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RNA silencing as a tool for exploring gene function in ascomycete fungi

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Abstract

We have developed a pHANNIBAL-like silencing vector, pSilent-1, for ascomycete fungi, which carries a hygromycin resistance cassette and a transcriptional unit for hairpin RNA expression with a spacer of a cutinase gene intron from the rice blast fungus *Magnaporthe oryzae*. In *M. oryzae*, a silencing vector with the cutinase intron spacer (147bp) showed a higher efficiency in silencing of the eGFP gene than did those with a spacer of a GUS gene fragment or a longer intron (850bp) of a chitin binding protein gene. Application of pSilent-1 to two *M. oryzae* endogenous genes, *MPG1* and polyketide synthase-like gene resulted in various degrees of silencing of the genes in 70-90% of the resulting transformants. RNA silencing was also induced by a pSilent-1-based vector in *Colletotrichum lagenarium* at a slightly lower efficiency than in *M. oryzae*, indicating that this silencing vector should provide a useful reverse genetic tool in ascomycete fungi.

1. Introduction

Recently, the complete genomes of a number of filamentous fungi including model organisms and common pathogens of human and plants have been sequenced (Galagan et al., 2003; <http://www-genome.wi.mit.edu/seq/fgi/candidates.html>; <http://www.broad.mit.edu/annotation/fungi/fgi/>). As fungal species are being sequenced at an ever increasing rate, we are faced with a wealth of genetic information that needs to be linked to biological function. Traditionally, analyses of gene function have been performed by examining the phenotypic or biochemical changes of organisms harboring a mutation in the gene of interest. This process is rather laborious and time-consuming and is therefore not feasible on a broader scale.

RNA-mediated gene silencing (RNA interference, co-suppression, quelling), here referred to as RNA silencing, is a posttranscriptional gene-silencing phenomenon in which double-stranded RNA (dsRNA) triggers degradation of cognate mRNA in a sequence-specific manner. This silencing mechanism has provided powerful reverse genetics tools for functional genomics in several eukaryotic organisms, including *Drosophila melanogaster* and *Caenorhabditis elegans* (Boutros et al., 2004; Kamath et al., 2003). Using an RNA silencing approach, approximately 90% of the predicted genes of both *D. melanogaster* and *C. elegans* were targeted for the loss-of-function analysis. The remarkable potency of RNA silencing has led to the development and commercial availability of various silencing vectors for mammalian cells (Wadhwa et al., 2004). In certain plants species, VIGS (virus-induced gene silencing), an RNA silencing-based strategy, has been an effective tool for high-throughput functional analyses. A target sequence carried by a virus vector is introduced into plants by inoculation and induces the silencing of homologous endogenous genes (Ruiz et al., 1998). In addition, Wesley *et al.* (2001) reported the construction of versatile silencing vectors, pHANNIBAL and pHELLSGATE, that provided a convenient way of generating hairpin constructs for stable transformation. In contrast, RNA silencing has not been widely used in the study of filamentous fungi, despite the demonstration that the silencing mechanism operates in several fungal species (Cogoni et al., 1996; Fitzgerald et al., 2004; Kadotani et al., 2003; Latijnhouwers et al.,

2004; Liu et al., 2002). This is partly due to the limited genetic tools and information available for RNA silencing research in filamentous fungi.

Here we have established an RNA silencing approach in filamentous fungi, using a pHANNIBAL-like silencing vector, pSilent-1, that facilitates the generation of hairpin constructs by PCR-based cloning. The applicability of the silencing vector was examined in two phytopathogenic Ascomycete fungi, *Magnaporthe oryzae* and *Colletotrichum lagenarium*. pSilent-1 induced gene silencing in both fungal species, with slightly higher efficiency in *M. oryzae* than in *C. lagenarium*.

2. Materials and Methods

2.1. Fungal strains, culture condition, and transformation

We used a rice infecting isolate of *Magnaporthe oryzae*, 1836-3 and a laboratory strain of *Colletotrichum lagenarium*, 104-T (Kubo and Furusawa, 1991). Strains were maintained on potato dextrose agar (PDA) for several months. For long-term storage, strains were cultured on barley seeds soaked with sucrose, dried and kept at 4 °C as described previously (Nakayashiki et al., 1999). For DNA or RNA isolation and protoplast preparation, fungal mycelia were grown in CM liquid broth (0.3% casamino acids, 0.3% yeast extract, 0.5% sucrose) at 26 °C.

Fungal transformation was performed by a polyethylene glycol (PEG)-mediated method as described previously (Nakayashiki et al., 1999). For antibiotics selection, hygromycin and geneticin were used both at a concentration of 200 µg/ml for *M. oryzae*, and at concentrations of 50 µg/ml, and 100 µg/ml, respectively for *C. lagenarium*.

2.2. Construction of silencing vectors

Three silencing vectors, pST-CUT, pST-CBP, and pST-GUS with different spacers were constructed using the fungal expression vector pBSTRP-PT (Kadotani et al., 2003), which contained the *trpC* promoter and terminator at *EcoRI/ClaI* and the *BamHI/KpnI* sites in pBluescript SK+ II (Stratagene), respectively. To add a hygromycin resistant marker to pBSTRP-PT, a hygromycin

resistant gene cassette was amplified by PCR with a pair of specific primers containing *NotI* restriction sites (5'-CCGCGGCCGCGACGTAACTGATATTGAAG-3', 5'-TTTGCGGCCGCAACCCAGGGCTGGTGACGG-3'), and inserted into the *NotI* site of pBSTRP-PT, resulting in pBSTRP-PT-H. The three spacer sequences, a 542bp fragment of the GUS gene (nt. #602-#1143; U12639) and two introns of *M. oryzae* chitin binding protein (CBP) (AB064264) and cutinase (X61500), respectively, were PCR-amplified using genomic DNA from a wheat isolate of *M. oryzae* (Br48) and the following three sets of primers; (GUS: 5'-GCGCAAGCTTGTTGACTGGCAGGTGGTGGC-3', 5'-GCGAGATCTGTTGACTGCCTCCTCGCTGTA-3'; CBP intron: 5'-GCGCTCGAGGTACGTACAAGCTTTGTTCTAACGCTGTAAGCTCCAGTATAACC-3', 5'-GGAGGGCCCAGGTACCAGATCTTCGTCACAGTATTTGCCTGACTTG-3' ; cutinase intron: 5'-GCGCAAGCTTGCTGGAGGATACAGGTGAGC-3', 5'-GCGAGATCTGCCGTTCCCTGGCTGTGTGTT-3'). Then, each of them was introduced into pBSTRP-PT-H or its derivative at appropriate restriction sites. The entire eGFP gene (U55762) was inserted twice into each of the pBSTRP-PT-H-derivatives with spacers using appropriate restriction sites to yield the overall gene organization: TrpC promoter, sense eGFP gene, spacer, antisense eGFP gene and TrpC terminator.

The silencing vector pSilent-1 was constructed by inserting a PCR-amplified fragment of the *M. oryzae* CUT intron with multiple cloning sites into pBSTRP-PT-H. The 5' multiple cloning site includes unique recognition sites for *XhoI*, *SnaBI*, and *HindIII*, and the 3' multi-cloning site includes those for *BglII*, *SphI*, *StuI*, *KpnI*, and *ApaI*.

2. 3. Imaging and measurements of GFP fluorescence

To quantify the GFP fluorescence of fungal transformants, 96 well plates were used to grow them on PDA media. Each transformant was cultured on 150ul of PDA in triplet for 5 days, and the GFP fluorescence was measured by the fluorescence plate reader 1420 ARVO SX (Perkin Elmer) with 485

nm excitation and 535 nm emission wavelengths. The average of the fluorescence signals of triplet cultures was calculated and used for the fluorescence intensity of the transformant.

2.4. Phenotypic assay of *MPG1* mutants

A cell surface hydrophobicity test was performed as described by Talbot *et al.* (Talbot et al., 1993) with some modifications. Five drops (10 μ l) of water or water containing 0.001% or 0.002% Tween 20 were placed on the surface of sporulation cultures of *M. oryzae* growing on oat meal agar media. The test was performed 3 days after removing aerial hyphae of the cultures and starting stimulating sporulation under BLB light. Tween 20 was added to increase the sensitivity of the test. The oat meal plates were incubated for 24 hr (water drop) or 3hr (water drop containing 0.001 and 0.002% Tween 20) at 28 C°, and then examined for a cell surface hydrophobicity of transformants. When more than three out of five water drops were absorbed into hyphae, the colony was scored as an "easily-wettable" phenotype.

2.5. RT-PCR analysis

Total RNA was prepared from fungal mycelia using the RNeasy kit (Qiagen). Total cDNA was generated by reverse transcription in a 20 μ l reaction using ReverTra Ace (Toyobo) with the oligo (dT)₁₅ primer. For PCR amplification, 1 μ l of 5X diluted RT mix was used as a template. The PCR reaction was carried out in 20 μ l with 0.5 unit of rTaq DNA polymerase (Toyobo), 1.2 mM MgCl₂, and 20 pmol of each primer. The PCR program was as follows: 10 min at 94 °C, 25 cycles of 45 s at 94 °C, 45 s at the optimal annealing temperature (50 °C to 55 °C), and 45 s at 72 °C, followed by 10 min at 72 °C. Sets of gene-specific primers used were as follows; *MPG1*-F: 5'-GCGCTCGAGATCATCCCAAATGCTCACCAT-3', *MPG1*-R: 5'-GCCAAGCTTAATCTGCTCGCCGAGCAGCA-3',

B-TUB-F: 5'-TTCCCCCGTCTCCACTTCTTCATG-3',
B-TUB-R: GACGAGATCGTTCATGTTGAACTC-3'.

2.6. Genomic Southern analysis

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). Genomic DNA was digested with appropriate restriction enzymes, and the digests were transferred to Hybond N⁺ (Amersham biosciences) after fractionation on a TAE agarose gel. The hybridization and detection procedures were performed according to the manufacture's instructions.

3. Results

3.1. Effect of spacer sequences in hairpin RNA on silencing efficiency

Recent work has demonstrated that a gene construct transcribed into single self-complementary hairpin RNA (hpRNA) efficiently induces RNA silencing in a wide range of eukaryotic organisms. It has been also reported that a spacer sequence, required to reduce the instability of an inverted repeat-containing construct in *Escherichia coli*, affects the efficiency of silencing induced by the construct (Lee and Carthew, 2003; Smith et al., 2000). In plants and animals, a spacer sequence encoding an intron results in a high efficiency of silencing (Lee and Carthew, 2003; Smith et al., 2000). Therefore, we first examined the effect of spacer sequences in hairpin RNA on silencing efficiency in the filamentous fungus *M. oryzae*. Three silencing constructs, all of which contained inverted repeats of the entire eGFP gene and a hygromycin phosphotransferase gene (*hph*) cassette, were made with three different spacer sequences, a fragment (542 bp) of the GUS gene (pST-GUS), intron 5 (850 bp) of *M. oryzae* chitin binding protein (AB064264) (pST-CBP), and intron 2 (147 bp) of *M. oryzae* cutinase (X61500) (pST-CUT). The size of the chitin binding protein intron is a bit different from that in the public databases since the intron was obtained from a different isolate of *M.*

oryzae from a rice isolate in the databases.

The silencing vectors and a control transformation vector (pSH75) with the *hph* gene (Kimura and Tsuge, 1993) were introduced into an eGFP-expressing *M. oryzae* strain, and the GFP fluorescence of randomly selected transformants grown on PDA in 96 well plates was measured using a fluorescence plate reader. Based on the intensity of GFP fluorescence relative to the parent strain, the transformants were categorized into five classes and the proportion of each class in total transformants was graphed (Fig 1). The results indicated that pST-CUT containing the cutinase intron spacer induced a higher silencing than that induced by pST-CBP or pST-GUS. The proportion of transformants showing some degree of silencing (< 80% GFP fluorescence of the parent strain) did not differ much between the different silencing constructs. This may be because most of the non-silenced transformants occurred due to a breakage of a transcriptional unit of hairpin RNA at the integration event, which is expected to occur at a similar frequency with each construct. More than half of the resulting drug-resistant transformants with pST-CUT exhibited less than 20% GFP fluorescence compared to the parent strain. This is more efficient compared to previous and current results obtained with the GUS spacer-containing silencing vectors such as pST-GUS and pEGFP-SA (Kadotani et al., 2003). Vector pST-CBP containing the longer intron spacer was less effective than pST-CUT but was comparable to pST-GUS in silencing induction. Therefore, we decided to employ the CUT intron for constructing a versatile silencing vector for filamentous fungi.

Figure 2 shows pSilent-1, which carries a hygromycin resistance cassette and a transcriptional unit for hairpin RNA expression consisting of the promoter and terminator from the *Aspergillus nidulans trpC* gene that flank a spacer of the CUT intron with multiple cloning sites (MCS) at both 5' and 3' ends. The MCS allows directional ligation of inserts into pSilent-1. In addition, MCS each include a site (*Sna*BI and *Stu*I, respectively) for a restriction endonuclease that leaves blunt ends, which allows direct ligation to blunt PCR fragments (eg., produced by *Pfu* polymerase). This is especially convenient for the first insert that does not require directional ligation.

3.2. Silencing of *M. oryzae* endogenous genes by pSilent-1-based constructs

As a demonstration of the effectiveness of pSilent-1 for determining gene function in *M. oryzae*, an endogenous gene, *MPG1* was targeted for silencing. The *MPG1* gene encodes a fungal hydrophobin involved in surface interactions during infection-related development of the fungus (Talbot et al., 1996). The gene knockout resulted in reduced cell surface hydrophobicity, reduced virulence on the host plant, and reduced appressorium formation (Talbot et al., 1993). The silencing vector expressing hairpin RNA of an *MPG1* fragment (416bp) was constructed in a two-step PCR-based cloning, and introduced into a rice infecting strain of *M. oryzae*. We initially monitored gene silencing of *MPG1* in resulting transformants by cell surface hydrophobicity tests. A reduction in cell surface hydrophobicity was observed in 34 and 45 out of the 51 transformants when examined with water drops containing 0.001% and 0.002% Tween 20, respectively (Fig 3A, Table 1), suggesting that the pSilent-1-based construct efficiently induced gene silencing of *MPG1* at different levels. When a cell surface hydrophobicity test was performed without adding Tween 20 as was used for detecting *mpg1* knockout mutants by Talbot et al. (1993), 7 out of 51 transformants showed the "easily-wettable" phenotype. This suggested that *MPG1* was silenced in the 7 transformants at a comparable level to *mpg1* null mutants (strongly silenced transformants). The expression of *MPG1* mRNA was examined by RT-PCR in 12 transformants with strongly silenced, moderately silenced and non-silenced phenotypes. Consistent with the phenotypic observation, *MPG1* mRNA expression was below the detection limit by RT-PCR in all the strongly silenced transformants tested whereas *MPG1* mRNA was detected at similar levels to wild type in the non-silenced transformants and at lower levels than wild type in the moderately-silenced transformants. Some representative samples are shown in Fig 3B.

We also targeted the polyketide synthase (PKS)-like gene (MG07219.4) for silencing by a pSilent-based vector. The PKS-like gene has sequence similarity to known fungal PKS genes involved in melanin synthesis (Takano et al., 1995). Introduction of the pSilent-based PKS silencing vector into the rice infecting strain of *M. oryzae* resulted in transformants with different colony colors

(Fig 3C), suggesting that the PKS-like gene was silenced in most transformants at various degrees. We observed a recognizable change in colony color in 34 of 48 transformants. In conclusion, these results clearly indicate that the pSilent-1-based silencing vector efficiently induced silencing of endogenous genes in *M. oryzae* with varying levels.

3.3. Applicability of pSilent-1 in *Colletotrichum lagenarium*

To examine the applicability of pSilent-1 in a wider range of ascomycete fungi, we used the phytopathogenic fungus *C. lagenarium*. *C. lagenarium* is a pathogen of cucumber plants and belongs to a different order of ascomycete fungi to *M. oryzae*. The silencing assay was performed using the model gene, eGFP as described above. Briefly, an eGFP-expressing strain of *C. lagenarium* was constructed by introducing pEGFP75 (Kadotani et al., 2003), and was subsequently transformed with the pSilent-based silencing construct for the eGFP gene, pST-GFP and the control plasmid pSH75 (Fig 4A). Based on the intensity of GFP, the double transformants were categorized into five classes (Fig. 4B). The introduction of pST-GFP triggered silencing of the eGFP gene in the resulting *C. lagenarium* transformants, suggesting that pSilent-1 could be a versatile tool for RNA silencing research in a wide range of ascomycete fungi. Interestingly, the efficiency of eGFP silencing induced by pST-GFP in *C. lagenarium* appeared to be a little lower than that in *M. oryzae* (see Figs 1 and 4A). Since the intron spacer was derived from an *M. oryzae* gene, the splicing efficiency might be the cause for the difference.

3.4. GFP silencing in protoplast regenerants and conidiospores derived from silenced transformants

Finally, we examined the stability or reversibility of a silent state in *M. oryzae* transformants and their conidiospores. An eGFP-expressing *M. oryzae* strain was transformed with the silencing vector pEGFP-SA (Kadotani et al., 2003). Microscopic observation revealed that, in a few moderately silenced transformants (50 to 75% GFP fluorescence), a portion of fungal mycelia

exhibited strong GFP fluorescence. To examine whether unstable silencing observed in the moderately silenced transformants is due to epigenetic reversion or genetic rearrangement, we made regenerants from single cells by protoplast isolation. The regenerants from the moderately silenced transformants were categorized into two discrete populations with regard to GFP fluorescence; one with strong GFP fluorescence and the other with very weak to no GFP fluorescence (Fig 5A). Unlike the parent strain, the mycelia uniformly exhibited GFP fluorescence in the GFP-expressing regenerants. We then selected four regenerants each from those with strong and weak GFP fluorescence, and carried out Southern blot analyses. A 3.2-kb fragment is expected upon digestion of the silencing vector pEGFP-SA with *EcoRI* and *XbaI* that contains a transcriptional unit of eGFP hairpin RNA. The Southern blots probed with the eGFP gene revealed that none of the GFP-expressing regenerants possessed the intact transcriptional unit of eGFP hairpin RNA whereas 3 out of 4 eGFP-silenced regenerants did (Fig. 5B). Therefore, loss of GFP silencing in the regenerants was due to loss of the intact transcriptional unit of eGFP hairpin RNA but was not due to epigenetic reversion of a silent state. It has often been reported that transgenes can be lost due to genome rearrangement after transformation in filamentous fungi. The risk of loss of a transgene can be reduced by the maintenance of antibiotics in the culture media. This should be effective for pSilent-1-based vectors since the vector carries a drug resistance gene. One regenerant showed a silenced phenotype without the intact transcriptional unit of eGFP hairpin RNA. Southern analysis revealed that this was likely due to some rearrangements at the eGFP-expressing transgene (data not shown).

In contrast to moderately silenced transformants, we observed very few fungal cells expressing GFP in any portion of the mycelia of strongly silenced transformants (0 to 25% GFP fluorescence). In fact, in the protoplast regenerants of strongly silenced transformants, no regenerants expressed GFP or had loss of the transcriptional unit of eGFP hairpin RNA (data not shown). We observed that the silencing state of the GFP gene was maintained even one year after transformation with pEGFP-SA (data not shown).

We also examined whether the silencing state of the eGFP gene was maintained in the conidiospores. Conidiospores of a strongly silenced transformant were produced under BLB light exposure and 30 spores were selected. Microscopic observation revealed that none of the spores expressed GFP fluorescence, suggesting that RNA silencing triggered by a silencing vector introduced in the genome by stable transformation is constant and transmittable to asexual progenies.

4. Discussion

RNA silencing approaches have been rapidly developed and employed in plants and animals as a tool for exploring gene function. The discovery by Fire *et al.* (1998) that dsRNA is a primary and essential trigger for this mechanism has led to an exponential increase in the number of publications on this subject (Frantz, 2003). In stark contrast to the situation in plants and animals, the RNA silencing approach has not been widely applied to filamentous fungi. This is partly because the gene knockout strategy by homologous recombination works with a relatively high targeting efficiency in many filamentous fungi. In addition, most fungal genomes are haploid which favors the elucidation of gene function by gene knockout strategy. This approach has also been successfully employed in genome-scale analysis of gene function in fission yeast *Saccharomyces cerevisiae* (Giaever *et al.*, 2002).

The RNA silencing approach has potential advantages and disadvantages over conventional gene knockout strategies. One major advantage of RNA silencing is its ability to suppress gene expression relatively easily and quickly. One of the rate-limiting steps in the gene knockout strategies is the inefficiency of homologous recombination. It has been reported in *Septoria lycopersici* that the targeting efficiency of homologous recombination was less than 1%, in attempts to mutate the tomatinase gene (Martin-Hernandez *et al.*, 2000). In model fungi such as *Aspergillus nidulans* and *Neurospora crassa*, gene targeting efficiency is relatively high (Asch and Kinsey, 1990; Miller *et al.*, 1985) but screening for the desired homologous recombination event is still generally laborious. Our

results indicated that 70% to 90% of drug-resistant transformants with the silencing constructs showed some degree of silencing in eGFP and endogenous gene expression. With the pSilent-1-based silencing construct, nearly half of the silenced transformants had GFP fluorescence reduced to less than 20% of the parent strain. Therefore, screening for silenced transformants does not require much effort. Secondly, suppression of gene expression by RNA silencing may be partial, not necessarily result in a null phenotype, unlike the gene knockout method. While this appears to be a weak point, it may also be advantageous. Imperfect silencing with reduced levels of gene expression allows the analysis of lethal genes that cannot be studied using the gene interruption approach. Finally, RNA silencing allows flexibility in gene inactivation experiments since it induces gene suppression in a sequence-specific, but not locus-specific, manner. For example, simultaneous silencing of homologous genes (ex. gene family) has been demonstrated by RNA silencing in various organisms (Thierry and Vaucheret, 1996; Baulcombe, 1999; Allen et al., 2004). This approach is considerably more efficient than performing multiple knockout experiments. In addition, silencing of specific alternatively spliced mRNA isoforms has been achieved by synthesized dsRNA treatment in *Drosophila* culture cells (Celotto and Graveley, 2002). Therefore, RNA silencing offers a tool for inactivating a precisely targeted gene with less effort.

Conversely, the disadvantages include that RNA silencing results in an incomplete and/or reversible mutation, and that a fraction of genes cannot be targeted by this method for as yet unknown reasons (Fraser et al., 2000). Our results suggested that stable transformants with a hairpin RNA-expressing construct showed stable silencing compared to siRNA-induced silencing or feeding RNAi since no reversion occurred due to instability of the silencing state itself. In addition, the pSilent-1-based constructs induced strong silencing (less than 20% of control) in nearly half of the resulting transformants. Ten to twenty percents of the PKS- and eGFP-silenced transformants exhibited almost "null phenotypes" with no visible melanization and no detectable GFP fluorescence, respectively. Therefore, the silencing induced by pSilent-1-based constructs was relatively stable, and seems to be sufficiently efficient for practical use. However in some cases, the varying degrees of

silencing can make experimental results difficult to interpret. Since RNA silencing and conventional gene knockout approaches have different advantages, use of RNA silencing in combination with the gene knockout approach will greatly facilitate exploring gene function in filamentous fungi.

Intron-enhanced silencing was originally reported in plants (Smith et al., 2000), and similar results were subsequently obtained in animal RNAi systems (Kalidas and Smith, 2002; Lee and Carthew, 2003). The results obtained in the present study were mostly consistent with those reports in animals and plants. Therefore, the molecular machinery responsible for intron-enhanced silencing might be conserved in filamentous fungi as well. However, the longer CBP intron did not increase silencing efficiency as did the shorter CUT intron. In general, fungal introns are shorter than those in animal and plant genes; the average size of an intron in *Aspergillus* genes is 72.2 bp, whereas in mouse and corn genes it is 1321.4 bp and 327.5 bp, respectively (Deutsch and Long, 1999). Exceptionally long introns such as the CBP intron (850bp) may have a lower splicing efficiency than that of a regular length intron in filamentous fungi, or higher silencing efficiency conferred by the CUT intron could be simply due to the size and/or sequence of the spacer.

RNA silencing promises to be a powerful tool for the study of filamentous fungi. In turn, filamentous fungi may provide an excellent model for the study of the mechanisms of RNA silencing. Certain fungal species provide excellent experimental systems for molecular biology because they have a small haploid genome, show rapid growth, and relatively high efficiency of gene targeting. In addition, some genes, such as Dicer, in which mutation results in a lethal or embryo lethal phenotype in higher eukaryotes, can be mutated without serious phenotypic changes in filamentous fungi, enabling easy handling of the mutant. Therefore, filamentous fungi may provide an excellent model for detailed studies of the molecular mechanisms of RNA silencing that are likely to be well conserved among eukaryotes, as has already been demonstrated in *N. crassa* and *M. oryzae* (Cogoni and Macino, 1999a, b; Kadotani et al., 2004).

Acknowledgments

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Figure Legends

Figure 1. A cutinase intron spacer in a hairpin-RNA producing plasmid gives higher efficiency of silencing

The effects of spacer sequences in hairpin-RNA producing plasmids on silencing induction were examined. Three plasmids expressing hairpin RNA of the eGFP gene with different spacer sequences, a fragment of the beta-glucuronidase (GUS) gene (pST-GUS), intron 5 of *M. oryzae* chitin binding protein (pST-CBP), and intron 2 of *M. oryzae* cutinase (pST-CUT), were constructed. The silencing vectors and a control plasmid carrying the hygromycin-resistant gene (pSH75) were introduced into an eGFP-expressing *M. oryzae* strain, and the GFP fluorescence of randomly selected transformants was measured using an image analyzer. Based on the relative intensity of GFP fluorescence to the parent strain, the transformants were categorized into five classes (0 to 20%, 20 to 40%, 40 to 60%, 60 to 80% and more than 80%), and the proportion of each class in total transformants was represented.

Figure 2. Schematic representation of the silencing vector pSilent-1, and the sequences of multicloning sites and splice junctions in the vector.

(A) A map of pSilent-1. Amp^r, ampicillin resistant gene; Hyg^r, hygromycin resistant gene; IT, intron 2 of the cutinase (CUT) gene from *M. oryzae*; PtrpC, *Aspergillus nidulans* trpC promoter; TtrpC, *A. nidulans* trpC terminator. (B) Restriction sites are underlined. Asterisks indicate unique sites in the silencing vector. Italic nucleotides indicate sequence from the cutinase gene and bold letters represent 5' and 3' splice sites.

Figure 3. Endogenous genes of *Magnaporthe oryzae*, *MPG1* and polyketide synthase (PKS)-like gene were targeted for silencing using the pSilent-1 vector

(A) Cell surface hydrophobicity of *M. oryzae* culture was assessed by placing a 10ul drop of water

containing 0.002% Tween20 on the surface of a plate culture for 3 hr. The water droplet remained suspended on the surface of the wild type culture but soaked into the mycelium of the *MPG1*-silenced transformant. (easily-wettable phenotype). (B) RT-PCR analysis of *MPG1* mRNA expression in the *MPG1*-silenced transformants. The beta-tublin gene was used for control. (C) Fungal colonies of the transformants with a pSilent1-based silencing vector targeted for the PKS-like gene. Various colony colors indicate that the PKS-like gene is silenced at varying degrees in the transformants.

Figure 4. A pSilent-based silencing vector (pST-GFP) induced GFP silencing in *Colletotrichum lagenarium*

(A) Bright field and GFP fluorescence images of representative fungal colonies of *C. lagenarium* transformants with the GFP silencing construct, pST-GFP and the parent strain (the center colony). The GFP fluorescence was detected using 485 nm excitation and 535 nm emission wavelengths. (B) A silencing test with *C. lagenarium* transformants was performed as described in Fig 1.

Figure 5. Southern blot analysis of protoplast regenerants of *Magnaporthe oryzae* with silenced and non-silenced GFP phenotypes

Genomic DNA of protoplast regenerants and the parent strain of *M. oryzae* was digested with *EcoRI* and *XbaI*, and probed with the GFP gene. An open triangle indicates the 2.6 kb fragment corresponding to the entire transcriptional unit of the eGFP gene in the GFP expressing construct, pEGFP75. A closed triangle indicates the 3.2 kb band representing the transcriptional unit of hairpin RNA of the eGFP genes in the GFP silencing construct, pEGFP-SA.

Table1

Table 1. Cell surface hydrophobicity tests of <i>M. oryzae</i> transformants targeted for <i>MPG1</i> silencing					
Treatments	Fungus	Transformant		Wild type	
	Phenotype	Easily-wettable	Normal	Easily-wettable	Normal
Water		7	44	0	7
Water + 0.001% Tween 20		34	17	0	7
Water + 0.002% Tween 20		45	6	0	7

Figure1

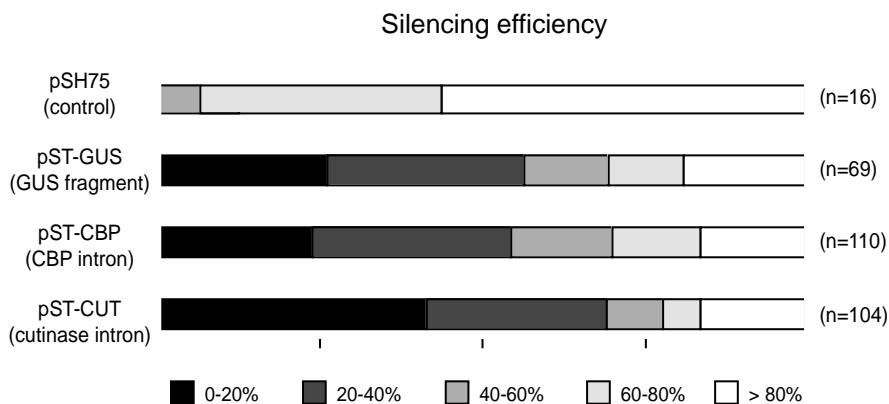


Figure 1. A cutinase intron spacer in a hairpin-RNA producing plasmid gives higher efficiency of silencing

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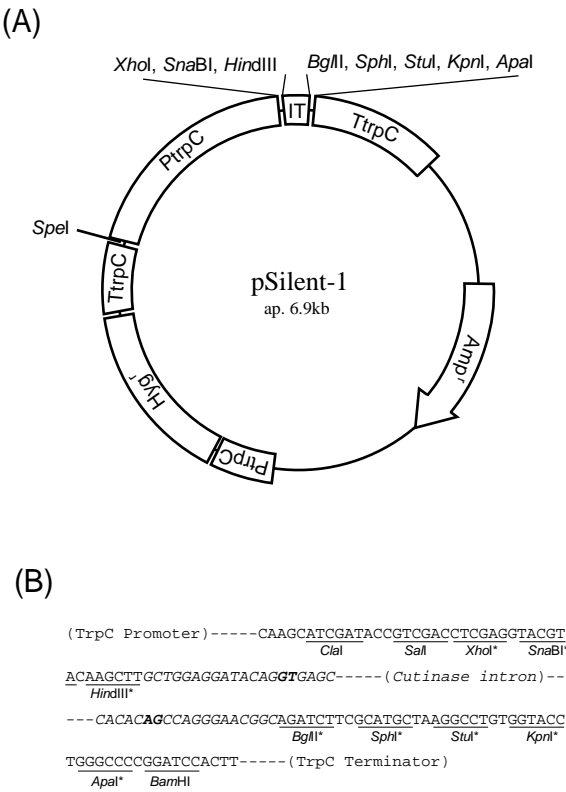


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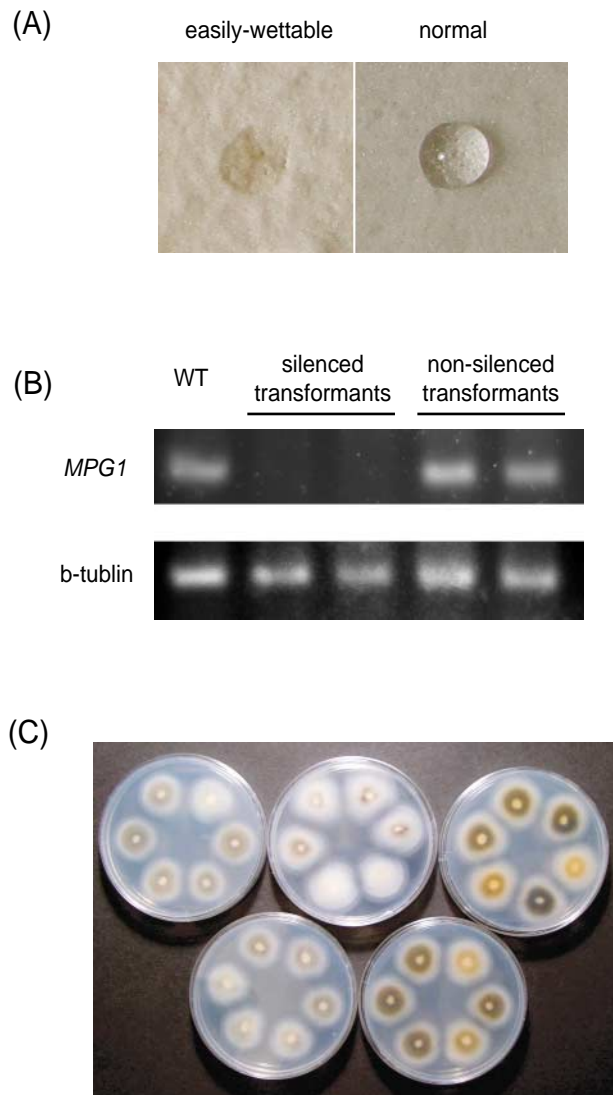


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Figure4

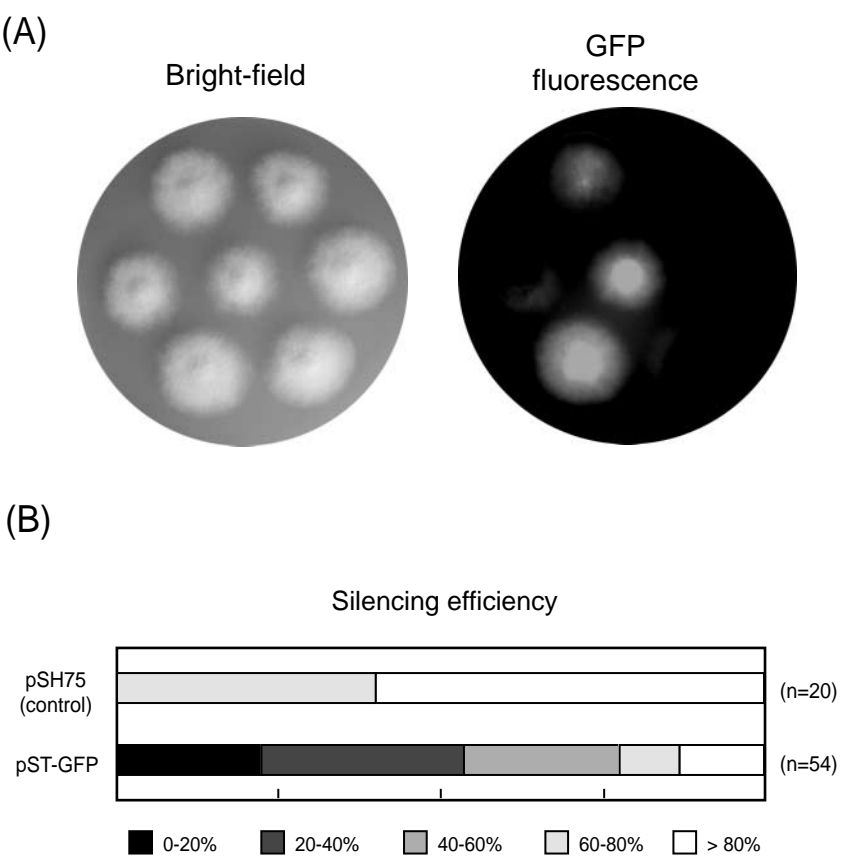


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Figure5

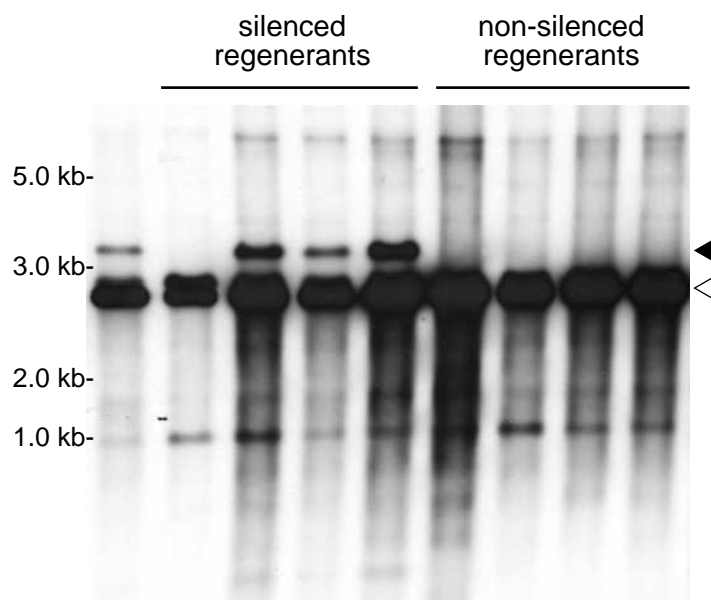


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