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Existence of retroelements in rubber (*Hevea brasiliensis*) genome

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Abstract

Retroelements are dispersed as interspersed repetitive sequences throughout the host genome and exploited as genetic tools for plant genome analysis. A reverse transcriptase (RT) gene fragment of *Hevea* was cloned indicating the presence of retrotransposons – a class of mobile genetic elements in the *Hevea* genome for the first time. In order to clone full-length retroelements as well as to identify their extent of diversity, a *Hevea* genomic library was screened for retroelements using reverse transcriptase (RT) gene fragment as the probe and consequently 23 positive clones were identified. Sequence analysis of positive clones screened for retroelements, showed homology of eight clones with nucleotide sequences of putative non-LTR retrotransposon RT in *Arabidopsis thaliana*, RT in *Medicago truncatula*, Ty3-Gypsy type of retrotransposons in *Oryza sativa*, viral gag/pol polyprotein from *Pisum sativum* and polyprotein of *Ananas comosus* suggesting abundance of retroelements in rubber genome. Existence of RT sequences in amplified NBS region (nucleotide-binding site of the resistance gene analogue) from rubber indicated insertion of retro-sequences in disease resistance genes, which might have a negative role on the functionality of the respective gene.

Key words : *Hevea brasiliensis*, retrotrasposons, resistance gene analogues (RGAs)

Introduction

Retroelements (class I transposable elements) are nobile genetic elements that are ubiquitous in ukaryotes and constitute a major portion of the nuclear enome. Retroelements are classed into two categories, etroviruses and retrotransposons. If a retroelement codes ully functional infective agent, it is classed as a etrovirus. If it has substantial missing functionality, it s a transposon. According to prevailing theory, etrotransposons are retroviruses, which got incorporated nto the ancestral germline and altered through mutation.

Retrotransposons (RTNs) encode the proteins needed or their own propagation, and through cycles of eplication have come to represent major fractions of he genome in many eukaryotes. Long terminal repeat LTR) RTNs are ubiquitous in plant genomes (Flavell *et l.*, 1992; Kubis *et al.*, 1998) and are often present in igh copy number. The replicative mode of transposition llows retrotansposons to generate genetic diversity by altering the size and organization of the host genomes and thus plays a major role in maintaining genome plasticity (Kumar and Hirochika, 2001). Because of their ubiquity and diversity, RTNs have great potential as genetic markers for plant genome and biodiversity analysis (Lee *et al.*, 1990; Kumar *et al.*, 1997; Waugh *et al.*, 1997; Ellis *et al.*, 1998; Kalendar *et al.*, 1999; Pearce *et al.*, 2000).

Although recent studies of *Hevea* genome provide some insight into the genome organization, there are no reports regarding characterization of retrotansposable elements and thereby no marker system has been developed based on retrotransposons in *Hevea brasiliensis*.

The aim of the present study is to demonstrate the existence of retrotransposons and their extent of diversity in rubber genome. Use of retro-sequences for generating potential markers for genome analysis has also been discussed.

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Materials and Methods

Plant material and DNA extraction

The clone 'RRII 105' of *H. brasiliensis* was chosen for the present study. Total genomic DNA was extracted from 1 g of young leaves following the CTAB (cetyl trimethyl ammonium bromide) protocol of Doyle and Doyle (1990) with minor modifications (Saha *et al.*, 2002).

Amplification and cloning of reverse transcriptase (RT) gene fragment

A primer-pair (5' CATCGACSAAATGTTAGA AAGG 3' and 5' TTTTTCCCA-ATTTAGGACCAG 3') for the amplification of RT was designed based on the conserved regions of the aligned RT sequences, derived from GenBank and synthesized at Sigma-Aldrich, Bangalore. PCR amplification was performed in a total volume of 50 µl containing 100 ng of template DNA with 0.5 µM of each primer, 0.2 µM of each dNTP, 2 units of Taq DNA polymerase (Amersham Biosciences) 5 µl of 10X DNA polymerase buffer [100 µM Tris-HCl (pH 9.0), 500 µM KCl, 20 µM $MgCl_2$]. Amplifications were performed in a thermal cycler (GeneAmp PCR System 9600, Perkin Elmer Cetus) with an initial denaturation at 94°C for 3 min, followed by 35 cycles of 30 sec at 94°C, 1 min at 58°C and 2 min at 72°C with a final extension of 10 min at 72°C and subsequent cooling at 4°C. Amplified products were separated on 1% agarose gel in 1X TAE buffer. The gels were stained and viewed on UV transilluminator.

The amplified putative RT fragment was purified using GFX column and ligated into pGEM-T vector. The ligated products were subsequently used for transformation of E. coli cells (DH5a). Nucleotide sequences of the cloned PCR products were determined by sequencing reaction using a BigDye Terminator Cycle Sequencing kit and the products were run with an Applied Biosystems ABI 3700 Sequencer. Sequencing reactions were primed on both the strands using either the T7 or SP6 promoter sequences of the pGEM-T vector.

Identity of the putative RT fragment

The nature of cloned sequences was confirmed by performing computer based homology searches with known retrotransposon sequences from other plants in the NCBI database using BLASTN and BLASTX algorithms (Altschul *et al.*, 1997).

Southern blot and hybridization

Genomic DNA (5 µg) of 'RRII 105' was completely digested with the restriction enzymes *Eco*RI and *Sau*3AI (New England Biolabs) separately, following the manufacturer's instructions, fractionated on 1% agarose gel and then transferred to Hybond-N+ (Amersham Biosciences) nylon membranes as described in Sambrook *et al.* (1989). The blotted membrane was hybridized with radioactively labelled RT probe. The RT fragment was released from the vector by *Eco*RI digestion, eluted from agarose gel, purified and used as probes after labeling with α^{32}P-dCTP by random priming method. Hybridization was performed in Rapid-Hyb buffer (Amersham Biosciences) at 60°C. The membrane was washed twice in a solution containing 0.5X SSC and 0.1% SDS at 60°C and then exposed to X-ray film.

Construction and screening of genomic library

Hevea genomic library of the clone RRII 105 was constructed in lambda vector (ZAP Express, Stratagene). Genomic DNA was completely digested with the restriction enzymes *Eco*RI and *Xho*I, purified through GFX column (Amersham Biosciences), cloned into ZAP Express vector and subsequently packaged in Gigapack III gold packaging extract following the manufacturer's protocol. This phage was plated after incubation with *E.coli* XL-1 Blue cells. Plaques were transferred to Hybond-N+ nylon membranes (Amersham Biosciences) according to standard procedures (Sambrook *et al.*, 1989), screened through plaque hybridization and probed with a radiolabelled (α^{32}P-dCTP) RT fragment. The positive plaques were recovered individually and were subjected to a second round of screening following the same procedure. The recombinant lambda vector from each of the positive plaque was converted into pBK-CMV phagemids by *in vivo* excision that allowed insert characterization in the plasmid system. All the positive clones were subjected to PCR amplification using vector directed T3 and T7 promoter primer-pair to determine the size of the inserts. Positive clones were sequenced from both ends of the inserts using a BigDye Terminator Cycle Sequencing kit and the products were run on a Applied Biosystems Sequencer ABI 3700 at Macrogen Inc., Korea, to validate the presence of retrotransposons in *Hevea* genome. All the sequences were subjected to homology search with known retrotransposon sequences from other plants in the NCBI database, as mentioned earlier.

Amplification and sequence analysis of resistance gene analogues (RGAs)

Putative RGAs were amplified from rubber using the primers based on conserved motifs existing in the nuclear binding site (NBS) regions of the disease resistance genes of several plant species. Amplified fragments were cloned and sequenced as described earlier and sequence analysis was performed to find out homology with

retrotransposons.

Results and discussion

Isolation of RT fragments from Hevea genome

A PCR product of 349 bp was obtained from *Hevea* genomic DNA using degenerate oligonucleotide primers, specific for reverse transcriptase (RT) gene (Fig.1). The amplified fragment was cloned and sequenced (Fig. 2). Encoded amino acids of the putative RT clone showed maximum sequence homology with the RT sequences (protein) of *Medicago truncatula* retrotransposon (ABE89984; E value 2e-54), followed by *Arabidopsis*, *Oryza*. *Pisum* and many more retro-sequences in the GenBank, which indicated the presence of retrotransposons – a class of mobile genetic elements in the *Hevea* genome. Presence of stop codon, detected in the translated sequences revealed a loss of reverse transcriptase activity of the RT gene fragment isolated from *Hevea*. Alignment of aminoacid sequences of the RT clone with other retrotrasposon sequences (Fig. 3) showed presence of properly translated primer sequences at both the ends (5' IDQMLER and 3' LVLNWEK).

Abundance of retrotansposons

The abundance of retrotransposons in *Hevea* genome was analyzed by Southern hybridization against genomic DNA of *Hevea brasiliensis* digested with *Eco*RI and *Sau*3AI. Cloned RT from *Hevea* was used as the probe. Very-strong hybridization signals were detected with the *Eco*RI restricted DNA at high molecular weight range, whereas with Sau3AI digested DNA, signals were at the low molecular weight range (Fig. 4) confirming abundance of RT sequences in *Hevea* genome.

A genomic library was constructed in lambda vector for isolation and characterization of retrotrasposons present in *Hevea* genome. A total of 23 positive clones were isolated from the genomic library after screening with homologous RT gene fragment. All these clones were converted to phagemid for further characterization. PCR amplification was carried out using T7 and T3 primer-pair to assess the size of the cloned fragments, which ranged between 0.6 kb to 4.0 kb (Fig. 5) Clones were designated as HRTN-1 to HRTN-23. Phagemids were isolated and sequenced. Putative amino acid sequences of each clone were compared with those reported from other plants for retrotransposons. Results of sequence analysis were presented in Table 1, which showed the presence of both LTR and non-LTR retrotransposons in rubber. Out of 23, eight genomic clones showed significant homology with retro-sequences. Two clones HRTN-3 and HRTN-15 showed maximum homology with putative non-LTR retroelement reverse transcriptase of *Oryza sativa* (japonica cultivar-group). Clones HRTN-9 and HRTN-13 shared sequence identity with putative 22 kDa kafirin cluster of Ty3-Gypsy type LTR retroelements from *Oryza sativa* (japonica cultivar-group) and also with polyprotein of *Ananas comosus*. Clone HRTN-11 showed maximum homology with reverse transcriptase of *Medicago truncatula* and clone HRTN-14 with retroelements from *Oryza sativa*. Sequences of the clones HRTN-17 and HRTN-20 were related to gag/pol polyprotein of *Pisum sativum*. Nucleotide variation was also noticed among similar sequences of retrotransposons from *Hevea* genome.

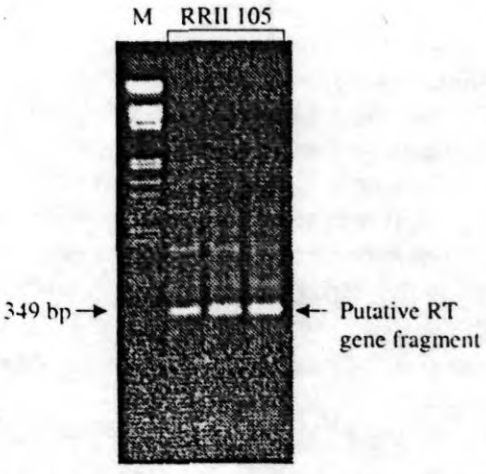

Fig. 1 PCR amplification of reverse transcriptase (RT) gene fragment from genomic DNA of the cultivated clone RRII 105 of *Hevea brasiliensis*. M, molecular weight marker (Lambda DNA/ *Eco*RI+*Hin*dIII).

Putative reverse transcriptase (RT); sequence length 349 bp

CATCGACCAAAATGTTAGAAAGGTTAGCGAAGCACTCTTATTTCTGTTATCTAGATGGGTA
TTCGGGATTCTTTCAAATTCCTATTCACCCAGAAGACTAAGAAAAGACAACATTCACTTA
TCCCTATGGAACATTTGCATATAGGAGAATGCCTTTTGGTCTTTGTAATGGCCCTGCTACC
TTTCAAAGATGCATGATAGCTATCTTTTCTAATTATATTGAAGATATCATGGAAGTTTTTA
TGGATGATTTTTCTGTCTATGGAACTACTTTTGATGATTGCCTAGCTAATTTATCTAAGGT
GTTGCAAAGATGTGAAGAATCAAACCTGGTCCTAAATTGGGAAAAA

Encoded amino acid sequence, number of amino acids 115

IDQMLERLAKHSYFCYLDGYSGFQIPIHPED*EKTTFTYPYGTFAYRRMPFGLCNGPATFQR
CMIAIFSNYIEDIMEVFMDDFSVYGTTFDDCLANLSKVLQRCEESNLVLNWEK

Fig. 2. Nucleotide sequence of the PCR amplified reverse trancriptase gene fragment from rubber (top) and their encoded amino acid sequences (bottom). Flanking underline sequences are primer-binding sites. Stop codon (*) is detected within the sequence.

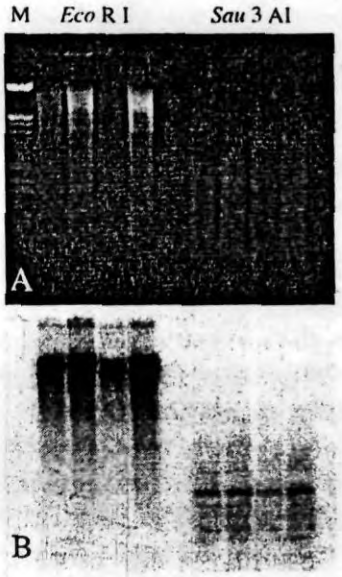

Fig. 4. Southern hybridization of *Hevea* genomic DNA digested with *Eco*RI and *Sau*3AI against RT probe. A, digested DNA on agarose gel; B, autoradiogram of the hybridized blot. M, molecular weight marker (Lambda DNA/ *Eco*RI+*Hin*dIII).

CLUSTAL W (1.83) multiple sequence alignment

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Arabidopsis RT          STWVSPVHCVPKKGGMTVVKNSKDELIPTRTITGHRKCIDYRKLNAASRKDHFPLPFIDQ 60
Pea RT                  SSWVSPVHVVPKKGGTSVILNEKNELIPTRTVTGWRVCIDHRRLNTATRKDHFPLPFIDQ 60
Medicago RT             SKWVSPVQVVPKKGGLTVIKNDKNESIATRTVTGWRMCIDYRKLNKATRKDHFPLPFIDQ 60
Rubber RT (putative)    ------------------------------------------------------------IDQ 3
Rice RT                 SEWVSPVQVVPKKGGMTAVANAQNELIPQRTVTGWRMCIDYRKLNKATKKDHFPLPFIDE 60
Cotton RT               SDWVSWVHVVPKKTGVTVVKNSSGELVPTRVQNRWRVCIDYRKLNAATRNDHFPLPFIDQ 60
                                                                                **:

Arabidopsis RT          MLERLANHPYYCFLDGYSGFFQIPIHPNDQEKTTFTCPYGTFAYKRMPFGLCNAPATFQR 120
Pea RT                  MLERLAGHEYYCFLDGYSGYNQIVVAPEDQEKTAFTCPYGIFAYRRMPFGLCNAPATFQR 120
Medicago RT             MLERLAKHSHFCYLDGYSGFFQIPIHPNDQEKTTFTCPFGTFAYRRMPFGLCNAPATFQR 120
Rubber RT (putative)    MLERLAKHSYFCYLDGYSGFFQIPIHPED-EKTTFTYPYGTFAYRRMPFGLCNGPATFQR 62
Rice RT                 MLERLANHSFFCFLDGYSGYHQIPIHPEDQSKTTFTCPYGTYAYRRMPFGLCNTPASFQR 120
Cotton RT               MLERLANKTHYCCLDGYSGLFQIPVAPEDQDKTTFTCPFGTFAYRRMSFGLCNAPATFQR 120
                        ****** : .:* ****** ** : *:* .**:** *:* :**:** ***** **:***

Arabidopsis RT          CMTSISSDLIEEMVEVFMDDFSVYGSSFSSCLLNLCRVLKRCEETNLVLNWEKCHFMVRE 180
Pea RT                  CMTSIFSDMLEKYMKVFMDDFSVFGSSFDNCLANLSLVLQRCQETNLVLNWEKCHFMVQE 180
Medicago RT             CMMSIFSDFVEKIMEVFMDDFSVHGSNFDDCLTNLEKVLERCEQVNLVLNWEKCHFMVRE 180
Rubber RT (putative)    CMIAIFSNYIEDIMEVFMDDFSVYGTTFDDCLANLSKVLQRCEESNLVLNWEK------- 115
Rice RT                 CMMSIFSDMIEDIMEVFMDDFSVYGKTLGHCLQNLDKVLQRCQEKDLVLNWEKCHFMVCE 180
Cotton RT               CMVSIFSDYVEKIIEFFMDDFTVYGNSFNECLDNLAKILQRCLEFNLVLNYEKCHFMVDK 180
                        ** :* *: :*. ::.*****:*.*..:. ** **  :*:** : :****:**

Arabidopsis RT          GIVLGHKI 188
Pea RT                  GIVLGHKI 188
Medicago RT             GIVLGHLV 188
Rubber RT (putative)    --------
Rice RT                 GIVLGHRV 188
Cotton Rt               GLILGHIV 188
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Fig. 3. Alignment of amino acid sequences of reverse transcriptase (RT) originating from *Hevea* with other plant species showing presence of properly translated primer sequences both the ends (5' IDQMLER and 3' LVLNWEK).

An interesting observation was made while working with resistance gene analogues (RGAs) in *Hevea*. A primer-pair based on NBS conserved motifs was used to amplify resistance gene analogues. Sequences of 16 putative RGAs clearly showed homology with retro-sequences (Table 2). Significant level of sequence heterogeneity was noticed among these clones. This observation reveals that retrotransposons are inserted within the disease resistance gene sequences of *Hevea*. However, association of retrotransposons with disease resistant marker or insertion of retrotransposon within the gene itself had already been reported in several plant species. Kenward *et al.* (1998) cloned and sequenced a randomly amplified polymorphic DNA (RAPD) marker associated with black root resistance in tobacco and found it to be a part of the retrotransposon Tnd-1. A BARE-1 RTN was found to be located 0.28 cM from the Mla locus, a member of a multigene family that confers resistance to powdery mildew (Wei *et al.*, 1999, 2002). Bhattacharyya *et al.* (1997) isolated and characterized the low copy copia-like RTN Tgmr, tightly linked to the Rps1-k allele that confers race-specific resistance to *Phytophtora sojae* in soybean.

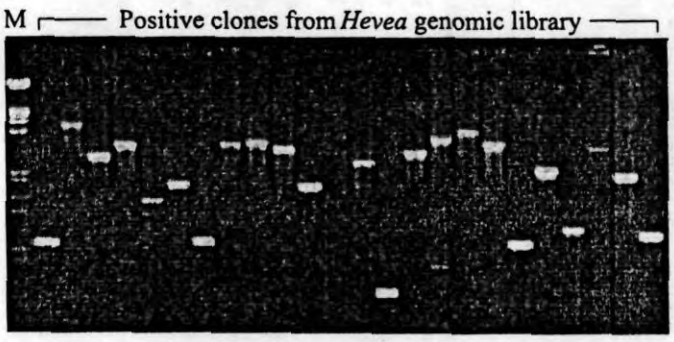

Fig. 5. PCR amplification of retro-positive *Hevea* genomic clones using vector directed T3 and T7 promoter primers for the determination of exact size of the inserts. Size of the inserts ranged between 0.6 kb to 4.0 kb.

Since the retrotransposon insertions are irreversible they are considered particularly useful in phylogenetic studies. In addition, their widespread occurrence throughout the genome can be exploited in genome mapping studies. Retrotransposons consist of long terminal repeats (LTR) with a highly conserved terminus, which is exploited for primer design in the development of retrotransposon-based markers. There have been several marker systems based on LTR retrotransposons including sequence-specific amplified polymorphism (SSAP), inter-retrotransposon amplified polymorphism (IRAP) and retrotransposon-microsatellite amplified polymorphism (REMAP) developed for plants (Waugh et al., 1997; Ellis et al., 1998; Kalendar et al., 1999). In the case of RTN-based markers, polymorphisms are generated by retrotransposition, which is a unidirectional process resulting in insertions of RTN daughter copies into new sites without removal of the parental copies from their sites. The consequence of retrotransposition is the alteration of a target site by integration of a fe

Table 1. Sequence analysis of *Hevea* genomic clones, screened with RT gene fragments

Hevea Genomic clone	Sequences Showing Maximum homology	Plant species	Accession numbers	E value
HRTN3	Non-LTR retroelement reverse transcriptase.	*Oryza sativa*	NP910568	7.00E-09
HRTN9-F	22 kDa kafirin cluster Ty3-Gypsy type	*Oryza sativa*	NP920981	7.00E-11
HRTN9-R	Polyprotein	*Ananas comosus*	CAA73042	9.00E-53
HRTN11	Reverse transcriptase	*Medicago truncatula*	ABE87837	2.00E-17
HRTN13-F	22 kDa kafirin cluster; Ty3-Gypsy type	*Oryza sativa*	AAP53268	1.00E-10
HRTN13-R	polyprotein	*Ananas comosus*	CAA73042	3.00E-64
HRTN14	Retroelement	*Oryza sativa*	NP919465	1.00E-16
HRTN15	Non-LTR retroelement reverse transcriptase	*Oryza sativa*	NP910568	7.00E-08
HRTN17	gag/pol polyprotein	*Pisum sativum*	AAQ82037	2.00E-18
HRTN20	gag/pol polyprotein	*Pisum sativum*	AAQ82037	7.00E-38

Table 2. Sequence analysis of *Hevea* resistance gene analogue (RGA) clones

Hevea RGA clone	Sequence showing Maximum homology	Plant species	Accession numbers	E value
RGA2	putative reverse transcriptase	*Cicer arietinum*	CAD59768	5.00E-12
RGA3	putative gag-pol polyprotein	*Citrus sinensis*	CAJ09951	5.00E-10
RGA7	RNA-directed DNA polymerase (RT)	*Medicago truncatula*	ABE83303	2.00E-15
RGA16	putative reverse transcriptase	*Cicer arietinum*	CAD59767	3.00E-10
RGA18	putative reverse transcriptase	*Cicer arietinum*	CAD59768	5.00E-12
RGA21	gag/pol polyprotein	*Pisum sativum*	AAQ82037	1.00E-87
RGA22	gag/pol polyprotein	*Pisum sativum*	AAQ82037	1.00E-86
RGA23	gag/pol polyprotein	*Pisum sativum*	NP910568	2.00E-84
RGA24	RNA-directed DNA polymerase (RT)	*Medicago truncatula*	ABE87222	5.00E-83
RGA26	RNA-directed DNA polymerase (RT)	*Medicago truncatula*	ABE87222	6.00E-87
RGA27	RNA-directed DNA polymerase (RT)	*Medicago truncatula*	ABE87222	1.00E-89
RGA28	gag/pol polyprotein	*Pisum sativum*	AAQ82037	5.00E-79
RGA29	Reverse transcriptase	*Medicago truncatula*	ABE91449	1.00E-89
RGA30	RNA-directed DNA polymerase (RT)	*Medicago truncatula*	ABE87222	3.00E-86
RGA31	RNA-directed DNA polymerase (RT)	*Medicago truncatula*	ABE87222	2.00E-82
RGA32	gag/pol polyprotein	*Pisum sativum*	AAQ82037	9.00E-89

hundred base pairs to a few kilobases. Genomic locations of MITE (miniature inverted transposable element) transposons are also variable within a species. These polymorphisms have been successfully exploited as genetic markers in maize and rice (Casa et al., 2000; Chang et al., 2001). However, efforts are being continued to develop retro-based marker system in rubber for genome analysis using the sequence information achieved in this study.

In the present study, reverse transcriptase domains of retrotransposon from *Hevea* genome was successfully amplified, which was further used as probe in screening of a genomic library to isolate genomic clones containing retrotransposons. Several retrotransposons were identified through sequence analysis. Positive clones bearing large inserts are practically important for cloning of full-length retrotransposons as their estimated size is around 5 kb (LTR retrotransposons). Therefore, efforts are being made towards full-length sequencing of large clones, which showed the presence of retro-sequences on both the ends. Presence of retrotransposons in association with resistance gene analogues was also evidenced through sequence analysis of RGAs derived from rubber. Sequence information of retrotransposons, isolated from rubber genome could be exploited as genetic tools for genome analysis. To our knowledge, this is the first report of the presence of retrotrasposons in rubber genome.

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