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Transposition of the Retrotransposon MAGGY in Heterologous Species of Filamentous Fungi

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ABSTRACT

MAGGY is a *gypsy*-like LTR retrotransposon isolated from the blast fungus *Pyricularia grisea* (teleomorph, *Magnaporthe grisea*). We examined transposition of MAGGY in three *P. grisea* isolates (wheat, finger millet, and crabgrass pathogen), which did not originally possess a MAGGY element, and in two heterologous species of filamentous fungi, *Colletotrichum lagenarium* and *P. zingiberi.* Genomic Southern analysis of MAGGY transformants suggested that transposition of MAGGY occurred in all filamentous fungi tested. In contrast, no transposition was observed in any transformants with a modified MAGGY containing a 513-bp deletion in the reverse transcriptase domain. When a MAGGY derivative carrying an artificial intron was introduced into the wheat isolate of *P. grisea* and *C. lagenarium*, loss of the intron was observed. These results showed that MAGGY can undergo autonomous RNA-mediated transposition in heterologous filamentous fungi. The frequency of transposition differed among fungal species. MAGGY transposed actively in the wheat isolate of *P. grisea* and *P. zingiberi*, but transposition in *C. lagenarium* appeared to be rare. This is the first report that demonstrates active transposition of a fungal transposable element in heterologous hosts. Possible usage of MAGGY as a genetic tagging tool in filamentous fungi is discussed.

EUKARYOTIC genomes contain long-terminal-repeat (LTR) retrotransposons, transposable elements that use an RNA intermediate during duplication, like retroviruses (Boeke *et al.* 1985; Garfinkel *et al.* 1985). Because LTR retrotransposons have been found in a wide range of eukaryotes, *e.g.*, insects (Saigo *et al.* 1984), yeast (Clare and Farabaugh 1985), nematodes (Aeby *et al.* 1986), plants (Voytas and Ausubel 1988), reptiles, amphibians (Flavell *et al.* 1995), fish (Tristem *et al.* 1995), and filamentous fungi (McHale *et al.* 1992), it is believed that they are ubiquitous components of eukaryotic genomes. LTR retrotransposons are divided into Ty1-*copia* and Ty3-*gypsy* groups. Two genes related to *gag* and *pol* of retroviruses have been identified in both groups. In addition, some elements of the latter group possess an additional open reading frame (ORF) or domain corresponding to the *env* gene of retroviruses, which encodes the envelope glycoprotein responsible for recognition of cell surface receptors and is essential for viral infection (Dorner *et al.* 1985; Dorner and Coffin 1986). A *gypsy* element (*gypsyDm*) containing a functional *env* gene has been shown to have infective properties and thus to be a retrovirus of *Drosophila melanogaster* (Kim *et al.* 1994a; Song *et al.* 1994), and other *gypsy* elements (*gypsyDs* and *gyspyDv*) containing defective *env* genes are thought to be degenerate forms of *D. melanogaster* retroviruses (Alberola and de Frutos 1996). It is unclear, however, whether all members of Ty3-*gypsy* group retrotransposons are derived from retroviruses of invertebrates.

In addition to Ty elements in yeast, several LTR retrotransposons have been identified recently in various species of fungi, especially in pathogenic filamentous fungi (Daboussi 1997). However, few of them have been proven to possess the ability to transpose. MAGGY is an LTR retrotransposon isolated from the blast fungus *Pyricularia grisea* (teleomorph, *Magnaporthe grisea*) and is comprised of two ORFs and 253-bp LTRs (Farman *et al.* 1996). ORF1 encodes a predicted peptide of 457 amino acids exhibiting a potential zinc finger domain (Cys-X2-Cys-X4-His-X4-Cys) that is widely conserved in the *gag* protein of retroelements. ORF2 has a typical character of a *pol* gene encoding a polypeptide with protease, reverse transcriptase, RNaseH, and endonuclease domains. Amino acid sequences of ORF2 and the order of its domains show that MAGGY belongs to the Ty3-*gypsy* group. Interestingly, most LTR retrotransposons found in filamentous fungi to date are classified into the Ty3-*gypsy* group with a few exceptions (Goyon *et al.* 1996; Tooley and Garfinkel 1996).

Pyricularia spp. consist of many subgroups that cause the blast disease on diverse plant species of Gramineae, Cannaceae, Cyperaceae, and Zingiberaceae (Kozaka and Kato 1980; Ou 1985). Genomic Southern analysis revealed that MAGGY was not ubiquitous in genomes of Pyricularia subgroups (Tosa *et al.* 1995). MAGGY was present in multiple copies in Pyricularia isolates from rice, foxtail millet, and some grasses but absent in those

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TABLE 1

Fungal isolates used in this study

Isolate	Species	Host	Locality	Collector
Br48	*Pyricularia grisea*	*Triticum aestivum* (L.) Thell (wheat)	Brazil	A. S. Urashima
Z2-1	*Pyricularia grisea*	*Eleusine coracana* (L.) Gacrtn (finger millet)	Japan	S. Ito *et al.*
Dig41	*Pyricularia grisea*	*Digitaria sanguinalis* (L.) Scop. (crabgrass)	Japan	Y. Iwamoto
HYZiM 101-1-1-1	*Pyricularia zingiberi*	*Zingiber mioga* (Thunb.) Rosc. (mioga)	Japan	M. Ogawa
104T	*Colletotrichum lagenarium*	*Cucumis sativus* L. (cucumber)	Japan	—[a]

[a] Stock culture of the laboratory of plant pathology, Kyoto University.

from wheat, finger millet, crabgrass, mioga, etc. The sporadic distribution of MAGGY in Pyricularia subgroups raises questions regarding the ability of the element to transpose in noncarriers of MAGGY and in heterologous species of filamentous fungi other than *Pyricularia* spp. Transposition of Ty1, a type member of the Ty1-*copia* group isolated from *Saccharomyces cerevisiae*, was not supported in *Schizosaccharomyces pombe* cells (Keeney *et al.* 1995), and no fungal retrotransposons have been reported to transpose in heterologous host species. Only Tad, a LINE-like element of Neurospora, was shown to undergo retrotransposition between nuclei in heterokaryons (Kinsey 1993).

In contrast to fungal retrotransposons, active transposition in heterologous host species was demonstrated in two plant Ty1-*copia* retrotransposons (Lucas *et al.* 1995; Hirochika *et al.* 1996a). One is the tobacco retrotransposon Tnt1, which is active in *Arabidopsis thaliana*, and the other is the tobacco retrotransposon Tto1, which actively transposes in *Oryza sativa.* Both elements transpose through an RNA intermediate, indicating that the whole transposition cycle of Tnt1 and Tto1 occurred even in heterologous plant host cells. These results suggested that all host factors required for retrotransposition are conserved in a wide range of plants including monocots and dicots. Although the whole cycle of transposition was not examined, it was also shown that the promoter activity of *D. melanogaster* retrotransposon 1731 was maintained in the amphibian *Pleurodeles waltl*, suggesting that the insect retrotransposon can be expressed in vertebrates (Kim *et al.* 1994b).

In this study we show that MAGGY is an active element that transposes autonomously via an RNA intermediate in fungal genomes that do not originally possess this element.

MATERIALS AND METHODS

Fungal isolates, bacterial strains, and plasmids: Fungal isolates used in this study are listed in Table 1. Three isolates of *P. grisea* (wheat, crabgrass, and finger millet isolates), one *P. zingiberi* isolate, and one *Colletotrichum lagenarium* isolate were examined for their ability to function as new hosts of MAGGY. All fungi tested did not possess a MAGGY element originally. *P. grisea* and *P. zingiberi* were maintained on potato dextrose agar (PDA) for several months. For long-time storage they were grown on sucrose containing sterilized barley seeds in a vial, then dried thoroughly at 25°, and kept in a case with silica gel at 5°. *C. lagenarium* 104 T was kindly provided by Dr. Yasuyuki Kubo and maintained on PDA agar media. For DNA isolation and protoplast preparation, *P. grisea* and *P. zingiberi* were grown in CM broth (0.3% casamino acids, 0.3% yeast extract, 0.5% sucrose) at 26°, while *C. lagenarium* was cultured in potato-sucrose liquid media at 26°.

Genomic subclone plasmids pMGY-RF and pMGY-LF2 containing partial MAGGY sequences were kindly provided by Dr. Sally Leong. All plasmids were maintained in *Escherichia coli* strains JM109 (TOYOBO, Osaka, Japan) and XL1-Blue (GIBCO BRL, Gaithersburg, MD). pBluescript SK+ II (Stratagene, La Jolla, CA) was used in subcloning procedures. Plasmids were extracted from *E. coli* cells using a Midi plasmid kit (QIAGEN GmbH) according to the manufacturer's instructions.

Recombinant DNA techniques: Restriction enzymes and other modification enzymes were obtained from Boehringer Mannheim (Tokyo, Japan) and Takara (Otus, Japan), and used according to the manufacturers' instructions. Cloning techniques were performed by standard procedures (Sambrook *et al.* 1989). A set of 50-bp oligonucleotides (5′-GGT AAGCTTACGCGATAGCTCAGGAGCTGGGAGCTGGCTGA CCATCTGCA-3′, 5′-GATGGTCAGCCAGCTCCCAGCTCGT GAGCTATCGCGTAAGCTTACCTGCA-3′) was synthesized according to intron 2 of a *THR1* reductase gene isolated from *C. lagenarium* (Perpetua *et al.* 1996). An artificial intron with cohesive *Pst*I ends was prepared by annealing these oligonucleotides under standard conditions (Sambrook *et al.* 1989).

Transformation of fungi: Fungal transformants with MAGGY plasmids were obtained via polyethylene glycol (PEG)-mediated cotransformation with plasmid pSH75 (Kimura and Tsuge 1993), which carries a hygromycin B phosphotransferase gene under the control of the *Aspergillus nidulans trp*C promoter. Transformation procedures were performed as described by Kubo and Furusawa (1991) with minor modifications. Fungal protoplasts were produced by digesting mycelia in a digestion buffer (10 mm Na_2HPO_4, 1.2 m $MgSO_4$) containing 5 mg/ml lysing enzymes (Sigma, St. Louis) and 3.5 mg/ml Zymolyase 20T (Seikagaku, Japan) for 3 hr. Kitalase (Wako Pure Chemicals, Japan) was added to the enzyme solu-

tion in the case of *P. zingiberi* at the concentration of 2 mg/ml. Digested protoplasts were filtered through sterile gauze and collected by centrifugation at 800 × *g*, then washed with STC solution (1 m sorbitol, 50 mm Tris-HCl, pH 8.0, 50 mm $CaCl_2$). Five micrograms of plasmid DNA were mixed with 3 μg of pSH75 and added to 150 μl of protoplast suspension. After 10 min incubation at room temperature, 200 μl, 400 μl, and 600 μl of PEG solution (60% polyethylene glycol 3350 in 50 mm Tris-HCl, pH 8.0, 50 mm $CaCl_2$) were added in a step-by-step manner and incubated for 10 min at room temperature. PEG solution was removed by centrifugation at 2000 × *g*. Collected protoplasts were suspended in 1 ml of STC solution and mixed with 3 ml of regeneration agar (PDA osmotically conditioned with 0.6 m glucose), then poured onto regeneration agar plates containing 100–400 μg/ml hygromycin B. The plates were incubated at 26° for 4–7 days. Hygromycin-resistant regenerants on the plates were transferred to new hygromycin-containing PDA media for the second screening.

Extraction of fungal genomic DNA and Southern blot hybridization: Frozen mycelia (0.3–0.4 g) were ground to a powder in liquid N_2 with a mortar and pestle, and 600 μl of DNA isolation buffer (100 mm LiCl, 100 mm EDTA, 10 mm Tris-HCl, pH 7.5, 0.5% SDS) was added and mixed thoroughly. After incubation at 55° for 30 min, the mycelial debris was pelleted by centrifugation at 10,000 × *g* for 10 min. The supernatant was carefully removed and extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The DNA was precipitated from the aqueous phase with 1 volume isopropanol and recovered by centrifugation at 10,000 × *g* for 15 min. The pellet was resuspended in 500 μl of TE (10 mm Tris-HCl, pH 8.0, 1 mm EDTA) and treated with 40 μg/ml RNase A at 37° for 30 min. Extractions with an equal volume of phenol, phenol:chloroform:isoamyl alcohol (25:24:1), and chloroform:isoamyl alcohol (24:1) were performed and repeated as needed. The aqueous phase was transferred to a new tube and 11/16 volume of D-PEG solution (20% polyethylene glycol 6000, 2.5 m NaCl) was added. After incubation on ice for 10 min, the DNA was recovered by centrifugation at 10,000 × *g* for 10 min, washed with 70% ethanol, and finally resuspended in an appropriate volume of TE.

Southern blot analysis was performed using the dioxetane chemiluminescence system Gene Images (Amersham, Arlington Heights, IL). Typically, 1 μg of genomic DNA was digested using a 3- to 10-fold excess of restriction enzyme to ensure complete digestion. The reaction mixture was fractionated on a 40 mm Tris-acetate, 1 mm EDTA (TAE) agarose gel. After denaturation and neutralization, the DNA was transferred to nylon membranes (Hybond N^+; Amersham) and fixed by ultraviolet irradiation. Fluorescein-labeled probes were prepared by a random prime labeling method using a kit included in Gene Images. The hybridization and detection procedures were performed according to the manufacturer's instructions.

PCR analysis: Template DNA for PCR analysis was prepared from fungal colonies at the stage of the second screening after transformation. Each fungal colony was picked up carefully from a PDA plate to avoid contamination with agar and homogenized with a mortar and pestle in 200 μl of extraction buffer containing 50 mm Tris-HCl, pH 8.0, 50 mm NaCl, 20 mm EDTA, 25 mm sucrose, and 1% Triton X-100. Mycelial debris was pelleted by centrifugation at 10,000 × *g* for 3 min. The supernatant was removed and extracted with an equal volume of phenol:chloroform:isoamyl alcohol. The DNA was precipitated from the aqueous phase with 1 volume isopropanol and recovered by centrifugation at 10,000 × *g* for 5 min. The pellet was resuspended in 50 μl of TE. The 100-fold dilution was made when it was used as a template for PCR analysis. Two primers, MAG5287 [5′-GACTGAACCTGCCGATTACC-3′; nucleotides (nt) 5267–5286] and MGYF0303-3 (5′-CAGGGTAGCAGGTGGTTGTTGACGAAGAC-3′; nt 5554–5532) were used to analyze the loss of an intron sequence during transposition. Amplification reactions were performed during 25 cycles of 30 sec denaturation at 94°, 20 sec primer annealing at 50°, and 15 sec elongation at 72° using "KOD dash" DNA polymerase (TOYOBO). For PCR-Southern analysis, samples were diluted 1:100 with TE, then subjected to TAE agarose gel electrophoresis.

Regeneration of fungi from a single protoplast: Fungal protoplasts were prepared as described above. Serial 10-fold dilutions of protoplast suspension were made with STC and mixed with regeneration agar medium, then poured onto regeneration agar plates containing 100–400 μg/ml of hygromycin B. After 3–5 days, single colonies of regenerants were picked and transferred to PDA slant media.

RESULTS

Construction of pMGY70 and its derivatives: Plasmids used in this study are shown in Figure 1A. The plasmid pMGY70 is ∼10 kb long and contains a full-length MAGGY copy. A *Dra*I-*Bam*HI fragment of pMGY-RF was cloned into pBluescript, then joined with the *Bam*HI-*Xba*I fragment of pMGY-LF2, another genomic subclone containing the 3′ half of MAGGY, resulting in pMGY70. pMGY70ΔEV is a plasmid with a deletion of a 513-bp *Eco*RV fragment (nt 2241–2754) corresponding to the protease and reverse transcriptase domains of MAGGY. Therefore it cannot provide proteins required for retrotransposition. Plasmid pMGY70-INT was constructed by inserting the synthetic artificial intron to the *Pst*I site (nt 5497) in 3′ LTR of pMGY70 through several subclonings. The position of the *Pst*I site is 112 bp downstream of the 5′ end in the 253-bp LTR and 30 bp upstream of the sequence TCGTCAAC, which follows the "+1 sequence consensus" identified around the transcription start point in *Neurospora crassa* (Bruchez *et al.* 1993). This indicates that the *Pst*I site occurs in the U3 domain of LTR, where the promoter of MAGGY would be located. Therefore, the corresponding domain in cDNA of MAGGY should be derived from that of 3′LTR. pMGY70-INT was designed so that a complete LTR sequence could be recovered after excision of the intron (Figure 1B). The consensus sequence of the donor site and the internal lariat were given in the synthetic sequence and that of the acceptor site, AG, was found in the recognition site of *Pst*I.

Transposition of MAGGY in the wheat isolate of *P. grisea*: First, to examine whether this MAGGY copy is active or not, the wheat isolate of *P. grisea*, which is phylogenically close to the rice and foxtail millet pathogens of *P. grisea*, native hosts of MAGGY, was used for a recipient of the MAGGY constructs. pMGY70, containing a complete MAGGY copy, and pMGY70ΔEV, containing a MAGGY copy with a deletion, were introduced to the wheat isolate by means of PEG-mediated transformation. Transformants were screened on PDA media containing 400 μg/ml hygromycin B. Initially,

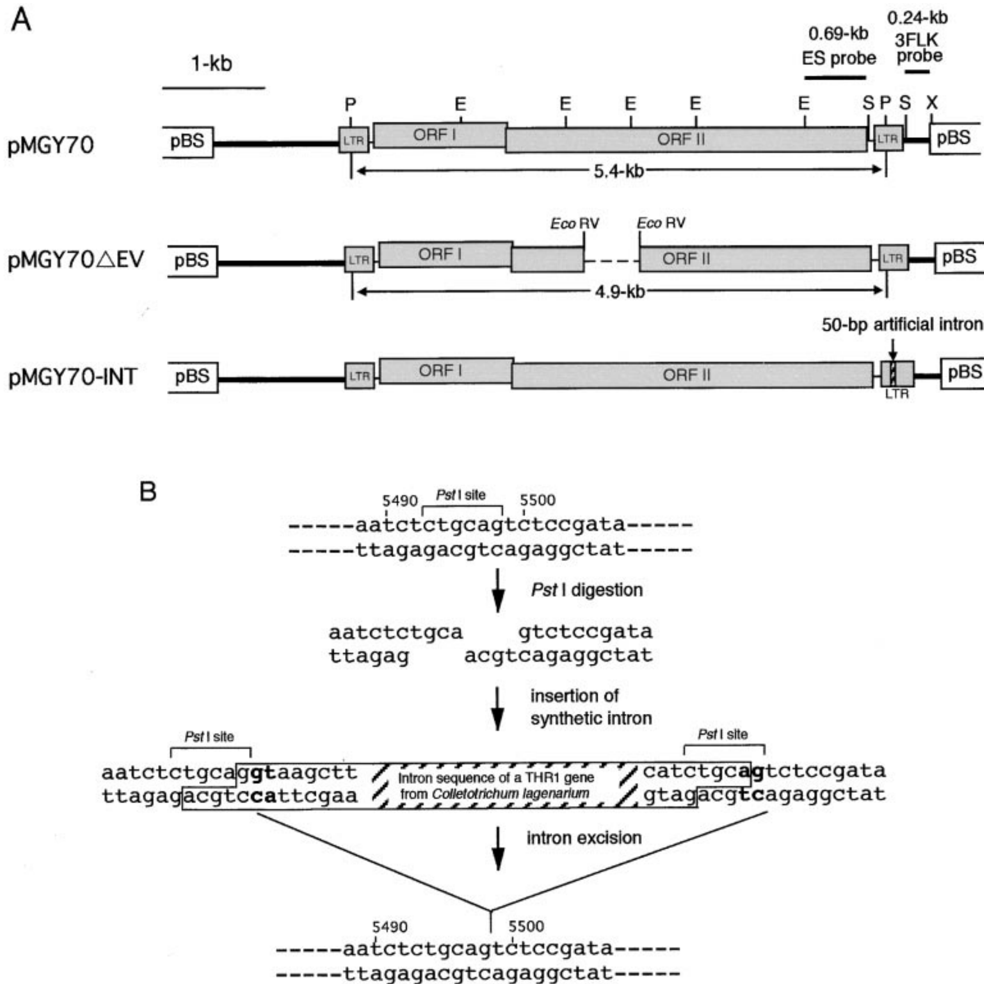

Figure 1.—Diagram of MAGGY constructs used in this study. (A) Structure of a plasmid containing MAGGY and its derivatives. The thick line between LTR and pBS sequence represents the flanking genomic sequence of *P. grisea*. An artificial intron was synthesized according to intron 2 of a *THR1* reductase gene isolated from *Colletotrichum lagenarium* (Perpetua *et al.* 1996). Segments used for probes are shown as bars. E, *Eco*RI; P, *Pst*I; S, *Sma*I; X, *Xba*I; pBS, pBluescript SK+ II. (B) Detailed diagram of pMGY70-INT construction and expected excision of the artificial intron. **gt** and **ag** represent the donor and acceptor sites of the intron, respectively.

the transposition of MAGGY was assessed by genomic Southern analysis with two restriction enzymes, *Eco*RI and *Pst*I, and two probes, ES and 3FLK (Figure 1A). The ES probe was a 0.69-kb *Eco*RI-*Sma*I fragment of pMGY70 corresponding to the 3′ region of ORF 2, and the 3FLK probe was a 0.22-kb *Sma*I-*Xba*I fragment of pMGY70 corresponding to the genomic sequence downstream of the 3′ LTR. When *Eco*RI-digested genomic DNA is probed with the ES fragment, the length of the detectable fragment varies depending on the position of the *Eco*RI site in the genomic sequence flanking the MAGGY insertion, resulting in detection of one unique band per MAGGY copy in the genomic DNA of a cell. Reprobing the blots with the 3FLK sequence enables us to distinguish the initially integrated pMGY70 because transposed MAGGY copies should have been excised from the flanked sequence containing the 3FLK sequence. On the other hand, *Pst*I digests hybridized with the ES probe yield a signal with a single molecular weight (5.4 kb), independent of the genomic position in which MAGGY inserted. The intensity of the band reflects the total copy number of MAGGY in all cells and thus is expected to correlate with total frequency of MAGGY transposition. In contrast, the intensity of the band detected with the 3FLK probe is thought to be constant irrespective of MAGGY transposition. Therefore, the blot probed with the 3FLK fragment was used as a quantitative control for the transposition assay.

Genomic DNA of three transformants with pMGY70 and one with pMGY70ΔEV were digested with *Eco*RI and *Pst*I and fractionated on a 0.7% TAE agarose gel. In the Southern blot using *Eco*RI and the ES probe, pMGY70 transformants produced smear signals with several intense bands, whereas the pMGY70ΔEV transformant produced a single band with no smear signal (Figure 2A). Smear signals were thought to be the products of MAGGY transposition because transposition events could occur independently in each cell at each stage of cell propagation. Intense signals can be produced when many cells of a transformant share the same integration site of MAGGY, resulting from the initial integration of pMGY70 or a transposition event at the relatively early stage of cell propagation. On the other hand, faint signals could be the products of MAGGY transposition at a relatively late stage of cell propagation. As a control, the membrane was stripped and reprobed with the 3FLK fragment. Because 3FLK is a genomic sequence of *P. grisea*, it hybridized with some fragments even in nontransformants. Detection of many signals suggested that the 3FLK fragment might contain some repetitive se-

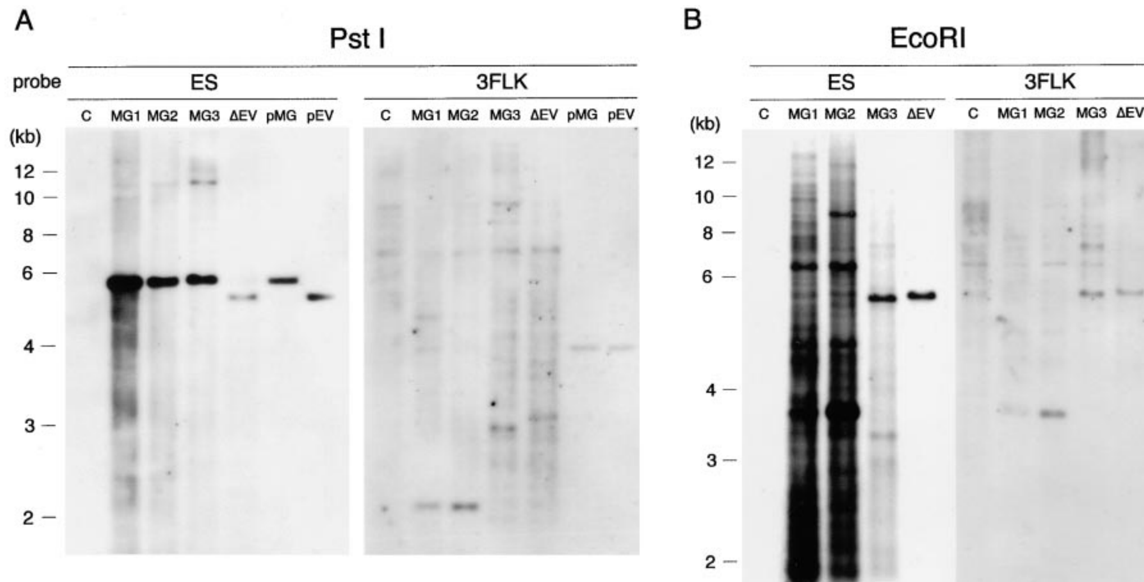

Figure 2.—Southern blot analysis of *P. grisea* wheat isolates transformed with pMGY70 or pMGY70ΔEV. Genomic DNA was digested with (A) *Pst*I or (B) *Eco*RI, fractionated on a 0.7% TAE agarose gel, and probed with the ES fragment of pMGY70. The Southern blot was then stripped and probed with the 3FLK fragment of pMGY70. Control plasmids were used as size markers in A. C, nontransformant; MG1–MG3, transformants with pMGY70; ΔEV, transformant with pMGY70ΔEV; pMG, pMGY70; pEV, pMGY70ΔEV.

quence. However, several intense bands hybridizing the 3FLK probe corresponded to those seen in the blots probed with the ES probe, indicating that these bands are the initially integrated pMGY70.

In the blot using *Pst*I and the ES probe, the intensity of the 5.4-kb bands in pMGY70 transformants was higher compared with that of a 4.9-kb band in a transformant with pMGY70ΔEV (Figure 2B). In contrast, there was not much difference in the intensity of signals between pMGY70 and pMGY70ΔEV transformants in the control blots reprobed with the 3FLK sequence. Theoretically, *Pst*I digests probed with the ES sequence should make a single signal of 5.4 kb in pMGY70 transformants. In fact, smear signals were reproducibly observed in all pMGY70 transformants. This might be due to well-known events concerning LTR retrotransposons such as recombination between elements, excision of an element leaving a solo LTR sequence, or nested integration of an element into another element.

The results described above strongly suggest that this MAGGY copy can transpose actively in the genome of *P. grisea* but that a derivative carrying a deletion in the *pol* gene cannot. Because transposition of pMGY70ΔEV was not effected by any other endogenous elements, MAGGY appears to undergo autonomous transposition. The frequency of transposition seemed to be somewhat different among pMGY70 transformants. This might be due to position effects or copy number of the original integration of pMGY70.

For further confirmation of MAGGY transposition in the wheat isolate of *P. grisea*, another experiment was carried out. A transformant with pMGY70 (R0) was cultured in liquid medium and subjected to protoplast isolation. Regenerants (R1) from a single protoplast were obtained, and their genomic DNA were digested with *Eco*RI and analyzed by Southern hybridization using an ES probe. The blot was stripped and reprobed with pBluescript to ensure that regenerants were derived from a single transformant. When probed with the ES fragment, 20–30 intense bands with a little smear signal were detected in all R1 regenerants, whereas more smear signals were present in the R0 transformant (Figure 3). Interestingly, band patterns of the blot were quite different among regenerants, indicating that the transposition of MAGGY occurred independently in each cell. A common band indicated by an arrow was observed in R0 and all R1 regenerants, which was thought to represent the initial integration site of pMGY70. These common fragments could be observed in the blot reprobed with pBluescript (Figure 3). The fact that most MAGGY copies in R1 detected with the ES probe did not hybridize with the vector sequence indicated active transposition of MAGGY in the genome of *P. grisea.* In addition to the common bands, some other fragments were detected in the blot probed with pBluescript. These fragments were thought to be derived from pSH75, the transformation vector carrying a hygromycin B phosphotransferase gene. The similar band pattern indicated that the regenerants were originated from a single transformant. However, some fragments in R1 regenerants differed from those in the R0 transformant, which might be due to recombination events after the original integration.

Transposition of MAGGY in the wheat isolate of *P. grisea* occurs via an RNA intermediate: To clarify whether MAGGY transposes through an RNA intermedi-

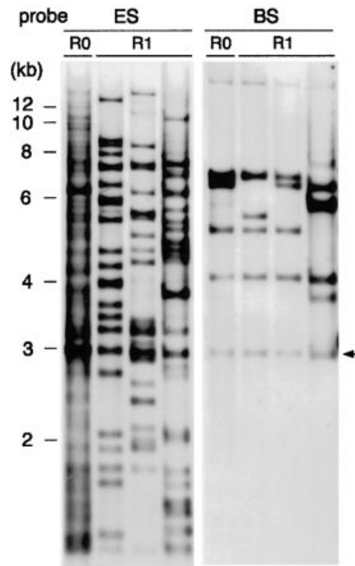

Figure 3.—Southern blot analysis of three independent protoplast regenerants of a MAGGY-transformed *P. grisea* wheat isolate. Each of the three regenerants (R1) was obtained from a single protoplast of the original MAGGY transformant (R0) of the *P. grisea* wheat isolate (Br48). Genomic DNA was digested with *Eco*RI, fractionated on a 0.7% TAE agarose gel, and probed with the ES fragment of MAGGY. The Southern blot was then stripped and probed with pBluescript SK+ II. Lane 1, an original MAGGY transformant (R0); Lanes 2–4, protoplast regenerants (R1). An arrow indicates the band observed in all lanes.

ate, pMGY70-INT, which carries a 50-bp artificial intron in the 3′ LTR region (Figure 1, A and B), was introduced into the wheat isolate of *P. grisea.* If MAGGY transposes through reverse transcription, we expect the appearance of intron-deleted elements in addition to the master intron-containing element. Loss of the intron in transformants was assayed by PCR using primers MAG-5287 and MGYF0303-3 (Figure 4A). Expected sizes of the amplified intronless and intron-containing fragments of 337 bp and 287 bp, respectively, were observed in lanes pMG and pIT of Figure 4B using pMGY70 and pMGY70-INT as templates. In all transformants, the fragment corresponding to the intronless MAGGY was amplified in addition to the fragment corresponding to the intron-containing MAGGY, whereas no fragment was observed from nontransformed *P. grisea* (Figure 4B).

To identify the amplified fragments, PCR-Southern analysis was performed with probes corresponding to the LTR and the intron regions. Both LTR and INT probes were synthesized using a random prime labeling kit (Amersham). A 0.3-kb *Sma*I fragment was used as a template for the LTR probe (Figure 4C). A single-strand oligonucleotide of the synthetic intron (see materials and methods) was used as a template for the INT probe, and a 20-bp intron-specific oligonucleotide (5′-CTTACGCGATAGCTCACGAG-3′) was used as a primer. Figure 4C clearly shows that both the upper and lower amplified fragments in the transformants are derived from the LTR domain but that the upper contains the intron sequence, whereas the lower does not. These results suggest that the intron sequence was excised during the transposition of MAGGY. Relatively low amplification of the intronless fragment could be due to the stage at which template DNA used for the PCR assay was extracted. Because it was prepared directly from the fungal colony at the second screening after transformation, there may not have been enough time for MAGGY to undergo multiple transpositions. PCR analysis of transformants after longer periods of growth (data not shown) revealed that, as expected, most copies of MAGGY are intronless. The transposition of MAGGY in pMGY70-INT transformants was further confirmed by genomic Southern analysis with the same strategy described above (data not shown), suggesting that a complete MAGGY copy was recovered. Thus the intron of *C. lagenarium* was removed precisely in *P. grisea.*

Transposition of MAGGY in *C. lagenarium*: To examine the transposition of MAGGY in a heterologous filamentous fungus, pMGY70-INT was introduced into a *C. lagenarium* isolate, 104T. Genomic DNA of four pMGY70-INT transformants and one nontransformant were extracted and the fragments corresponding to the 3′ LTR region were amplified by PCR as described above. TAE agarose gel electrophoresis (Figure 5) followed by Southern analysis using LTR and intron probes (data not shown) revealed that the intron-containing fragment was amplified in all transformants and the intronless fragment was in three of the four transformants. When genomic DNA was digested with *Pst*I and probed with the ES fragment, a fragment of 5.4-kb *Pst*I fragment of the complete MAGGY was not detected in the transformant from which the intronless fragment was not amplified (data not shown). This indicated that the integrated MAGGY copy in this transformant had been disrupted because of cleavage within the MAGGY sequence during the original integration. Genomic Southern analysis of regenerants from a single protoplast derived from the same pMGY70-INT transformant was performed. Genomic DNA of six protoplast regenerants was digested with *Eco*RI and probed with the ES fragment. One or two new bands appeared in all regenerants in addition to the band observed in the original transformant, indicating that transposition of MAGGY occurred in *C. lagenarium* (Figure 6). Some of the regenerants seemed to share the same transposition sites, for example, lanes 3, 4, and 6, suggesting that the transposition event might occur at an early stage of cell propagation. Weak bands observed in several regenerants, especially in lane 4, indicated further transposition of MAGGY. These results clearly showed that MAGGY could transpose into the genome of *C. lagenarium.* However, the transposition frequency seemed to be much lower compared with that of the *P. grisea* wheat isolate because there were fewer newly appearing bands

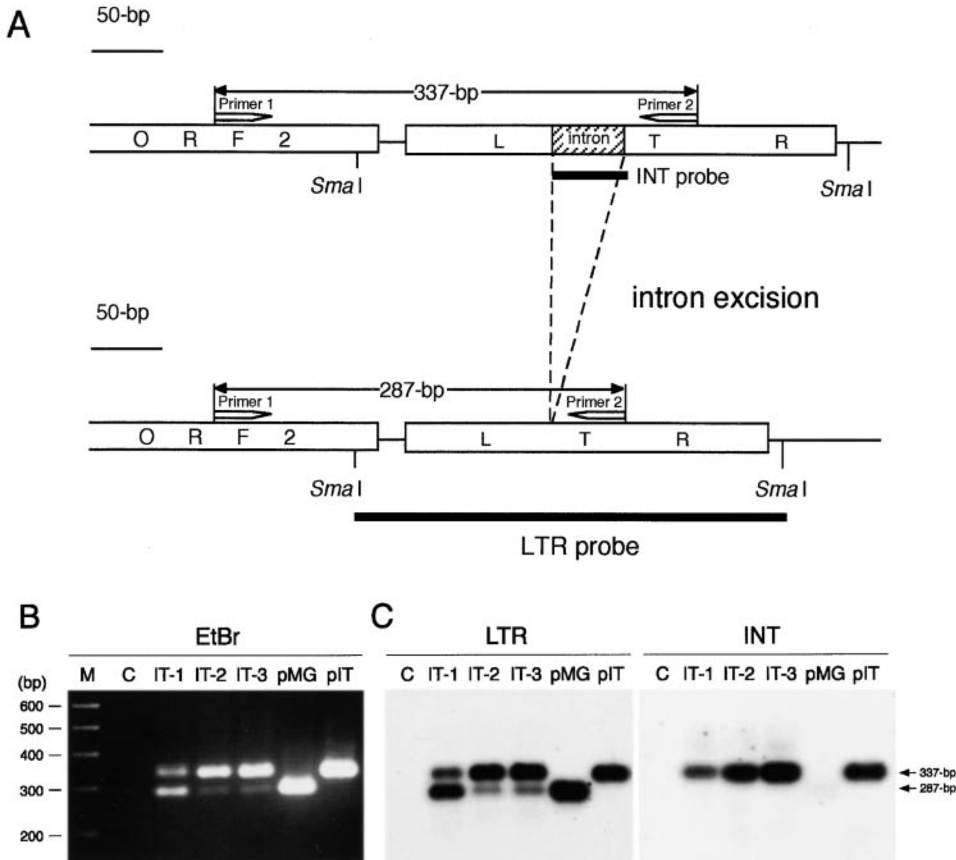

Figure 4.—PCR analysis for the loss of an artificial intron during MAGGY transposition in the wheat isolate of *P. grisea* (Br48). (A) Map of 3′ LTR region of pMGY70-INT and its expected intronless derivative, showing the positions of primers used in the transposition assay by PCR and segments used for probing blots. Sizes of the intronless and intron-containing PCR products are indicated. Primer 1, MAG5287; primer 2, MGYF0303-3. (B) PCR transposition assay of pMGY70-INT transformants of the wheat isolate. Genomic DNA of three individual transformants, one nontransformant, and control plasmids pMGY70 and pMGY70-INT were subjected to PCR amplification using primers 1 and 2 shown in A. Amplified fragments were fractionated on a 3.5% TAE agarose gel and visualized by ethidium bromide staining (EtBr). M, 100-bp ladder; C, nontransformant; IT-1–3, transformants with pMGY70-INT; pMG, pMGY70; pIT, pMGY70-INT. (C) Southern blot analysis of PCR products in B using LTR probe and INT probe shown in A. Amplified fragments were fractionated on a 3.5% TAE agarose gel and probed with INT probe (INT). The Southern blot was then stripped and probed with LTR probe (LTR). Abbreviations are identical to those in B.

in the MAGGY transformants of *C. lagenarium* than in those of *P. grisea* (Figures 3 and 6).

Frequency of MAGGY transposition in various filamentous fungi: MAGGY has been shown to transpose in heterologous species of filamentous fungi, even though the frequency seemed to be lower than in homologous species. The frequency of MAGGY transposition was assessed in various filamentous fungi by using Southern analysis. Two *P. grisea* isolates (the finger millet and the crabgrass isolates), a *P. zingiberi* isolate, and the *C. lagenarium* isolate were used. Several studies suggested that the finger millet pathogen of *P. grisea* was much closer to the rice pathogen than was the crabgrass pathogen (Shull and Hamer 1994; Kusaba *et al.* 1999). *C. lagenarium* belongs to a different order from Pyricularia. These fungal isolates were transformed with pMGY70 or pMGY70ΔEV, and genomic DNA of two independent pMGY70 transformants and one pMGY70-ΔEV transformant per fungus were analyzed by Southern blots. When the *Eco*RI digest was probed with the ES fragment, smear signals indicating the transposition of MAGGY were observed in pMGY70 transformants of *P. zingiberi*, moderately so in those of the finger millet and crabgrass isolates of *P. grisea*, but not in all pMGY70-ΔEV transformants (Figure 7A). The smear signal was not detected distinctly in *C. lagenarium* by this assay, even though transposition of MAGGY had been demonstrated in the experiment using pMGY70-INT. This

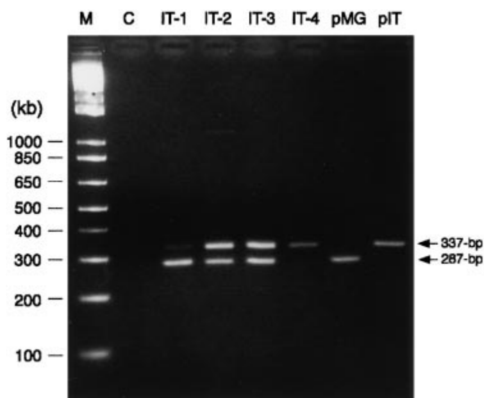

Figure 5.—PCR analysis for the loss of an artificial intron during MAGGY transposition in *C. lagenarium.* Genomic DNA of four individual transformants, one nontransformant, and the control plasmids pMGY70 and pMGY70-INT were subjected to PCR amplification using primers 1 and 2 shown in Figure 4A. Amplified fragments were fractionated on a 3.5% TAE agarose gel and visualized by ethidium bromide staining. M, 100-bp ladder; C, nontransformant; IT-1–4, transformants with pMGY70-INT; pMG, pMGY70; pIT, pMGY70-INT.

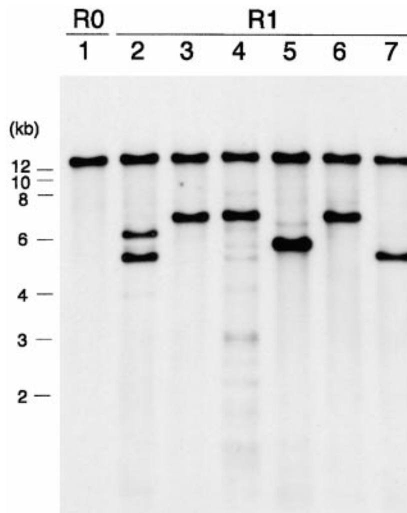

Figure 6.—Southern blot analysis of six independent protoplast regenerants derived from a *C. lagenarium* transformant with MAGGY. Each of the six individual regenerants (R1) was obtained from a single protoplast of the original MAGGY transformant (R0) of *C. lagenarium.* Genomic DNA was digested with *Eco*RI, fractionated on a 1.2% TAE agarose gel, and probed with the ES fragment of pMGY70. Lane 1, an original MAGGY transformant (R0); Lanes 2–7, protoplast regenerants (R1).

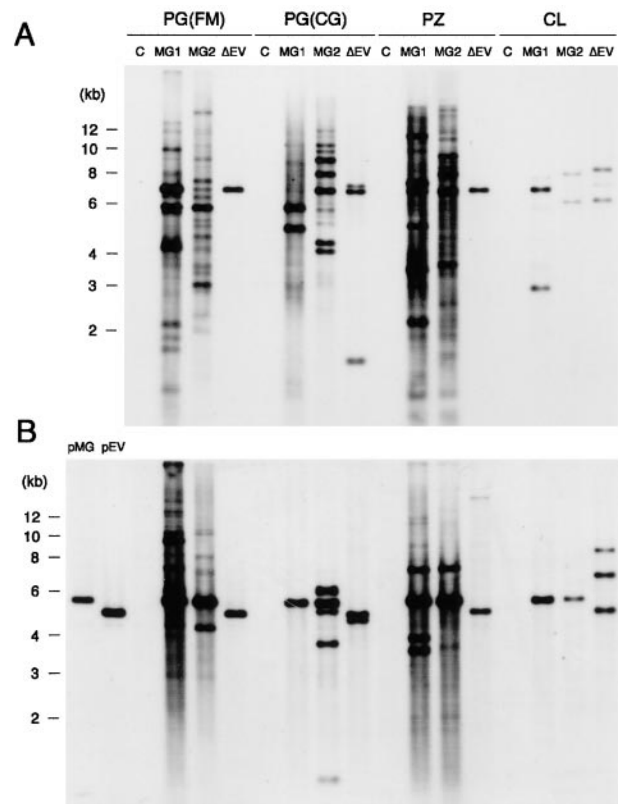

Figure 7.—Southern blot analysis of various filamentous fungi transformed with pMGY70 or pMGY70ΔEV. Genomic DNA were digested with *Eco*RI (A) or *Pst*I (B), fractionated on a 0.7% TAE agarose gel, and probed with a 0.69-kb *Eco*RI-*Sma*I fragment (ES) of MAGGY. One nontransformant, two pMGY70 transformants, and one pMGY70ΔEV transformant were analyzed for each fungal isolate. Control plasmids were used as size markers in B. C, nontransformant; MG1 and MG2, transformants with pMGY70; ΔEV, transformants with pMGY70ΔEV; pMG, pMGY70; pEV, pMGY70ΔEV; PG(FM), finger millet isolate of *Pyricularia grisea*; PG(CG), crab grass isolate of *P. grisea*; PZ, *P. zingiberi*; CL, *Colletotrichum lagenarium.*

might be due to low-frequency MAGGY transposition in *C. lagenarium.* When the *Pst*I digest was probed with the ES fragment, a 5.4-kb signal was observed in all pMGY70 transformants, and its intensity was much higher compared with that of 4.9 kb in a corresponding pMGY70ΔEV transformant in the *P. grisea* finger millet isolate and *P. zingiberi* (Figure 7B). In two pMGY70 transformants of *P. grisea* crabgrass isolate and one of *C. lagenarium* (transformant 1), the intensity of the 5.4-kb band was slightly higher than that of the 4.9-kb band in a corresponding pMGY70ΔEV transformant. In the other pMGY70 transformant of *C. lagenarium* (transformant 2), no significant difference was observed in the intensity of bands from the pMGY70ΔEV transformant, indicating that frequency of the MAGGY transposition in this transformant was, if any, very low. These results indicate that, as shown in the previous experiment using protoplasts, the frequency of MAGGY transposition in *C. lagenarium* is low compared with that in homologous species. Frequency of MAGGY transposition within Pyricularia species, however, was not necessarily correlated with the phylogenical closeness to native hosts of MAGGY.

DISCUSSION

In the last decade the whole spectrum of transposable elements identified in eukaryotic genomes has been found in fungal genomes, for example, DNA transposons including Tc1/*mariner* and *Ac*-like groups, LTR retrotransposons including Ty1/*copia* and Ty3/*gypsy* groups, LINEs, SINEs, and unclassified elements (Daboussi 1997). Only a few elements, however, were proven to be active even in their own hosts. This is the first report of active transposition of a fungal transposable element in heterologous species of fungus. MAGGY, a retrotransposon of the rice blast fungus, can undergo autonomous transposition via an RNA intermediate even in C. *lagenarium*, which belongs to an order of filamentous fungi different from *P. grisea*, the original host of MAGGY. In plants, DNA transposons, exemplified by the maize *En/Spm* and *Ac/Ds* elements, are used as gene tagging tools in a wide range of heterologous plant species in addition to their original host (Fedoroff *et al.* 1984; Baker *et al.* 1986; Aarts *et al.* 1993; Bancroft *et al.* 1993; Chuck *et al.* 1993; Long *et al.* 1993; Tacke *et al.* 1995). In other eukaryotic organisms such as *S. cerevisiae* and *D. melanogaster*, several genes have been tagged by their own LTR retrotransposons Ty1 and *copia*, and successfully isolated (Bingham *et al.*

1981; Garfinkel *et al.* 1988). Our results demonstrated that MAGGY is a candidate for a gene-tagging tool in filamentous fungi, especially in Ascomycota. We have found that RNA-mediated transposition of MAGGY occurred in *Alternaria longipes* (data not shown). However, transposition of MAGGY in Basidiomycota or Zygomycota has not been examined yet. Some plant retrotransposons have been shown to be stable under normal growth conditions and to be activated in response to various kinds of stresses (Pouteau *et al.* 1991, 1994; Hirochika 1993; Mhiri *et al.* 1997; Takeda *et al.* 1998). Furthermore, the tobacco retrotransposon Tnt1 maintained its responsive induction upon pathogen-related stress even in Arabidopsis and tomato (Moreau-Mhiri *et al.* 1996). Analysis of the regulation of MAGGY transposition and target site specificity will be important in establishing MAGGY as a tagging tool in filamentous fungi.

Several phylogenic studies of retrotransposons based on their conservative reverse transcriptase domains revealed that the distribution of closely related retrotransposons does not always follow the phylogenic relationship of their host species (Doolittle *et al.* 1989; Xiong and Eickbush 1990; Flavell *et al.* 1995). Therefore, many researchers have pointed out the possibility of horizontal transfer of retrotransposons (Mizrokhi and Mazo 1990). However, no direct evidence has been obtained up to now. In Pyricularia species, the distribution of MAGGY, *fosbury*, and *grh* is sporadic (Dobinson *et al.* 1993; Tosa *et al.* 1995; Farman *et al.* 1996; Shull and Hamer 1996). MAGGY and *fosbury* are present in rice and Setaria pathogens of Pyricularia, whereas the distribution of *grh* is localized in Eluesine pathogens only. Based on a dendrogram constructed by rDNA RFLP (Kusaba *et al.* 1999), Pyricularia isolates of MAGGY carriers were classified into the same cluster and all *grh* carriers were classified into two rDNA types that stood apart from the cluster of MAGGY carriers. The results presented here support the concept that MAGGY was acquired horizontally because it diminishes the alternative explanation that MAGGY has been lost in non-MAGGY carriers.

The activity of MAGGY in heterologous hosts seemed to be different depending on the host species. Active transposition of MAGGY occurred in the wheat isolate of *P. grisea* and *P. zingiberi* (Figures 2 and 7) but appeared to be a rare event in *C. lagenarium* (Figure 6). Several host factors have been proposed to regulate or restrict the transposition of retrotransposons. It has been shown that activated transposition of plant retrotransposons in protoplasts or tissue culture reflects transcriptional activation (Pouteau *et al.* 1991; Hirochika *et al.* 1996b). It might be possible that the promoter activity of MAGGY is higher in Pyricularia than in Colletotrichum. Another factor is known to be correlated with transposition of Ty1 in heterologous species of yeast. Ty1, a retrotransposon of *S. cerevisiae*, cannot transpose in *S. pombe* cells because of its inability to use a heterologous tRNA for priming the minus DNA synthesis (Keeney *et al.* 1995). Database searching enabled us to find that the sequence just 3′ to the 5′ LTR of MAGGY shares significant similarity with an internal sequence of several tRNAs of *S. cerevisiae* (data not shown). However, the molecular species used as a primer in the reverse transcription process of MAGGY has not yet been determined. The problem of primer annealing might be involved in the different activity of MAGGY in heterologous hosts. In addition, a specific host gene has been reported to have the ability to control retrotransposition. A single allele, called *flamenco*, repressed the transposition of *gypsy* in *D. melanogaster* (Prud'homme *et al.* 1995). Even though its gene and product have not been isolated so far, it was shown that its restrictive effects on transposition occurred at the transcriptional level (Pelisson *et al.* 1994); *gypsy* transcripts were reduced 5- to 10-fold in *D. melanogaster* strains that carried a restrictive *flamenco* allele. In another interesting report, the mouse *Fv1* gene, which restricts the replication of murine leukemia virus, encodes the *gag* region of an endogenous retrovirus unrelated to the murine leukemia virus (Best *et al.* 1996). Although interference between retrotransposons has not yet been reported, one can expect that analogous restriction may exist in the relationship between retrotransposons, especially in the case of Ty3/*gypsy* LTR retrotransposons that are closely related to retroviruses (Doolittle *et al.* 1989; Xiong and Eickbush 1990). MAGGY appeared to transpose more actively in *P. zingiberi* than in the finger millet and crabgrass isolates of *P. grisea.* The activity of MAGGY in Pyricularia species, therefore, may not depend solely on the general genetical similarity of the new host to the original one. Some factors other than compatibility to cellular machinery might be involved in the different activity of MAGGY in Pyricularia species.

Pathogenic fungi isolated from the field often show changes in their phenotype in culture, including morphological changes such as colony color or shape, loss of pathogenicity, and reduction of spore production (Giatgong and Frederiksen 1969). These observations lead us to hypothesize that such phenotypic unstableness results from active transposition of transposable elements in field isolates. Indeed, most active fungal transposable elements are derived from field isolates. Transposable elements could contribute to genetic variation in fungi under natural conditions, which might result in the survival of the fittest. On the other hand, fungal transposable elements found in laboratory strains are commonly inactive. Laboratory strains are usually established as a result of continuous selection for phenotypic stability. In such fungal strains, transposable elements might be neutralized by host suppression mechanisms.

Little is known about the molecular mechanism of MAGGY transposition. Studies on retrotransposons are

sometimes complicated by their high copy number and relic copies in host genomes. Introduction of MAGGY into a new host should facilitate the elucidation of mechanisms of retrotransposition and the interaction between a retrotransposon and a host at the molecular level.

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