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

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Abstract

The distribution of an LTR-retrotransposon, MAGGY, is sporadic among Pyricularia grisea isolates. Based on a dendrogram constructed by RFLP of rDNA, the isolates of MAGGY-carriers were classified into a single cluster that comprised four rDNA types. However, a few members belonging to this cluster, exemplified by the isolates from common millet (Panicum miliaceum), showed distinct features regarding the MAGGY element. Southern analysis suggested that they possessed a single copy of MAGGY-related sequence showing different restriction patterns from MAGGY. Sequence analysis revealed that the MAGGY-related sequence was a degenerate form of MAGGY caused by numerous C:G to T:A transitions which are often reported in RIP (Repeat-induced point mutation) or RIP like processes. However, the favored target sites of the C:G to T:A transitions determined by examining a total of 500 sites were (A/T)pCp(A/T) in this fungus, which was different from that of the original RIP, CpA, or that reported in Aspergillus, CpG. The fact that certain members of the MAGGY-carriers' cluster retain a single copy of degenerate MAGGY implies that an ancestor of these isolates captured successfully the genetic invader, MAGGY.

Key Words  retrotransposon • filamentous fungi • Pyricularia grisea • RIP • gene inactivation

Introduction

Pyricularia grisea (teleomorph, Magnaporthe grisea), a causal filamentous fungus of blast disease, shows intraspecific variation on pathogenicity in spite of a uniform morphological feature (Kozaka and Kato 1980). Several attempts to classify P. grisea isolates infecting different host plants have been carried out 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 have genetic diversity and could be classified, to a certain extent, into several subgroups depending on the host plants they were isolated from. Recently we reported that seventy-four *P. grisea* isolates from twenty-nine host plants were reduced into fourteen subgroups according to rDNA types (Kusaba et al. 1999). Most isolates from a given host plant belonged to a subgroup of a single rDNA type, indicating that they are genetically close and might be derived from a clonal lineage.

In *P. grisea*, the distribution of some transposable elements were restricted to certain subgroups. An LTR-retrotransposon, *grasshopper* was found exclusively in a subgroup of *Eleusine* isolates (Dobinson et al. 1993). Concerning MAGGY, an another LTR-retrotransposon of *P. grisea*, the situation appeared to be rather complex because it was present in multiple copies in isolates from several plants belonging to different genus, 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 isolates of MAGGY-carriers are classified into four rDNA types, which form a single cluster in a dendrogram of *P. grisea* isolates including MAGGY-free isolates from various host plants (Kusaba et al. 1999). These phenomena imply that the elements, *grasshopper* and MAGGY, were acquired horizontally by each common ancestor of the clusters. Support for this notion was obtained from transformation experiments in which MAGGY were introduced into MAGGY-free isolates of *P. grisea*. MAGGY has been proved to have an ability to transpose and amplify the copy number even in MAGGY-free isolates, suggesting that the absence of MAGGY in these isolates might be due to the reason that their genomes have not ever encountered the element (Nakayashiki et al. 1999).

Horizontal transmission has been proposed in several transposable elements because of their sporadic or discontinuous distribution (Mizrokhi and Mazo 1990; Flavell et al.
A possibility of horizontal transmission of MAGGY between \textit{P. grisea} isolates was also pointed out based on an exceptional presence of MAGGY in a buffel grass isolate which belonged to a genetically distant cluster from that of MAGGY-carriers (Kusaba et al. 1999). A remarkable example is \textit{gypsy} that was found in \textit{Drosophila melanogaster} and thought to be a type member of LTR-retrotransposon for long time. One of \textit{gypsy} elements, \textit{gypsyDm} has been proved to have infective properties, thus apparently undergo 'horizontal transmission' between \textit{Drosophila} species (Kim et al. 1994; Song et al. 1994).

Most transposons have been regarded as intragenomic parasites that could cause several types of genetic variations of hosts. Most of them, despite some exceptions, could be harmful for hosts. Some 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 \textit{Neurospora crassa} (Selker et al. 1987) and MIP (methylation induced premeiotically) in \textit{Ascobolus immersus} (Goyon and Faugeron 1989) are well-known processes which inactivate repeated sequences in a specific period of the sexual cycle (Selker 1997). Epigenetic changes such as cytosine methylation seemed to be associated with both the inactivation processes. Moreover, RIP causes genetic changes, C:G to T:A transitions, mainly at CpA dinucleotides in duplicated sequences and inactivate affected sequences irreversibly (Cambareri et al. 1989).

In this study we cloned a MAGGY-related sequence in a \textit{P. grisea} isolate from common millet (\textit{Panicum miliaceum}), which belonged to the MAGGY-carriers' cluster but showed distinct features regarding MAGGY; i) a single copy per genome, ii) different internal restriction patterns from 'authentic' MAGGY. We found that the sequence was a degenerate form of MAGGY caused by numerous C:G to T:A transitions. The origin and implication of the sequence were discussed.
Materials and methods

Fungal strains and culture media

Field isolates of *P. grisea* used in this study are shown in Table 1. All of the isolates were purified by monoconidial isolation and maintained on PDA media for short-time storage or on sterilized barley seeds for long-time 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* strains JM109 (TOYOBO, Osaka, JAPAN) and XL1-Blue (GIBCO-BRL, Gaithersburg, Md., USA). pBlurescript SK+ II (Stratagene) or pUC19 (Takara, Otsu, Japan) was used in subcloning procedures. Plasmids were extracted from *E. coli* cells using QIAGEN Midi plasmid kit™ (QIAGEN GmbH, Germany) according to the manufacturer's instruction.

DNA isolation and analysis

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

Construction of a genomic library

Fifty micrograms of total DNA of a *P. miliaceum* isolate (NNPM3-1-1) was partially digested with *Sau3AI*. 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 fluorescein-labeled 5.4kb-*XhoI* fragment of MAGGY using Gene Images™ detection system (Amersham).

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 sequence was determined on double-stranded DNA using a SequiTherm EXCEL II Long-Read DNA kit (Epicentre technologies) in conjunction with fluorescent-labeled universal primers and using a Perkin-Elmer-Cetus DNA thermal cycler PEC480 and a DSQ1000 DNA sequencer (Shimadzu).

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

In addition to rice isolates, *P. grisea* isolates from several crops and grasses have been shown to possess MAGGY homologues in their genomes (Tosa et al. 1995; Farman et al. 1996; Kusaba et al. 1999). A MAGGY homologue detected in a *Panicum repens* isolate showed weak homology with MAGGY and a distinct feature in respect to internal restriction fragments (Farman et al. 1996). To examine the distribution and structure of the MAGGY homologue in *Panicum* isolates of *P. grisea*, we carried out Southern analysis of twelve isolates from four plant species belonging to *Panicum* genus. Genomic DNA was digested with *Pst*I and probed with a 4.6-kb *Sal*I-*Pst*I fragment of MAGGY (SP fragment). *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 is shown in a rice isolate (Fig. 1A, lane 13). In *P. maximum* and *P. bisulcatum* isolates, the fragment of 5.4-kb was detected, indicating that these isolates possess elements closely related with 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 than 5.4-kb. Although two fragments (estimated as 20-kb, 13-kb) were observed in most *P. miliaceum* isolates, it has been revealed that the isolates possess only the 13-kb fragment actually when the fungal DNA was extracted from mycelia cultured in CM liquid broth containing 5mM 5-azacytidine (Fig. 1B). This indicated that the 20-kb fragment was a partial digest with *Pst*I on account of methylation in the recognition site. Thus, the MAGGY homologue seemed to be 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 homologues in *Panicum* isolates, isolates showing distinct band patterns in Fig. 1 were picked up, and their DNA
were digested with EcoRI and hybridized with the SP fragment. 'Authentic' MAGGY contains four internal EcoRI 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 homologues found in P. grisea isolates from various host plants (Farman et al. 1996; Kusaba et al. 1999). P. maximum and P. bisulcatum isolates as well as rice and foxtail millet isolates were shown to contain all the four fragments (Fig. 2), whereas P. miliaceum and P. repens isolates showed different hybridization patterns. Only one fragment (1.19-kb) in the P. miliaceum isolates showed the same mobility in electrophoresis as the fragment of 'authentic' MAGGY. These results indicated that sequences of the MAGGY homologues in the P. miliaceum and P. repens isolates were different from that of 'authentic' MAGGY.

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

To isolate the MAGGY homologue, a genomic library was constructed from a P. miliaceum isolate NNPM3-1-1 that showed a typical hybridization pattern of the P. miliaceum isolates in the Southern blots. The genomic library was screened with 5.4-kb XhoI fragment of MAGGY. Five thousands phage plaques were screened, from which two positive phage clones, #8-1 and #8-2, were isolated. Southern blot analysis of these phage clones and genomic DNA of the NNPM3-1-1 isolate revealed that inserts of the phage clones were overlapped each other and contained partial fragment of the same MAGGY homologue (data not shown). The MAGGY homologue was called MRPAN (MAGGY related sequence in Panicum isolate). The physical map of cloned MRPAN and its flanking genomic region is shown in Fig. 3. Nine kilobases SalI fragment of MRPAN#8-1 was subcloned into SalI-digested pUC19 and sequenced. This fragment also contained a homologue of MGSR1, a SINE like element (Sone et al. 1993).
MRPAN is degenerate MAGGY resulting from C:G to T:A transitions.

The sequenced region contained domains corresponding to protease, reverse transcriptase, RNaseH, and integrase of LTR-retrotransposon. Predicted polypeptides were, however, truncated by several stop codons. Fig. 4 represents the comparison of nucleotide and deduced amino acids sequences between MRPAN and MAGGY in reverse transcriptase and integrase domains. Overall identity between MRPAN and MAGGY was 86.6% in 3895 nucleotides. Interestingly, 96.0% of base changes between MRPAN and MAGGY were C:G to T:A transitions that were responsible for most stop codons embedded in 'ORF' of MRPAN (Table 2). Excepting the C:G to T:A transitions, MRPAN and MAGGY were identical at 99.5%. Thus, MRPAN was not a distinct retrotransposon from MAGGY but a degenerate form of MAGGY caused by C:G to T:A transitions. C:G to T:A transitions are often reported in events like RIP, with which cytosine methylation is associated (Cambereri et al. 1989). The most probable explanation for a mechanism of the C:G to T:A transitions is deamination of 5-methyl cytosine that leads to generate thymine, and consequently to produce base changes of guanine to adenine in the complementary strand during a duplication process.

Five hundred G-C base pairs out of 3895-bp in MAGGY were converted to A-T base pairs in MRPAN. More detail, 235 out of 1183 cytosine residues (19.9%) and 265 out of 939 guanine residues (28.2%) on a sense strand of MAGGY were replaced with thymine and adenine residues in MRPAN, respectively. The frequency of occurrence of G to A transitions was significantly higher than that of C to T transitions. To determine whether the C:G to T:A transitions occurred randomly or not, target site specificity was examined by comparing sequences flanking the transitions between MAGGY and MRPAN. The bases immediately 5’ and 3’ of the site of transitions were categorized and counted.
When the bases flanking the transitions were also changed on account of C:G to T:A transitions or other base replacements between MAGGY and MRPAN, we eliminated them from further analysis, because we could not determine which change was earlier, leading us being uncertain of the target context. In general, a cytosine or guanine residue flanked with A:T base pair was preferred site of C:G to T:A transition. Particularly, the base 3’ of changed cytosine and 5’ of changed guanine were very restricted to A:T base pairs. On the other hand, restriction to A:T base pairs at 5’ of changed cytosine and 3’ of changed guanine seemed to be rather looser. This symmetric target specificity leads us to assume that the C to T and G to A transitions represented the same event in different complementary strands of nucleic acids. Thus, we summed up the results from both transitions with a view from the strand showing C to T transitions. When just 5’ of cytosine was A, C, G, and T, respectively, the C to T transitions occurred in 32.6%, 6.0%, 13.1%, and 33.3%. While just 3’ of cytosine was A, C, G, and T, respectively, the C to T transitions occurred in 41.5%, 2.9%, 1.2%, and 59.0%. It is noteworthy that 80.3% of cytosine residues was subjected to the C to T transition when both 5’ and 3’ of it were A:T base pairs. However, no preference of the symmetrical context, like 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, especially A:T base pair 3’ of the target cytosine was a critical factor.

Methylation of MRPAN in the genomic DNA.

In order to analyze the methylation status of MRPAN in the genomic DNA of the isolate NNPM3-1-1, we carried out Southern hybridization using two sets of isoschizomers of restriction enzymes showing different sensitivity to cytosine methylation, HapII and MspI or Sau3AI and MboI. HapII and Sau3AI are sensitive to cytosine methylation in the
recognition site but MspI and MboI are not. The HapII-digests probed with the SP fragment created many additional bands of higher molecular weight compared with these in the MspI-digests, indicating that the MRPAN sequence was methylated in the fungal genome (Fig. 5). Likewise, additional bands of higher molecular weight appeared in the Sau3AI-digests in the Southern blot compared with the MboI-digests. As a control, the blot was stripped and reprobed with pEBA18 which contained a LINE-like retrotransposon, MGR583 homologue (Urashima et al. 1999). No difference was detected in hybridization patterns between the isoschizomers, indicating that no GATC nor CCGG sites in the MGR583 homologue was 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.

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Discussion

Emerging evidence from studies on inactivation or silencing of repetitive sequences, transposable elements, and transgenes in fungi and plants suggest that all genomes are endowed with defense systems against intrusive DNA (Kumpatla et al. 1998). At least in several respects, common features are shared with resistance response to viral infection in plants (Ratcliff et al. 1997). In fungi, mechanisms to 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. An another phenomenon in N. crassa, "quelling", which describes the inhibition of gene expression by the homologous transgene in vegetative cells, seemed to occur at posttranscriptional level independent of cytosine methylation (Pandit and Russo 1992; Cogoni et al. 1996).

In this study, M. grisea isolates from P. miliaceum and possibly those from P.
repens possessed only degenerate MAGGY, indicating that an ancestor of these isolates captured successfully the genetic invader, MAGGY. Involvement of two mechanisms in the inactivation process are suggested, cytosine methylation and C:G to T:A transitions. Both are typical features of RIP. Several degenerate fungal transposons have been found, which seemed to be 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; Hua Van et al. 1998).

MRPAN is, however, distinct from previous reports in two points.

First, MRPAN exist in the genomic DNA of the isolate NNPM3-1-1 as a single copy element as far as judged from Southern analysis. We could not identify apparent other MAGGY homologues in this isolate by Southern analysis under low stringency wash condition (data not shown). It is possible that numerous C:G to T:A transitions make other homologues degenerated beyond cross-hybridization under high stringency condition. However, if 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 crosshybridize with MAGGY under high stringency condition, because RIP always occurs in a pair-wise manner; not just one are altered (Selker and Garrett 1988).

There are two possible explanations for MRPAN to be a single copy in the genome of the isolate NNPM3-1-1. One is the involvement of an elimination process after amplification of the element, and the other is that an ancestor of this P. grisea subgroup inactivated MAGGY before the element spread throughout its genome. It is more likely that MRPAN is a survived copy from a homology-dependent elimination mechanism after being inactivated, because RIP always occurs in repeated sequences and the elimination of one copy of duplicated sequences is reported in several organisms (Selker 1997). However, we can not rule out the possibility that fungal genome inactivated the genetic invader before amplification. It is proposed that the genome has defense systems in which intrusive DNA is monitored or detected by certain structural features of integration intermediates, sequence compositional heterogeneity, ectopic pairing or disruption of normal genome functions.
(Kumpatla et al. 1998). Most of these points except ectopic pairing seemed not to depend on the duplication of intrusive DNA.

Secondly, preferred target site of C:G to T:A transitions determined by examining total 500 sites in MRPAN was (A/T)pCp(A/T), which differed from that found in previous reports, CpA in original RIP of N. crassa (Cambareri et al. 1989) and CpG indicated in Aspergillus fumigatus by their significant depletion in Afut1 transposable element (Neuveglise et al. 1996). This was also different from symmetrical target sites of cytosine methylation in mammalian or plant cells, CpG or CpNpG (Gruenbaum et al. 1981). The target site, (A/T)pCp(A/T) could form symmetrical context like ApCpT or TpCpA. However, the target sites were not restricted to symmetric context in MRPAN. It is surprising that target sites of the C:G to T:A transitions in RIP or RIP like processes in fungi have many varieties whereas that of cytosine methylation seems to be widely conserved in mammals and plants despite some exceptions (Meyer et al. 1994). If the target sites of the RIP like processes are determined by specificity of some enzyme, a possible example of which is cytosine methylase, the enzyme in fungi might have wide varieties in respect to the target site specificity. Indeed, when cytosine methylation was examined in duplicated sequences in Neurospora and Ascobolus by bisulphite genomic sequencing, it was not restricted to CpG and affected 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 by isoschizomers, Sau3AI and MboI, showed that CCGG sites which did not fit the target context of the C:G to T:A transitions, were also methylated in this fungus (Fig. 5).

RIP occurs in a specific period of the sexual cycle when haploid nuclei of the two mating types are in a common cytoplasm (Selker 1997). Pyricularia is proved to have a sexual stage (Kato et al. 1976) and its teleomorph, Magnaporthe, belongs to ascomycete. However, the sexual stage has been reported only under laboratory condition and never in
a field so far. Thus, it is unclear whether *Pyricularia* has a functional sexual cycle under natural condition. 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 an attractive explanation that C:G to T:A transitions found in these fungal species are the relic of RIP at a time when they had a sexual cycle. However, taken together the target site specificity, there might be more general processes extended from the characteristics of original RIP in the defense mechanisms of 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 being active is unclear. Because MAGGY rapidly amplified its copy number in the isolate NNPM3-1-1 when it was introduced by PEG-mediated transformation (unpublished data). Genomic position or copy number of the initial integration of MAGGY might cause the difference, or an ancestor of this subgroup might have different nature from the present isolate.

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Figure Legends

Fig. 1 Southern blot analysis of *P. grisea* isolates from *Panicum* plants and rice. (A) Genomic DNA was digested with *Pst*I, fractionated on a 0.7% TAE agarose gel, and probed with a 4.6-kb *Sal*I-*Pst*I fragment of MAGGY. An arrow indicates a 5.4-kb fragment of MAGGY. 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 containing no (C) or 5mM 5-azacytidine (Aza).

Fig. 2 Southern blot analysis of *P. grisea* isolates from rice, foxtail millet, and *Panicum* plant species. Genomic DNA was digested with *Eco*RI, fractionated on a 1.5% TAE agarose gel, and probed with a 4.6-kb *Sal*I-*Pst*I fragment of MAGGY. 1, pMGY70 (a control plasmid containing a copy of MAGGY); 2, 1836-3 (rice); 3, GFSI1-7-2 (foxtail millet); 4, NI922 (*P. bisulcatum*); 5, IN77-33-1-1 (*P. maximum*); 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 to the cloned MAGGY sequence.

Fig. 3 Physical map of genomic sequence of an isolate NNPM3-1-1 containing MRPAN. Isolated genomic clones are shown by arrows. Open box indicates the MRPAN sequence which hybridizes with the MAGGY probe. Shaded box indicates a MGSR1 homologue. Recognition sites for *Apa*I, *Bam*HI, *Cla*I, *Pst*I, *Sal*I, *Sma*I are indicated.

Fig. 4 Nucleotide and deduced amino acid alignments of reverse transcriptase (A) and integrase (B) domains of MAGGY and MRPAN. Stop codons are indicated by asterisks. The hyphens indicate identical nucleotides with the reference sequence (MAGGY). Differences are given by letters.
Fig. 5 Analysis of methylation in MRPAN and MGR583 homologue regions of the isolate NNPM3-1-1.

Genomic DNA was digested with isoschizomers, *Sau3AI* (S) and *MboI* (B), or *HapII* (H) and *MspI* (M). *HapII/Sau3AI* and *MspI/MboI* are methylation sensitive and insensitive enzymes, respectively. Hybridization was performed using MAGGY and MGR583 probes.
Table 1 *Pyricularia grisea* isolates used in this study

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<td><em>Panicum repens</em> L.</td>
<td>Bangalore</td>
<td>H. Kato</td>
</tr>
<tr>
<td>IN77-33-1-1</td>
<td><em>Panicum maximum</em> Jacq.</td>
<td>Bangalore</td>
<td>H. Kato</td>
</tr>
<tr>
<td>NI922</td>
<td><em>Panicum bisulcatum</em> Thmb.</td>
<td>Tochigi</td>
<td>N. Nishihara</td>
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<tr>
<td>1836-3</td>
<td><em>Oryzae sativa</em> L.</td>
<td>Niigata</td>
<td>M. Yamada</td>
</tr>
<tr>
<td>GFSI1-7-2</td>
<td><em>Setaria Italica</em> Beauv.</td>
<td>Gifu</td>
<td>H. Kato</td>
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</tbody>
</table>
Table 2  Nucleotide base replacements found between MAGGY and MRPAN

<table>
<thead>
<tr>
<th>MAGGY</th>
<th>MRPAN</th>
<th>Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>C</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>A</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>235</td>
</tr>
<tr>
<td>G</td>
<td>A</td>
<td>265</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>3</td>
</tr>
<tr>
<td>T</td>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 3  Frequency of occurrence of C:G to T:A transitions in each sequence context

<table>
<thead>
<tr>
<th></th>
<th>C to T</th>
<th></th>
<th>G to A</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5'</td>
<td>3'</td>
<td></td>
<td>5'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Occurrence</td>
<td>Sites</td>
<td>(%)</td>
<td>Occurrence</td>
<td>Sites</td>
<td>(%)</td>
<td>Occurrence</td>
</tr>
<tr>
<td>A a</td>
<td>93</td>
<td>297</td>
<td>31.3</td>
<td>84</td>
<td>199</td>
<td>42.2</td>
<td>139</td>
</tr>
<tr>
<td>C</td>
<td>15</td>
<td>417</td>
<td>3.6</td>
<td>12</td>
<td>417</td>
<td>2.9</td>
<td>3</td>
</tr>
<tr>
<td>G</td>
<td>33</td>
<td>255</td>
<td>12.9</td>
<td>5</td>
<td>327</td>
<td>1.5</td>
<td>5</td>
</tr>
<tr>
<td>T</td>
<td>46</td>
<td>166</td>
<td>27.7</td>
<td>100</td>
<td>206</td>
<td>48.5</td>
<td>87</td>
</tr>
</tbody>
</table>

a Nucleotide 5' or 3' of changed C or G
b Number of the target sites in the sequenced region of MRPAN