase which is generally conserved well in many retroelements. Site-specific mutagenesis in the amino acid sequence Y-X-D-D in the conserved region has decreased the reverse transcriptase activity severely, indicating that the sequence is one of the active sites (Lader et al., 1987). In *RIRE1*, the coding region corresponding to *rt* existed at positions 5023–6288 (Fig. 4), and contained this sequence (Fig. 6). The *rh* gene encodes RNase H which contains the D (34) E (19) D (51) D motif which is involved in metal-binding (Davies et al., 1991). The coding region corresponding to this gene in *RIRE1* existed at positions 6289–6777 (Fig. 4), and contained this motif (Fig. 6).

The size of LTR in *RIRE1* was similar to those in two plant retrotransposons of *copia* type, *BARE-1* in barley (Manninen and Schulman, 1993) and *Wis-2-1A* in wheat (Moore et al., 1991; Lucas et al., 1992; Murphy et al., 1992), which carry long LTR of 1829 and 1755 bp, respectively. The LTR sequence in *RIRE1*, however, showed poor homology with those of *BARE-1* and *Wis-2-1A* (less than 50%), although the internal region showed good homology with those in the retrotransposons (see Fig. 5B for the homologous relationship between *RIRE1* and *BARE-1*). Mouse mammary tumor virus (MMTV) carries a long LTR, 1330

Fig. 5. Dot matrix between the nucleotide sequences of *RIRE1* and *copia* (A) or *BARE-1* (B). Dots are placed at locations of the identical nucleotides when more than 25 nucleotides out of 50 are identical. Structures of retrotransposons with LTR at the 5' end and the internal region are shown at top and on the right side.

[illegible]

Fig. 6. Amino acid sequences containing motifs or particular sequences conserved in the proteins encoded by retrotransposons. Amino acids boxed indicate those important in motifs or sequences. Asterisks indicate amino acids conserved in more than 4 retrotransposons.

bp in length, but this LTR did not show any homology with LTR of *RIRE1*.

LTR of retrotransposons are divided into three functional regions, U3, R, and U5 (Temin, 1981, Birnstiel et al., 1985; Varmus, 1983): The U3 region contains a promoter for RNA polymerase II, and the R region is defined to be the region from a cap site to a polyadenylation site; the U5 region is defined to be the region downstream of the polyadenylation site. It has been pointed out that variations in size occur in the U3 region in retroviruses (Temin, 1981). LTR of *RIRE1* contained sequence CCACT (see Fig. 4) at positions 1053–1057, which is a promoter sequence in Rous sarcoma virus (RSV) (Swanstrom et al., 1981). Another promoter sequence, TATAA, was present in the region at positions 1079–1083, 19 bp downstream of the CCACT sequence in *RIRE1* (Fig. 4). The termination signal, TTGT, for retroviral RNA synthesis (Temin 1981) existed at positions 1334–1487 in the LTR sequence of *RIRE1* (see Fig. 4).

Insertion of one *RIRE1* member into another. As described earlier, PCR using a pair of primers, which hybridize to the end regions of LTR and prime DNA synthesis towards outside of the sequence, produced the fragments containing a portion of *RIRE1* due to the presence of many *RIRE1* members with an insertion of another *RIRE1* member (see Fig. 1). We investigated the insertion sites of *RIRE1* further by PCR using several pairs of primers, which hybridize to either end region of LTR and with a region

inside of LTR (Fig. 7). Electrophoresis of the PCR products in a polyacrylamide gel and sequencing of some of them could locate many insertion sites in LTR of *RIRE1* (Fig. 7). We could also locate the insertion sites of *RIRE1* in the internal region of *RIRE1* by analyzing the PCR products using primers which hybridize to an end region of LTR and to a region in the internal region (data not shown). The insertion sites determined do not seem to be clustered in a particular site or region (see Fig. 7).

DISCUSSION

In this paper, we have determined the entire nucleotide sequence of *RIRE1*, and shown that *RIRE1* is a typical *copia*-like retrotransposon. *RIRE1* becomes the first retrotransposon of rice whose entire sequence has been determined. A clone (pRN134; Fig. 2) screened from a genomic DNA library contained solo LTR which was flanked by direct repeats of a 5-bp sequence. It is known that retroposition of retrotransposons and retroviruses generates a duplication of a target site sequence of several bp in length (Bingham and Zachar, 1989). Since solo LTR is supposed to be produced by recombination between two LTR sequences present at the ends of retrotransposons (Roeder and Fink, 1980), the result above indicates that *RIRE1* generates a duplication of a 5-bp sequence at the target upon retroposition.

We have described here that *RIRE1* has a long LTR se-

Fig. 7. Insertion sites of *RIRE1* in the LTR sequence of *RIRE1*. Short vertical bars indicate insertion sites mapped by lengths of the PCR-amplified fragments which were determined by gel electrophoresis. Long vertical bars indicate insertion sites mapped by cloning and sequencing of the PCR-amplified fragments. The PCR fragments were amplified using primer E14', which hybridizes to the 3'-end region of LTR and primes DNA synthesis towards the outside of LTR, and the other primer, such as E1, E6 or E10 (open arrowheads), which hybridizes to the sequence inside of LTR and primes DNA synthesis from right to left (see Table 1). These fragments were used to map the insertion sites shown above the structure of LTR. The PCR fragments were also amplified using primer E13, which hybridizes to the 5' end region of LTR and primes DNA synthesis towards outside of LTR, and the other primer, such as E5' E6' or E12' (solid arrowheads), which hybridizes to the inside sequence of LTR and primes DNA synthesis from left to right (see Table 1). These fragments were used to map the insertion sites shown below the structure of LTR. Horizontal lines with an arrowhead indicate the largest PCR fragments analyzed.

quence like *BARE-1* in barley and *Wis-2-1A* in wheat. LTR of *RIRE1* shows poor homology with those of *BARE-1* and *Wis-2-1A*, although those in the latter retrotransposons are highly homologous each other (Manninen and Schulman, 1993). In the LTR sequence of *Wis-2-1A*, there exist many direct and inverted repeats of short DNA sequences. It is supposed that genetic alteration caused by an imprecise excision of a transposon happened to be inserted into LTR of *Wis-2-1A* or slippage occurring during reverse transcription have elongated the LTR sequence (Lucas et al., 1992). *RIRE1* also contained many direct and inverted repeats like *Wis-2-1A*, suggesting that the same kinds of events described above might have occurred in an ancestor retrotransposon of *RIRE1* to lengthen its LTR.

Retroposition of retrotransposon *Tnt1* in tobacco is activated by treatment with cell wall hydrolases used to release protoplasts (Pouteau et al., 1991). Retroposition of retrotransposon *Tos17* in rice is activated during culturing and regeneration of rice tissues (Hirochika et al., 1996). To see whether retroposition of *RIRE1* is activated during culturing and regeneration of tissues of *O. sativa* like *Tos17*, we carried out Southern hybridization analysis of total DNA prepared from the regenerated rice plants from protoplasts, and observed no extra bands attributed to retroposition of *RIRE1* (H. Hirochika, H. Ohtsubo and E. Ohtsubo, unpublished results). From this experiment, however, we cannot determine whether *RIRE1* is activated or not, since the *O. sativa* genome contains about 180 copies of *RIRE1* (Nakajima et al., 1996), and the new bands, even if appeared, might be overlapped with the bands of the fragments derived from each copy of *RIRE1* preexisted in the rice genome.

The LTR sequences of *RIRE1* are present in *O. australiensis* in about 7500 copies per haploid genome (Nakajima et al., 1996). In this paper, we have shown that there existed many *RIRE1* members with an insertion of another *RIRE1* member. This indicates that these *RIRE1* members have received another *RIRE1* member to make an extraordinary number of copies of it in the *O. australiensis* genome without giving a deleterious effect on the growth of rice cells. Many retrotransposons isolated in eukaryotes are inserted at a specific region, such as the 5' regions of genes (for a review, see Sandmeyer et al., 1990). We do not know at present whether *RIRE1* is also inserted in the 5' region of genes or not. We know, however, that *RIRE1* is not inserted into any particular sites in the LTR sequence of itself. We know also that *RIRE1* resides throughout the chromosomes in *O. australiensis* as revealed from a study of fluorescence in situ hybridization (FISH) using LTR of *RIRE1* as a probe (Ohmido, Fukui, E. Ohtsubo and H. Ohtsubo, unpublished results).

The results of flow cytometry indicate that the size of the haploid genome of *O. sativa* is 434 Mb, while the size of *O. australiensis* is 960 Mb, about 2 times larger than that of *O. sativa* (Martinez et al., 1994). The number of copies

of LTR of *RIRE1* in the haploid genome of *O. sativa* is about 180, while it is 7500 in *O. australiensis* (Nakajima et al., 1996). This indicates that *RIRE1* caused the increase of size of 11 Mb (if all the LTRs are present as solo LTR) or 31 Mb (if all the LTRs are portions of *RIRE1* with the internal region) in *O. australiensis*. It is, therefore, considered that the increase of *RIRE1* copies has contributed in part to the increase of size of the genome of *O. australiensis*.

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