Construction of a BAC Library of the Rice Blast Fungus *Magnaporthe grisea* and Finding Specific Genome Regions in which Its Transposons Tend to Cluster

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We have constructed a BAC library of the rice blast fungus *Magnaporthe grisea* consisting of 5760 clones. The insert size ranged from 35 to 175 kbp, with an average of 120 kbp. The library is about 18 genomes equivalent, therefore covering more than 99.999% of the genome. This library is the first to be constructed using a rice pathogenic wild type isolate. Improved high molecular weight DNA size fractionating helped to construct the library with high efficiency. Total library clones were arranged onto two nylon membranes for efficient screening. Test hybridization with a single-copy RFLP marker showed ten positive clones, of which restriction patterns indicated no chimerality or deletions. As a model case of application of this library, the distribution of the well-studied fungal retrotransposons MGR583, and MAGGY and DNA transposons MGR586 and Pot2 was analyzed. Of all the BAC clones, 10%, 13%, 18%, 12%, and 23% contained MGR583, MGR586, MAGGY, MGR586 and Pot2, respectively. The percentage of clones possessing more than five kinds of transposons was 1.4%, 215 times greater than the expected number. The results show that these transposons were distributed in clusters in the *M. grisea* genome.

Key words: *Magnaporthe grisea*; BAC library; transposon distribution

The filamentous ascomycete fungus, *Magnaporthe grisea* (Hebert) Barr, shows wide and variable pathogenicity against many grassy plants including important crops such as rice, wheat, and barley. In rice, especially the fungus often has a serious economic effect.

To control this bio-stress, the use of resistance genes of plants against pathogens is prospective. For that purpose, analysis of the resistance mechanism is important. Rice and rice blast fungus is one of the most studied of systems on the 'gene for gene' relationship between resistance and avirulence genes.17 As we are trying to clone blast resistance genes in rice,2,3 characterization of the avirulence counterpart in the rice blast fungus is desirable for further study of the interaction between these genes.

Genetic maps of the fungus were constructed using RFLP markers and repetitive DNA sequences.4,5,6,7 Based on the maps, a host specificity gene was cloned from a cosmids library.8 However, for efficient positional cloning, the use of cosmids libraries presents some problems such as instability and small size of the insert DNAs (<40 kbp).

Recently, the bacterial artificial chromosome (BAC) was developed as a vector for construction of large insert-sized libraries.9 BAC libraries of many kinds of organisms have been constructed, e.g., human,9,10,11 rat,2 mouse,13 plants,14,15 and fungi.16,17 BACs are superior to YACs and cosmids because of their low rate of chimerism, high efficiency of cloning or recovery of long insert DNA, easy handling, stability of the insert DNA, and readiness for direct sequencing. In addition, the digital hybridization (DH) method was developed to facilitate rapid construction of genome-wide BAC contigs18 and direct gene complementation using BAC clones succeeded.19,18,19 BAC libraries have become an essential tool for positional cloning, genomic sequencing, and functional analysis of genes.

We used our experience in constructing a rice BAC library of excellent quality (average insert size: 155 kbp, 7-genomes equivalent),2 in the construction of a rice blast fungal genome library. Rice-pathogenic Chinese field isolate CHNOS60-2-3 was chosen for the construction because it showed strong and stable fertility towards other rice pathogenic *M. grisea* strains. Furthermore, various progenies of this strain have been obtained to analyze avirulence genes.

Two *M. grisea* BAC libraries have already been constructed by Diaz-Perez et al. (insert size = 66 kbp, 7 × genome coverage),16 and by Zhu et al. (insert size = 130 kbp, 25 × genome coverage).17 However *M. grisea* shows great diversity in fertility, host specificity, and avirulence to various cultivars. As most of the rice blast fungal strains have no or weak fertility, it is difficult to cross rice pathogenic *M. grisea*. The two *M. grisea* BAC libraries so far reported were constructed using strains obtained from crosses between non-rice and rice pathogenic fungal strains. For the analysis of avirulence genes corresponding to rice resistance genes against rice blast,
it is desirable to use crosses between rice pathogenic strains. Hence, it is desirable to construct a BAC library using the rice-pathogenic Chinese field isolate, CHNOS60-2-3.

Here we report the construction of a BAC library of *M. grisea* CHNOS60-2-3. To show the usability of the library, the distributions of five kinds of *M. grisea* transposons were analyzed. These are known to be present in high copy numbers in the rice-pathogenic *M. grisea* genome. Our results show that transposons have a tendency to cluster in this fungal genome.

**Materials and Methods**

*M. grisea* isolate, *E. coli* strain, and plasmid. Chinese *M. grisea* isolate CHNOS60-2-3 (*MatI*), which has strong fertility, was used to prepare High Molecular Weight (HMW) DNA for use in the construction of the BAC library. This isolate was obtained from Yunnan Province of China, where most of the isolates are highly fertile. The *E. coli* strain DH10B (BRL) was used for electroporation of the BACs. A modified BAC vector named *pBAClAc* carrying an inserted *lacZ* fragment of M13mp18, was used as a vector.1) Purification, digestion by HindIII, and dephosphorylation by calf intestine phosphatase (Pakara) of *pBAClAc* were done as described by Nakamura et al.2)

**Preparation of high molecular weight DNA.** HMW DNAs were prepared following the protocols of Orbach et al.20 and Shizuya et al.21 with some modifications. A small piece of mycelia (1 cm²) from a three-day-old culture grown on YG agar (0.5% w/v yeast extract, 2% w/v glucose, 2% w/v agarose) was inoculated in 100 ml of YG liquid medium in 300-ml flasks with gentle shaking (90 rpm) for four days at 25°C. Mycelia were harvested by vacuum filtration on a filter paper, resuspended in 50 ml of Novozyme treatment buffer (10 mg/ml CaCl₂, 0.7 M sorbitol, 2 mg/ml Novozyme 234, Novo Nordisk) and incubated at 28°C with agitation at 100 rpm for 3 h to generate protoplasts. Protoplasts were filtered through Miracloth (Calbiochem) and pelleted at 1600 × g for 15 min. The protoplasts were rinsed three times with 0.7 M sorbitol and resuspended, and then mixed with an equal volume of 1% low melting point agarose (In Cert, FMC) in sorbitol buffer (0.7 M sorbitol, 0.5 M EDTA). The mixture was poured into plastic molds (Bio-Rad) and solidified at 4°C for 20 min. The final density of protoplasts in agarose was 2.4 × 10⁷/ ml. The DNA/agarose plugs were treated with proteinase K in 50 ml of digestion buffer (1% w/v sodium laurel sarcosinate, 0.5 M EDTA pH 8.0, 1 mg/ml proteinase K, Clontech) at 50°C for 48 h with gentle agitation and one change of digestion buffer after 24 h. The digested plugs were washed with 50 ml of 0.5 M EDTA for 6 hours and then with 50 ml of TE, pH 8.0 for 6 h. At each wash, EDTA and TE were renewed once every hour.

Partial digestion and size fractionation of DNA. For partial digestion, 25 mg of the DNA/agarose plug was equilibrated with 200 µl of *HindIII* digestion buffer (10 mM Tris-HCl, pH 7.5, 1 mM DTT, 50 mM NaCl, 0.01% w/v BSA) containing 0.1 unit of *HindIII* at 4°C, overnight. One hour before the digestion, 10 mM MgCl₂ was added to the buffer on ice and diffused into the plug. Then the DNA in the plug was digested by incubating at 37°C for 1 h, and the reaction was stopped on ice by addition of EDTA to a final concentration of 50 mM. The digested DNA was fractionated in a single 1% low melting point (LMP) agarose gel (Sea plaque GTG, FMC) by CHEF electrophoresis in 0.5 × TBE at 10°C, with two-phase pulse time: a constant pulse time of 90 sec for 5 h followed by 6 sec for 12 h at 0.6 V/cm.

**BAC library construction.** A BAC library was constructed following the procedures of Nakamura et al.2) and Asakawa et al.23 with some modifications. The fractionated DNA fragments ranging from 200 kb to 500 kb were excised from the electrophoresed LMP agarose and equilibrated with TE (Tris-HCl 10 mM, EDTA 1 mM), pH 7.5, for 6 h with a buffer change every hour. The agarose slice was melted at 68°C for 10 min after NaCl was added to a final concentration of 50 mM, and then digested with 1 U of GELase (Epicentre)/25 mg of agarase at 42°C for 1 h. The solution was directly used for ligation with depophosphorylated *pBAClAc*. Insert DNA (added as 50 µl at 10 ng/µl with an average size of 350 kb) was ligated to the vector DNA, at the insert to vector ratio of 1:1, using 0.042 Weiss units of T4 DNA ligase (NEB) in a final volume of 70 µl of 1 × ligation buffer supplied by the manufacturer. After overnight incubation at 16°C, the ligation mixture was drop-dialyzed against 0.2 × TE for 1 h on a 0.025-µm pore size filter (Millipore VSWP 02500) at 4°C. Electrocompetent *E. coli* DH10B cells were prepared as recommended by the supplier (BRL). Forty microliters of thawed DH10B cells was mixed with 5 µl of the DNA and placed in a 0.1 cm cuvette for electroporation using a BTX Electroe Cell Manipulator 600 m at 129 Ohm, 1.25 kV and 25 µF. Following electroporation, cells were immediately incubated with agitation in 1 ml of SOC medium for 1 h at 37°C and plated onto LB plates containing 12.5 µg/ml chloramphenicol (CM), 50 µg/ml X-gal and 12.5 µg/ml IPTG. After 24 h of incubation, white colonies were selected and placed in wells of microtitre plates each containing 150 µl of LB freezing buffer (36 mM K₂HPO₄, 13.2 mM KH₂PO₄, 10% v/v glycerol, 12.5 µg/ml CM, LB). After 24 h of growth at 37°C, the microtitre plates were stored at −80°C.

**Preparation and analysis of BAC DNA.** The BAC DNA was prepared from overnight culture in 1.5 ml of LB containing CM (12.5 µg/ml) using an automatic plasmid isolator (Kurabo Model Pl 100 Sigma, Tokyo) and dissolved in 100 µl of TE. For the insert size estimation, 10 µl of BAC DNA was digested with *N*ol for 1 h and electrophoresed in 1% CHEF gel in 0.5 × TBE at 6 V/cm with a ramped pulse time of 5 to 15 sec for 17 h.

To check the copy number of MGR 586, BAC inserts were digested with *HindIII*, *BamHI* and *XhoI*. The digested DNA was transferred onto Hybond N⁺ (Amerham) for Southern hybridization.
Preparation of high-density replica membranes. For hybridization analysis, high-density replica membranes were prepared as described by Nakamura et al. A total of 6144 clones (5760 BAC + 384 pBR 328 clones) was replicated from 56 microtiter plates onto two sheets of Biodyne B nylon membrane (8 cm × 12 cm) using the BioMek2000 automated workstation (Beckman).

DNA probes. A single-copy RFLP marker, NH165, linked to an avirulence gene av t a was used to test the representation of the genomes in the rice blast BAC library. *M. grisea* transposons were amplified from CHNOS60-2-3 genomic DNA by PCR using primers located in the transposon sequences; (5′-gaccacaccttgtagcat-3′) and (5′-gtcgtaatactgggac-3′) for MGR583, (5′-catatctctctcctgccgt-3′) and (3′-ctgtagtacgtaacctgc-3′) for MGR586, and (5′-acaggaaccctgacatct-3′) for MGR586, and (5′-taacgtaatccgccgt-3′) for Pot2. Those sequences were obtained from GenBank. The transposons were amplified under the following cycling conditions: 94°C for 5 min, followed by 30 cycles of 94°C, 1 min; 57°C, 1 min; 72°C, 1.5 min. Final extension was done at 72°C for 7 min. The clone pMGY23, which contains *M. grisea* retrotransposon MAGGY, was kindly provided by Dr. Tosa of Kobe University.

Hybridization analysis. Labeling of probes for Southern hybridization was done by ECL direct nucleic acid labeling and detection systems (Amersham) according to the manufacturer's instructions except that hybridization was done overnight at 50°C to reduce the backgrounds. After hybridization, membranes were first, washed with the primary wash buffer (6 M urea, 0.4% SDS, 0.5 × SSC) (1 × SSC: 0.015 M Na₃ citrate, 0.15 M NaCl, pH 7.0) at 42°C, then with the secondary wash buffer (2 × SSC) at room temperature, before being used to expose X-ray film.

Results and Discussion

Construction of a Magnaporthe grisea BAC library

A BAC library of *M. grisea* consisting of 5760 clones was constructed from mycelial protoplasts of strain CHNOS6-0-2-3. The mycelia were harvested before melanization to allow a high efficiency of digestion with Novozyme234. The partially digested HMW DNA was CHEF electrophoresed on a single gel with two phases of pulse time (90 sec and 6 sec) to fractionate the region of 200-500 kbp. The cloning efficiency using this region as an insert was approximately 1 × 10⁶ clones/µg vector.

Our single-gel method had the following improvements that gave the high transformation efficiency of 1 × 10⁶ clones/µg vector: first, HMW DNA was compressed and separated from low-molecular weight components by a single step; second, the amount of lost HMW DNA was decreased compared to the method using two CHEF gels.

Insert size distribution of the BAC clones

The insert size of 100 randomly selected clones was estimated by NotI digestion (Fig. 1). Ninety-five percent of the clones contained inserts. The estimated insert size ranged from 35 to 175 kbp, with an average of 120 kbp (Fig. 2). The observation that the average insert size was lower than the size of fractionated DNA has been made by others.

Size fractionation of the digested HMW DNA greatly affected the insert size of the library. Before this study, we tested the relationship between average insert size and size fractionation conditions. Using HMW DNA compressed to more than 50 kbp, the average insert size was 60 kbp, while using compressed DNA ranging from 200 to 500 kbp gave an insert size of 120 kbp.

Stability is one of the advantages of BAC libraries. Our *M. grisea* BAC library was also found to be stable as shown by the same NotI digestion patterns even after inoculation of more than one hundred generations (data not shown).

The coverage of the library

The library size was estimated to be about 18 genomes equivalent, considering the genome size to be 38 Mbp.
Fig. 3. Hybridization of the BAC Clones on the High Density Membrane with an RFLP Marker.

On one membrane, 2880 BAC clones were arrayed in $6 \times 6$ point-matrices on the 96 well microplate. The size of the membrane is $8 \times 12$ cm. Clones hybridized with the single-copy RFLP marker NH165 are indicated by arrows. The content of DNA in one row of the matrices corresponds to the content of one genome of *M. grisea*. Circles in a minimatrix on the top row indicate marker positions.

The probability of covering the genome is greater than 99.999%. Based on estimated genome size and total genetic distances (38 Mbp: Hamer *et al.*, 22) 802 cM: Romao and Hamer, 4 840 cM: Sweigard *et al.*, 6), the average ratio of physical/genetic distances in *M. grisea* is approximately 50 kbp/cM. Therefore, the insert size of 120 kbp of our BAC library corresponds to more than 2 cM. With this insert size, about 100 individuals are sufficient for $F_2$ analysis.

**High-density replica membranes**

For efficient colony hybridization, the BAC clones were replicated at high density on two nylon membranes of microplate size. Each membrane is composed of 96 ($8 \times 12$) matrices each of which contained a $6 \times 6$ point-matrix lacking two points. It is arranged with 2880 BAC clones at 30 clones per matrix and with four position markers of pBR328 in a matrix (Fig. 3). The two membranes represent a total of 5760 BAC clones. All BAC clones were easily screened by a one-step method using the membranes.

**Representation of genome coverage in the BAC library**

To test the representability of the library, the membranes were screened with a single-copy RFLP marker linked to *av ta*, which corresponds to the *Pi-ta* gene for blast resistance in rice (Fig. 3). Ten clones hybridized with the marker. This result is consistent with the estimated coverage of the library. The *NorI* digestion patterns of these clones showed that they share 25-kbp and 23-kbp fragments (Fig. 4A). The RFLP marker hybridized with the 23-kbp fragment. The restriction enzyme map of these clones was constructed with *NorI* digestion patterns and Southern hybridization patterns using these clones as probes. The maps showed that the ten clones covered a 240-kbp region around the RFLP marker (Fig. 4B). This result indicated the absence of chimerism or deletion.

**Distribution of transposons in the BAC library**

Transposable elements have been found in a wide range of organisms from bacteria to higher eucaryotes. 23) In rice-pathogenic *M. grisea*, six transposable elements, originally isolated by cloning bands of repetitive DNAs, have been reported: four retrotransposons, MGR58, 20) MGR583, 20) MAGGY, 25) and Mg-SINE 20) and two DNA transposons, MGR586 22,27) and Pot2 29)
These transposons are thought to be responsible for the generation of variability of this fungus. MGR1, MGR583, MAGGY, and MGR 586 were reported to be present in high copy number in rice-pathogenic M. grisea, and Mg-SINE and Pot2, in both rice and non-rice pathogens. These five transposons are not similar in sequence to each other at the DNA level.

As a model case of application of this BAC library, we analyzed the distribution of these transposons in M. grisea genome. The five transposons were hybridized with the BAC membranes using as probes; MGR1, MGR583, MAGGY, MGR586, and Pot2 (Fig.5). Mg-SINE was not present in our isolate.

As shown in Table 1., among the 5760 BAC clones, 10% contained MGR1, 13% MGR583, 18% MAGGY, 12% MGR586, and 23% Pot2.

**Table 1. The Percentage of the BAC Clones Containing Each Transposon**

<table>
<thead>
<tr>
<th>Name of transposons</th>
<th>Percentage of positive clones (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGR1</td>
<td>10</td>
</tr>
<tr>
<td>MGR583</td>
<td>13</td>
</tr>
<tr>
<td>MAGGY</td>
<td>18</td>
</tr>
<tr>
<td>MGR586</td>
<td>12</td>
</tr>
<tr>
<td>Pot2</td>
<td>23</td>
</tr>
</tbody>
</table>

*Analysis of copy number of MGR586 in the BAC clones.*

![Fig. 5. Hybridization of High Density Membranes of the BAC Clones with M. grisea Transposon Sequences.](image)

(A) Hybridization pattern of MGR1, 10% of the BAC clones are positive. (B) Hybridization pattern of MGR583, 13% of the BAC clones are positive. (C) Hybridization pattern of MAGGY, 18% of the BAC clones are positive. (D) Hybridization pattern of MGR586, 12% of the BAC clones are positive. (E) Hybridization pattern of Pot2, 23% of the BAC clones are positive.

![Fig. 6. Analysis of MGR586 Copy Numbers in the Positive BAC Clones.](image)

Forty one positive clones hybridizing with MGR586 in the fourth row of Fig. 5 (D) were digested with a mixture of HindIII, BamHI, and XhoI, and hybridized with MGR586 probe (lane 1–41). No restriction site of these enzymes exists in the MGR586 sequence. Forty one percent of the clones contained more than two copies of MGR586. The estimated copy number of MGR586 in one genome of this strain is about 60.
On the fourth row of the membrane, forty-one BAC clones were hybridized with MGR586 (indicated by the arrow in Fig. 5D). For further analysis of copy number, 41 clones were digested with an enzyme mixture of HindIII, BamHI, and XhoI, as these enzymes have no restriction sites in the MGR586 sequence. Of 360 clones in the fourth row, 17 had more than two copies and 24 one copy of MGR586; totalling 64 copies per 43 Mbp (=120 kbp × 360 clones) of genome (Fig. 6). The copy number of MGR586 in CHNOS60-2-3 genome was about 60 by counting the number of hybridized bands of MGR586 probe in the Southern blot of the genomic DNA (data not shown). The copy number of MGR586 obtained using BAC membranes matched the result of genomic Southern hybridization.

**Clustering of the transposons.**

It was reported that some transposable elements are distributed differentially in genomes from bacteria to higher eukaryotes: IS110 and IS211 elements in the genome of E. coli, Ty elements in S. cerevisiae,22 Ty1-copia retrotransposon in allium,23 and LINEs and SINEs in the mouse.24 In addition, analysis of a 280-kbp region of the maize genome25 and some cosmids clones of M. grisea genomic DNA,26 suggested that several transposons were clustering in the genome. We became interested in how the transposons are clustered in our BAC library. Using the BAC membrane, it was possible to study the portion of clones with several transposons simultaneously and the transposon clustering pattern.

As shown in Table 2, the proportion of BAC clones that contained several transposons obtained in this study was greater than the probable values obtained by calculation. The figures of A/B in Table2. indicate that MGR583 was closely associated with MGR1 and MGR586. MAGGY and Pot2 were likely to co-occur at high frequency as they were both reported to insert in the AT-rich DNA region.25,27 However, the results of this study showed that they occurred together at a lower rate than expected. This suggested that these two transposons were highly amplified in the genome compared to others.

The percentage of BAC clones having five kinds of transposons simultaneously was 1.4% (Table .2C). If these transposons are randomly dispersed in the genome, the probability of containing five of them would be 0.0065%. The result was 215 times the probable value obtained by estimation. Our results give the first clear indication that these transposons are not randomly distributed in the M. grisea genome, but have a tendency to cluster. Further analysis of the BAC clones with several transposons simultaneously will give detailed information of regions in the genome where the transposons are.

**Conclusion**

The BAC library is an essential tool for positional cloning and genomic sequencing. In addition, as shown in this study, BACs are also helpful for the study of gene distribution. To date, studies of gene distribution in the genome and rearrangement of genomes have been mainly dependent on gene mapping. BACs with simplified procedures will greatly facilitate the study of genome dynamics.

In the future, we are planning to construct contigs of the whole M. grisea genome with our BAC library.

**Acknowledgment.**

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Distribution of Transposons in M. grisea BAC Library

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