

Mg-SINE: A short interspersed nuclear element from the rice blast fungus, *Magnaporthe grisea*

(transposon/repeated DNA)

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ABSTRACT A short interspersed nuclear element, Mg-SINE, was isolated and characterized from the genome of the rice blast fungus, *Magnaporthe grisea*. Mg-SINE was isolated as an insertion element within *Pot2*, an inverted-repeat transposon from *M. grisea* and shows typical features of a mammalian SINE. Mg-SINE is present as a 0.47-kb interspersed sequence at ≈ 100 copies per haploid genome in both rice and non-rice isolates of *M. grisea*, indicating a common evolutionary origin. Secondary structure analysis of Mg-SINE revealed a tRNA-related region at the 5' end which folds into a cloverleaf structure. Genomic fusions resulting in chimeric Mg-SINEs (Ch-SINEs) composed of a sequence homologous to Mg-SINE at the 3' end and an unrelated sequence at its 5' end were also isolated, indicating that this and other DNA rearrangements mediated by these elements may have a major effect on the genomic architecture of this fungus.

Genomes of diverse eukaryotes harbor interspersed repeated sequences (1). These repetitive elements may be mobile or maintained stably in the genome (2). Mobile DNA elements are classified broadly into two categories, based on the intermediates formed during their replication (3). The elements that replicate via an RNA intermediate are subgrouped further into either retrotransposons, which have structural features similar to retroelements, or retroposons, which duplicate via RNA intermediates but do not have retrovirus-like structure (4). The majority of mammalian interspersed retroposons are classified as short interspersed nuclear elements (SINEs) or long interspersed nuclear elements (LINEs) (5, 6).

Typical features of a generic SINE are the presence of an RNA polymerase III promoter, an adenine-rich 3' end varying from 8 to 50 bp, and direct repeats at the ends (6). The dispersion and amplification of SINEs are thought to occur by retroposition in which the RNA polymerase III-dependent SINE transcripts are reverse transcribed into cDNA which then integrates into new genomic sites (7). Since SINEs lack open reading frames, they have been hypothesized to depend on reverse transcriptase function in trans in order to make cDNA (6).

SINEs form a unique group of transposable elements and although no single functional role has been demonstrated for them unequivocally, they have been shown to be actively involved in insertional inactivation of genes (8, 9) and in the formation of chimeric sequences (10). Insertion of these elements at previously unoccupied sites has been shown to result in mRNA truncation, altered polyadenylation, and modified protein structure (11–13). These elements can also undergo homologous and nonhomologous recombination which can contribute to genomic flux and may even result in genetic disorders (14).

Magnaporthe grisea, an ascomycetous fungus responsible for the blast disease of rice (*Oryza sativa*), is known to generate new pathogenic variants at a high frequency (15). Various mechanisms have been postulated to explain pathogenic variability in this fungus (15–17), although its molecular basis remains elusive. We initiated an analysis of repeated DNA sequences of *M. grisea* in order to delineate their role in genome organization and generation of variability. We report here the presence of a SINE sequence in the genome of *M. grisea* and further show that these elements are capable of causing insertions and possibly other genomic rearrangements.§

MATERIALS AND METHODS

Bacterial Strains and Fungal Isolates. *Escherichia coli* strains JM101 and DH5 α were used for bacterial transformations and plasmid propagation. *M. grisea* isolates (35 isolates, 18 of which were rice pathogens) representing diverse geographical regions were used in the present study.

Nucleic Acid Analysis. Genomic DNA isolation, Southern hybridizations, contour-clamped homogeneous electric field (CHEF) analysis, and copy-number determination were carried out as described (18). Plasmid DNA was prepared by the mini-maxi procedure (19). The complete sequence of 5-B101 (1.4 kb), 32-5-E H13 (1.1 kb), 23-4-D H7 (2.3 kb) was determined by making subclones in pUC19 and carrying out double-strand sequencing with Sequenase version 2.0 (United States Biochemical). Sequence of Mg-SINE was determined from 5-B101 (PCR amplicon), 9-rep (*Bam*HI genomic clone of pUC19), and cosmid 32-12-A (20) by using the primers P1 to P4 (see Fig. 2). Sequence of Ch-SINE was determined from cosmid clones 32-5-E and 23-4-D. Sequence comparisons were carried out with DNA INSPECTOR version 3.25 (Textco, West Lebanon, NH) and SEQAID II version 3.70 (Rhoads and Roufa, Molecular Genetics Laboratory, Kansas State University) software programs.

PCR analysis used a 5' primer (5'-GTCATTGAAA-GAGAAGTAAA-3') 13 bp upstream of a *Pot2* insertion in a single-copy region (CRP) (18) and a 3' primer (5'-GAAATTGCCAATTATCG-3') located 0.9 kb downstream from the start of *Pot2*. PCR was carried out as described (21).

Total RNA was extracted from isolate Guy 11 by an acid phenol method (22). RNA was fractionated on glyoxal/1.7% agarose gels in 10 mM phosphate buffer and blotted onto MSI nylon membranes. Northern blotting was carried out as described (23).

Abbreviations: CHEF, contour-clamped homogeneous electric field; LINE, long interspersed nuclear element; SINE, short interspersed nuclear element.

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§The sequences reported in this paper have been deposited in the GenBank database (accession nos. U35313 and U35230).

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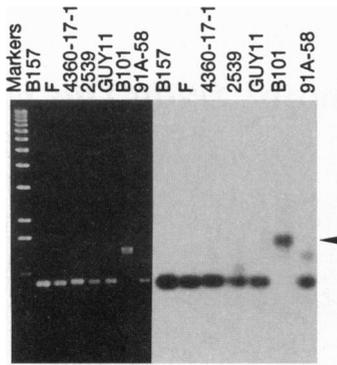


FIG. 1. PCR analysis of CRP, an insertion of *Pot2* within a single-copy region. The isolates are listed above each lane. Isolate B101 amplified a polymorphic band of 1.4 kb (shown by arrowhead), while all other isolates amplified a monomorphic band of 0.9 kb. (A) Ethidium bromide-stained gel. The 1-kb ladder (BRL) was used as molecular size markers. (B) Autoradiogram of the Southern blot of the gel shown in A, probed with an internal 0.9-kb *Bam*HI fragment of *Pot2* (18).

RESULTS

Sequence Analysis. The distribution of specific insertions of *Pot2*, an inverted repeat transposon of *M. grisea* (18), in various rice- and non-rice-infecting isolates was analyzed by PCR. Amplification of a *Pot2* insertion within a single-copy region, CRP, revealed the expected 0.9-kb band in most of the isolates tested except for the isolate B101, in which a band of 1.4 kb was identified (arrowhead, Fig. 1). Sequence analysis of this fragment (p5-B101) showed the presence of an additional 0.5 kb of DNA within *Pot2*, resulting in a duplication of 16 bp at the target site (*Pot2* region flanking the insertion site). The duplications were present as direct repeats and were A+T-rich. (13 of 16 nt being A or T). The 0.5-kb DNA showed many features common to SINE sequences, such as the presence of A- and B-box consensus sequences which showed an exact match to the tRNA polymerase III promoter consensus sequence (Fig. 2A) (6). The A box starts 8 bp downstream of the 5' end of the element, and the two conserved regions are separated by 32 nt. This element was called Mg-SINE (*M. grisea* SINE).

Mg-SINE, excluding the 16-bp direct repeats, is 472 bp in size in the insertion clone p5-B101 (Fig. 2B). The target-site duplication for these elements was found to vary from 1 bp in insertion 9-rep (isolate 2539) or 2 bp in 35-12-A (isolate 2539) to 16 bp in insertion 5-B101 (isolate B101) (Fig. 2C). Complete

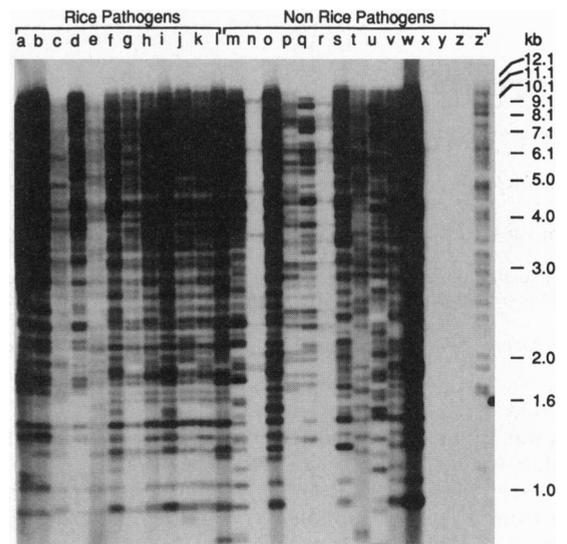


FIG. 3. Southern hybridization analysis of genomic DNA of rice- and non-rice-infecting isolates of *M. grisea* probed with Mg-SINE. Genomic DNA (2 µg) was digested with *Pst* I, electrophoresed through a 0.8% agarose gel, transferred to MSI nylon membrane and hybridized with the radiolabeled 258-bp PCR amplicon of Mg-SINE (prepared as in Fig. 2B). *Pst* I does not have any site within Mg-SINE. The lanes contain genomic DNA from isolate F (India), lane a; B (India), lane b; B101 (India), lane c; B157 (India), lane d; 4360-17-1 (United States), lane e; PO6-6 (Philippines), lane f; JMB840610 (Philippines), lane g; 101 (Philippines), lane h; 102 (Philippines), lane i; 103 (Philippines), lane j; V8601 (Philippines), lane k; 104 (Philippines), lane l; 91-A-58 (United States), lane m; Pd 8824 (Philippines), lane n; Ec522 (Philippines), lane o; Pr886 (Philippines), lane p; Pr342 (Philippines), lane q; Pr8988 (Philippines), lane r; Cb334 (Philippines), lane s; Pg3393 (India), lane t; Cd88215 (Philippines), lane u; Lc454 (Philippines), lane v; Ei 476 (Philippines), lane w; 4091-5-8 (laboratory strain), lane x; Bd8401 (Philippines), lane y; Dc88428 (Philippines), lane z; Lh88490 (Philippines), lane z'.

sequence analysis of four individual Mg-SINEs from isolate B101 (5-B101) and 2539 (9-rep, 35-12-A, and 42-1-B) revealed only a single base difference, a transition mutation (T → C) at position 419 in 9-rep, 35-12-A, and 42-1-B, indicative of the conserved nature of this sequence. Mg-SINEs terminate with a characteristic trinucleotide repeat, TAC, which is reiterated, with different clones showing variable numbers of iterations. Sequence analysis of the 3' end of 5-B101, 9-rep, 35-12-A, 7-3-B, and 42-1-B revealed five, five, nine, six, and six trinucleotide repeats, respectively. Mg-SINE did not show any

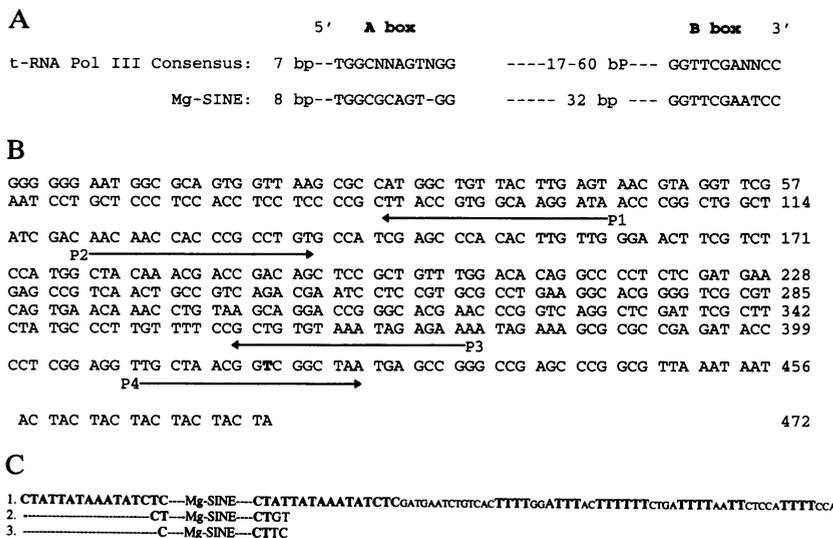


FIG. 2. Sequence analysis and target-site characterization of Mg-SINE. (A) Alignment of conserved sequence of RNA polymerase III A- and B-box regions of Mg-SINE with tRNA polymerase III promoter consensus. (B) Nucleotide sequence of Mg-SINE primers P1, P2, P3, and P4 were used for sequence analysis. P2 and P3 were also used for PCR amplification of a 258-bp fragment which was used as a probe for Southern and Northern analysis. Sequence comparison of three individual Mg-SINE clones—5-B101, 35-12-A, and 9-rep—revealed a transition mutation from T (marked as bold type) to C at position 419 in 35-12-A and 9-rep. Numbers at right mark the nucleotide positions. (C) Target site and the flanking region of Mg-SINE insertions. Sequences 1, 2, and 3 represent insertion-site sequence of 5-B101, 35-12-A, and 9-rep, respectively. The target-site duplication, conserved dinucleotide CT, and the poly(T) stretch downstream of the insertion site are shown in bold type.

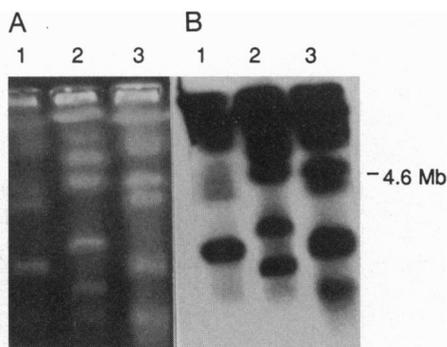


FIG. 4. Southern hybridization analysis of electrophoretically separated chromosomes of *M. grisea* probed with Mg-SINE. Chromosomal DNA from isolate A, lane 1; B101, lane 2; and B157, lane 3, was fractionated in a 0.8% agarose gel, transferred to MSI nylon membrane, and hybridized with the radiolabeled 258-bp PCR amplicon of Mg-SINE (prepared as in Fig. 2B). The molecular size 4.6 Mb corresponds to chromosome 6 of isolate Guy 11 (24). (A) Ethidium bromide-stained gel. (B) Autoradiogram of the Southern blot of the gel shown in A.

prominent open reading frames, as commonly observed in other SINEs (6).

DNA and RNA Analysis. The presence and distribution of Mg-SINE within the genomes of various isolates of *M. grisea* were studied by Southern hybridization and CHEF analysis. Genomic DNA of 35 isolates representing rice pathogens, non-rice pathogens, and laboratory strains of *M. grisea* was digested with *Pst* I or *EcoRV* (data are shown for 27 isolates) and hybridized to a 258-bp internal fragment generated by PCR amplification of Mg-SINE with primers P2 and P3 (Fig. 2B). The genomic DNA of all the rice pathogens and 10 out of 15 non-rice pathogens showed intense hybridization signals, indicating Mg-SINE to be a multicopy, repeated DNA element (Fig. 3). This repeat element was present in a very low copy number in 5 non-rice pathogens—Pd8824, Pr8988, 4091-5-8, Bd8401, and Dc88428, the last three isolates showing a copy number of <5. These possibly represent a group of isolates which may have escaped amplification of Mg-SINE during the evolution of *M. grisea*.

Distribution of Mg-SINE on various chromosomes was studied by CHEF analysis of two rice isolates and one non-rice isolate of *M. grisea*. All the isolates used in the present study showed the presence of B chromosomes which were polymorphic in size as well as number (Fig. 4A). A CHEF gel (Fig. 4A) probed with Mg-SINE showed hybridization to all resolvable chromosomes and B chromosomes of *M. grisea*, indicating it to be a dispersed repeated DNA sequence (Fig. 4B). The copy number of Mg-SINE as determined by quantitative dot blot analysis was ≈ 100 for isolates B157 and Pg3393.

Transcription of Mg-SINE was studied by Northern blot analysis. When hybridized to the 258-bp PCR amplicon from Mg-SINE, total RNA from isolate Guy 11 revealed a transcript of 0.5 kb size in the lanes containing 18 and 30 μ g of total RNA (Fig. 5).

Secondary Structure. The possible secondary structure of Mg-SINE derived by use of the Genetics Computer Group programs FOLD and SQUIGGLE (25) showed a tRNA-related region, a tRNA-unrelated region, and an A+T-rich region. The 5' end of Mg-SINE (bp 1–73) folds in a tRNA-like structure (Fig. 6). The cloverleaf structure terminates with nucleotides CCA at the 3' end, a characteristic feature of tRNAs, although unlike tRNA, the cytosine of CCA in Mg-SINE shows pairing with guanosine at the 5' end, adenine staying unpaired. The positions of the D loop and T Ψ C loops of tRNA correspond, respectively, to the A and B boxes of Mg-SINE, both of which also form a loop-like structure, while the anticodon arm forms the middle loop. Several highly

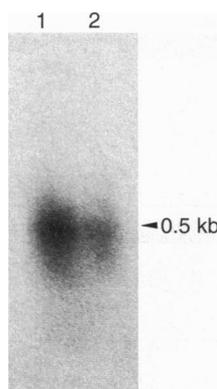


FIG. 5. Northern hybridization analysis of total RNA from *M. grisea* with Mg-SINE as probe. Total RNA from a rice-infecting isolate of *M. grisea* (Guy11) was fractionated in a 1.7% agarose gel, blotted onto MSI nylon membrane, and hybridized with the 258-bp PCR amplicon of Mg-SINE (prepared as in Fig. 2B). Lane 1 and 2 represent 30 and 18 μ g of total RNA, respectively.

conserved nucleotide positions—e.g., those involved in the formation of the stem structure (C-G, C-G, and U-A) of the T Ψ C loop were also found to be conserved in Mg-SINE. This analysis also revealed an unpaired A+T-rich end toward the 3' end of the element and a tRNA-unrelated region (data not shown).

Isolation and Characterization of Chimeric Elements. When a genomic library of isolate 2539 in the cosmid vector pMLF1 (20) was probed with Mg-SINE, hybridization signals of varied intensities were observed. Cosmid DNA digested with several restriction enzymes and probed with Mg-SINE indicated that the weakly hybridizing cosmid clones may represent either a divergent family of a similar class of elements or defective Mg-SINEs (data not shown). Sequence analysis of fragments from the cosmid clones 35-12-A, 7-3-B, and 42-1-B, which hybridize intensely to Mg-SINE, revealed sequences identical to that of Mg-SINE, whereas the cosmid clones 32-5-E and 23-4-D, which hybridized weakly, showed only a partial homology to Mg-SINE, restricted to the 3' end. Sequencing of these partially homologous regions and their flanking sequences revealed that homology to Mg-SINE resides in the 240 bp at the 3' end (Fig. 7A and B, shown within box). The 452-bp sequence preceding the region homologous to Mg-SINE was identical in both 32-5-E and 23-4-D cosmid subclones; beyond this region, the two subclones showed divergent sequence. Both the cosmid subclones terminated with the trinucleotide repeat TAC at the 3' end, a characteristic feature of Mg-SINE, followed by a divergent sequence.

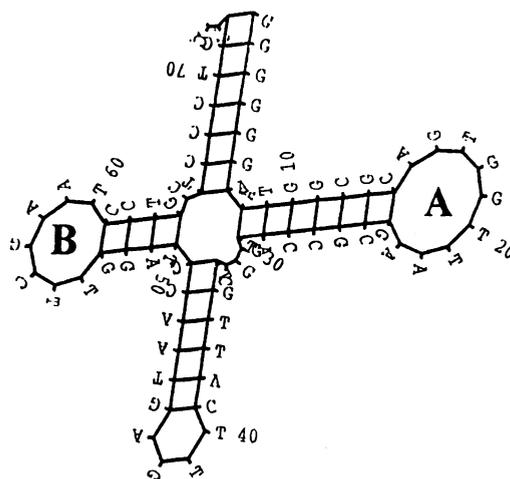


FIG. 6. Secondary structure analysis of Mg-SINE. The structural analysis was carried out with the SQUIGGLE and FOLD programs of the University of Wisconsin Genetics Computer Group (25). Nucleotide numbers are given from the 5' end of Mg-SINE. A and B designate the conserved polymerase III consensus promoter sequence. The 5' region (bp 1–73) folds into a cloverleaf structure typical of tRNA.

cytosine residue in the upper strand, the cleavage site for the bottom strand being variable. Several topoisomerase II enzymes possess specific asymmetric recognition/cleavage sites and are known to cleave the top strand 3- to 10-fold more efficiently than the bottom strand (28).

The presence of a transcript of ≈ 0.5 kb strongly suggests that Mg-SINE may be a functional retroposon. The diffuse nature of the signal possibly indicates transcripts of various sizes produced by several active Mg-SINE sequences. *In vivo* and *in vitro* transcription experiments have shown that *Alu* and other related SINE families from mammals are transcribed by RNA polymerase III (29, 30). Since SINEs do not possess any open reading frames, the reverse transcriptase function should be available in trans for the synthesis of cDNA to take place (6). The *M. grisea* genome has been shown to possess a functional retroelement (M. Farman, Y. Tosa, N. Nitta, and S.L., unpublished results; refs. 31 and 32) and a LINE-like sequence hybridizing to various size transcripts in a Northern blot (33). Our unpublished results indicate the presence of more than one class of retroelements in the genome of *M. grisea*, and it seems likely that Mg-SINE utilizes the reverse transcriptase produced by any of the retroelements or LINE sequences to generate a cDNA copy which could be inserted at a region containing a double-strand break in the genome.

The high copy number of Ch-SINE in the genome of *M. grisea* indicates that a fusion/insertion event followed by deletion of a major region of inserted sequence or a recombination event leading to generation of Ch-SINE must be followed by the amplification of this repeat. The fusion event could be the consequence of premature termination of reverse transcriptase followed by joining to a genomic sequence and subsequent amplification of the fusion structure. This could also represent a possible mechanism for the generation of repeated DNA sequences in the genome of eukaryotes. The presence of Ch-SINE in both rice and non-rice pathogens of *M. grisea* indicates that these elements evolved before the divergence of the two host-specific forms. Ch-SINE resembles the type II Galago SINE family (10), which is a chimeric element composed of sequences related to the Monomer family in its left half and sequences identical to the *Alu* family in the right half.

The dispersed nature of the repetitive element as evidenced by CHEF analysis, the insertion of Mg-SINE into *Pot2*, the detection of a transcript in a Northern blot, and the restriction fragment length polymorphisms seen in the Mg-SINE-hybridizing bands indicate that Mg-SINE is possibly functional. PCR analysis of 30 different isolates showed that only B101 carried a Mg-SINE insertion within *Pot2*, which in turn is inserted within a single-copy region, CRP. This suggests that the Mg-SINE insertion within *Pot2* may be a recent event. The amplification and widespread presence of these elements throughout the genome may have major effects on genome structure and function and could be one of the ways by which pathogenic variability is generated among isolates of *M. grisea*.

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