We found the orthologous genes required for RNA interference (RNAi) in the *Aspergillus oryzae* genome database, and constructed a set of tools for gene silencing using RNAi in *A. oryzae*. This system utilizes compatible restriction enzyme sites so that only a single target gene fragment is required to create the hairpin RNA cassette. For ease of handling, we also separated the construction of the hairpin RNA cassette for the target gene from its subsequent introduction into the expression vector. Using the *brlA* gene as a target for RNAi, we detected decreased mRNA levels and a delayed conidiation phenotype in the transformants. Furthermore, even though *A. oryzae* possesses three copies of the *α*-amylase gene, a single copy of an *α*-amylase RNAi construct was sufficient to downregulate the mRNA levels and decrease the enzymatic activity to 10% of control levels. Gene silencing by RNAi should provide a powerful genetic tool for post-genomic studies of the industrially important fungus *A. oryzae*.

**Key words:** *Aspergillus oryzae*; gene silencing; RNA interference

*Aspergillus oryzae* is an important filamentous fungus in the Japanese fermentation industry, used in the manufacture of such products as sake, soy sauce, and miso, as well as in commercial enzyme production. In these industries, *A. oryzae* is used in making koji, which provides various hydrolytic enzymes and nutrients required in the fermentation process. Given its history of safety during more than 1,000 years of use in the food industry, *A. oryzae* is also classified as a generally regarded as safe (GRAS) organism by the United States Food and Drug Administration (FDA), and is currently recognized as a promising host cell for recombinant enzyme production. In this study, we constructed a set of tools for gene silencing by RNAi in *A. oryzae*. We utilized compatible restriction enzyme sites and the Gateway system for the construction of a hairpin RNA cassette and its subsequent introduction into the expression vector. With the *brlA* and *α*-amylase genes as the silencing target, we confirmed the efficacy of our tools in gene silencing. We conclude that gene silencing by RNAi provides an efficient method for the post-genomic functional analysis of genes in the industrially important fungus *A. oryzae*.

**Materials and Methods**

Strains, media, and DNA manipulations. *Escherichia coli* strain DH5α and *A. oryzae* niaD300(15) were used as...
hosts. *E. coli* was grown in LB medium with or without 2% agar, and supplemented with the appropriate antibiotics. Czapek-Dox (CD) medium, consisting of 0.3% NaNO₃, 0.05% KCl, 0.1% KH₂PO₄, and 0.05% MgSO₄·7H₂O (pH 5.5), with or without 2% agar, and supplemented with the appropriate carbohydrate (fructose, glucose, or maltose), was used for cultivation of *A. oryzae*. *A. oryzae* transformation was performed as described by Gomi *et al.*[^16] Gateway LR clonase II mix (Invitrogen, Carlsbad, CA) was used to introduce the hairpin RNA cassette into the expression vector. All DNA manipulations were performed using standard methods, as described by Sambrook *et al.*[^17]

**Construction of RNAi plasmids.** The EGFP gene was PCR-amplified from the template pEGFP (BD Biosciences Clontech, Shiga, Japan) with primers iGFP1 and iGFP2, and subcloned into pENTR/D-TOPO (Invitrogen). After digestion with primers MtA1 and MtA2, and subcloned into pCR-Blunt (Invitrogen), iGFP2, and subcloned into pENTR/D-TOPO (Invitrogen, Carlsbad, CA) with primers iGFP1 and iGFP2 to express those hairpin RNA cassettes under the control of the *amyB* gene promoter.

**Hairpin RNA cassette for the α-amylase genes.** EST clones JK0115 and JK0475 were treated with *SalI* and *NotI*, and the *α-amylase*[^19]−encoding cDNA fragments were introduced into the *XhoI*-PspOMI sites and *NotI*-SalI sites of pMtLi to generate pMtLi-aL2 and pMtLi-aS2. A polyA tail-deleted one, pamyB, was also constructed by digestion of pMtLi-aS2 with *MunI* and ligation of the fragment into the *EcoRI*-MunI sites of pGFPi.

**Introduction of hairpin RNA cassette into the expression vector.** The hairpin RNA cassette in plasmids pbrlAi, pMtLi-aL2, pMtLi-aS2, and pamyB was introduced into the expression vector pUNAgate by the LR reaction, to express those hairpin RNA cassettes under the control of the *amyB* gene promoter.

**Southern blot analysis of the *A. oryzae* transformants.** Genomic DNA from the *A. oryzae* transformants was extracted according to Lee *et al.*[^20] The genomic DNA samples were digested with *XbaI*, separated by electrophoresis on a 0.8% agarose gel, and transferred onto Hybond-N+ (Amersham, Tokyo, Japan). To make the probe, a nitrate reductase gene (*niaD*) fragment was PCR-amplified from *A. oryzae* RIB40 genomic DNA, using the oligonucleotide primers niaD-S and niaD-X and a PCR DIG probe synthesis kit (Roche Diagnostics, Tokyo, Japan). Hybridization was carried out according to the manufacturer’s instructions.

**Northern blot analysis of the brlA gene.** The *A. oryzae* transformants were inoculated onto the surface of a Versapor-3000 membrane (PALL, Tokyo, Japan) on a CD plate supplemented with 1% maltose to a final concentration of 2.5 × 10⁻⁶ conidia/cm², and incubated at 30 °C for 48 h. After the mycelia were scraped off from the membrane, total RNA was extracted using Isogen (Nippongene, Tokyo, Japan). Hybridization analysis was carried out as described by Akao *et al.*[^21] To make the probe, the *brlA* gene fragment was PCR-amplified with the oligonucleotide primers brlA-DIG1 and brlA-DIG2 and an *A. oryzae* RIB40 genomic DNA template, using a PCR DIG probe synthesis kit (Roche).

<table>
<thead>
<tr>
<th>Primers Used in This Study</th>
<th>Sequence (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>iGFP1</td>
<td>CACCGAATTCGACATGTTGACAAAGGCGAG</td>
</tr>
<tr>
<td>iGFP2</td>
<td>GGGGCCCAATTTGCTGAGTTACTTGACAGCTGCATGT</td>
</tr>
<tr>
<td>MaA1</td>
<td>GTCGACATGGGCTGTGATTGCCGAAAATACC</td>
</tr>
<tr>
<td>MaA2</td>
<td>CTCTGAGCTCACTTTCCGGAGTTCTTCGACTG</td>
</tr>
<tr>
<td>ENT-mut1</td>
<td>CCAGTCTTAAAGCTGCCCACAAATAATGATTATTATTGT</td>
</tr>
<tr>
<td>ENT-mut2</td>
<td>CAAAATAAATACATTATTTGGAACGCGGACCTAAAGACTGG</td>
</tr>
<tr>
<td>niaD-S</td>
<td>CTTGGGATATTGGCGGAAAATC</td>
</tr>
<tr>
<td>niaD-X</td>
<td>GAATAGCAAGGAATTCCTCA</td>
</tr>
<tr>
<td>brlA-DIG1</td>
<td>CTTCGCGCCATGATTCCCCCTAC</td>
</tr>
<tr>
<td>brlA-DIG2</td>
<td>GGCGAATGGTCTGAGGGCGCAATGGT</td>
</tr>
</tbody>
</table>

Restriction sites are indicated in bold.
Northern blot analysis of α-amylase genes and enzymatic assay. The *A. oryzae* transformants were inoculated into CD medium supplemented with 2% maltose and 10 mM phosphate buffer (pH 7.0) to a final concentration of 1 × 10⁶ conidia/ml, and incubated at 30°C for 48 h at 100 rpm. After harvesting the mycelia, the total RNA was extracted and used in Northern blot analysis, as described above. The probe was PCR-amplified with the plasmid pBluescriptSK-amyBS, which includes the *Sal*I-*Mun*I *amyB* cDNA fragment, and the oligonucleotide primers M13 P7 and P8 (Hokkaido System Science, Sapporo, Japan), using a PCR DIG probe synthesis kit (Roche). The *C11*-amylase activities of the culture supernatants were measured using an *C11*-amylase assay kit (Kikkoman, Chiba, Japan).

Results

Orthologs of RNAi in *A. oryzae* genome

In *N. crassa*, three genes involved in post-transcriptional transgene-induced gene inactivation, termed quelling, have been identified and characterized. The first gene, *qde-1*, encodes an RNA-dependent RNA polymerase, and its overexpression results in a dramatic increase in the efficiency of quelling. The second gene encodes QDE-2, an argonaute family protein that is an essential component of RISC and is homologous with AGO1, which is required for gene silencing in *Arabidopsis thaliana*, and RDE-1, which is required for RNAi in *C. elegans*. The last gene, *qde-3*, encodes a member of the RecQ helicase family. Orthologous predicted proteins for all three of these genes have been found by searching the *A. oryzae* genome database with blastp, under accession nos. AO090020000563 (e-value, 5 × 10⁻⁹³), AO090005000621 (1 × 10⁻¹³⁵), and AO09003000299 (1 × 10⁻¹⁵⁵). Recently, the Dicer genes, responsible for siRNA production, have been cloned from *N. crassa* and *M. oryzae* as *dcl-1* and *mdl-2*²⁹,³⁰ respectively, and a homologous gene was also found in the *A. oryzae* genome under no. AO09001000193. The presence of these orthologs suggests that RNAi should be functional in *A. oryzae*.

Constructs for RNAi in *A. oryzae*

Nakayashiki et al. developed a silencing vector for a wide range of ascomycete fungi, pSilent-1, which carries the *A. nidulans trpC* gene promoter and terminator for expression of the hairpin RNA cassette, and a hygromycin resistance gene for selection of the transformants. Unfortunately, however, this vector cannot be used in *A. oryzae*, because of the high endogenous resistance of this fungus to hygromycin. To overcome this problem, we constructed vectors for the hairpin RNA cassette pGFPi, and pMtli. These plasmids include a 720-bp EGFP or 93-bp metallothionein gene as a spacer sequence for the hairpin structure. We also converted *A. oryzae* expression vector pUNA to the Gateway expression vector, pUNAgate, by insertion of Gateway vector conversion cassette A between the promoter and terminator of the *amyB* gene.
These RNAi vectors were designed with three strategies in mind. First, since over 10,000 clones from the *A. oryzae* EST project are stocked at our institute, we planned to use them as gene resources for silencing targets. Second, to reduce the time and costs associated with subcloning, we designed the vectors for the hairpin RNA cassette with restriction sites that generate compatible cohesive ends, so that only one restriction fragment of the target gene is needed to construct the hairpin RNA cassette. Lastly, in consideration of the large size of the *A. oryzae* expression plasmid, we separated the construction of the hairpin RNA cassette from its introduction into the expression vector using Gateway technology.

Because the gene fragments can be digested out from our EST clones with *Not* I and *Eco* RI or *Sal* I, these restriction sites were included in pGFPi and pMtli. The two vectors also include three other restriction sites: *Xho* I, which generates cohesive ends compatible with *Sal* I; *Mun* I, compatible with *Eco* RI; and *Psp* OMI, compatible with *Not* I. Consequently, a *Not* I-*Sal* I (or *Eco* RI) digested gene fragment from an EST clone or other sources can be inserted into both the *Not* I-*Sal* I (or *Eco* RI) and *Psp* OMI-*Xho* I (or *Mun* I) sites of these vectors in opposite orientations to generate a hairpin RNA cassette. The vectors also include Gateway attL sequences around the cassette, so that the hairpin RNA cassettes can easily be inserted into an expression vector for the Gateway system, pUNAgate, to generate RNAi vectors for *A. oryzae* in which we can induce expression of the hairpin RNA cassette under control of the strong *amyB* gene promoter with an appropriate carbohydrate, such as maltose.

### RNAi of the *brlA* Gene

To test the efficacy of gene silencing using RNAi in *A. oryzae*, we selected the *brlA* gene, which encodes a transcription factor for conidiation, as a target. The 556-bp *Sal* I-*Mun* I *brlA* gene fragment was used in the hairpin RNA cassette in the gene-silencing experiment (Fig. 2A). The cassette was introduced into pUNAgate and transformed into an *A. oryzae* niaD/C0 strain, niaD300. The empty cassette from pGFPi was also cloned into pUNAgate and transformed into niaD300 as a control. After several rounds of purification, the transformants, NA-GFPi1 and NA-brlAi1, were confirmed by Southern blot to have a single copy integration of the plasmid into the *niaD* gene locus (data not shown). As shown in Fig. 2B, NA-brlAi1 showed delayed conidia development on CD supplemented with 1% maltose at 48 h as compared to control strain, NA-GFPi1. Northern blot analysis also showed a decreased signal for the *brlA* gene in NA-brlAi1 (Fig. 2C). Based on these results, we concluded that the RNAi is also effective in *A. oryzae*.

### RNAi of *α*-amylase genes

Next we investigated the effect of gene silencing by RNAi on *α*-amylase gene expression, because *A. oryzae* possesses three copies of this gene, and they are known to be among the most strongly expressed genes in *A. oryzae*. In this case, we also used two types of EST clones for the expression of hairpin RNA cassettes. The first included a 1,656-bp fragment carrying almost the entire *α*-amylase coding region and a 15-bp polyA tail from EST clone JK0115, and the other included a 750-bp fragment carrying about half of the coding region and
We also prepared a hairpin RNA cassette that included only a 341-bp α-amylase gene fragment and no polyA tail (Fig. 3A). In this case, vector pMtli was used, to shorten the spacer length. These plasmids and pMtli were introduced into pUNAgate to generate RNAi vectors. After transformation into niaD300, transformants NA-Mtli1, NA-αL2-1, NA-αS2-1, and NA-amyBi-1 were obtained and confirmed by Southern blot analysis to have a single-copy integration of the plasmid into the niaD gene locus (data not shown). As shown in Fig. 3B, NA-αS2-1 and NA-amyBi1 showed drastically decreased signals for α-amylase mRNA in CD medium supplemented with 2% maltose. In these strains, α-amylase activity was also reduced to about 6.4 and 10.2%, relative to the control strain, NA-Mtli1, respectively (Table 2). These data suggest that one copy of a RNAi cassette has the potential to silence expression from multiple copies of the gene. It is also suggested that some of the EST clones, but not all, would be useful as resources for gene silencing in RNAi experiments.

Discussion

We developed gene-silencing vectors using RNAi for A. oryzae, named pGFPi, pMtli, and pUNAgate. This system for RNAi has two important features. First, we utilized compatible restriction enzyme sites to create hairpin RNA cassettes, allowing us to use our EST clones as a resource for silencing. These compatible sites would also be useful for cloning of target gene fragments amplified by PCR, because only one set of primers would be required for PCR amplification. This cuts down on time and costs as compared with the conventional method, in which two sets of primers (and two separate reactions) are usually used for gene amplification. A second feature of our system is that the hairpin RNA cassette is introduced into an expression vector by Gateway technology. This strategy requires one more step than the usual method, in which the gene fragments are ligated into the expression vector directly. However, because the expression vectors for A. oryzae are relatively large, we believe that the increased ease of constructing hairpin RNA cassettes in the smaller vectors compensates for the extra cloning step. This method also has the advantage of using readily available restriction enzymes. Furthermore, once a hairpin RNA cassette has been constructed for target gene silencing, it can easily be introduced into other expression vectors with different promoters for gene expression and/or markers for transformation, adding

**Table 2.** RNAi of α-Amylase Genes

<table>
<thead>
<tr>
<th>Strain</th>
<th>α-amylase activity (U/g wet mycelia)</th>
<th>% of control</th>
</tr>
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<tbody>
<tr>
<td>NA-Mtli1</td>
<td>729 ± 107</td>
<td>100</td>
</tr>
<tr>
<td>NA-αL2-1</td>
<td>609 ± 48</td>
<td>83.5</td>
</tr>
<tr>
<td>NA-αS2-1</td>
<td>46 ± 15</td>
<td>6.4</td>
</tr>
<tr>
<td>NA-amyBi-1</td>
<td>75 ± 9</td>
<td>10.2</td>
</tr>
</tbody>
</table>

The α-amylase activities of culture broth were measured in three independent experiments.
flexibility to the system. Because the Gateway system is used widely in gene expression experiments, our RNAi system might be applicable to gene-silencing studies in organisms besides *A. oryzae*.

In this paper, we selected the *brlA* and α-amylase genes as targets to investigate a gene-silencing effect using RNAi in *A. oryzae*. The expression of a RNAi construct for *brlA* under the control of the *amyB* gene promoter caused delayed conidia development and a decreased signal for the *brlA* gene in Northern blot analysis (Fig. 2B, C). Based on these results, we concluded that RNAi is also effective in *A. oryzae*. Next, we used two types of EST clones in construction of a hairpin RNA cassette for α-amylase genes. In this case, effective gene silencing was observed in transformants NA-aS2-1, expressing a hairpin RNA cassette with a 660-bp EST sequence and a 90-bp polyA tail, and NA-amyBi-1, expressing a cassette with no polyA tail (Fig. 3B). These results indicate that the inclusion of a 90-bp polyA tail has little negative impact on the effectiveness of RNAi, and that a 341-bp length of DNA fragment is sufficient for gene silencing in *A. oryzae*. It is also suggested that the 93-bp fragment of the metallothionein gene is a sufficient spacer sequence for the formation of hairpin structures. On the other hand, the transformant, NA-al2-1, expressing an RNAi cassette with almost the entire α-amylase gene, had little effect on α-amylase production (Table 2). There might be some problem in the transcription and/or formation of the hairpin RNA with large RNAi constructs. More investigation is needed on the effective use of EST clones as a resource for RNAi experiments.

The transformation system for *A. oryzae* based on the *niaD* gene results in a high frequency of homologous single-copy integration events. Because we planned to test the potential of one hairpin expression cassette to silence a gene and multiple genes, the transformants used in this study were all selected from strains confirmed by Southern blot analysis to have a single copy integration of the hairpin RNA cassette into the *niaD* gene locus. If we used another selection marker for transformation, such as the ATP sulfurylase gene, *sc*; or the pyrithiamine resistance gene, *ptrA*, both of which are reported to be integrated in the genome randomly and sometimes multiply, the silencing efficiencies would vary between transformants, and we might be able to obtain strains with a stronger silencing phenotype.

Generally, gene silencing by RNAi has certain advantages and disadvantages. The major disadvantage is that the effects of RNAi are partial and, unlike the conventional gene replacement approach, result in an incomplete knockout phenotype. The delayed condiation phenotype of the *brlA* gene-silencing strain and the remaining enzymatic activities of the α-amylase gene-silencing strains indicate that this disadvantage also applies to *A. oryzae*. However, the partial phenotype of RNAi allows us to analyze essential genes that cannot be approached by the gene-knockout method. The inclusion of an inducible promoter for expression of the RNAi construct also might be advantageous in this context, because the target gene can be downregulated conditionally. Another advantage of the RNAi technique is that it works in a sequence-specific manner and can simultaneously downregulate multiple homologous genes. In *A. oryzae*, we reduced the α-amylase activity to 10% of the control strain using only one copy of an RNAi expression cassette, even though three copies of this gene exist in the genome. Finally, the RNAi method of target gene-suppression is easier and faster than the conventional knockout approach. This feature should be a major advantage in *A. oryzae*, because the efficiency of homologous recombination is quite low in this organism.

**References**


