Mol Gen Genet (1999) 261: 958–966

ORIGINAL PAPER

H. Nakayashiki · N. Nishimoto · K. Ikeda
Y. Tosa · S. Mayama

Degenerate MAGGY elements in a subgroup of *Pyricularia grisea*: a possible example of successful capture of a genetic invader by a fungal genome

Received: 1 February 1999 / Accepted: 8 April 1999

Abstract The LTR-retrotransposon MAGGY is found sporadically in isolates of *Pyricularia grisea* (*Magnaporthe grisea*). Based on a dendrogram constructed by RFLP analysis of rDNA, isolates that carry MAGGY elements were classified into a single cluster that comprised four rDNA types. However, in a few members of this cluster, exemplified by isolates from common millet (*Panicum miliaceum*), the MAGGY element has distinct features. Southern analysis suggested that these isolates possessed a single copy of a MAGGY-related sequence whose restriction map differed from that of MAGGY itself. Sequence analysis revealed that the MAGGY-related sequence was a degenerate form of MAGGY, characterized by numerous C:G to T:A transitions, which have often been reported to result from RIP (Repeat-induced point mutation) or RIP-like processes. However, the favored target site for C:G to T:A transitions in this fungus, determined by examining a total of 501 sites, was (A/T)pCp(A/T), which differs from that for the RIP process originally reported in *Neurospora* (CpA), and from that reported in *Aspergillus* (CpG). The fact that certain members of the cluster of MAGGY carriers retain a single copy of a degenerate MAGGY element implies that the ancestor of these isolates successfully "captured" the invading MAGGY element.

Key words Retrotransposon · Filamentous fungi · *Pyricularia grisea* · Repeat-induced point mutation (RIP) · Gene inactivation

Communicated by H. Saedler

H. Nakayashiki (✉) · N. Nishimoto · K. Ikeda · Y. Tosa
S. Mayama
Laboratory of Plant Pathology,
Faculty of Agriculture,
Kobe University, Rokkodai-cho 1-1,
Kobe 657-8501, Japan
e-mail: hnakaya@kobe-u.ac.jp
Tel.: +81-78-803-5867; Fax: +81-78-803-5867

Introduction

Pyricularia grisea (teleomorph *Magnaporthe grisea*), the filamentous fungus that causes blast disease in plants, shows intraspecific variation in pathogenicity, in spite of its morphological uniformity (Kozaka and Kato 1980). Several attempts have been made to classify *P. grisea* isolates that infect different host plants by examining sexual compatibility, host range, and enzyme polymorphisms (Matsuyama et al. 1977; Leung and Williams 1986; Urashima et al. 1993; Kato 1994), and more recently by using molecular genetic techniques like restriction fragment length polymorphisms (RFLPs) of nuclear or mitochondrial DNA (Borromeo et al. 1993; Shull and Hamer 1994). These studies have revealed that *P. grisea* isolates are genetically diverse and can be classified into several subgroups, with some exceptions, depending on the host plants from which they were isolated. Recently we reported that seventy-four *P. grisea* isolates from twenty-eight host plant species could be grouped into fourteen types on the basis of rDNA haplotypes (Kusaba et al. 1999). Most isolates from a given host plant belonged to a single rDNA type, indicating that they are genetically closely related and might be derived from a clonal lineage.

In *P. grisea*, some transposable elements appear to be restricted to certain subgroups. An LTR-retrotransposon, *grasshopper*, was found exclusively in a subgroup of *Eleusine* isolates (Dobinson et al. 1993). With MAGGY, another LTR-retrotransposon of *P. grisea*, the situation appeared to be rather complex, because this element was present in multiple copies in isolates from several plants belonging to different genera, such as rice, foxtail millet, guinea grass, Italian ryegrass, tall fescue etc. (Tosa et al. 1995; Farman et al. 1996; Kusaba et al. 1999). It has been shown, however, that almost all MAGGY carriers fall into one of four rDNA types, which form a single cluster in a dendrogram of *P. grisea* isolates from various host plants (Kusaba et al. 1999). These phenomena imply

that the elements *grasshopper* and MAGGY were acquired horizontally by the common ancestors of each cluster. Support for this notion was obtained from transformation experiments in which MAGGY was introduced into MAGGY-free isolates of *P. grisea*. MAGGY has been demonstrated to have the ability to transpose and increase its copy number even in MAGGY-free isolates, suggesting that MAGGY may be absent from these isolates simply because their genomes have never encountered the element (Nakayashiki et al. 1999).

Horizontal transmission has been proposed for several transposable elements because of their sporadic or discontinuous distribution (Mizrokhi and Mazo 1990; Flavell et al. 1995). The possibility of horizontal transmission of MAGGY between *P. grisea* isolates was also suggested based on the discovery of MAGGY in a buffelgrass isolate belonging to a genetic cluster that is remote from the major group of MAGGY carriers (Kusaba et al. 1999). A remarkable example concerns *gypsy*, which was first found in *Drosophila melanogaster* and thought to be a typical member of LTR-retrotransposon group. One of the *gypsy* elements, *gypsyDm*, has been shown to be infectious; thus, it apparently undergoes horizontal transmission between *Drosophila* species (Kim et al. 1994; Song et al. 1994). For this reason, some *gypsy* elements are now considered to be retroviruses of invertebrates.

Most transposons have been regarded as intragenomic parasites that can cause several types of genetic variation in their hosts. Most of them, with a few exceptions, are probably harmful to their hosts. Most organisms appear to have evolved defense systems against intragenomic parasites or foreign sequences like transposable elements, repetitive sequences, viruses and introduced exogenous genes (Kumpatla et al. 1998). In fungi, RIP (repeat-induced point mutation) in *Neurospora crassa* (Selker et al. 1987) and MIP (methylation induced premeiotically) in *Ascobolus immersus* (Goyon and Faugeron 1989) are well-known processes which inactivate repeated sequences during a specific period in the sexual cycle (Selker 1997). Epigenetic changes such as cytosine methylation seem to be associated with both of these inactivation processes. Moreover, RIP causes genetic changes – C:G to T:A transitions – mainly at CpA dinucleotides in duplicated sequences and inactivates the affected sequences irreversibly (Cambareri et al. 1989).

In this study we cloned a MAGGY-related sequence from a *P. grisea* isolate isolated from common millet (*Panicum miliaceum*), which belongs to the cluster of MAGGY carriers, but this sequence is found in a single copy per genome and differs in the pattern of internal restriction sites from "authentic" MAGGY. We found that the sequence represented a degenerate form of MAGGY characterized by numerous C:G to T:A transitions. The origin and implications of this sequence are discussed.

Materials and methods

Fungal strains and culture media

Field isolates of *P. grisea* used in this study are listed in Table 1. All of the isolates were purified by monoconidial isolation and maintained on PDA (potato-dextrose agar) media for short-term storage or on sterilized barley seeds for long-term storage, as described previously (Nakayashiki et al. 1999). For DNA extraction, fungal mycelia were grown in CM liquid broth (0.3% Casamino acids, 0.3% Yeast extract, 0.5% sucrose) at 26° C.

Bacterial strains and plasmids

All plasmids were maintained in *Escherichia coli* JM109 (Toyobo) or XL1-Blue (GIBCO-BRL). pBluescript SK + II (Stratagene) and pUC19 (Takara) were used in subcloning procedures. Plasmids were extracted from *E. coli* cells using the Qiagen Midi plasmid kit (Qiagen) according to the manufacturer's instructions.

DNA isolation and analysis

Total fungal DNA was isolated as described previously (Nakayashiki et al. 1999). Southern analysis was performed using a dioxethane chemiluminescence system (Gene Images; Amersham). Fluorescein-labeled probes were prepared by the random-primer labeling method. Hybridization was performed in 5 × SSC, 0.1% (w/v) SDS, 5% (w/v) dextran sulfate, and 5% (v/v) liquid block (Amersham) at 60° C overnight. After hybridization, membranes were washed twice in 1 × SSC containing 0.1% SDS for 15 min at 65° C and twice in 0.5 × SSC containing 0.1% SDS for 15 min at 65° C. Detection procedures were performed according to the kit manufacturer's instructions.

Construction of a genomic library

Fifty micrograms of total DNA from a *P. miliaceum* isolate (NNPM3-1-1) was partially digested with *Sau*3AI. A genomic library was constructed by ligating the digests into *Bam*HI-digested Lambda EMBL3 (Stratagene) as described by Sambrook et al. (1989). The library was screened by plaque hybridization with the fluorescein-labeled 5.4-kb *Xho*I fragment of MAGGY and the results were analyzed using the Gene Images detection system (Amersham).

Table 1 *Pyricularia grisea* isolates used in this study

Isolate	Host	Origin	Collector
NNPM1-2-4	*Panicum miliaceum* L.	Nagano, Japan	N. Hayashi
NNPM2-1-2	*P. miliaceum* L.	Nagano, Japan	N. Hayashi
NNPM3-1-1	*P. miliaceum* L.	Nagano, Japan	N. Hayashi
NNPM7-1-1	*P. miliaceum* L.	Nagano, Japan	N. Hayashi
NRPM1-1-1	*P. miliaceum* L.	Nara, Japan	H. Kato
STPM4-2-3	*P. miliaceum* L.	Saitama, Japan	N. Hayashi
STPM4-3-5	*P. miliaceum* L.	Saitama, Japan	N. Hayashi
YNPM2-1-1	*P. miliaceum* L.	Yamanashi, Japan	H. Kato
IN77-28-1-1	*P. repens* L.	Mysore, India	H. Kato
IN77-45-1-1	*P. repens* L.	Bangalore, India	H. Kato
IN77-33-1-1	*P. maxicum* Jacq	Bangalore, India	H. Kato
NI922	*P. bisulcatum* Thumb.	Tochigi, Japan	N. Nishihara
1836-3	*Oryza sativa* L.	Niigata, Japan	M. Yamada
GFSI1-7-2	*Setaria italica* Beauv.	Gifu, Japan	H. Kato

DNA sequencing

Based on restriction analysis of the region sharing homology with MAGGY, subclones encompassing the MAGGY-related region were made in pBluescript SK+ II and pUC19. DNA sequences were determined on double-stranded DNA using a SequiTherm EXCEL II Long-Read DNA kit (Epicentre Technologies) in conjunction with fluorescence-labeled universal primers and using a Perkin-Elmer-Cetus DNA thermal cycler PEC480 and a DSQ1000 DNA sequencer (Shimadzu). The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under the Accession No. AB024423.

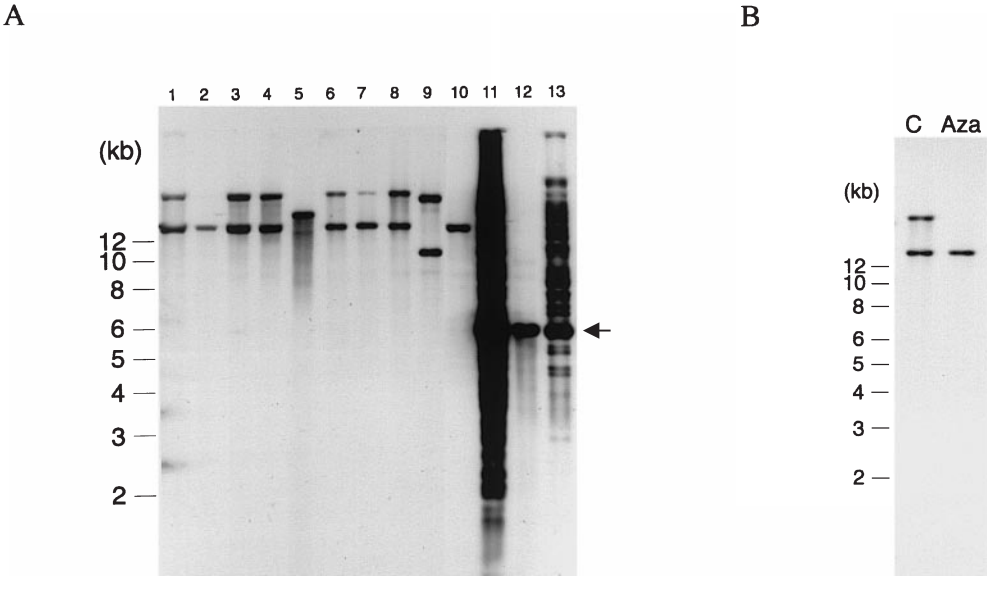
Results

Distribution and structure of MAGGY-related sequences in *P. grisea* isolates from *Panicum* plants

In addition to strains obtained from rice, *P. grisea* isolates from several crops and grasses have been shown to possess MAGGY homologs in their genomes (Tosa et al. 1995; Farman et al. 1996; Kusaba et al. 1999). A MAGGY homolog detected in an isolate from *Panicum repens* showed weak homology with MAGGY and some distinct features with respect to internal restriction fragments (Farman et al. 1996). To examine the distribution and structure of the MAGGY homolog in *Panicum* isolates of *P. grisea*, we carried out a Southern analysis on twelve isolates from four plant species belonging to the genus *Panicum*. Genomic DNA was digested with *Pst*I and probed with the 4.6-kb *Sal*I-*Pst*I fragment of MAGGY (SP fragment). A *Pst*I site is located in each LTR of MAGGY, thus the digestion of MAGGY DNA with *Pst*I excises a 5.4-kb fragment, as shown for a rice isolate (Fig. 1A, lane 13). In isolates from *P. maximum* and *P. bisulcatum*, the fragment of 5.4 kb was also detected, indicating that these isolates possess elements that are closely related to MAGGY (Fig. 1A). On the other hand, *P. miliaceum* and *P. repens* isolates shared no 5.4-kb fragment but had hybridizing fragments of higher molecular weight. Although two fragments (estimated as 20 kb and 13 kb long) were observed in most *P. miliaceum* isolates, the 20-kb fragment was not observed when the fungal DNA was extracted from mycelia cultured in CM liquid broth containing 5 mM 5-azacytidine (Fig. 1B). This indicated that the 20-kb fragment resulted from incomplete digestion by *Pst*I as a consequence of methylation of its recognition site. Thus, the MAGGY homolog appeared to be present in a single copy per genome – at least in the *P. miliaceum* isolates. The 13-kb fragment was widely conserved in isolates from *P. miliaceum* and *P. repens*, with a few exceptions.

In order to examine the internal structure of the MAGGY homologs in *Panicum* isolates, isolates showing distinct band patterns in Fig. 1 were picked, and their DNAs were digested with *Eco*RI and hybridized with the SP fragment. "Authentic" MAGGY contains four internal *Eco*RI fragments of 1.19, 1.00, 0.66, and 0.61 kb, respectively (Fig. 2, lane 1). These four fragments were widely conserved in MAGGY homologs found in *P. grisea* isolates from various host plants (Farman et al. 1996; Kusaba et al. 1999). Isolates from *P. maximum* and *P. bisulcatum*, as well as rice and foxtail millet isolates, were shown to contain all four fragments (Fig. 2), whereas *P. miliaceum* and *P. repens* isolates showed different hybridization patterns. Only

Fig. 1A–B Southern analysis of *P. grisea* isolates from *Panicum* species and rice. **A** Genomic DNA was digested with *Pst*I, fractionated on a 0.7% agarose gel, and probed with the 4.6-kb *Sal*I-*Pst*I fragment of MAGGY. The *arrow* indicates the 5.4-kb fragment characteristic of MAGGY. Lanes: 1, NNPM1-2-4 (*P. miliaceum*); 2, NNPM2-1-2 (*P. miliaceum*); 3, NNPM3-1-1 (*P. miliaceum*); 4, NNPM7-1-1 (*P. miliaceum*); 5, NRPM1-1-1 (*P. miliaceum*); 6, STPM4-2-3 (*P. miliaceum*); 7, STPM4-3-5 (*P. miliaceum*); 8, YNPM2-1-1 (*P. miliaceum*); 9, IN77-28-1-1 (*P. repens*); 10, IN77-45-1-1 (*P. repens*); 11, IN77-33-1-1 (*P. maximum*); 12, NI922 (*P. bisulcatum*); 13, 1836-3 (rice). **B** NNPM3-1-1 cultured in CM broth without (C) or with (Aza) 5 mM 5-azacytidine



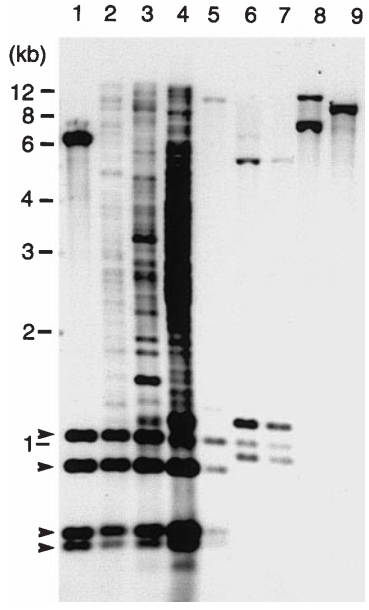


Fig. 2 Southern analysis of *P. grisea* isolates from rice, foxtail millet, and *Panicum* species. Genomic DNA was digested with *Eco*RI, fractionated on a 1.5% agarose gel, and probed with the 4.6-kb *Sal*I-*Pst*I fragment of MAGGY. Lanes: 1, pMGY70 (a control plasmid containing a copy of MAGGY); 2, 1836-3 (rice); 3, GFSI1-7-2 (foxtail millet); 4, IN77-33-1-1 (*P. maximum*); 5, NI922 (*P. bisulcatum*); 6, NNPM3-1-1 (*P. miliaceum*); 7, NRPM1-1-1 (*P. miliaceum*); 8, IN77-28-1-1 (*P. repens*); 9, IN77-45-1-1 (*P. repens*). *Arrows* indicate internal fragments characteristic of the cloned MAGGY sequence

one fragment (1.19 kb) in the *P. miliaceum* isolates showed the same electrophoretic mobility as a fragment of "authentic" MAGGY. These results indicated that the MAGGY homologs in the *P. miliaceum* and *P. repens* isolates differed in sequence from "authentic" MAGGY.

Isolation of a MAGGY-related sequence (MRPAN) from a *P. miliaceum* isolate of *P. grisea*

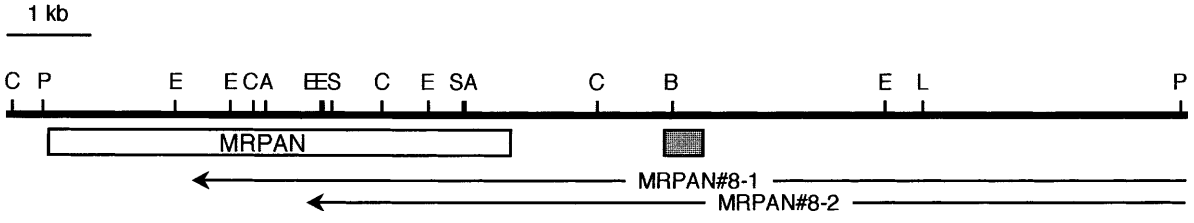
To isolate the MAGGY homolog, a genomic library was constructed from a *P. miliaceum* isolate, NNPM3-1-1, that showed a hybridization pattern typical of the *P. miliaceum* isolates in Fig. 1A. The genomic library was screened with the 5.4-kb *Xho*I fragment of MAGGY. Five thousand phage plaques were screened and two positive clones, #8-1 and #8-2, were isolated. Southern analysis of these phage clones and of genomic DNA from the NNPM3-1-1 isolate revealed that the inserts in the phage clones contained overlapping fragments of the same MAGGY homolog (data not shown). The MAGGY homolog was called MRPAN (MAGGY-related sequence in *Panicum* isolate). The physical map of cloned MRPAN and its flanking genomic region are shown in Fig. 3. The 9-kb *Sal*I fragment of MRPAN#8-1 was subcloned into *Sal*I-digested pUC19 and sequenced. This fragment also contained a homolog of MGSR1, a SINE-like element (Sone et al. 1993).

MRPAN is a degenerate form of MAGGY resulting from C:G to T:A transitions

The sequenced region contained domains corresponding to protease, reverse transcriptase, RNaseH, and integrase genes of LTR-retrotransposons. The predicted polypeptides were, however, truncated by several stop codons. Fig. 4 presents a comparison of nucleotide and deduced amino acid sequences between MRPAN and MAGGY in the reverse transcriptase and integrase domains. The overall identity between MRPAN and MAGGY was 86.6% in a stretch of 3895 nucleotides. Interestingly, 96% of the differences between MRPAN and MAGGY were due to C:G to T:A transitions: such changes were responsible for all the stop codons embedded in the "ORF" of MRPAN (Table 2). If the C:G to T:A transitions are disregarded, MRPAN and MAGGY are 99.4% identical. Thus, MRPAN is not a distinct retrotransposon, but rather represents a degenerate form of MAGGY characterized by numerous C:G to T:A transitions. C:G to T:A transitions have often been reported among sequence alterations caused by RIP, which is associated with cytosine methylation (Cambereri et al. 1989; Singer et al. 1995). The most probable explanation for the C:G to T:A transitions is deamination of 5-methyl cytosine to generate thymine, leading subsequently to a base change from guanine to adenine in the complementary strand during duplication.

Five hundred and one G-C base pairs (in the 3895-bp stretch corresponding to the MRPAN sequence) in MAGGY were converted to A-T base pairs in MRPAN. Specifically, 235 out of 1183 cytosine residues (19.9%) and 266 out of 939 guanine residues (28.3%) on the sense strand of MAGGY were replaced by thymine and adenine residues, respectively, in MRPAN. The frequency of G to A transitions was significantly higher than that of C to T transitions. To determine whether

Fig. 3 Physical map of the genomic region of isolate NNPM3-1-1 that contains MRPAN. Isolated genomic clones are indicated by the *arrows*. The *open box* indicates the MRPAN sequence which hybridizes with the MAGGY probe. The *shaded box* indicates a MGSR1 homolog. Recognition sites for *Apa*I (A), *Bam*HI (B), *Cla*I (C), *Eco*RI (E), *Pst*I (P), *Sal*I (L) and *Sma*I (S) are indicated



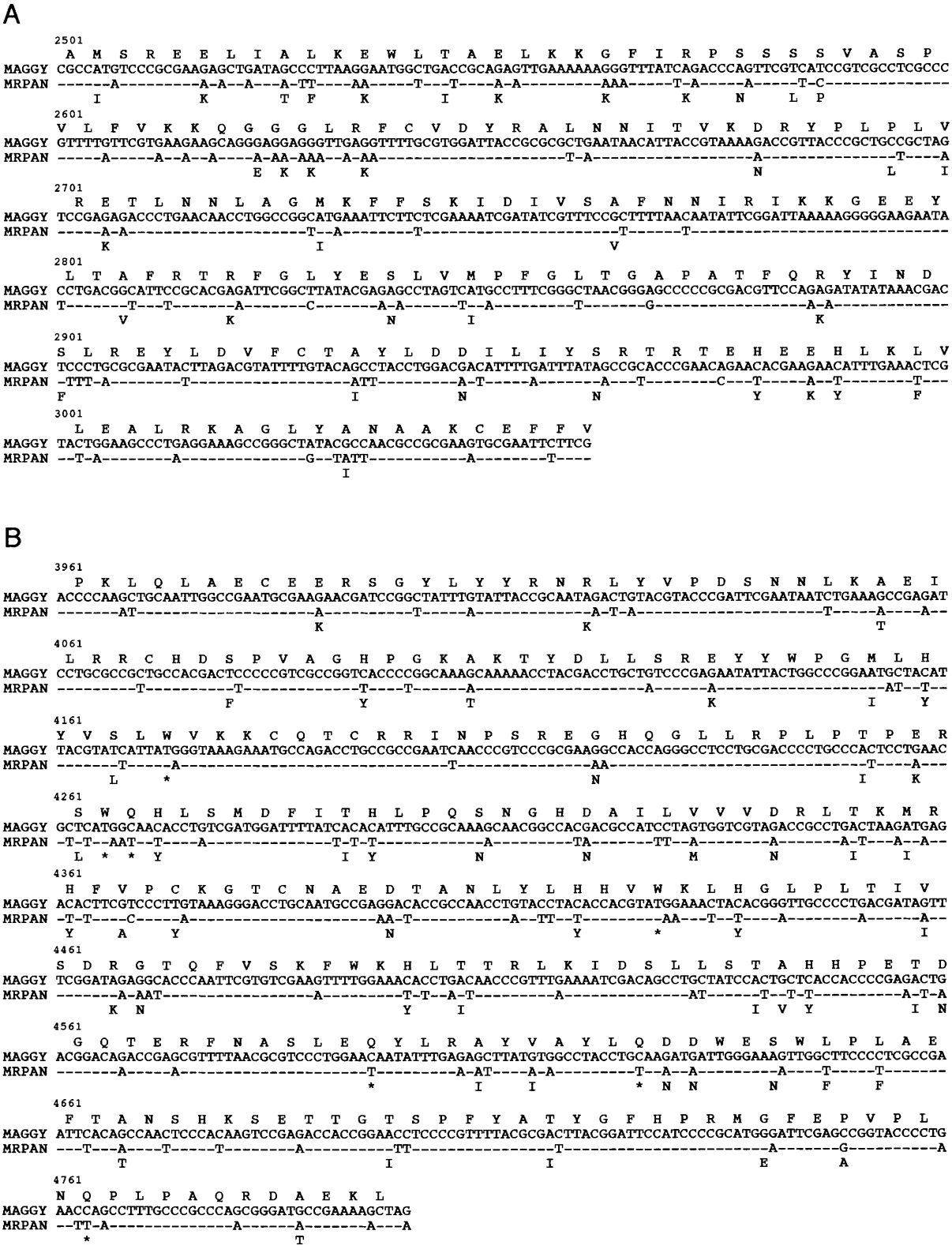


Fig. 4A, B Nucleotide and deduced amino acid alignments of the reverse transcriptase (**A**) and integrase (**B**) domains of MAGGY and MRPAN. Stop codons are indicated by *asterisks*. The *hyphens* indicate identity to the reference sequence (MAGGY). Differences are indicated by *letters*

the C:G to T:A transitions occurred randomly or not, target site specificity was examined. The bases immediately 5′ and 3′ of the sites of transitions were categorized and counted (Table 3). When the bases flanking the transitions had also been changed owing to C:G to T:A transitions or other base replacements between MA-

Table 2 Distribution of sequence differences between MAGGY and MRPAN

Base in MAGGY	Replacement in MRPAN	Frequency
A	C	1
	G	7
	T	1
C	A	3
	G	1
	T	235
G	A	266
	C	1
	T	3
T	A	0
	C	4
	G	0

GGY and MRPAN, we eliminated them from further analysis, because we could not determine which change had occurred first, and thus the target context was uncertain. In general, a cytosine or guanine residue flanked by A:T base pairs was the preferred site for C:G to T:A transitions. Particularly, the bases 3′ of altered cytosines and 5′ of altered guanines were very likely to be A:T base pairs. On the other hand, the restriction to A:T base pairs immediately 5′ of altered cytosines and 3′ of altered guanines seemed to be a little less pronounced. This symmetrical target specificity leads us to assume that the C to T and G to A transitions represent the same event in complementary strands of nucleic acids. Thus, we summed the results for both transitions with respect to the strand showing C to T transitions. When the base immediately 5′ of a cytosine was A, C, G, or T, C to T transitions occurred in 33.7%, 4.7%, 14.1%, and 33.3% of cases, respectively. If the base just 3′ of the cytosine was A, C, G, or T, respectively, the C to T transitions occurred in 41.3%, 2.7%, 1.2%, and 59.0% of cases. It is noteworthy that 80.3% of cytosine residues that were flanked on both sides by A:T base pairs were subjected to the C to T transition. However, no preference for the symmetrical context, ApCpT or TpCpA, was observed (data not shown). Based on the data presented above, we concluded that (A/T)pCp(A/T) was the preferred target site for the C to T transitions in this fungus, the presence of an A:T base pair immediately 3′ of the target cytosine being especially critical.

Methylation of MRPAN in the genomic DNA

In order to analyze the methylation status of MRPAN in genomic DNA of the isolate NNPM3-1-1, we carried out Southern hybridization using two pairs of isoschizomers that show differential sensitivity to cytosine methylation – *Hpa*II and *Msp*I or *Sau*3AI and *Mbo*I. *Hpa*II and *Sau*3AI are sensitive to cytosine methylation in the recognition site but *Msp*I and *Mbo*I are not. The *Hpa*II digests probed with the SP fragment revealed many additional bands of higher molecular weight compared with the *Msp*I digests, indicating that the MRPAN sequence was methylated in the fungal genome (Fig. 5). Likewise, additional bands of higher molecular weight appeared in the blot prepared with *Sau*3AI compared with that for which *Mbo*I was used. As a control, the blot was stripped and reprobed with pEBA18, which contains a LINE-like retrotransposon – an MGR583 homolog (Urashima et al. 1999). No difference was detected in hybridization patterns between the isoschizomers, indicating that no GATC or CCGG sites in the MGR583 homolog were heavily methylated in this fungus. These results suggested that cytosine residues in MRPAN were selectively methylated in the genomic DNA of the isolate NNPM3-1-1.

Discussion

Evidence emerging from studies on inactivation or silencing of repetitive sequences, transposable elements, and transgenes in fungi and plants suggests that all genomes are endowed with systems for defense against intrusive DNA (Kumpatla et al. 1998). At least in several respects, these share common features with the resistance response to viral infection in plants (Ratcliff et al. 1997). In fungi, mechanisms that inactivate transposable elements or duplicated sequences have been studied intensively in certain species. RIP in *N. crassa* (Selker et al. 1987) and MIP in *A. immersus* (Goyon and Faugeron 1989) are well-known processes. Another phenomenon observed in *N. crassa* – known as "quelling" – involves the inhibition of expression of a transgene by an endogenous homolog in vegetative cells, and

Table 3 Frequency distribution of C:G to T:A transitions with respect to sequence context

Nucleotide[a]	C to T transitions						G to A transitions					
	5′ flank			3′ flank			5′ flank			3′ flank		
	Frequency	Total sites[b]	Percentage	Frequency	Total sites[b]	Percentage	Frequency	Total sites[b]	Percentage	Frequency	Total sites[b]	Percentage
A	94	297	31.6	83	199	41.7	140	199	70.4	118	329	35.9
C	15	417	3.6	11	417	2.6	3	327	0.9	39	250	15.6
G	32	255	12.5	5	327	1.5	5	170	2.9	13	179	7.3
T	47	166	28.3	99	206	43.1	87	213	40.8	54	142	38.0

[a] Nucleotide immediately adjacent to an altered C or G
[b] Total number of potential target sites in the region of MAGGY corresponding to the MRPAN sequence

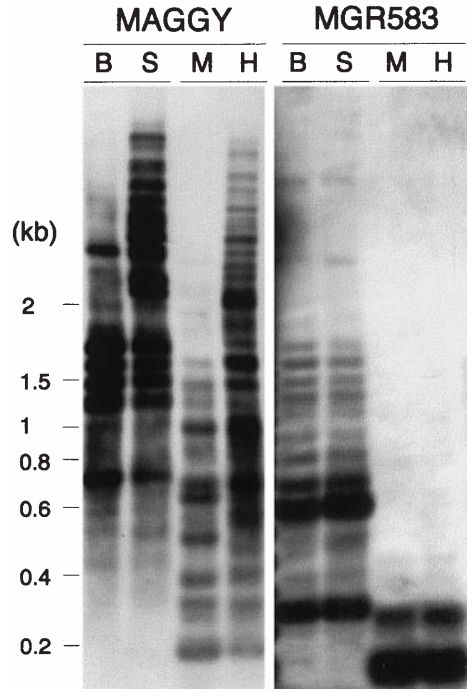


Fig. 5 Analysis of methylation in the genomic region containing MRPAN and MGR583 homologs isolated from the isolate NNPM3-1-1. Genomic DNA was digested with the isoschizomers *Sau*3AI (S) and *Mbo*I (B), or *Hpa*II (H) and *Msp*I (M). *Hpa*II and *Sau*3AI are methylation sensitive, while *Msp*I and *Mbo*I are insensitive to methylation of their recognition sites. Hybridization was performed using probes for MAGGY and MGR583 probes

seems to occur at the posttranscriptional level and to be independent of cytosine methylation (Pandit and Russo 1992; Cogoni et al. 1996).

In this study, *P. grisea* isolates from *P. miliaceum* and possibly those from *P. repens* were found to have only degenerate MAGGY elements, indicating that the ancestor of these isolates successfully captured the genetic invader MAGGY. Two mechanisms are suggested to be involved in the inactivation process, cytosine methylation and C:G to T:A transitions, both of which are typical features of RIP. Several degenerate fungal transposons have been found, which appear to have been subjected to RIP or RIP-like processes (Schechtman 1990; Julien et al. 1992; Kinsey et al. 1994; Neuveglise et al. 1996; Bibbins et al. 1998; HuaVan et al. 1998). MRPAN is, however, distinct from the previously reported cases in two respects.

First, MRPAN is present in the genome of the isolate NNPM3-1-1 as a single-copy element, judging from the results of Southern analysis. We could not identify any other MAGGY homologs in this isolate by Southern hybridization, even under low-stringency washing conditions (data not shown). It is possible that numerous C:G to T:A transitions make other homologs so degenerate that they do not cross-hybridize. However, if this were so, we might need to consider the involvement of some process other than RIP to explain why only one copy of MRPAN remains so unchanged that it can still cross-hybridize with MAGGY under high-stringency conditions; because RIP always occurs in a pair-wise manner; not just one of the extra copies is altered (Selker and Garrett 1988).

There are two possible explanations for the presence of a single copy of MRPAN in the genome of NNPM3-1-1. One invokes the involvement of elimination processes after amplification of the element, and the other postulates that the ancestor of this *P. grisea* subgroup inactivated MAGGY before the element could spread throughout its genome. It is more likely that MRPAN has survived a homology-dependent elimination mechanism after being inactivated, because RIP always occurs in repeated sequences and the elimination of one copy of duplicated sequences has been reported in several organisms (Selker 1997). However, we cannot rule out the possibility that the fungal genome inactivated the genetic invader before amplification. It has been proposed that the genome has defense systems which can detect intrusive DNA on the basis of certain structural features of integration intermediates, compositional heterogeneity of sequence composition, ectopic pairing or disruption of normal genome functions (Kumpatla et al. 1998). Most of these mechanisms, with the exception of ectopic pairing, do not seem to depend on the duplication of intrusive DNA.

Secondly, the preferred target site for C:G to T:A transitions, determined by examining a total 501 sites in MRPAN, was (A/T)pCp(A/T), which differs both from the target site originally determined for RIP in *N. crassa* (CpA; Cambareri et al. 1989) and from the site (CpG) identified for insertions of the *Afut1* transposable element in *Aspergillus fumigatus* (Neuveglise et al. 1996). It also differs from the symmetrical target sites for cytosine methylation in mammalian or plant cells, CpG or CpNpG (Gruenbaum et al. 1981). The target site (A/T)pCp(A/T) can occur in a symmetrical context – as ApCpT or TpCpA. However, the transitions were not restricted to the symmetrical context in MRPAN. It is surprising that the target sites for C:G to T:A transitions in RIP or RIP-like processes in fungi show such variation, whereas that for cytosine methylation seems to be widely conserved in mammals and plants, with some exceptions (Meyer et al. 1994). If the target sites for RIP-like processes are determined by the specificity of a particular enzyme, for example a cytosine methylase, the enzymes in fungi might vary widely with respect to their target site specificity. Indeed, when cytosine methylation was examined in duplicated sequences in *Neurospora* and *Ascobolus* by bisulphite-mediated genomic sequencing, it was found not to be restricted to CpG but could affect any cytosine residues (Selker et al. 1993; Goyon et al. 1996). It is also possible, however, that the enzyme involved in determining the target site of RIP-like processes might be different from that used in cytosine methylation, because the analysis using the isoschizomers *Hpa*I and *Msp*I showed that CCGG sites, which did not fit the target context of the C:G to T:A transitions in MRPAN, were also methylated (Fig. 5).

RIP occurs during a specific period of the sexual cycle when haploid nuclei of the two mating types are in a common cytoplasm (Selker 1997). *Pyricularia* has been shown to have a sexual stage (Kato et al. 1976) and its teleomorph, *Magnaporthe*, belongs to Euascomycetes. However, the sexual stage has been reported only under laboratory conditions and has never been seen in a natural field context. Thus, it is unclear whether *Pyricularia* has a functional sexual cycle under natural conditions. There are several reports that RIP-like processes occur in fungal species in which sexual reproduction has not been found (Julien et al. 1992; Neuveglise et al. 1996; Bibbins et al. 1998). It is attractive to assume that C:G to T:A transitions found in these fungal species are relics of RIP that occurred at a time when they had a functional sexual cycle. However, more general processes might exist that exploit characteristics of the original RIP process that were retained for use in the defense of the fungal genome against "non-self" or repeated sequences.

The reason why only isolates from *P. miliaceum* and *P. repens* have succeeded in preventing the MAGGY element from actively transposing is unclear, because the copy numbers of MAGGY rapidly increased in the isolate NNPM3-1-1 when it was introduced by PEG-mediated transformation (unpublished data). Genomic position or the copy number of the initial integration of MAGGY might account for the difference, or the ancestor of this subgroup might have been quite different from the present isolate.

Acknowledgements We are much indebted to Hajime Kato, the former professor of Kobe University, for valuable suggestions. This work was partly supported by a grant from the Ministry of Education, Japan (No. 10660049).

References

Bibbins M, Cummings NJ, Connerton IF (1998) *DAB1*: a degenerate retrotransposon-like element from *Neurospora crassa*. Mol Gen Genet 258:431–436

Borromeo ES, Nelson RJ, Bonman JM, Leung H (1993) Genetic differentiation among isolates of *Pyricularia* infecting rice and weed hosts. Phytopathology 83:393–399

Cambareri EB, Jensen BC, Schabtach E, Selker EU (1989) Repeat-induced G-C to A-T mutations in *Neurospora*. Science 244:1571–1575

Cogoni C, Irelan JT, Schumacher M, Schmidhauser TJ, Selker EU, Macino G (1996) Transgene silencing of the *al-1* gene in vegetative cells of *Neurospora* is mediated by a cytoplasmic effector and does not depend on DNA-DNA interactions or DNA methylation. EMBO J 15:3153–3163

Dobinson KF, Harris RE, Hamer JE (1993) *Grasshopper*, a long terminal repeat (LTR) retroelement in the phytopathogenic fungus *Magnaporthe grisea*. Mol Plant Microbe Interact 6:114–126

Farman ML, Tosa Y, Nitta N, Leong SA (1996) MAGGY, a retrotransposon in the genome of the rice blast fungus *Magnaporthe grisea*. Mol Gen Genet 251:665–674

Flavell AJ, Jackson V, Iqbal MP, Riach I, Waddell S (1995) Ty1-copia group retrotransposon sequences in Amphibia and Reptilia. Mol Gen Genet 246:65–71

Goyon C, Faugeron G (1989) Targeted transformation of *Ascobolus immersus* and de novo methylation of the resulting duplicated DNA sequences. Mol Cell Biol 9:2818–2827

Goyon C, Rossignol JL, Faugeron G (1996) Native DNA repeats and methylation in *Ascobolus*. Nucleic Acids Res 24:3348–3356

Gruenbaum Y, Naveh Many T, Cedar H, Razin A (1981) Sequence specificity of methylation in higher plant DNA. Nature 292:860–862

HuaVan A, Hericourt F, Capy P, Daboussi MJ, Langin T (1998) Three highly divergent subfamilies of the impala transposable element coexist in the genome of the fungus *Fusarium oxysporum*. Mol Gen Genet 259:354–362

Julien J, Poirier Hamon S, Brygoo Y (1992) Foret1, a reverse transcriptase-like sequence in the filamentous fungus *Fusarium oxysporum*. Nucleic Acids Res 20:3933–3937

Kato H (1994) Phylogenetic aspects of *Pyricularia grisea* on cereals – origin and dissemination (in Japanese). Ann Phytopathol Soc Jpn 60:266–268

Kato H, Yamaguchi T, Nishihara N (1976) The perfect state of *Pyricularia oryzae* Cav. in culture. Ann Phytopathol Soc Jpn 42:507–510

Kim A, Terzian C, Santamaria P, Pelisson A, Prud'homme N, Bucheton A (1994) Retroviruses in invertebrates: the gypsy retrotransposon is apparently an infectious retrovirus of *Drosophila melanogaster*. Proc Natl Acad Sci USA 91:1285–1289

Kinsey JA, Garrett Engele PW, Cambareri EB, Selker EU (1994) The *Neurospora* transposon Tad is sensitive to repeat-induced point mutation (RIP). Genetics 138:657–664

Kozaka T, Kato H (1980) Morphology, host range, and classification of the blast fungus, In: Yamasaki Y, Kozaka T (eds) Rice blast disease and breeding of resistant cultivars. Hakuyusha, Tokyo, pp 47–80

Kumpatla SP, Chandrasekharan MB, Iyer LM, Li GF, Hall TC (1998) Genome intruder scanning and modulation systems and transgene silencing. Trends Plant Sci 3:97–104

Kusaba M, Eto E, Tosa Y, Nakayashiki H, Mayama M (1999) Genetic diversity in *Pyricularia* isolates from various hosts revealed by RFLP of nuclear ribosomal DNA and the distribution of the MAGGY retrotransposon. Ann Phytopathol Soc Jpn, in press

Leung H, Williams PH (1986) Enzyme polymorphism and genetic differentiation among geographic isolates of the rice blast fungus. Phytopathology 76:778–783

Matsuyama N, Kato H, Yamaguchi T (1977) Comparison of the isozyme patterns of extracellular enzymes in *Pyricularia* strains from gramineous and zingiberaceous plants. Ann Phytopathol Soc Jpn 43:419–425

Meyer P, Niedenhof I, ten Lohuis M (1994) Evidence for cytosine methylation of non-symmetrical sequences in transgenic *Petunia hybrida*. EMBO J 13:2084–2088

Mizrokhi LJ, Mazo AM (1990) Evidence for horizontal transmission of the mobile element jockey between distant *Drosophila* species. Proc Natl Acad Sci USA 87:9216–9220

Nakayashiki H, Kiyotomi K, Tosa Y, Mayama S (1999) Transposition of the retrotransposon MAGGY in heterologous species of filamentous fungi. Genetics, in press

Neuveglise C, Sarfati J, Latge JP, Paris S (1996) *Afut1*, a retrotransposon-like element from *Aspergillus fumigatus*. Nucleic Acids Res 24:1428–1434

Pandit NN, Russo VE (1992) Reversible inactivation of a foreign gene, *hph*, during the asexual cycle in *Neurospora crassa* transformants. Mol Gen Genet 234:412–422

Ratcliff F, Harrison BD, Baulcombe DC (1997) A similarity between viral defense and gene silencing in plants. Science 276:1558–1560

Sambrook J, Tritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual (2nd edn). Cold Spring Harbor Laboratory Press, N.Y

Schechtman MG (1990) Characterization of telomere DNA from *Neurospora crassa*. Gene 88:159–165

Selker EU (1997) Epigenetic phenomena in filamentous fungi: useful paradigms or repeat-induced confusion? Trends Genet 13:296–301

Selker EU, Garrett PW (1988) DNA sequence duplications trigger gene inactivation in *Neurospora crassa*. Proc Natl Acad Sci USA 85:6870–6874

Selker EU, Cambareri EB, Jensen BC, Haack KR (1987) Rearrangement of duplicated DNA in specialized cells of *Neurospora*. Cell 51:741–752

Selker EU, Fritz DY, Singer MJ (1993) Dense nonsymmetrical DNA methylation resulting from repeat-induced point mutation in *Neurospora*. Science 262:1724–1728

Shull V, Hamer JE (1994) Genomic structure and variability in *Magnaporthe grisea*. In: Zeigler R, Leong SA, Teng PS (eds) Rice blast disease. CAB International, Wallingford, UK, pp 65–86

Singer MJ, Marcotte BA, Selker EU (1995) DNA methylation associated with repeat-induced point mutation in *Neurospora crassa*. Mol Cell Biol 15:5586–5597

Sone T, Suto M, Tomita F (1993) Host species-specific repetitive DNA sequence in the genome of *Magnaporthe grisea*, the rice blast fungus. Biosci Biotech Biochem 57:1228–1230

Song SU, Gerasimova T, Kurkulos M, Boeke JD, Corces VG (1994) An *env*-like protein encoded by a *Drosophila* retroelement: evidence that gypsy is an infectious retrovirus. Genes Dev 8:2046–2057

Tosa Y, Nakayashiki H, Hyodo H, Mayama S, Kato H, S AL (1995) Distribution of retrotransposon MAGGY in *Pyricularia* species. Ann Phytopathol Soc Jpn 61:549–554

Urashima AS, Igarashi S, Kato H (1993) Host range, mating type, and fertility of *Magnaporthe grisea* from wheat in Brazil. Plant Dis 77:1211–1216

Urashima AS, Hashimoto Y, Don LD, Kusaba M, Tosa Y, Nakayashiki H, Mayama S (1999) Molecular analysis of the wheat blast population in Brazil with a homologue of retrotransposon MGR583. Ann Phytopathol Soc Jpn, in press