RNA Silencing in the Phytopathogenic Fungus *Magnaporthe oryzae*

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Systematic analysis of RNA silencing was carried out in the blast fungus *Magnaporthe oryzae* (formerly *Magnaporthe grisea*) using the enhanced green fluorescence protein (eGFP) gene as a model. To assess the ability of RNA species to induce RNA silencing in the fungus, plasmid constructs expressing sense, antisense, and hairpin RNAs were introduced into an eGFP-expressing transformant. The fluorescence of eGFP in the transformant was silenced much more efficiently by hairpin RNA of eGFP than by other RNA species. In the silenced transformants, the accumulation of eGFP mRNA was drastically reduced, but no methylation of the promoter or coding region was involved in it. In addition, we found small interfering RNAs (siRNAs) only in the silenced transformants. Interestingly, the siRNAs consisted of RNA molecules with at least three different sizes ranging from 19 to 23 nucleotides, and all of them contained both sense and antisense strands of the eGFP gene. To our knowledge, this is the first demonstration in which different molecular sizes of siRNAs have been found in filamentous fungi. Overall, these results indicate that RNA silencing operates in *M. oryzae*, which gives us a new tool for genome-wide gene analysis in this fungus.

Additional keywords: RNA interference (RNAi).

RNA-mediated gene silencing is a relatively newly discovered gene regulatory mechanism that suppresses gene expression in a sequence-specific manner at the posttranscriptional level. This unique mechanism has been independently discovered and studied in different classes of eukaryotes as cosuppression or posttranscriptional gene silencing in plants (Napoli et al. 1990; van der Krol et al. 1990), quelling in the fungus *Neurospora crassa* (Romano and Macino 1992), and also RNA interference (RNAi) in animals (Fire et al. 1998). Even the mechanisms of these phenomena remain incompletely elucidated; diverse lines of evidence suggest that common genetic and biochemical mechanisms underlie them (Hammond et al. 2001). Therefore, we here use the generic term RNA silencing as proposed recently (Baulcombe 1999; Ding 2000). RNA silencing is now regarded as an intrinsic defense mechanism of most eukaryotes against invasive nucleic acids, such as viruses, transposable elements, and transgenes (Waterhouse et al. 2001). A simplified model for the general processes of RNA silencing is as follows. Double-stranded RNA (dsRNA) is the initiator and is subsequently processed into short RNAs, called small interfering RNAs (siRNAs), by the action of RNase III-related endonuclease. The resulting siRNAs containing both

sense and antisense strands of the target RNA are then incorporated into the multicomponent RNase complex RISC (RNA interference silencing complex) to serve as guide molecules for targeted mRNA degradation.

In plants, two distinct classes of siRNAs were reported to have different roles in the RNA silencing process (Hamilton et al. 2002). The shorter species is 21 to 22 nucleotides (nt) long, while the longer is 24 to 26 nt. Through the use of viral suppressors of RNA silencing and *Arabidopsis* mutants, Hamilton and associates (2002) demonstrated that the shorter siRNAs correlated with sequence-specific mRNA degradation and that the longer class triggered methylation of homologous DNA and systemic signaling of silencing throughout the plant. In animal cells such as a *Drosophila* in vitro system, however, no discrete size classes of siRNAs were observed even though some size variations from 21 to 23 nt appeared in the length of the siRNAs (Elbashir et al. 2001; Tang et al. 2003; Zamore et al. 2000). In the fungus *N. crassa*, siRNAs have been demonstrated to be involved in the silencing pathway with a single molecular size reported at 25 nt (Catalanotto et al. 2002). Therefore, siRNAs seem to have some variations in size depending on the organism and may have one or more of the same or different roles in the RNA silencing pathway.

Despite numerous reports on RNA silencing in a variety of organisms, only two species among filamentous fungi to date have been proven to have this mechanism. One is *N. crassa*, as described earlier, and the other is the human pathogenic fungus *Cryptococcus neoformans* (Cogoni and Macino 1999a; Liu et al. 2002). No application of RNA silencing has been reported in phytopathogenic fungi so far. Rice blast disease, caused by the ascomycete *Magnaporthe oryzae* (formerly *Magnaporthe grisea*) (Couch and Kohn 2002), is one of the most devastating threats to rice production worldwide. This filamentous fungus represents a model fungal pathogen for elucidating plant-microbe interactions. The rice blast genome project was launched in 1998 by the International Rice Blast Genome Consortium, and the almost-complete genome sequence of this fungus was published and became available on a web database in 2002 (Martin et al. 2002). An effective means to exploit the wealth of the genome information is now anticipated. In this study, we show the operation of RNA silencing in the rice blast fungus, using enhanced green fluorescence protein (eGFP) as a model, and perform a detailed analysis on its molecular mechanisms.

RESULTS

Hairpin RNA efficiently induces genetic interference in *M. oryzae*.

Various species of RNA, such as sense, antisense, double strand, and hairpin, are all known to have the ability, at least to some extent, to trigger RNA silencing in plants and animals. In

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filamentous fungi, quelling (RNA silencing in *N. crassa*) occurred in transformants with multiple transgenes having no promoter (Romano and Macino 1992). A second example of RNA silencing in fungi has been found in the human pathogenic fungus *C. neoformans* by expressing the hairpin RNA of the cryptococcal genes (Liu et al. 2002). In those experiments, however, only one kind of strategy was adopted, and no systematic analysis was done to determine what RNA species is the best trigger for RNA silencing in filamentous fungi. Therefore, we first focused on this point.

To establish a model system for elucidating RNA silencing in *M. oryzae*, we first made an eGFP expressing transformant by introducing a plasmid (pEGFP75) carrying the eGFP gene under the control of the *Aspergillus nidulans trpC* promoter (Fig. 1). A wheat isolate of *M. oryzae* was used as a recipient. One of the transformants exhibiting strong GFP fluorescence and possessing about ten copies of pEGFP75 was designated as GFP10 and was used in subsequent transformations. Next, we made double transformants with various silencing constructs expressing a single or duplicated GFP-RNA in different orientations, using GFP10 as a recipient (Fig. 1). One of the silencing constructs, pEGFP-A, expresses a single anti-sense eGFP RNA, and the others (pEGFP-AA, pEGFP-SS, and pEGFP-SA) produce double eGFP RNAs in three orientations, sense-sense, antisense-antisense, and sense-antisense. All of these have a partial sequence of the beta-glucuronidase (GUS) gene as an internal spacer. Each of the constructs was introduced by cotransforming them with pII99 carrying the geneticin resistance gene as a selective marker (Namiki et al. 2001). For controls, we also made double transformants with only pII99.

Double transformants (80) were randomly picked from each transformation, and their GFP fluorescence was measured with an image analyzer. The double transformants were then classified into five categories based on relative fluorescence to the original single-transformant GFP10, and the number of the double transformants in each category was plotted (Fig. 2). The loss of GFP fluorescence was most frequently observed in the double transformants with pEGFP-SA that expressed a hairpin eGFP RNA with a double-stranded structure at the stem. It is noteworthy that various degrees of silencing were observed in the pEGFP-SA double transformants. In contrast, a few silenced double transformants were recognized with other con-

structs expressing antisense (pEGFP-A), antisense-antisense (pEGFP-AA), and sense-sense (pEGFP-SS) eGFP RNAs. However, the number of silenced transformants among them was low.

Genomic Southern analysis was carried out to examine if genetic changes occurred in the pEGFP75 loci during the second transformation. Five silenced and four nonsilenced double transformants with pEGFP-SA as well as GFP10 were subjected to the analysis. Genomic DNA was digested with *Eco*RI and *Xba*I and was probed with the coding region of the eGFP gene (CD probe). The entire transcriptional unit of eGFP in pEGFP75 should be detected at 2.6 kb by this restriction digestion (Fig. 1). As shown in Figure 3, almost all the pEGFP75 copies seemed to be retained intact in both the silenced and nonsilenced double transformants, eliminating the possibility that eGFP silencing in the double transformants was caused by genetic changes in the pEGFP75 loci. eGFP silencing in the double transformants is notably correlated with the presence of a 3.2-kb band that corresponds to the eGFP-containing fragment in pEGFP-SA (Fig. 1), indicating that most, if not all, double transformants that possess intact pEGFP-SA exhibited eGFP silencing in varying degrees.

In conclusion, these results indicated that hairpin RNA (dsRNA) was a potent trigger of gene silencing in *M. oryzae*, as reported previously in other eukaryotes exhibiting RNA silencing (Fire et al. 1998; Kennerdell and Carthew 1998; Nasevicius and Ekker 2000; Sanchez Alvarado and Newmark 1999; Wesley et al. 2001). In addition, homology-dependent transcriptional gene silencing in the promoter region did not seem to function efficiently in *M. oryzae*, because significant silencing was induced only by pEGFP-SA among the constructs used with the same promoter including drag resistant plasmids.

eGFP silencing in *M. oryzae* is caused by low level accumulation of mRNA and is associated with siRNAs.

Because RNA silencing in plants and animals has been described as being due to posttranscriptional degradation of targeted mRNA, we tested whether the loss of GFP fluorescence correlates with a lower level of eGFP mRNA accumulation. Total RNA was isolated from the GFP10 and the pEGFP-SA double transformants with or without the GFP fluorescence, followed by Northern blot analysis. Images of their mycelia and GFP fluorescence are given in Figure 4A. High levels of eGFP mRNA were detected in the single transformant GFP10, in addition to the double transformants exhibiting strong GFP fluorescence (Fig. 4B). In contrast, significantly reduced levels of eGFP mRNA accumulation were detected in the silenced transformants with little or no GFP fluorescence. In some of the silenced transformants (e.g., no. 1), mRNA accumulation was detected at a very low level; however, it was below the levels detectable by Northern analysis in the other silenced transformants. The different levels of mRNA accumulation in the silenced transformants may be the cause of various degrees of silencing.

We also examined the involvement of small interfering RNAs (siRNAs) in eGFP silencing of *M. oryzae*, to gain further insight into its molecular mechanisms. siRNAs are known as key molecules in the RNA silencing process, thus recognized as its hallmark. Fractionated total RNA from the double transformants and GFP10 were separated on a 15% denatured polyacrylamide gel and were transferred electronically to a nylon membrane. When hybridized with an antisense eGFP probe, small hybridizing RNA molecules were detected only in silenced double transformants with at least three different molecular sizes ranging from 19 to 23 nt (Fig 4C). Interestingly, the amount of siRNA accumulation was correlated with the de-

Fig. 1. Diagram of transformation plasmids used in this study. Arrows indicate the orientation of the enhanced green fluorescence protein gene. Segments used as probes in Southern analysis are indicated by thick bars. A *Hinc*II fragment of the beta-glucuronidase (GUS) gene was used as a spacer. All the constructs were under the control of the *Aspergillus nidulans trpC* promoter (*PrrpC*) and terminator (*TrpC*).

gree of silencing in the transformants. In contrast with mRNA accumulation, more siRNA accumulation of eGFP was observed in the transformants showing strong eGFP silencing, such as numbers 28 and 31, than in transformant number 1, which showed moderate silencing. Similarly, hybridizing with a sense eGFP probe allowed the detection of eGFP siRNAs with slightly different molecular sizes in the silenced double transformants (Fig. 4D). In filamentous fungi, siRNAs have so far been reported only in *N. crassa* as a single band 25 nt long on the Northern blot (Catalanotto et al. 2002). In this report, different sizes of siRNAs were detected for the first time in filamentous fungi, just as they had been reported in animals and plants.

DNA methylation is not involved in GFP silencing in *M. oryzae*.

DNA methylation has been clearly demonstrated to be involved in transcriptional silencing in various species of eukaryotes. In addition, DNA methylation is sometimes reported to be associated with RNA silencing, especially in plants (Ingelbrecht et al. 1994; van Houdt et al. 1997). However, quelling found in the fungus *N. crassa* is known to be a methylation-independent process (Cogoni et al. 1996). Thus, we examined whether DNA methylation is involved in the GFP silencing observed in *M. oryzae*. We used a set of restriction endonuclease isoschizomers, *HapII* and *MspI*, to analyze the methylation in the double transformants. Both of the restriction enzymes recognized and digested an identical 4-bp target CCGG. However, the former is methylation-sensitive, and the latter is not. Therefore, if the target sequence is methylated, undigested higher DNA molecules can be observed only in *HapII* digests in the Southern blots probed with the target. We checked the DNA methylation by this approach in both the coding and promoter regions of the eGFP genes, using three silenced and two nonsilenced double transformants as well as the single transformant GFP10. As shown in Figure 5A and B, no difference was observed between the *HapII* and *MspI* digests in any of the DNA samples, not only from the nonsilenced transformants but also those from the silenced ones

when they were probed with either the coding (CD) or promoter (PR) region (Fig. 1). These results suggest that DNA methylation was not involved in the GFP silencing in *M. oryzae*.

DISCUSSION

RNA silencing in *M. oryzae*.

RNA silencing has been discovered in every eukaryotic kingdom, animal, plant, alga, protozoan, and fungus (Fire 1999); thus, it is considered to be conserved through a long period of evolution as an eukaryotic natural defense system against viruses and transposons (Matzke et al. 2000). In these eukaryotes, various forms of foreign nucleic acids have been shown to trigger RNA silencing. Cosuppression in plants, which is now regarded as being caused via the RNA silencing pathway, was originally discovered in *Petunia* plants over-expressing the chalcone synthase mRNA (Napoli et al. 1990; van der Krol et al. 1990), therefore induced by sense RNA. Quelling in the fungus *N. crassa* occurred in transformants with multiple promoterless transgenes (Romano and Macino 1992). Injecting dsRNA into nematode (RNAi) results in silencing a homologous endogenous gene with a very high efficiency (Fire et al. 1998). The antisense strategy, which has been used to attenuate the expression of a gene of interest in a wide range of organisms, is also believed to function, at least partly, through the processes of RNA silencing.

To establish RNA silencing as a gene analysis tool in a given organism, it is important to know which trigger induces the silencing most efficiently. However, such systemic analysis has never been done in filamentous fungi. Consequently, we examined various RNA species with different orientations to find a potent trigger for RNA silencing in *M. oryzae*. The results indicated that dsRNA (hairpin RNA) worked best for the purpose in *M. oryzae*, which is also the case with every other organism examined (Fire et al. 1998; Kennerdell and Carthew 1998; Nasevicius and Ekker 2000; Sanchez Alvarado and Newmark 1999; Wesley et al. 2001). It was surprising that not much difference between sense and antisense RNAs was evident in the efficacy of the silencing induction in *M. oryzae*, because an-

Fig. 2. Hairpin RNA is potent at inducing genetic interference in *Magnaporthe oryzae*. The recipient strain exhibiting green fluorescent protein (GFP) fluorescence (GFP10) was transformed with different constructs. Double transformants with only pII99 were used for control. Double transformants (80) were picked from each transformation and their GFP fluorescence was measured. The double transformants were then classified into five categories (0 to 20, 20 to 40, 40 to 60, 60 to 80, and above 81%) based on relative fluorescence to GFP10. Number of the double transformants in each category was plotted in the graph.

tisense RNA was expected to readily form dsRNA by annealing with the target mRNA in vivo. In the current model of RNA silencing, an occurrence or a synthesis of dsRNA is believed to be the first and key step in initiating the following processes. Our results implied that most of the dsRNA molecules induced by both sense and antisense RNAs may be produced by a host-encoded RdRP (RNA-dependent RNA polymerase)-mediated process with some recognition system for "aberrant RNA." A direct interaction of RNA molecules with the target mRNA, on the other hand, may rarely be involved.

As a whole, our results indicated that RNA silencing operates in *M. oryzae* through processes very similar to those reported in other eukaryotes. Actually, counterparts of *qde-1*, *qde-2*, and *qde-3* genes required for quelling in *N. crassa* (Cogoni and Macino 1997, 1999b) are present in the genome database of *M. oryzae* (data not shown). This information is consistent with our conclusion.

siRNAs in filamentous fungi.

siRNAs are known as characteristic molecules in RNA silencing processes. siRNAs were originally found in plants as 25-nt-long RNAs associated with posttranscriptional gene silencing (Hamilton and Baulcombe 1999). The biochemical and biological roles of siRNAs in the process are not fully understood but now are becoming clearer because of intensive research in animals and plants. Hamilton and associates (1999) detected siRNAs in a multicomponent protein complex called RISC, which had RNase activity. Thus, siRNAs are supposed to serve guide molecules in targeting cognate mRNA for degradation. Lipardi and associates (2001) demonstrated that, in *C. elegans*, siRNAs functioned not only as guide molecules for sequence-specific targeting but also as primers to amplify

themselves, using homologous RNA as a template, namely, random degradative polymerase chain reaction (PCR). However, Schwarz and associates (2002) reported that siRNAs functioned as guides but not as primers in human and *Drosophila* cells. In plants, two different classes of siRNAs were detected with discrete sizes of 21 to 22 and 24 to 26 nt. The shorter siRNAs were suggested to be involved in sequence-specific mRNA degradation, and those in the longer class were correlated with the methylation of homologous DNA and the systemic signaling of silencing (Hamilton et al. 2002). With respect to molecular size, animal siRNAs corresponded to shorter siRNAs in plants (Tang et al. 2003). In our results, at least three molecular sizes of siRNAs from 19 to 23 nt were detected in the Northern blots, but they were not discrete in size and appeared to correspond to plant short siRNAs based on molecular size. Given that the role of siRNAs in *M. oryzae* is the same as it is in plants, this trend is consistent with the fact that little or no accumulation of eGFP mRNA without DNA methylation was observed in the silenced transformants of *M. oryzae*. However, this is merely speculation based on molecular size. Further study will be required to determine the roles of these siRNAs in *M. oryzae*. In fact, a diffusible trans-acting molecule was shown in *N. crassa* to be able to mediate gene silencing between nuclei in heterokaryotic strains, indicating that a systemic signaling molecule of silencing was present in the fungus (Cogoni et al. 1996). Because *N. crassa* and *M. oryzae* are relatively close fungal species, a similar signal translocation may exist also in *M. oryzae*. If so, some of the siRNAs may have been responsible for the intercellular signaling.

DNA methylation is not involved in RNA silencing in *M. oryzae*.

In our results, DNA methylation was not essential for RNA silencing in *M. oryzae* (Fig. 5A and B). A significant role of methylation on promoter sequences in transcriptional gene silencing has been indicated in plants and in some species of filamentous fungi, such as *Ascotholium immersus* (Selker 1997; Vaucheret and Fagard 2001). Also in the case of posttranscriptional gene silencing (RNA silencing), methylation was often observed in the coding or 3' untranslated region sequence of the silenced gene in plants (Ingelbrecht et al. 1994; van Houdt et al. 1997). Surprisingly, RNA and, more recently, dsRNA have been demonstrated to have the ability to direct the methylation of homologous genomic DNA in plants (Mette et al. 2000; Wassenegger et al. 1994). This RNA-directed DNA methylation was shown to be mediated by the longer class of siRNAs, as described earlier. In contrast with these reports on plants, quelling in the fungus *N. crassa* was reported as a methylation-independent process, because this silencing phenomenon occurred even in a DNA methylation-defective mutant (Cogoni et al. 1996). Similarly in *C. elegans* and *Drosophila melanogaster*, DNA methylation is not regarded to be involved in RNA silencing, because these model organisms have almost no DNA methylation. Thus, the requirements of DNA methylation for RNA silencing appeared to differ among organisms. In *M. oryzae*, we showed previously that the repression of the retrotransposon MAGGY seemed to be mostly methylation-independent but was likely associated with a post-transcriptional process (Nakayashiki et al. 2001). This observation is consistent with the results obtained in the present study. Therefore, in *M. oryzae*, DNA methylation is not likely to play a crucial role in suppressing invasive nucleic acid elements.

RNA silencing for genomics research in *M. oryzae*.

In several model organisms (e.g., *C. elegans*), RNA silencing has been shown to provide a powerful reverse genetic tool

Fig. 3. Southern blot analysis of the double-transformants with pEGFP-SA. Genomic DNA was digested with *EcoRI* and *XbaI* and was probed with the green fluorescent protein (GFP) gene. Numbers on the top of the blot indicate transformant numbers. An open triangle indicates the 2.6-kb fragment corresponding to the entire transcriptional unit of the eGFP gene in pEGFP75. A closed triangle indicates the 3.2-kb band representing the *EcoRI-XbaI* fragment containing the eGFP genes in pEGFP-SA.

to convert accumulating genomic information into biological functions by silencing a gene of interest using dsRNA homologous to the target (Ashrafi et al. 2003; Gonczy et al. 2000). In contrast, RNA silencing is not commonly used as a gene analysis tool in filamentous fungi, probably because gene targeting by homologous recombination works with a relatively high, in most cases, targeting efficiency. However, RNA silencing has several advantages over the conventional method. First, this technology can provide rapid and efficient methods to attenuate gene expression in vivo. For example, in nematode, RNA silencing is induced by injecting the homologous dsRNA in the worm or even dipping the worm into solution containing the dsRNA (Fire et al. 1998). In plants, virus-induced gene silencing, in which a target sequence carried by a virus vector is introduced into plants simply by inoculation, is widely used (Angell and Baulcombe 1997). These methods do not require time-consuming transformation. In addition, the targeting efficiency of homologous recombination is not very high even in yeast or filamentous fungi (less than 10% in most cases). Our results indicated that almost 80% of the double transformants with the construct expressing the hairpin form of eGFP RNA showed

some degrees of silencing. Thus, the efficiency of the RNA silencing was much higher than that of the gene targeting reported previously in *M. oryzae* (Sweigard et al. 1992). Second, RNA silencing can be applicable to genes that are difficult to study with the gene disruption method (e.g., multigene families and lethal genes), because it even functions against homologous RNA species with some sequence diversity and usually results in various but not always total degrees of silencing. Therefore, we expect that using RNA silencing in *M. oryzae* in conjunction with other methods will facilitate the genome-wide gene analysis of this economically important phytopathogenic fungus.

MATERIALS AND METHODS

M. oryzae strains and culture conditions.

M. oryzae Br48 isolated from wheat, *Triticum aestivum* (L.) Thell, was used as a recipient in all transformation experiments. Br48 and its transformants were maintained on potato dextrose agar (PDA) for several months. An appropriate antibiotic compound was added into PDA with transformants. For long-time storage, fungi were grown on sucrose containing

Fig. 4. siRNAs are associated with enhanced green fluorescence protein (eGFP) silencing in *Magnaporthe oryzae*. **A**, Images of the mycelia and GFP fluorescence of silenced and nonsilenced double transformants. Numbers on the top of the figure indicate transformant numbers. **B**, Northern blot analysis of the accumulation of eGFP mRNA in the double transformants. The rRNA, stained with ethidium bromide and photographed before blotting, is shown below the blot. siRNAs of eGFP were also analyzed by Northern blots. Total RNA was separated on a 15% polyacrylamide gel and was hybridized with **C**, antisense and **D**, sense probes. The size markers presented on the blots are FITC-labeled DNA oligonucleotides with the molecular sizes indicated. The positions of synthetic RNA oligonucleotides (21 and 25 nt) are indicated by arrowheads.

sterilized barley seeds in a vial, were dried in a plastic box with silica gel, and were stored at 5°C. 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.

Plasmid constructions.

A summary of plasmid constructs used in this study is shown in Figure 1. The eGFP gene was derived from pCB16EGFP (Takano et al. 2001). pEGFP75 carrying the eGFP gene under the control of the *Aspergillus nidulans trpC* promoter and terminator was obtained by inserting a 0.72-kb PCR product corresponding to the complete eGFP gene into *ClaI* and *ApaI* sites of pBSTRP-PT, which contained the *trpC* promoter and terminator at *EcoRI/ClaI* and the *BamHI/KpnI* sites, respectively, in pBluescript SK+ II (Stratagene, La Jolla, CA, U.S.A.). pEGFP-SA was made by inserting another eGFP gene into an *XhoI* site of pEGFP75 in the opposite orientation to the preexisting eGFP gene, using a *HincII* fragment of the GUS gene (607 to 1140 nt) as a spacer. In detail, the GUS spacer and the EGFP gene were once cloned into pBluescript SK+ II in an orientation designed, then amplified by PCR with primers attached, with an *XhoI* site at the 5' end. pEGFP-A expressing antisense GFP RNA was made by self-ligation of a larger fragment of *ClaI*-digested pEGFP-SA, which resulted in removal of one copy of the eGFP gene and the GUS spacer from pEGFP-SA. pEGFP-AA and pEGFP-SS were constructed in a similar manner to pEGFP-SA, with a modification in the orientation of the gene. In the case of pEGFP-AA, pEGFP-A instead of pEGFP75 was

used as a recipient plasmid. The orientation of one or more eGFP genes in each construct was confirmed by restriction analysis together with PCR analysis and sequencing.

Transformation of *M. oryzae*.

Fungal transformants were obtained via polyethylene glycol (PEG)-mediated cotransformation with plasmid pSH75 or pII99. Plasmids pSH75 and pII99 contain the hygromycin B phosphotransferase gene and the neomycin phosphotransferase gene, respectively, as a selective maker (Kimura and Tsuge 1993; Namiki et al. 2001). Transformation was performed as described previously, with minor modifications (Nakayashiki et al. 1999). Fungal protoplasts were produced by digesting fungal mycelia in digestion buffer (10 mM Na₂HPO₄, 1.2 M MgSO₄, pH 5.8) containing 10 to 15 mg lysing enzyme per ml (Sigma, St. Louis) for 3 to 5 h. Fungal protoplasts were filtered through sterile Kimwipe paper, and two volumes of STC solution (20% sucrose, 25 mM Tris-HCl, pH 7.5, 25 mM CaCl₂) were added to the filtrate. The protoplasts were collected by centrifugation at 800 × g for 10 min and then washed with STC solution. Plasmid DNA (3 µg) and pSH75 or pII99 were added to 200 µl of protoplast suspension and then mixed. After 10 min of incubation on ice, 200 µl, 400 µg, and 800 µg of PEG solution (60% polyethylene glycol 3350 in 25 mM Tris-HCl, pH 8.0, 25 mM CaCl₂) were added in a step-by-step manner, and suspensions were incubated for 10 min on ice. PEG solution was removed by centrifugation at 2,000 × g. Collected protoplasts were suspended in 1 ml of STC solution and mixed with 40 ml of regenerated agar media (20% sucrose, 0.3% yeast extract, 0.3% casamino acids, 1.5% agarose) kept at 50°C. After overnight culture at 26°C, selective agar media (regenerated agar containing 400 µg of hygromycin B or 800 µg of geneticin per ml) was overlaid. After incubation at 26°C for 4 to 7 days, antibiotic resistant regenerants on the plates were transferred to new PDA media containing antibiotics for secondary screening.

Imaging and measurements of GFP fluorescence.

The GFP fluorescence of *M. oryzae* transformants was observed with the Intensified PentaMAX System (Princeton Instruments Inc., Trenton, NJ, U.S.A.), which contains a camera, the Olympus IX70 fluorescent microscope, and Polychrome II (Photronics, Planegg, Germany). The GFP fluorescence was excited at 488 nm and was detected using an FITC optical filter. The fluorescent images were recorded by the PentaMAX camera and analyzed with METAMORPH software (Nippon Roper, Chiba, Japan).

The GFP fluorescence of transformant colonies on PDA media was measured by the ProXPRESS Proteomic Imaging System and ProFinder software (PerkinElmer Life Sciences, Boston). The GFP fluorescence was detected using 485 nm excitation and 535 nm emission wavelengths.

Southern and Northern blots.

Fungal genomic DNA was extracted as described previously (Nakayashiki et al. 1999). Southern blot analysis was performed using the dioxetane chemiluminescence system Gene Image (Amersham Biosciences, Piscataway, NJ, U.S.A.). Genomic DNA was digested with one or more appropriate restriction enzymes. The digests were fractionated on a Tris-acetate-EDTA agarose gel and transferred to Hybond N⁺ (Amersham Biosciences). The hybridization and detection procedures were performed according to the manufacture's instructions.

For Northern analysis, total RNA was extracted from mycelia as follows. Frozen mycelia were homogenized with a mortar and a pestle in liquid N₂. After grinding, the powder was transferred to a polypropylene tube, and 2 ml of extraction buffer (0.1M LiCl, 0.1 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1%

Fig. 5. Analysis of methylation status of the promoter and enhanced green fluorescence protein (eGFP) coding regions. Genomic DNA extracted from the silenced and nonsilenced double transformants was digested with isoschizomers *HapII* (H) and *MspI* (M). *HapII* is sensitive to cytosine methylation in the recognition sequence CCGG, but *MspI* is not. The genomic DNA digested was fractionated on a Tris-acetate-EDTA 3.0% agarose gel and was probed with **A**, the eGFP gene or **B**, the promoter. Molecular size markers are shown in base pairs.

sodium dodecyl sulfate [SDS]) and 2 ml of phenol (80°C) were added. The homogenates were shaken with vortex for 30 s, and 2 ml chloroform-isoamylalcohol (24:1) was added and mixed. The mycelial debris was pelleted by centrifugation at 10,000 × g at 4°C for 20 min. The supernatant was carefully removed and extracted twice with an equal volume of chloroform-isoamylalcohol (24:1). Total RNA was precipitated from the aqueous phase with 1× volume of 4 M LiCl and was recovered by centrifugation at 10,000 × g for 20 min. The pellet was washed with 70% ethanol and was dried. Finally, the pellet was dissolved in an appropriate volume of distilled water treated with diethyl pyrocarbonate.

Total RNA (20 µg) was separated on a 1.2% denaturing agarose gel. After electrophoresis, RNA was transferred to Hybond N⁺. Hybridization and detection were performed using Gene Image. RNA probe was labeled with UTP-fluorescein, using the T3 and T7 transcriptional systems (Roche Diagnostics, Mannheim, Germany). A plasmid template for labeling was used after digesting with an appropriate restriction enzyme. Prehybridization and hybridization were performed in ULTRA-hyb (Ambion, Austin, TX, U.S.A.) at 68°C. After hybridization, membranes were washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% SDS for 15 min at 68°C and, subsequently, were washed twice in 0.1× SSC containing 0.1× SDS for 15 min at 68°C. Equal loading of total RNA was estimated by ethidium bromide staining of rRNAs.

Small RNAs were extracted and detected mainly as described by Dalmay and associates (2000), with a modification of the use of Gene Image. The UTP fluorescein-labeled GFP RNA probe was hydrolyzed by adding 300 µl of 200 mM carbonate buffer (80 mM NaHCO₃, 120 mM Na₂CO₃) to a 20-µl probe and incubating the solution at 60°C for 3 h. To stop the hydrolysis reaction, 20 µl of 3 M NaOAc, pH 5.0, was added. The probe was then added to hybridization buffer (50% formamide, 7% SDS, 50 mM NaHPO₄/NaH₂PO₄, pH 7.0, 0.3 M NaCl, 5× Denhardt's solution, and 100 µg of sheared, denatured salmon sperm DNA per ml). The hybridization was performed at 35°C overnight, and the membrane was washed twice at 40°C in 2× SSC containing 0.2% SDS. Two kinds of molecular size makers were used to estimate the sizes of siRNAs. One kind is FITC-labeled DNA oligonucleotides 19, 23, and 30 nt long. The other is synthetic RNA oligonucleotides with 21 (5'-UGCGCUCCUGGACGUAGCCUU-3') and 25 (5'-GUACAGCUCGUCCAUGCCGUGAGUG-3') bases of the GFP sequence.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

International Rice Blast Genome Consortium *Magnaporthe grisea* database: www-genome.wi.mit.edu/annotation/fungi/magnaporthe/