Molecular Analysis of the Wheat Blast Population in Brazil with a Homolog of Retrotransposon MGR583

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Abstract

We have identified a family of dispersed repetitive DNA sequences in the genome of a Magnaporthe grisea isolate from finger millet (Eleusine coracana). Southern blot analyses showed that this element is present in a moderate copy number (30-40 copies) in the genome of blast isolates from wheat (Triticum aestivum). DNA sequence data suggested that this element contains a region highly homologous to the reverse transcriptase domain of MGR583, a poly A-type retrotransposon. Using the reverse transcriptase domain of this element as a molecular probe, the genetic structure of the wheat blast population was examined. DNA fingerprinting analyses revealed that the wheat-infecting isolates constitute a separate, single lineage of their own, suggesting that they are derived from a single origin.

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Key words: Magnaporthe grisea, blast fungus, retrotransposon, wheat, DNA fingerprinting.

INTRODUCTION

Magnaporthe grisea (anamorph, Pyricularia grisea) is a common pathogen of monocots that infects more than 50 grass species. Among them, rice (Oryza sativa) is the most important staple crop for which this fungus causes major losses, with frequent reports of devastating damage worldwide. Recently, another crop of vital importance has been reported to be suffering from high yield loss because of this fungus in Brazil. In 1985 some wheat (Triticum aestivum) fields in Northern Parana state were severely damaged by this pathogen. Since this first report, the wheat blast disease has spread to other regions, and it is now present in all major wheat-growing regions of this country. In the state of Mato Grosso do Sul the yield reduction has been estimated to be 10 to 11%. It is currently considered to be one of the major wheat diseases in Brazil as a result of the damage it inflicts, its geographical distribution, the poor performance of chemicals in protecting wheat spikes in the field and the lack of resistant cultivars.

Because this disease occurred in a region where wheat and rice are cultivated in close proximity, wheat blast was soon suggested to be caused by the rice blast pathogen. Later reports, however, have provided information on differences between these pathogens. Studies on the host range and sexual fertility have shown that the wheat pathogen is distinct from the rice pathogen. This difference between the rice pathogen and the wheat pathogen has raised questions regarding the origin of the wheat pathogen and its relationship to isolates from other hosts. Based on the pathogenic pattern on a set of gramineous plants, Urashima et al. have noted that blast isolates from finger millet (Eleusine coracana) are the most similar to the wheat pathogen. Restriction fragment length polymorphism detected by single-copy probes has also shown a close relationship between isolates from finger millet and those from Brazilian wheat when compared with those from corn or rice. These results were curious because finger millet is an unknown crop in Brazil.

MGR586 is the most frequently employed probe in molecular studies of the blast fungus. It belongs to the inverted repeat (IR) class of transposon and is present in a high copy number in rice isolates but in a low copy number in isolates from wheat and weeds. Using this probe, Valient has demonstrated the difference between the rice pathogen and the wheat pathogen. Further, MGR586 has proved to be a reliable genetic marker for studies of epidemiology and population structure of the rice blast pathogen in various parts of the world, allowing the identification of clonal lineages. However, its usefulness in studying the genetic aspect of the wheat pathogen is limited by its low copy number in the wheat pathogen.

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Besides MGR586, many repetitive DNA sequences have been found in the genome of *M. grisea* Fosbury and MAGGY, retrotransposons containing LTRs, have common characteristics of high copy number in isolates from rice and foxtail millet, but being absent in those from other hosts, which make them adequate markers for studies of the rice blast population but not for the wheat blast population. *Grasshopper*, another retroelement present in multiple copies, is dispersed throughout the genome of some isolates from *Eleusine* spp. but is absent in isolates from other hosts. MGR583 is present in a high copy number in rice isolates and in a majority of non-rice isolates but shows a less polymorphic hybridization pattern compared with that of MGR586.

The present work provides a molecular approach to the study of the wheat blast population. For this purpose, we isolated and characterized a molecular probe to use to examine the population structure of the wheat pathogen and its genetic relationship to isolates from other hosts.

### MATERIALS AND METHODS

#### Collection, identification and culture conditions of isolates
A total of 77 isolates of *M. grisea* were collected from wheat [*Triticum aestivum* (L.) Thell.] and weeds in Parana (PR), Sao Paulo (SP) and Mato Grosso do Sul (MS), Brazil, where the wheat blast disease is prevalent. The following diseased grass weeds were gathered: *Digitaria horizontalis* Wild., *Brachiaria plantaginea* (Link) Hitchc., *Cenchrus echinatus* L., *Setaria geniculata* (Lam.) Beauv. and *Echinochloa colonum* Link. Isolates from rice [*Oryza sativa* L.] collected in Brazil or Japan and those from finger millet [*Eleusine coracana* (L.) Gaertn.] collected in Japan, Nepal or India were also used. The designation, original hosts, geographic origin and date of collection of the isolates are shown in Table 1. Three samples were taken from each wheat field, which was surrounded by rice fields or had rice plants grown in the same field. All isolates were confirmed to be pathogenic to its own host. In the test of pathogenicity of wheat isolates, *Triticum aestivum* cv.

#### Table 1. Host and locality of *Magnaporthe grisea* isolates tested

| Host                     | Locality and year of collection | Isolate/
<table>
<thead>
<tr>
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<tbody>
<tr>
<td><em>Triticum aestivum</em></td>
<td>Parana, Brazil (1990)</td>
<td>Br1, Br2, Br3, Br4, Br5, Br6, Br7, Br8, Br9, Br39</td>
</tr>
<tr>
<td>(Wheat)</td>
<td>Sao Paulo, Brazil (1990)</td>
<td>Br46, Br47, Br48, Br49, Br50, Br52</td>
</tr>
<tr>
<td></td>
<td>Mato Grosso do Sul, Brazil (1992)</td>
<td>Br10, Br13</td>
</tr>
<tr>
<td><em>Oryza sativa</em></td>
<td>Parana, Brazil (1990)</td>
<td>Br27, Br28, Br30, Br31, Br32, Br33</td>
</tr>
<tr>
<td>(Rice)</td>
<td>Tochigi, Japan (1976)</td>
<td>Br29</td>
</tr>
<tr>
<td><em>Digitaria horizontalis</em></td>
<td>Parana, Brazil (1990)</td>
<td>Br34, Br35, Br54</td>
</tr>
<tr>
<td></td>
<td>Sao Paulo, Brazil (1990)</td>
<td>Bp3a</td>
</tr>
<tr>
<td><em>Brachiaria plantaginea</em></td>
<td>Parana, Brazil (1990)</td>
<td>Br36</td>
</tr>
<tr>
<td></td>
<td>Sao Paulo, Brazil (1996)</td>
<td>Br37</td>
</tr>
<tr>
<td><em>Cenchrus echinatus</em></td>
<td>Parana, Brazil (1990)</td>
<td>Br38</td>
</tr>
<tr>
<td><em>Setaria geniculata</em></td>
<td>Parana, Brazil (1990)</td>
<td>Z2-1</td>
</tr>
<tr>
<td><em>Echinochloa colonum</em></td>
<td>Parana, Brazil (1990)</td>
<td>G10-1</td>
</tr>
<tr>
<td><em>Eleusine coracana</em></td>
<td>Kagawa, Japan (1979)</td>
<td>NP10-17-4-1-3</td>
</tr>
<tr>
<td>(Finger millet)</td>
<td>Tochigi, Japan (1979)</td>
<td>IN77-31-1-1</td>
</tr>
</tbody>
</table>

a) Isolates with the same number followed by different alphabetical letters were collected from the same field.
Anahuac was used as a test plant. Monoconidial cultures of these isolates were grown on sterilized barley seeds in a vial, then dried thoroughly at 25°C and kept in a case with silica gel at 5°C.

**DNA manipulation** Isolates were grown in CM broth (3 g Casamino acids, 3 g Yeast extract, 5 g sucrose/) on a rotary shaker at 25°C. After 4-5 days incubation, mycelia were harvested and stored at -80°C. DNA was extracted following the procedure described by Liu et al.\(^\text{14}\) with a few adaptations.

A repetitive DNA sequence for the probe was isolated from genomic DNA of an isolate from finger millet, G10-1. It was inserted into the plasmid pUC19 and established as pEBA18 in *E. coli* strain C75 (Takara Shuzo Co.). Its 0.9-kb fragment was subcloned into pUC19, established as pEBA18-09 and subsequently sequenced using the Auto Read Sequencing Kit (Pharmacia Biotech.), according to manufacturer's instruction. The subclone was labeled with biotin using NEBlot Phototope Kit (New England Biolabs) to use as a probe for fingerprinting.

One microgram of genomic DNA was digested to completion with *EcoRI* and separated by horizontal agarose gel (0.8%) electrophoresis at 2V per centimeter in a 0.5X TBE buffer for 18 hr. DNA fragments were denatured and transferred to a nylon membrane (Magnograph, Micron Separations, Inc.) by overnight capillary blotting. Prehybridization and hybridization were conducted in 6X SSC containing 5X Denhardt's solution, 0.5% SDS and 100 μg/ml of sonicated salmon sperm DNA in a sealed plastic bag at 68°C. Hybridization reactions were allowed to continue for 12-15 hr with the biotin-labeled probe. After hybridization, the membrane was washed twice in 2X SSC, 0.1% SDS for 5 min each time at room temperature, followed by two washes in 0.1X SSC, 0.1% SDS for 15 min at 68°C. DNA blots were submitted to chemiluminescent detection using Phototope™ Star Detection Kit (New England Biolabs).

**Analysis of fingerprints** The band pattern of DNA fingerprints was scored visually by checking the presence (value 1) or absence (value 0) of bands in the 1-7 kb range. Bands with ambiguous interpretation were not evaluated in order to minimize unexpected errors. Similarity coefficients were calculated by Nei and Li's method\(^\text{27}\): \(S_{xy} = 2N_{xy}/(N_x+N_y)\), where \(N_{xy}\) = number of fragments shared by two isolates, and \(N_x\) and \(N_y\) = number of fragments in isolate \(x\) and \(y\). A phenogram was constructed using the "unweighted pair group method using arithmetic means" (UPGMA)\(^\text{34}\) with a computer program developed by M. Okuda, Kyushu National Agricultural Experiment Station, Japan. A bootstrap analysis was carried out with the WINBOOT program\(^\text{42}\), and the robustness of each cluster was verified in 1000 replications.

**RESULTS**

**Isolation and partial characterization of pEBA18**

Genomic DNA of a finger millet isolate, G10-1, was digested with *HindIII* and cloned into pUC19. One of these clones, pEBA18, proved to be present in multiple copies in wheat isolates (Fig. 1). The insert of pEBA18 was a 5.5-kb *HindIII* fragment (Fig. 2). A 0.9-kb *EcoRV-HindIII* fragment of the insert was then subcloned into pUC19 and established as pEBA18-09 for sequencing. Its complete sequence and deduced peptide sequence are shown in Fig. 3. pEBA18-09 showed a very high homology (98.6% in nucleotide sequence) to the reverse transcriptase domain of a non-LTR retrotransposon, MGR583, reported by Hamer et al.\(^\text{11}\), and Valent and Chumley\(^\text{12}\) (Figs. 2 and 3).
Fingerprinting with the MGR583 homolog

Genomic DNA of blast isolates from various hosts was digested with EcoRI, electrophoresed, and hybridized with pEBA18-09. Isolates from rice carried this element in a high copy number (>40 copies), whereas those from wheat, finger millet and Digitaria, etc., carried it in a moderate copy number (20–40 copies) (Fig. 1). An isolate from Setaria geniculata carried only a single copy (Fig. 1). When hybridization profiles were examined in detail, isolates from Digitaria horizontalis, Echinochloa colomum, Cenchrus echinatus and finger millet appeared to be distinct from wheat isolates because they produced no common bands with wheat isolates. Isolates from Brachiaria plantaginea produced four common bands with wheat isolates. To clarify the relationship between Brachiaria isolates and wheat isolates, a phenogram was constructed using UPGMA (Fig. 4). Each population formed a robust cluster with a high bootstrap value, and the similarity coefficient between them was only 16%, suggesting that each represents a distinct genetic group. However, confidence of clusters within the wheat blast population was low, indicating that the wheat blast population has a high variation and cannot be separated into lineages.

DNA fingerprints with pEBA18-09 were further analyzed to elucidate the population structure of wheat isolates collected in major regions of Brazil where blast disease was prevalent. All these isolates shared more than 75% of the bands (Figs. 1 and 4). However, patterns of uncommon bands showed a high degree of diversity. Diverse haplotypes occurred even in a population collected from the same city and year. For example, isolates Br130.1B, Br130.1D, Br130.1E, Br130.8, Br130.9B and Br130.9O collected in Rolanda (PR) in 1992, formed a group with only 81% similarity. Such diversity was more evident when isolates from the same field showed a similarity as low as 78% (isolates Br119.1B, Br119.1C, Br119.1F). On the other hand, 100% similarity occurred among isolates collected in different locations of the same state (Br48 from Itapora, MS, and Br50 from Rio Brilhante, MS), those gathered in the same state but in different years (Br2 and Br3 from Londrina, PR, sampled in 1990, and Br130.1E from Rolanda, PR, collected in 1992) and those collected in the same year, but in different states (Br202.1D from MS and Br123.1D, Br127.11C, Br127.11D, and Br128.1B from PR).

DISCUSSION

The main objectives of this work were to isolate a clone that allows DNA fingerprinting of non-rice isolates of M. grisea and to examine the genetic variation in M. grisea populations that infect wheat and weeds in Brazil. Analyses of the population structure of the blast fungus using DNA fingerprinting have been conducted in many parts of the world. Those studies have focused exclusively on the rice pathogen, and most of them have used MGR586 as a molecular probe. This probe and others described so far have not been suitable for examining genetic variation in the wheat blast population. In the present study we isolated a repetitive element from a finger millet isolate as a molecular marker for the wheat blast population. A 0.9-kb fragment of this element has proven to be a homolog of the reverse transcriptase domain of MGR583, a non-LTR retrotransposon found in rice isolates by Hamer et al. (13). It was present in a moderate number (30-40) in the genome of wheat isolates, and its use as a molecular probe allowed us to examine their genetic variability.

DNA fingerprinting with this probe revealed that wheat isolates collected in different regions of Brazil were clustered into a single group; all the isolates tested shared more than 75% of the bands with one another, but no or few bands with isolates from weeds or finger millet. In other words, the wheat isolates constituted a separate, single lineage of their own. These results suggest that the wheat blast population in Brazil is derived from a single origin. In the subsequent dissemination to new regions, seed transmission seems to have played an important role because wheat seeds have frequently been found to be contaminated with the blast fungus.

Nevertheless, the wheat blast population showed a high degree of variation among isolates. Unique (uncommon) bands appeared in various combinations. Consequently, no lineages were detected within the wheat blast population in contrast to the structure of the rice blast population that is composed of distinct clonal lineages. We can point out two factors which may be related to this difference between the rice blast and wheat blast populations. One is the history of the blast diseases. The rice blast pathogen, with its long
Fig. 3. Comparison of nucleotide sequences and putative amino acid sequences between pEBA18-09 (abbreviated as pEBA18, accession # AB025252) and a corresponding region of MGR583 (MGL, accession # AFO18033).
An isolate from *S. geniculata* produced only one hybridization band with pEBA18-09, suggesting that it is remote from wheat isolates. Although this isolate has been shown to be pathogenically similar to the wheat blast pathogen with positive cross-infection\(^\text{39}\), there was no sexual compatibility between them\(^\text{38}\). Isolates from *D. horizontalis*, *C. echinatus* and *E. colonum* produced no common bands with wheat isolates (Fig. 1), suggesting that those isolates are not close to wheat isolates. This result is in accordance with pathogenicity data\(^\text{39}\) that isolates from those weeds are incompatible with wheat and vice versa. Finger millet isolates also had no bands in common with wheat isolates, suggesting that they are not so closely related to wheat isolates. This result was unexpected because the former work\(^\text{39}\) has verified a close resemblance between wheat isolates and finger millet isolates in terms of host range and sexual compatibility. However, the present result appears to be logical, since finger millet, which is a staple food in India, Nepal and other regions of Asia, is absent in Brazil. *Brachiaia plantaginea* isolates shared four bands with wheat isolates but produced a cluster with only 16% similarity to the wheat blast population, suggesting that they form a distinct group.

The usefulness of pEBA18-09 for studying the population structure of the wheat blast pathogen has been demonstrated in the present work. This probe also appeared to be applicable to fingerprinting of various weed isolates. We suggest that the MGR583 homolog, and probably MGR583 itself, promise to be useful probes for fingerprinting *M. grisea* isolates from a broad range of hosts and to be good complements to the MGR586 element which is somewhat limited in its usefulness.

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**Literature cited**


和文摘要

Alfredo S. URASHIMA・横本容子・Le D. DON・草場基章・土佐幸雄・中屋敷均・倉山滋志：ブラジルにおけるコムギもち病菌個体群のレトロトランスポーザンMGR583ホモログによる分子解析

コムギもち病菌個体群の分子解析のためのプローブとして、シコクピエロー病菌のゲノムから散在反復配列のファミリーをクローニングした。この因子はコムギもち病菌にゲノムあたり30から40コピー存在した。シークエンスの結果、本因子はポリAタイプレトロトランスポーザンMGR583の逆転写酵素ドメインと高い相関性を有することが判明した。本因子をプローブとしてコムギもち病菌個体群のフィンガープリント解析を行ったところ、本菌群はそれ自身で独立かつ単一のリネージを形成していることが明らかとなり、単一起源であることが示唆された。