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Ikeda, K.
Nakayashiki, Hitoshi
Takagi, M.
Tosa, Yukio
Mayama, Shigeyuki

(Citation)
Molecular Genetics and Genomics,266(2):318-325

(Issue Date)
2001-10

(Resource Type)
journal article

(Version)
Accepted Manuscript

(URL)
https://hdl.handle.net/20.500.14094/90000028

Heat shock, copper sulfate and oxidative stress activate the retrotransposon MAGGY resident in the plant pathogenic fungus, *Magnaporthe grisea*

K Ikeda, H Nakayashiki, M Takagi, Y Tosa, S Mayama

Laboratory of Plant Pathology, Faculty of Agriculture, Kobe University, Rokkodai, Nada, Kobe 657-8501, Japan

Corresponding author: Hitoshi Nakayashiki; Laboratory of Plant Pathology, Faculty of Agriculture, Kobe University, Rokkodai, Nada, Kobe 657-8501, Japan

Telephone and Fax: +81-78-803-5867

E-mail: hnakaya@kobe-u.ac.jp

Abstract

MAGGY is a *gypsy*-like retrotransposon isolated from the plant pathogenic fungus, *Magnaporthe grisea*. Activation of MAGGY by various stresses was tested in the original and heterologous hosts of MAGGY, *M. grisea* and *Colletotrichum lagenarium*, with ß-glucuronidase (GUS) used as a reporter. The MAGGY promoter was activated in *M. grisea* by either heat shock, copper sulfate, and oxidative stress but not by the antifungal substance, para-coumaric acid. Transcriptional up-regulation of MAGGY RNA was also observed by heat shock and oxidative stress. The MAGGY promoter maintained response to the above-mentioned stresses in a *M. grisea* isolate that originally does not possess the MAGGY element. In *C. lagenarium*, however, the MAGGY promoter showed only basal expression of GUS and no further up-regulation was induced by any of the stress treatments, suggesting that the stress-responding *cis*-element(s) in the MAGGY promoter is not functional in a wide range of fungi. The relationship between the activation of MAGGY by stress and diversification of phenotypes of *M. grisea*, including pathogenicity, is discussed.

Key words Activation, MAGGY, *Magnaporthe*, Retrotransposon, Stress

Introduction

Transposable elements (TEs) are ubiquitous and abundant in eukaryotic genomes. There

are two classes of TEs, defined according to their mode of propagation. Class I elements transpose via an RNA intermediate, and class II elements transpose directly from DNA to DNA (Finnegan 1989). Class I elements are further divided into three types (Kempken and Kück 1998), i.e. long terminal repeats (LTR) retrotransposons, LINE and SINE elements. MAGGY is an LTR retrotransposon isolated from the blast fungus *Magnaporthe grisea* and is comprised of two ORFs and 253-bp LTRs (Farman et al. 1996). ORF1 encodes a *gag*-like protein exhibiting a zinc finger domain (Cys-X2-Cys-X4-Cys), and ORF2 encodes a *pol*-like protein with domains sharing sequence homology with protease, reverse transcriptase, RNaseH and endonuclease. Our previous studies demonstrated that MAGGY was the most active element among *Magnaporthe* TEs examined during sexual hybridization and following vegetative growth (Eto et al. 2000), and that MAGGY transposed via an RNA intermediate not only in various isolates of *M. grisea* but also in heterologous species of fungi such as *Pyricularia zingiberi* and *Colletotrichum lagenarium* (Nakayashiki et al. 1999).

Despite a large number of TEs found in various species of eukaryote, transpositional activity has been reported for only a few elements. Since TEs pose a serious threat to the host genome, it is likely that they are repressed or regulated by some mechanisms of the hosts. McClintock (1984) proposed that the TEs might be activated by ‘genomic shock’ as an adaptive mechanism of the genome. Activation of TEs could facilitate reorganization of the genome and might lead to a new phenotype that can survive under severe conditions. Actually, various transposons have been shown to be activated under stress conditions. Transposition of the most well characterized plant LTR retrotransposons, Tnt1 and Tto1, were stimulated by tissue culture, protoplast formation or pathogen infection (Pouteau et al.

1991, Hirochika 1993, Mhiri et al. 1997). Heat shock modulated transcription of *gypsy* and *copia*-like elements in *Drosophila melanogaster* (Ziarczyk and Best-Belpomme 1991; Lyubomirskaya et al. 1993; Ratner et al. 1992). Moreover, Ty element in *Saccharomyces cerevisiae*, 1731 in *D. melanogaster*, and *MuDR* in maize were activated by ultraviolet irradiation (Rolfe et al. 1986; Bradshaw and McEntee 1989; Faure et al. 1996; Walbot 1999). These results might support McClintock's hypothesis. In this study, we examined activation of the MAGGY promoter in response to various kinds of stresses, i.e., heat shock, oxidative stress, copper sulfate, and antifungal compounds, in the original and heterologous hosts of MAGGY, *M. grisea* and *C. lagenarium*.

Materials and methods

Fungal strains and culture media

Fungal materials used were an isolate of *Colletotrichum lagenarium* [104-T] and two of *Magnaporthe grisea* from *Oryza sativa* (rice) [1836-3] and *Triticum aestivum* (wheat) [Br48]. The *Oryza* isolate of *M. grisea* possesses MAGGY in multiple copies but the others do not. Fungal mycelia were grown in CM liquid broth (0.3% Casamino acids, 0.3% Yeast extract, and 0.5% sucrose) at 26˚C unless noted otherwise.

Plasmid constructions and fungal transformations

pMGY70 is a plasmid carrying a copy of MAGGY in pBluescript SK+ (Nakayashiki et al. 1999). pUC-GUS-Nsi containing the GUS gene with a *Nsi*I restriction site at the translation start site was provided by Dr. Furusawa (Mori et al. 1993). pNOM102 expressing GUS under the control of the glyceraldehydetriphosphate dehydrogenase promoter (Roberts et al. 1989) was kindly gifted by Dr. Oliver. pLTR-GUS was constructed by replacing MAGGY ORFs in pMGY70 with the GUS gene by introducing a synthetic linker at the translation start site of MAGGY ORF1 (Fig. 1). The details of the plasmid construction are as follows. A *Sna*BI and *Eco*52I fragment (nucleotide #257-#5207 in MAGGY) of pMGY70 was replaced with the synthetic linker with an *Eco*52I cohesive end (upper strand, 5'-GTAGCTCCTTCATTAGGTGCCCGCGATGCCTGAGCTCACCGCGACGTCCGGATC C-3'; lower strand, 5'-GGCCGGATCCGGACGTCGCGGTGAGCTCAGGCATCGCGGGCACCTAATGAAGG AGCTAC-3'), resulting in pMGY-G. Then, a *Nsi*I-*Sac*I fragment of pUC-GUS-Nsi containing the GUS gene was inserted at the *Eco*52I site in pMGY-G by blunt-end ligation. In the resulting plasmid pMGY-GUS, the GUS gene is inserted so that translation begins at the translation start site of MAGGY ORF1. The final construct, pLTR-GUS was made by inserting a 2.2kb *Xho*I fragment of pMGY-GUS at the *Xho*I site in an LTR fragment (nucleotide #5365-#5638 in MAGGY) cloned in pUC19. Genomic sequence upstream of 5' LTR in pMGY-GUS, which originally came from pMGY70, was removed by this subcloning procedure. pLTR-GUS and pMGY-GUS were sequenced to confirm the structures of the inserts using ABI PRISM 310 Genetic Analyzer (PE Biosystems) according to the manufacturer's protocols.

Plasmids to be tested were introduced into a fungal genome by a polyethylene glycol

(PEG)-mediated co-transformation with a plasmid pSH75 as described previously (Nakayashiki et al. 1999). pSH75 carries a hygromycin B phosphotransferase gene as a selective marker. Transformants were screened for the integration of the GUS gene by PCR using GUS-specific primers (forward primer, CCCCAACCCGTGAAATCAAA; and reverse primer, ACGCCGTATTCGGTGATGAT). PCR (1min at 95˚C, 1min at 55˚C, and 1min at 72˚C; 30 cycles) was performed in a Thermal Cycler, PERSONAL (Takara) using 0.25 units of rTaq polymerase (TOYOBO) and 100 ng of total genomic DNA as a template. Transformants were maintained on potato dextrose agar (PDA) slant media for several months.

Southern blot analysis

Fungal genomic DNA was extracted as described previously (Nakayashiki et al. 1999) and digested with *Eco*T22I (for transformants with pLTR-GUS) or *Cla*I (for transformants with pNOM102). The digests were fractionated on a 0.9% TAE agarose gel and transferred to Hybond N^+ (Amersham). Signals were detected by probing the blot with a dUTP-fluorescein-labeled *Hinc*II fragment (nt. #607-#1140) of the GUS gene. Hybridization and detection were performed using a dioxethane chemiluminescence system (Gene Images^{TM}, Amersham).

Stress treatments

Transformants were subjected to stress treatments after culturing in 40ml of liquid CM

broth for 4 to 5 days. Heat shock treatment was performed at 32, 37, and 42˚C in a water bath for 45 minutes. Chemical compounds used were prepared as follows. Copper sulfate (0.01, 0.1, and 1mM), and methyl viologen (0.1, 1, 10mM) were dissolved in sterilized water. Para-coumaric acid (10, 50, 100µg/ml) was dissolved in methanol: acetone (1: 1). Chemical solution was added to culture media at less than 2% (V/V). Fungal mycelia were cultured in liquid CM broth containing each chemical for 16-18 hours, then subjected to GUS assay.

GUS assays

Fungal mycelia were ground to a powder in liquid N_2 with a mortar and pestle and suspended in extraction buffer (50mM phosphate buffer, pH7.0, including 10mM EDTA and 10mM ß-mercaptoethanol). Samples were centrifuged at 10,000g and supernatant was collected. Protein concentration in the supernatant was estimated according to Bradford (1976). GUS activity was measured using 4-MUG (methylumbelliferyl ß-D-glucuronide) as a substrate by following the protocol of Jefferson et al. (1987).

RNA isolation and Northern blot analysis

Northern blot analysis was carried out as described previously (Nakayashiki et al. 2001). Fungal mycelia were collected 6 hours after stress treatment. Total RNA was extracted from mycelia using the RNeasy plant mini kit (Qiagen). Twenty micrograms of total RNA was separated on a 1.2% denaturing agarose gel. Hybridization and detection were

performed using the dioxetane chemiluminescence system (Gene Images™, Amersham). RNA probes were labeled with UTP-fluorescein using T3 and T7 transcriptional system (Roche diagnostics). A *Sal*I - *Bam*HI fragment of MAGGY (nt. #885-#1445) and the *Hin*cII fragment within the GUS gene (nt. #607-1140) cloned in pBluescript were used as templates for transcription after digesting with an appropriate restriction enzyme.

Results

Transformation of an *Oryza* isolate of *Magnaporthe grisea* with the GUS reporter driven by MAGGY LTR

Stable fungal transformants with two kinds of plasmids with the ß-glucuronidase (GUS) reporter were used in this study. Each of pLTR-GUS containing the GUS gene under the control of the MAGGY LTR promoter (Fig. 1) and the control GUS plasmid pNOM102 with the glyceraldehydetriphosphate dehydroganase promoter was introduced into an *Oryza* isolate of *M. grisea*, a natural host of MAGGY, using a PEG-mediated co-transformation method. The transformants were first screened for the GUS gene by PCR. Five transformants with pLTR-GUS (LGL-O) and three with pNOM102 (PGD-O) were, then, subjected to Southern blot analysis to examine the copy number and structure of the integrated GUS plasmid. Fungal genomic DNA was digested with a restriction enzyme (*Eco*T22I or *Cla*I) that does not have a recognition site in the integrated GUS plasmid so that the length of detectable fragments varies depending on recognition sites in the adjacent

genomic sequence. By probing with the GUS gene, one to five fragments with different sizes were detected in the LGL-O transformants, suggesting that pLTR-GUS inserted into the fungal genome at different locations with various copy numbers among the transformants (Fig. 2). Likewise, the integration of pNOM102 (one to four copies) into the genome seemed to occur at different chromosomal locations among the PGD transformants (Fig. 2).

Effect of stress treatments on the promoter activity of MAGGY LTR assessed by GUS expression

Heat shock treatment; We first tested the activation of the MAGGY promoter by heat shock. Heat shock is known to activate the expression of several LTR retrotransposons (e.g. 1731, *gypsy*) (Ziarczyk and Best-Belpomme 1991; Lyubomirskaya et al. 1993; Ratner et al. 1992). The LGL-O and PGD-O transformants were cultured at 42˚C for 45 min and subjected to fluorometric GUS assay. All of the LGL-O transformants showed increased GUS activity by the heat shock treatment (Fig. 3A), indicating that the MAGGY promoter was up-regulated by heat shock regardless of the genomic position. The induction factors by the heat shock treatment in the five LGL-O transformants (1 to 5) were 5.53, 5.27, 3.27, 9.07, and 11.27, respectively, which were calculated by dividing a mean of GUS activities of each transformant at 42˚C by that at 26˚C (non-treatment control). On the other hand, the PGD-O transformants (1 to 3) showed relatively low activation of GUS expression by heat shock (induction factors of PGD-O1 to PGD-O3 = 1.46, 2.00, and 2.10, respectively).

Copper sulfate treatment; Heavy metals are well-known agents that activate a stress

responding pathway (O'Halloran 1993; Ruis and Schüller 1995). To test the effect of copper sulfate on the promoter activity of MAGGY LTR, the LGL-O and PGD-O transformants were cultured in CM liquid broth containing 0.1mM $CuSO_4$. The induction factors of GUS expression by $CuSO_4$ in LGL-O1 to LGL-O5 were 1.78, 1.97, 2.50, 4.52, and 2.52 while those in PGD-O1 to PGD-O3 were 0.50, 0.91, and 1.46, respectively (Fig 3B). The results indicated that the promoter activity of MAGGY was significantly up-regulated by the $CuSO_4$ treatment.

Oxidative stress; The activity of the MAGGY promoter under oxidative stress condition was examined by treatment of the transformants with methyl viologen, which is commonly known as the bipyridyl herbicide, paraquat. Methyl viologen generates reactive oxygen species in the cytoplasm (Bus and Gibson 1984). Figure 3C shows the effect of methyl viologen treatment (10mM) on GUS expression in the LGL-O and PGD-O transformants. Treatment with methyl viologen led to significant increase in the MAGGY promoter activity in most of the LGL-O transformants (induction factors are 2.27, 2.07, 1.99, 3.89, and 1.28) while no significant increase of GUS activity was observed in any of the PGD transformants (induction factors are 0.33, 0.90, and 1.49). In addition to methyl viologen, we tried hydrogen peroxide as another oxidative stressor. High concentration of H_2O_2 (100mM) also induced 7.4 times higher GUS activity than untreated control in LGL-O4. Therefore, activation of the MAGGY promoter by oxidative stress was strongly suggested.

Para-coumaric acid; Para-coumaric acid is a phenylpropanoid compound of cinnamate found in plants and known to inhibit fungal growth. Para-coumaric acid inhibited spore germination of *M. grisea* at a concentration of 50 ppm or more (data not

shown). As shown in Fig 3D, the induction factors by 100μg/ml para-coumaric acid in LGL-O1 to LGL-O5 were 1.33, 1.05, 0.67, 1.44, and 0.88 while those in PGD-O1 to PGD-O3 were 2.15, 2.89, and 2.58, respectively. Contrary to the other treatments, para-coumaric acid induced GUS expression in the PGD transformants but not in the LGL transformants. The promoter of glyceraldehydetriphosphate dehydroganase could have a cis-element responsive to para-coumaric acid. However, this seemed to be, at least partly, due to the solute of para-coumaric acid (acetone: methanol=1: 1) since the solute itself significantly induced GUS activity at a concentration of 1 % in the PGD transformants (data not shown).

To study further the activation of the MAGGY promoter by heat shock, copper sulfate and oxidative stress, GUS expression in LGL-O4 and PGD-O3 was examined under various temperatures or concentrations of the stresses (Fig. 4). Generally, LGL-O4 treated with the stresses exhibited increased GUS activity in a temperature- or dose-dependent manner within the ranges we tested. As an exception, GUS activity was higher (induction factor = 4.5) at a concentration of 0.1mM $CuSO_4$ than that at 1mM $CuSO_4$ (induction factor = 2.2) in LGL-O4. Since $CuSO_4$ is a toxic compound, higher concentration of $CuSO_4$ such as 1mM might cause some dysfunction in the basic metabolism of *M. grisea*, leading to lower GUS activity. Actually, GUS activity in PGD-O3 was much reduced by treatment with 1mM $CuSO_4$ (Fig. 4). These results again showed that the MAGGY promoter is up-regulated by heat shock, copper sulfate and oxidative stress. Activation of the MAGGY promoter by para-coumaric acid was also examined at 10, 50, 100μg/ml. None of the para-coumaric acid treatments up-regulated the MAGGY promoter (data not shown).

With PGD-O3, significant increase in GUS activity relative to non-treatment control

was observed only by heat shock at 42 ˚C. The promoter of glyceraldehydetriphosphate dehydroganase might be responsive to heat shock, or higher stability of GUS protein might contribute to the increase in GUS activity per mg protein.

Northern blot analysis of GUS and MAGGY RNA driven by the MAGGY promoter under stress conditions

Transcription of the GUS gene driven by the MAGGY promoter was examined under heat shock condition by Northern blot analysis. LGL-O4 and PGD-O3 were treated with heat shock (42˚ C for 45 min) and total RNA was extracted 6 hr after treatment. GUS mRNA was detected at a position of 2.0 kb in LGL-O4 treated with heat shock but was not observed in that of untreated control, indicating induction of GUS transcription by heat shock in LGL-O4 (Fig. 5). In the case of PGD-O3, GUS mRNA expression was higher than that in LGL-O4, but level of the mRNA accumulation was similar between heat shock treated and untreated transformants (Fig. 5). These results were consistent with the analysis of GUS activity in the transformants under heat shock stress.

To analyze expression of MAGGY RNA under various stress conditions, Northern blot analysis was carried out using a *M. grisea* transformant (*Triticum* isolate) with MAGGY (Nakayashiki et al. 1999). The transformant was treated with heat shock (42˚C, 45min), copper sulfate (0.1mM), methyl viologen (10mM), and para-coumaric acid (100µg/ml). Total RNA was extracted 6hr after treatment and subjected to Northern analysis. MAGGY RNA of 5.4kb in size, which corresponds to the full length of MAGGY, was detected by all the treatments (Fig. 6). Transcriptional up-regulation of MAGGY RNA was observed by

heat shock and methyl viologen treatments but not by copper sulfate and para-coumaric acid treatments. Mostly, the results of Northern analysis were consistent with those of GUS analysis in the LGL-O transformants but the exception was copper sulfate treatment. As shown in Fig. 3B and Fig. 4, GUS activity was induced by copper sulfate in the LGL-O transformants 16 hr to 18 hr after treatment but up-regulation of MAGGY RNA was not detected 6 hr after treatment. This might be due to timing of activation of the MAGGY promoter by copper sulfate. Transcription of MAGGY RNA could be activated earlier or later than 6hr after treatment.

Effects of the stress treatments on the activity of the MAGGY promoter in heterologous hosts

To examine whether the MAGGY promoter maintains response to stress in heterologous hosts, pLTR-GUS and pNOM102 were introduced into a *Triticum* isolate of *M. grisea* [Br48] and *Colletotrichum lagenarium*, both of which originally do not possess the MAGGY element but can support MAGGY transposition when MAGGY is introduced into them by PEG-mediated transformation (Nakayashiki et al. 1999). Three independent transformants with each plasmid were obtained with the fungal isolates. They were designated as LGL-T1 to 3 (*Triticum* transformants with pLTR-GUS), PGD-T1 to 3 (*Triticum* transformants with pNOM102), LGL-C1 to 3 (*Colletotrichum* transformants with pLTR-GUS), and PGD-C1 to 3 (*Colletotrichum* transformants with pNOM102), respectively. These transformants were treated with heat shock (42˚C, 45min), copper

sulfate (0.1mM) and methyl viologen (10mM), and then subjected to fluorometric GUS assay. Every LGL-T transformant showed significant increase in GUS activity by all the treatment tested while none of the PGD-T transformants showed increased GUS activity by any of these stress treatments (Fig. 7A). This indicated that the stress responding *cis*-element(s) in the MAGGY promoter is functional not only in the original host but also in the non-MAGGY-carrier of *M. grisea*.

On the other hand, increase in GUS activity by the stress treatments was, generally, not found in the LGL-C transformants (Fig 7B). Even though a little up-regulation of the MAGGY promoter was detected in some LGL-C transformants (ex. LGL-C1 and LGL-C2) by either of heat shock or copper sulfate, this seemed to be due to chromosomal position effects but not to the MAGGY promoter since response to the stresses differed among the transformants. The results presented here indicated that the stress responding *cis*-element(s) in the MAGGY promoter is not effective in *C. lagenarium*. It is noteworthy, however, that the basal expression level of GUS was not much different between the LGL-T and LGL-C transformants (data not shown), indicating that the promoter of MAGGY worked in *C. lagenarium* as well as in *M. grisea* without response to the stresses tested here.

Discussion

We demonstrated that MAGGY, a retrotransposon found in the pathogenic fungus *Magnaporthe grisea*, was activated by either heat shock, $CuSO_4$, and oxidative stress. On

the other hand, treatment with the antifungal substance, para-coumaric acid did not activate the MAGGY promoter. We also examined UV irradiation, protoplast formation and other antifungal substances such as sakuranetin (rice phytoalexin), and isoprothiolane (fungicide) for stressors. None of the stressors, however, induced GUS expression driven by the MAGGY promoter (data not shown), indicating that the stress responding cis-element(s) in the MAGGY promoter is responsive to the specific kinds of stress. A multistress-responding *cis*-element or several stress-specific *cis*-elements in the LTR promoter could be involved in the activation of MAGGY. In *Saccharomyces cerevisiae*, three stress-responding *cis*-elements have been identified: heat shock elements (HSEs), stress response elements (STREs), and AP-1 responsive elements (AREs) (Ruis and Schüller 1995). All of them are multistress responding elements. Ruis and Schüller (1995) proposed that the three types of control elements have overlapping but distinct functions: HSEs regulates genes required under moderate stress, STRE-activated genes appears to be important under severe stress and ARE-controlled genes may mainly function in response to oxidative stress and toxic condition. Searching for a core sequence of these stress-responding *cis*-elements in the MAGGY LTR resulted in detection of only a CCCCT motif, the core sequence of STREs. This CCCCT motif may be involved in the stress response of the MAGGY promoter since the promoter seemed to be responsive to multistress and to be activated under severe stress conditions. Deletion and site directed mutation analyses in the MAGGY promoter will be addressed in the future.

Transposable elements have been considered as selfish parasites, but several reports showed that transposable elements acted as beneficial factors against stress conditions (Schmid 1998; Teng et al. 1996). *M. grisea*, the causal pathogen of blast disease, is known

to have a variety of pathotypes or races (Kato et al. 2000). Each of them is pathogenic on only a restrict range of plant species or their cultivars (Ou 1985). It has been often reported, however, that a new race of *M. grisea* that overcome the defenses of a previously resistant plant arose after introduction of the resistant plant cultivar in rice fields. It is attractive to assume that transposable elements may play a role in the emergence of a new race of *M. grisea*. Actually, molecular analysis of a spontaneous mutant of *M. grisea* that overcame the resistance of the rice cultivar Yashiro-mochi (carrying the *Pi-ta* gene) revealed that the DNA transposon, Pot3 (MGR586), was inserted into the upstream of the corresponding avirulence gene, *Avr-Pita* in the mutant (Orbach et al. 2000). Nishimura et al. also showed that the insertion of a LINE-like element MGL into the ACR1 gene, which is involved in spore formation, led to loss of pathogenicity of *M. grisea* toward rice. Although the biological significance of the activation of MAGGY by stress is unclear to date, it could contribute to produce diversity in phenotypes of *M. grisea* including pathogenicity since stress conditions we used in the experiments might take place in the field. Fungicides containing copper or other heavy metals (ex. mercury) were used in the past. It was reported that *M. grisea* mutants resistant to copper sulfate were obtained by culturing the fungi in PDA media containing copper sulfate at a low concentration (Yamazaki and Tsuchiya 1964). Such a genetic change might involve transposition of transposable elements activated by the stress. In addition, accumulation of H_2O_2 at a concentration of 1M or even more was suggested in lettuce cell walls in response to inoculation with an incompatible bacterial pathogen, *Pseudomonas syringae* pv. *Phaseolicola* (Bestwick et al. 1997). The relationship between activation of MAGGY by stress and emergence of a new race of *M. grisea* is an interesting point to investigate and requires future work.

MAGGY was activated by the stresses even in the *M. grisea* isolates that originally do not possess MAGGY. In *C. lagenarium*, however, only basal expression of GUS driven by the promoter was observed and no further up-regulation was induced by any of the stress treatments we tested. The stress-responding *cis*-element(s) in the MAGGY promoter is not likely to be functional in a wide range of fungi, and could be specific to *M. grisea*. The promoters of some retrotransposons have been shown to maintain their response to stress in heterologous hosts as in the original hosts. The tobacco retrotransposon Tnt1 was activated by protoplast formation and pathogen elicitors in *Arabidopsis* as well as in the original host (Pauls et al. 1994). Induction of the promoter of the *Drosophila* retrotransposon 1731 by UV-B, previously found in *Drosophila* cells, was maintained in a human epithelial cell line (Faure et al. 1996). On the other hand, an interesting report is that the promoter of Tnt1 was activated by the plant hormone auxin in a heterologous host *Arabidopsis* but not in the original host tobacco (Pauls et al. 1994). Difference in activation of the promoters between the original and heterologous hosts may be due to difference in the binding sequence of the corresponding transcription factor or presence/absence of the stress-responding pathway of the hosts.

How retrotransposons have acquired stress responsiveness is an interesting point to address. From this point of view, intriguing examples are plant retrotransposons Tnt1 and Tto1, both of which were activated by pathogen-related stresses. The promoters of Tnt1 and Tto1 showed striking similarity to those of cellular defense genes such as PR-proteins and chalcone synthase but showed no similarity between their stress-response motifs (Takeda et al. 1999; Vernhettes et al. 1997). Therefore, it could be possible that stress responsiveness or other specific expression features of retrotransposons have been acquired

by capturing adjacent genomic *cis*-element(s) into the transposons by mechanisms similar to retroviral transduction as suggested previously (Jin and Bennetzen 1994). Alternatively, the stress responding *cis*-elements in retrotransposons have developed through inter-element selection in the host as proposed for the enhancer motif in LTR retrotransposon (McDonald et al. 1997).

Acknowledgements

We thank Dr. R. P. Oliver for providing pNOM102, Dr. Iwao Furusawa for providing the pUC-GUS-Nsi, and Dr. Motoichiro Kodama for technical advice of fungal transformation. We are much indebted to Hajime Kato, the former professor of Kobe University, for valuable suggestions for improvement. This work was partly supported by a grant from the Ministry of Education, Japan (No. 10660049)

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FIGURE LEGENDS

Figure 1 Schematic representation of MAGGY (**A**) and pLTR-GUS (**B**). pLTR-GUS was constructed by replacing MAGGY ORFs with the GUS gene. The GUS gene was inserted between LTRs so as to be translated from the translation start site of ORF1 (*gag*) of MAGGY (see Materials and Method). LTR, long terminal repeats; ORF, open reading frame; GUS, ß-glucuronidase; pBS, pBluescript (SK+); Ec, *Eco*RI; Sc, *Sac*I; X, *Xho*I

Fig. 2 Southern blots of the pLTR-GUS and pNOM102 transformants of an *Oryza* isolate of *Magnaporthe grisea*. Genomic DNA was digested with *Eco*T22I (LGL-O) or *Cla*I (PGD-O) and probed with the GUS gene. Lane 1 to 5, transformants with pLTR-GUS (LGL1-5); lane 6 to 8, transformants with pNOM102 (PGD1-3)

Fig. 3A-D GUS activity in the transformants of an *Oryza* isolate of *Magnaporthe grisea*

under various stress conditions. **A**, heat shock (42˚C); **B**, $CuSO_4$(0.1mM); **C**, Methyl viologen (10mM); **D**, p-coumaric acid (100µg/ml). Black bars, pLTR-GUS transformants (LGL-O1 to 5); white bars, pN0M102 transformants (PGD-O1 to 3). GUS activity was examined in at least three independent experiments. A mean of GUS activity and standard deviation are represented

Fig. 4 Effects of heat shock, methyl viologen (MV) and copper sulfate ($CuSO_4$) treatments on GUS activity in the *Magnaporthe grisea* transformants (*Oryza* isolate) with the GUS constructs, pLTR-GUS (LGL-O4, black bars) and pNOM102 (PGD-O3, white bars). GUS activity was examined in at least three independent experiments. A mean of GUS activity and standard deviation are represented

Fig. 5 Northern blot analysis of GUS in the transformants with pLTR-GUS (LGL-O4) and pNOM102 (PGD-O3) after heat shock treatments. Fungal mycelia were treated with heat shock (42˚C, 45 min) and total RNA was extracted. Twenty micrograms of total RNA was run on a 1.2% formaldehyde agarose gel, transferred to a nylon membrane, then probed with the GUS gene. The18s rRNA stained with ethidium bromide and photographed before blotting is shown below the blot. C, control (26˚C); H, heat shock (42˚C)

Fig. 6 Northern blot analysis of MAGGY under various stress conditions. Fungal mycelia were treated with various stresses (see below) and total RNA was extracted. Twenty micrograms of total RNA was run on a 1.0% formaldehyde agarose gel, transferred to a nylon membrane, then probed with MAGGY. The18s rRNA stained with ethidium bromide

and photographed before blotting is shown below the blot. Lane 1, control; lane 2, heat shock (42˚C); lane 3, copper sulfate (0.1mM); lane 4, methyl viologen (10mM); lane 5, p-coumaric acid (100μg/ml)

Fig. 7A, B GUS activities in the pLTR-GUS and pNOM102 transformants of a *Triticum* isolate of *Magnaporthe grisea* and *Colletotrichum lagenarium*. **A**, Transformants of *M. grisea* (*Triticum* isolate); **B**, Transformants of *C. lagenarium*. Black bars, transformants with pLTR-GUS; white bars, transformants with pNOM102. GUS activity was examined in at least three independent experiments. A mean of GUS activity and standard deviation are represented

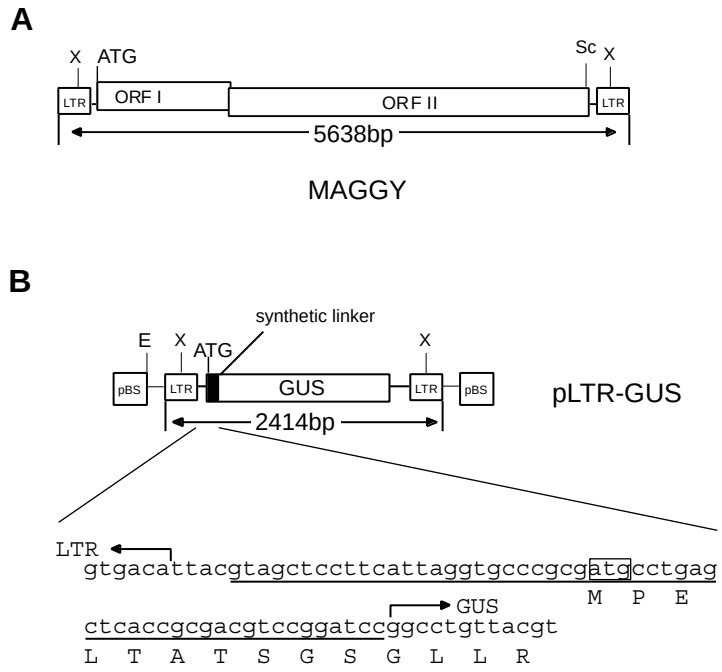
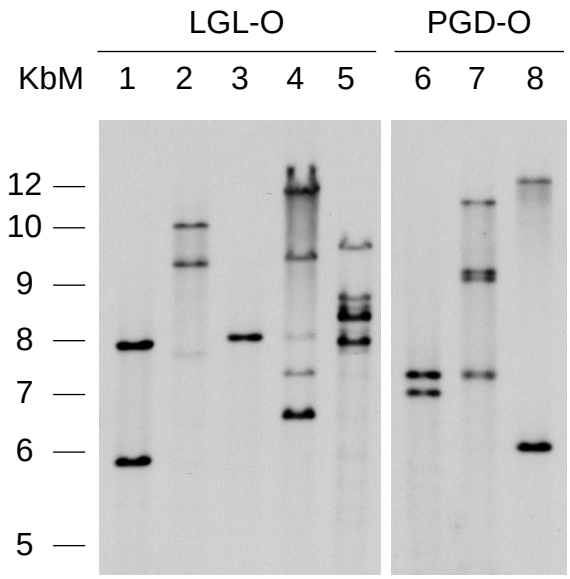


Fig. 1A, B Schematic representation of MAGGY (**A**) and pLTR-GUS (**B**). pLTR-GUS was constructed by replacing MAGGY ORFs with the GUS gene. The GUS gene was inserted between MAGGY LTRs so as to be translated using the original translational start site of MAGGY ORF1 (boxed). Nucleotide sequence of the synthetic linker is underlined. Deduce amino acid sequence is shown under the nucleotide sequence.
LTR, long terminal repeats; ORF, open reading frame; GUS, β-glucuronidase; pBS, pBluescript (SK+); Ec, *Eco*RI; Sc, *Sac*I; X, *Xho*I



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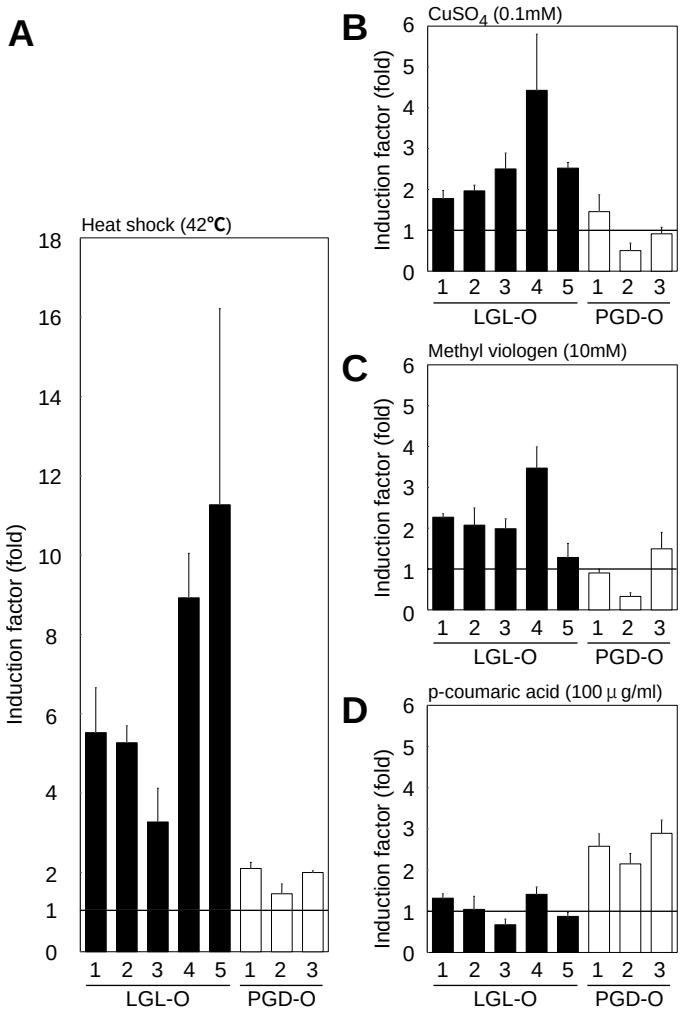

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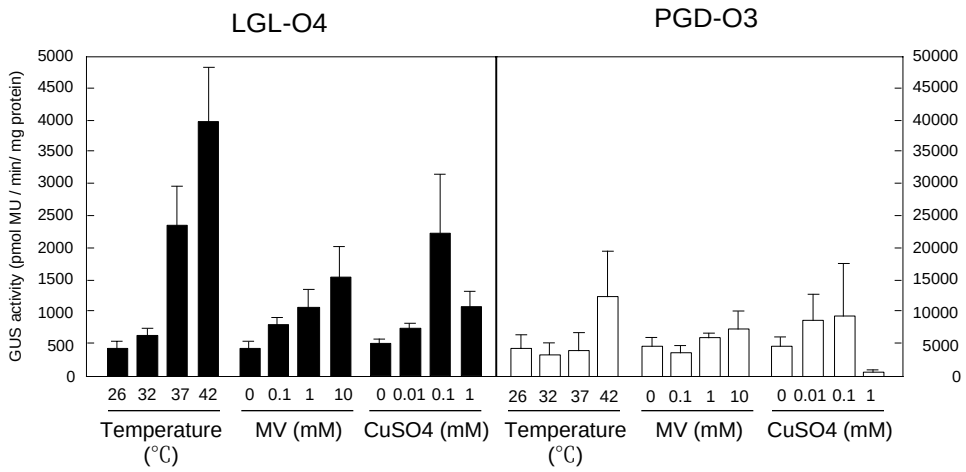

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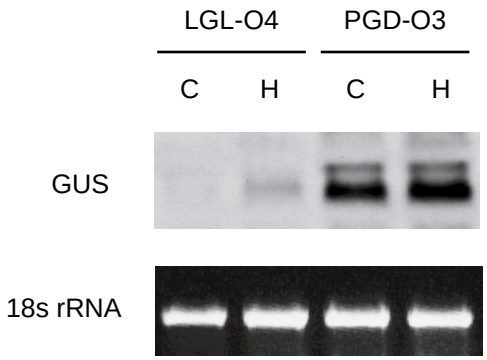

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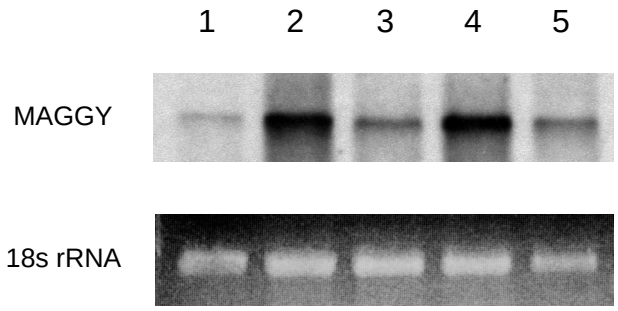

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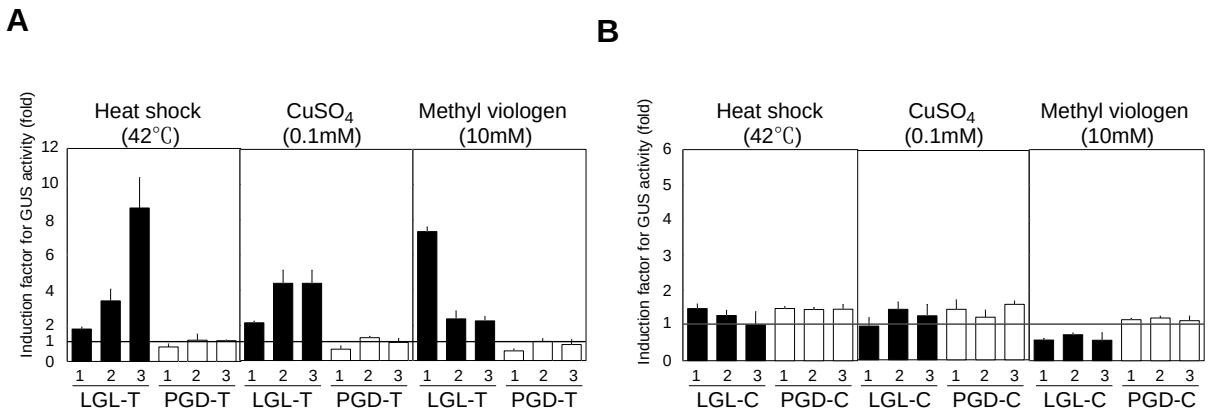

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