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The C-terminal chromodomain-like module in the integrase domain is crucial for high transposition efficiency of the retrotransposon MAGGY

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Abstract

MAGGY is a Ty3/Gypsy retrotransposon, which was identified in the rice blast fungus *Magnaporthe oryzae*. Some Ty3/Gypsy retrotransposons, including MAGGY, contain a chromodomain-like module (CLM) in the C-terminus of the integrase domain. We have made a series of MAGGY mutants to examine the role of the CLM in the transposition activity of the element. Introduction of a mutation at different positions in the MAGGY integrase revealed that a loss or alteration of the CLM resulted in a drastic decrease in the transposition activity of the element. Our results indicate that the CLM may confer high transposition activity to the element.

Keywords: LTR-retrotransposon, integrase, chromodomain, transposition activity

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Abbreviations: CLM, chromodomain-like module; LTR, long terminal repeat; ORF, open reading frame

1. Introduction

Long-terminal-repeat (LTR) retrotransposons form a ubiquitous group of retrotransposons and are widely distributed in eukaryotic genomes [1]. The structure of LTR-retrotransposons is comparable to that of retroviruses, which consists of gag, pol, and, in some cases, env genes flanked by LTRs at both extremities of the element. The gag gene of retroviruses encodes structural proteins of the virus particle and the pol locus encodes a polyprotein with protease, reverse transcriptase, RNaseH, and integrase domains. Based on the order of the domains in pol and the sequence similarity of the domains, LTR-retrotransposons are further classified into two major classes, the Ty1/Copia and Ty3/Gypsy groups.

Malik and Eickbush [2] performed a phylogenetic analysis of the integrase sequences of Ty3/Gypsy LTR-retrotransposons and showed that some contain a chromodomain-like module (CLM) in the C-terminus of the integrase domain. Chromodomain is a stretch of amino acids conserved in proteins that interact with chromatin [3,4], and possibly functions as an assembling module for a variety of macromolecular complexes in chromatin. However, no information is available on the biological role of the CLM found in the integrases of LTR-retrotransposons. Since only a portion of LTR-retrotransposons has the CLM in their integrase domain, it is not clear whether the CLM is an essential module for transposition activity or is just an accessory component of the element.

MAGGY is a Ty3/Gypsy LTR retrotransposon isolated from the blast fungus, *Magnaporthe oryzae* (formerly *Magnaporthe grisea*), comprising two ORFs and 253-bp LTRs [5]. ORF1 encodes a gag-like protein of 457 amino acids exhibiting a potential

zinc finger domain (CCHC) in the C-terminus. ORF2 has a typical character of a pol gene with the known catalytic domains of protease, reverse transcriptase, RNaseH and integrase. The integrase of MAGGY contains a CLM in the C-terminus in addition to known catalytic motifs of integrase such as HHCC and DD(35)E motifs. By examining transposition events during a sexual cross, Eto et al. [6] showed that MAGGY was the most active element among five major *M. oryzae* transposable elements. MAGGY elements are stress-responsive [7] and can be activated during a sexual cross [6]. It is of interest to know which components are responsible for the high activity of the element.

In this study, we have made a series of MAGGY integrase domain mutants to study the role of the CLM in the transposition activity of the element. Our results indicate that a mutation in the CLM drastically decreased, but not completely abrogated, the transposition activity, suggesting a role for the CLM in the high activity of the element.

2. Materials and Methods

2. 1. Fungal strains and growth conditions

M. oryzae strain Br48 isolated from wheat, *Triticum aestivum* (L.) Thell, was used as a recipient throughout the experiments. Br48 does not carry endogenous MAGGY elements. The fungus was maintained on potato dextrose agar (PDA) for several months. For long-term storage, the fungus was cultured on barley seeds soaked with sucrose, dried and kept in 4 ˚C as described previously [8]. For DNA or RNA isolation and protoplast preparation, *M. oryzae* was grown in CM liquid broth (0.3% casamino acids, 0.3% yeast extract, 0.5% sucrose) at 26 ˚C for 5 days on an orbital shaker (120rpm).

2.2. Transformation of M. oryzae

Fungal protoplasts were prepared as described previously [8], and fungal transformation were performed using the co-transformation method with the plasmid pSH75 carrying the hygromycin B phosphotransferase gene as a selective maker as described previously [9]. For selection, hygromycin was used at a concentration of 400 μg/ml.

2. 3. Site-directed mutagenesis

A 1.3kb *Mlu*I-*Xba*I fragment containing an integrase domain of MAGGY in pMGY70-INT [8] was subcloned into pBluescript SK II$^+$, establishing pMlu-X-int. pMlu-X-int was subjected to site-directed mutagenesis experiments using the "QuikChange" site-directed mutagenesis kit (Stratagene, La Jolla, USA). A mutation made was confirmed by sequencing, and then the 1.3kb *Mlu*I-*Xba*I fragment with the mutation was subcloned back to pMGY70-INT to establish a MAGGY mutant. Sequencing reactions were performed using the ABI Prism Big-dye terminator ready reaction sequencing kit

(Applied Biosystems, Foster City, USA) and analyzed by ABI310 sequencer.

2. 4. Intron-excision transposition assay

Intron-excision transposition assay was performed as described previously [8]. Two primers, MAG5287 (5'-GACTGAACCTGCCGATTACC-3'; nt. 5267 to 5286) and MGYF0303-3 (5'-CAGGGTAGCAGGTGGTTGTTGACGAAGAC-3'; nt. 5554 to 5532) were used to analyze the loss of an intron sequence during transposition. Template fungal DNA was prepared directly from a fungal colony on a PDA plate as described previously [8]. Amplification reactions were performed during 25 cycles of 30 seconds denaturation at 94˚, 20 seconds primer annealing at 50˚ and 15 seconds elongation at 72˚.

2. 5. Transposition assay by single protoplast isolation

Approximately 50 days after transformation of *M. oryzae* with MAGGY plasmids, a piece of PDA culture of the transformants was picked up and grown in CM broth (0.3 % Casamino acids, 0.3% yeast extract, 0.5% sucrose) at 26˚C for a week. Fungal protoplasts were produced by digesting mycelia in a digestion buffer (10 mM Na_2HPO_4, 1.2 M Mg_2SO_4) containing 5 mg/ml Lysing enzymes (Sigma) for 3 hr. Serial 10 fold dilutions of protoplast suspension were made with STC (1 M sorbitol, 50 mM Tris-HCl, pH 8.0, 50 mM $CaCl_2$) and mixed with regeneration agar medium, then poured onto regeneration agar plates containing 400 µg/ml of hygromycin B. After 3 to 5 days, single colonies of regenerants were transferred to PDA slant media, and subjected to Southern analysis. Total fungal DNA was isolated as described previously [8]. DNA gel blot analysis was performed using a dioxetane chemiluminescence system, Gene

Images™ (Amersham, Arlington Heights, USA). *Eco*RI-digested fungal DNA was separated on a 0.8% TAE agarose gel and transferred to a nylon membrane. A 0.68-kb *Eco*RI-*Sma*I fragment (ES probe), which corresponds to the 3' region of ORF 2 (nucleotide number #4660-#5342) of MAGGY, was labeled with fluorescein by the random prime labeling method and used as a probe. Hybridization was performed in 5x SSC, 0.1% (w/v) SDS, 5% (w/v) dextran sulfate, 5% (v/v) liquid block (Amersham) at 68 ˚C overnight. Southern blots were then 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.

3. Results and Discussion

3. 1. Construction of MAGGY mutations in the integrase domain

The series of mutations introduced in the integrase domain of MAGGY are depicted in Fig 1A. The 3' truncated integrase mutants were made by introducing a stop codon, either by site-directed mutagenesis or by insertional mutagenesis. The MAGGY integrase sequence exhibits the GPY/F and chromodomain-like modules in addition to typical conserved motifs found in integrases, such as the C2H2 zinc finger and DD(35)E motifs. Rough positions of the motifs and modules in the MAGGY integrase are shown.

MGY-ΔE65 has a 5-bp insertion at the *Eco*O65I site (nt. #4092) that was made by EcoO65I digestion, klenow filled-in, and re-ligation. The insertion results in a breakage of the C2H2 zinc finger motif by eliminating the last cysteine residue in the motif, and in generating a stop codon approximately 20 amino acids downstream of the insertion. MGY-ΔBsi has a 4-bp insertion at the BsiWI site (nt. #5020), which was made in a similar way to MGY-ΔE65. The MGY-ΔBsi insertion is located within the GPY/F module and causes the protein to be truncated by 75 residues, with the last 25 residues of this protein differing from the wild-type integrase sequence. MGY-C5170A, MGY-T5234A and MGY-G5289T were made by site-directed mutagenesis so that a stop codon was introduced at the positions indicated in Fig 1A. A stop codon was introduced in MGY-C5170A and MGY-T5234A just upstream and in the middle of the predicted CLM, respectively. In MGY-G5289T, a stop codon occurs immediately downstream of the conserved CLM sequence.

In addition to the 3' truncated MAGGY mutants, we made four missense MAGGY mutants in two conserved amino acid residues of the CLM to more specifically examine

the role of the CLM in the transposition activity of MAGGY. Fig 1B shows the core domain of the CLM found in integrases of several Ty3/Gypsy retrotransposons and chromoproteins, and the mutation positions are indicated by closed triangles. In MGY-T5239C and MGYT5239G, a conserved valine residue was converted to alanine and glycine, respectively. Similarly, a conserved tryptophan residue was altered to arginine and glycine in MGYT5244C and MGY5244G, respectively.

3. 2. Transposition activity of the MAGGY integrase mutants assessed by an intron excision assay

The transposition activity of the MAGGY integrase mutants was first examined using a PCR-based intron excision assay as described previously [8]. A 50-bp artificial intron was introduced in the 3' LTR region of MAGGY (Fig. 2A). Since MAGGY is a retrotransposon transposing through reverse transcription, intron-deleted (spliced) elements in addition to the master intron-bearing (unspliced) element should appear in the fungal genome upon a transposition event. The frequency of transposition in the fungal genome can be roughly estimated by comparing the signal intensity of unspliced and spliced bands amplified by PCR. Using PEG-mediated transformation, each of the deletion mutants and wild-type MAGGY was introduced into Br48, a wheat-infecting isolate of *M. oryzae* that possesses no endogenous MAGGY element, and subjected to the intron excision assay. Four to five independent transformants each of the mutated and wild-type MAGGY elements were analyzed to reduce bias due to position effects of the master plasmid integration sites in the genome. As shown in Fig 2B, both unspliced

and spliced bands were amplified when genomic DNA from transformants of wild-type MAGGY was used as a template. The intensity of the spliced bands was stronger than that of the unspliced bands in some transformants, indicating a high rate of transposition events in those transformants. In contrast, no spliced band was detectable when genomic DNA from transformants from MGY-ΔE65, MGY-ΔBsi and MGY-C5170A, all of which lack the entire CLM, was used (Fig. 2B), indicating that the transposition activity of the mutants appeared to be almost abolished. With MGY-T5234A, which has a stop codon in the middle of the CLM, a spliced band was detected in two out of five transformants with much lower intensity than the corresponding unspliced band, indicating that the transposition activity of the mutant was largely impaired but still present. Compared with the other MAGGY mutants, MGY-G5289T, which encodes the entire CLM, appeared to retain relatively high transposition activity since spliced bands were observed in all the transformants. However, lower intensity of the spliced bands suggested that the MGY-G5289T transformants still had a lower transposition activity than wild-type MAGGY. To confirm that the mutations were maintained in the active mutants, MGY-T5234A and MGY-G5289T, we PCR-amplified and cloned spliced DNA fragments containing the mutated sites. Sequencing of the fragments revealed the correct mutations in the spliced fragments (data not shown). In summary, these results showed that deletion mutants bearing CLM, even in part, had some degree of transposition activity but none of the mutants lacking the CLM retained detectable transposition activity.

We further examined the conserved amino acid residues of the missense MAGGY mutants to directly evaluate the effect of the CLM module on the transposition activity of the element. The precise positions of the missense mutations are shown in Fig. 1B.

Intron excision assay of the *M. oryzae* transformants revealed that all of the missense mutations negatively affected the activity of MAGGY transposition (Fig. 2C). In particular, some of the MGY-T5244G transformants, which had the conserved tryptophan converted to glycine, showed low to undetectable intensity of the spliced bands, indicating that the tryptophan residue is crucial to the function of CLM in the MAGGY integrase domain. The other missense mutations demonstrated milder effects than seen with MGY-T5244G, although still concerted a considerable negative effect on the transposition activity of the MAGGY elements, strongly implicating CLM as important in the transposition activity of the element.

3. 3. Transposition activity analysis of the MAGGY integrase mutants by single protoplast isolation

To directly assess the transposition activity of the MAGGY integrase mutants, the number of transpositions in a cell of the mutants was estimated using regenerants from a single protoplast as described previously [10]. Generally, to detect a transposition event in a cell is technically difficult since MAGGY transposition occurs independently in each cell, making the number of DNA molecules sharing the MAGGY insert too small to be detected. However, by making regenerants from a single protoplast, we detected MAGGY inserts as a band on a Southern blot that has already appeared in the genome of the isolated single protoplast. We used *Eco*RI-digested fungal genomic DNA and the ES probe (see Materials and Methods) so that the length of the detectable fragment varied depending on the position of the *Eco*RI site in the genomic sequence flanking the MAGGY insertion [8]. In this way, we could detect one unique band per MAGGY copy in the genomic DNA of a cell.

One representative transformant was chosen for analysis from each of the four to five transformants with wild-type MAGGY and all the mutants with the exception of MGY-ΔE65. Protoplasts of the representative transformants were made one month after introduction of the elements into the fungus by transformation. Five protoplast regenerants were selected for genomic DNA extraction and analysis by Southern blotting (Fig 3). A band in a regenerant that is absent in the other regenerants indicated a new insert of transposition, and the average number of transposition events in five regenerants was calculated (Table 1). Wild-type MAGGY showed the highest transposition activity of an average of 3.8, and the MGY-G5289T mutant exhibited relatively high transposition events per regenerant (1 transposition events per regenerant on average) compared to the other mutants. This result was comparable to those with the intron excision assay. MGY-C5170A, MGY-T5239C and MGY-T5244C showed low levels of transposition activity, and no transposition was detected in the MGY-ΔBsi, MGY-T5234A, MGY-T5239G and MGY-T5244G regenerants by this assay. The sensitivity of the assay used may not be high enough to precisely evaluate the transposition frequency among the mutants with low activities such as the CLM missense mutants. Nevertheless, those results were consistent with the conclusion obtained with the intron excision assay, in that the CLM module is crucial for the transposition activity of the element. All the CLM missense mutants showed lower transposition activity than MGY-G5289T that lacks the C-terminal 22 amino acid residues in the integrase domain. This evidence indicates that a point mutation in the CLM is more important for the transposition activity of the element than loss of the C-terminal 22 amino acid integrase residues. Notably, one additional band was observed in a regenerant of the MGY-C5170A mutant (Fig 3), which lacks the entire CLM and

showed no detectable transposition in the intron excision assay. Although it is possible that the additional band occurred by some genomic rearrangement such as duplication, it is more likely that the MGY-C5170A mutant retains some transposition activity at a very low level since a spliced band was detected by the intron excision assay using a genomic DNA template of the regenerant with the additional band (data not shown). Therefore, the CLM might not be absolutely indispensable for the transposition activity of the element. This hypothesis is further supported by the fact that only a portion of, but not the majority of, retrotransposons possesses the CLM module.

Integrase is a multidomain enzyme that is essential for the integration of retroelement cDNA into the host genome. Using the yeast LTR-retrotransposon Ty3, Sandmeyer and colleagues reported the surprising findings that mutations in the integrase domain affected multiple stages of retrotransposition such as reverse transcription, nuclear localization, processing/stability of reverse transcriptase and the integrase itself, as well as 3'-end processing of extrachromosomal Ty3 DNA [11-15]. Therefore, the integrase of Ty3 mediates not only integration but also multiple other processes of retrotransposition. However, since Ty3 integrase does not possess a recognizable CLM motif, the role of the "additional module" CLM in retrotransposition remains unknown.

The chromodomain was originally defined as a motif of 37 amino acid residues conserved between two *Drosophila* proteins, HP1 and Polycomb [4]. HP1 is a non-histone chromosomal protein with dosage-dependent effects on heterochromatin-mediated gene silencing [16,17], and the Polycomb protein is a silencer of homeotic genes [18]. The domain has been found in single and multiple copies in a variety of proteins from a range of organisms such as fungi, protists, amphibians, plants, and animals [3]. Since chromoproteins comprise an array of chromosomal proteins with

diverse functions and some of the members interact with nucleic acids and different types of proteins, the domain is proposed to mediate nucleic acid-protein and/or protein-protein interactions that target chromatin modifiers to their specific sites of action [19, 20]. Therefore, it is attractive to assume that the CLM module in the MAGGY integrase may interact with one or more of the chromosomal proteins to facilitate targeted integration of MAGGY cDNA into the genome. In fact, the MAGGY element tends to integrate into AT-rich sequences and form a transposon cluster in the genome [5, 21] even though no specific integration site for this tendency has been identified. Thus, the MAGGY CLM might contribute to this feature of the element. It is also possible, however, that the MAGGY CLM interacts with some basic chromosomal protein or DNA to increase the overall integration efficiency.

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657-666.

Figure Legends

Fig. 1. (A) Diagram of wild type MAGGY and its integrase mutants used in this study. The positions of known zinc finger and DD(35)E motifs and proposed GPY/F and CLM (chromodomain-like) modules are given in expanded view of wild type MAGGY integrase (second top). Mutations made in the integrase domain were schematically shown in expanded views thereunder. (B) Alignment of chromodomains from known chromoproteins and chromodomain-containing LTR-retrotransposons. Colouring was generated by Clustal X using default values. The positions of conserved valine (V) and tryptophan (W) in the chromodomain, in which site-directed mutations are made, are indicated by arrowheads. The chromoproteins used are *Drosophila melanogaster* Polycomb (P26017) and Su(Var)3-9 (S47004), *Homo sapiens* HP1 (AAB26994) and *Mus musculus* MOD3 (CAA44398). The chromodomain-containing LTR-retrotransposons are *Cladosporium fulvum* Cft1(CAA77890), *Fusarium oxysporum* Skippy (AAA88790), *Lilium henryi* Del (X13886) and *Hordeum vulgare* Retra (Y14573). The amino acid sequences of Del and Retra are conceptually translated from their DNA sequences.

Fig. 2. (A) Map of 3' LTR region of pMGY70-INT and its expected intron-less derivative, showing the positions of primers used in the intron excision assay. (B) Intron excision assay of the integrase deletion mutants of the MAGGY element. Two plasmids

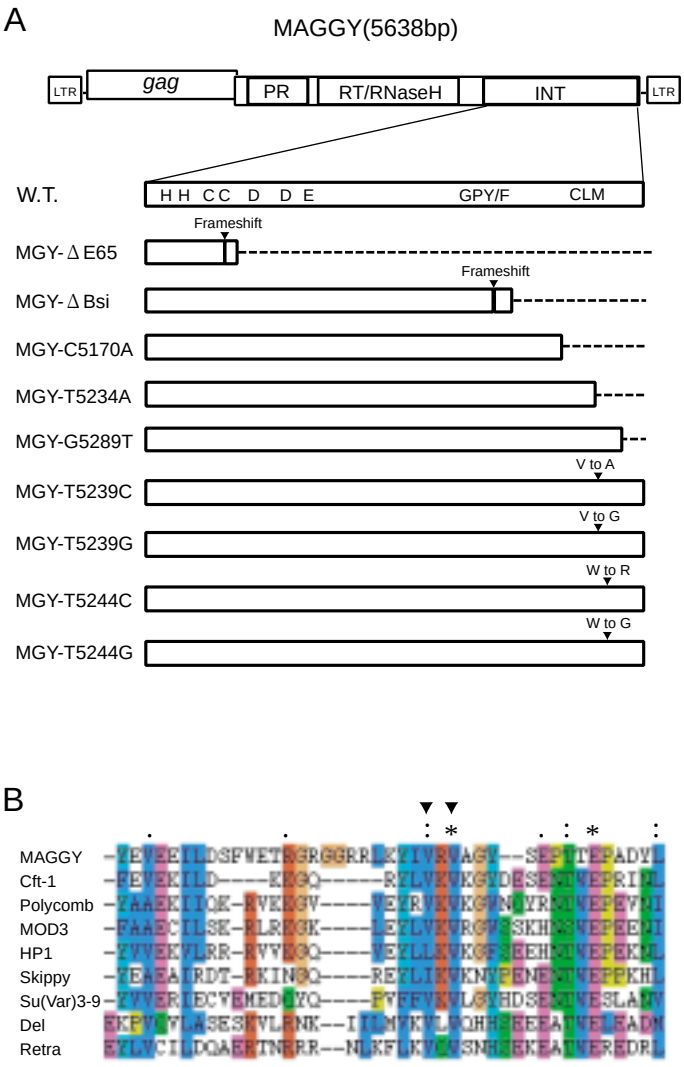
pMGY70 (intron less MAGGY plasmid; MG) and pMGY70-INT (intron containing MAGGY plasmid; IT) were used as templates to amplify unspliced and spliced MAGGY fragments for size makers. (C) Intron excision assay of the integrase missense mutants of the MAGGY element. Molecular sizes of unspliced and spliced MAGGY fragments are indicated by arrowheads.

Fig. 3. An example of transposition assay of *M. oryzae* transformants with wild type MAGGY and its integrase mutants by Southern blots. Five protoplast regenerants were employed for each transformant in the analysis. *Eco*RI-digested fungal genomic DNA and the ES probe were used to detect a unique band per MAGGY copy in the genome as described by Nakayashiki et al. (1999). M, molecular maker; MGY-T5170A and MGY-G5289T, MAGGY integrase mutants; W.T., wild type MAGGY

Table 1 Transposition frequency of the MAGGY integrase mutants assessed by the single protoplast isolation assay

Element	No of transposition/regenerant*
W.T.	3.8±1.79
MGY-ΔBsi	0±0
MGY-C5170A	0.2±0.45
MGY-T5234A	0±0
MGY-G5289T	1.0±1.22
MGY-T5239C	0.4±0.55
MGY-T5239G	0±0
MGY-T5244C	0.2±0.45
MGY-T5244G	0±0

*Five protoplast regenerants from a representative transformant were used for genomic DNA extraction and analysis by Southern blotting. A band in a regenerant that was absent in the other regenerants was regarded as a new insert of transposition, and the average number of transposition events per regenerant was calculated



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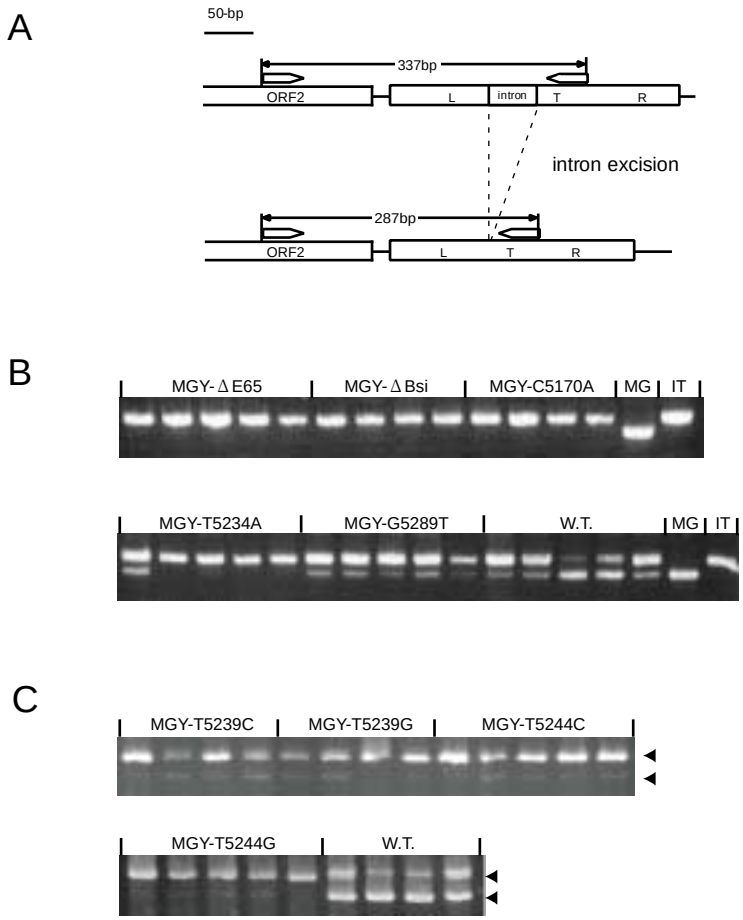

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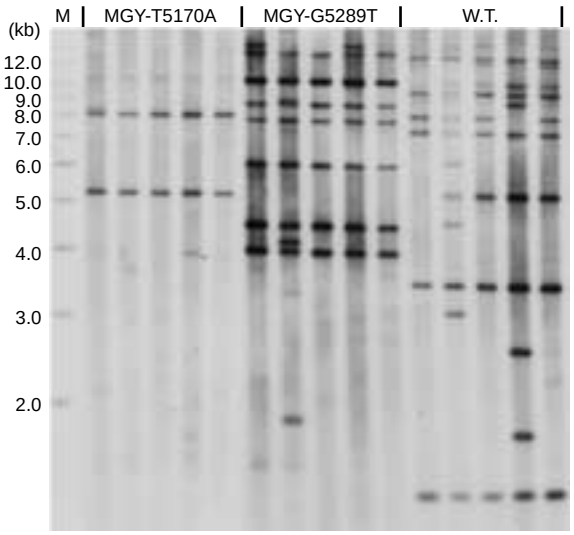

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