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Polymorphism and Genetic Relationships Among *Magnaporthe grisea* Isolates Obtained from Various Hosts by Using Repetitive DNA Sequences

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기주가 다른 *Magnaporthe grisea* 균주간의 Polymorphism과 유전적 유연관계 분석

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ABSTRACT : DNA polymorphisms of the isolates of blast fungus, *Magnaporthe grisea*, were analyzed to show the genetic relationships among these isolates. Korean isolates of *M. grisea* were obtained from various gramineous hosts such as rice, common millet, foxtail millet, reed canary grass, green foxtail, crab grass, and barnyard grass. Random genomic clones of the race KJ 201 isolated from rice were used as probes to detect DNA polymorphisms within the isolates of the fungus, and a clone containing a repetitive sequence, pMJ6, was found. pMJ6 hybridized to about 30 *Hin*dIII restriction fragments in the isolates from rice plants, and to 20 to 33 bands in the other hosts, but it hybridized to only a few fragments in the isolate of *M. grisea* infecting barnyard grass, suggesting that the isolates of *M. grisea* infecting barnyard grass and the other plants are genetically less related. When *Eco*RI-digested fungal DNA was hybridized with pMJ6, the band pattern was similar to that with MGR sequence as probes. This study clearly demonstrated the feasibility of use of this repeated copy clone in genetic relationship analysis among isolates of *M. grisea*.

***Key words* : polymorphism, genetic relationship, *Magnaporthe grisea*, repetitive sequence, barnyard grass.**

The blast fungus, *Magnaporthe grisea* Berr., is one of the most important plant pathogenic fungi in the world. It belongs to Ascomycetes and is heterothallic. Although this fungus infects over 50 gramineous plants including rice, foxtail millet, cultivated and feral grass, individual isolates can infect only a few limited hosts (15). Hundreds of races have been identified in the population of rice-infecting isolates (8, 15). Moreover, new races frequently occur in the rice fields, and this in turn makes it difficult to breed resistant rice varieties against blast (14).

Several programs were conducted to investigate the genetic variation and relationship among isolates obtained from different hosts using various techniques. The isozyme profiles of blast fungus isolates of rice from 12 regions of the world were nearly uniform and not much differed from those of *M. grisea* isolates that infect other grass hosts (11). Genetic relationship between isolates having different hosts is still not clear. In spite of that *M. grisea* can infect several gramineous plants, it was considered as different species of genus *Pyricularia* in classification for a long time. As the perfect stage of this fungus was discovered from the crossing over between rice and the other hosts, it was tried to reclassify this pathogen as a single species without much success (1, 16).

M. grisea easily forms the perfect stage and mutants in the laboratory, thus several investigators are trying to study the genetic variation using the repetitive DNA sequences in this fungus (2, 3, 5, 9, 12). These studies provided at DNA sequence level some of the evidences for the genetic evolution, variation and re-

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lationship analysis among isolates in many countries. Additionally RFLP techniques which developed recently offered a new tool to monitor the genetic characteristics in plant pathogenic fungi, and contributed a lot in many phytopathological aspects (4, 7, 10, 13, 18). They are especially useful for the genetic study of pathogens that do not possess the clearly defined virulence or physiological markers.

We have performed RFLP analysis using repetitive DNA sequences of *M. grisea* isolated in Korea in order to obtain the basic informations for genetic relationship and difference among isolates of *M. grisea.*

MATERIALS AND METHODS

Fungal isolates. Ten isolates of *Magnaporthe grisea* used in this study were provided from Department of Plant Pathology, Agricultural Science and Technology Institute, Korea, and are listed in Table 1. All cultures were maintained on potato dextrose agar slant at 4°C.

Cloning of *M. grisea* DNA. Total genomic DNA from one *M. grisea* isolate belonging to KJ 201 race was digested to completion with *Hin*dIII, ligated into *Hin*dIII-digested pUC118 with T-4 DNA ligase and transformed into *Escherichia coli* strain DH5α as described by Vieira and Messing (20). Plasmid DNAs containing *M. grisea* DNA inserts were selected from ampicillin resistant clones, and then extracted by the alkali lysis method (17).

DNA isolation. The fungi used in this study were grown in potato dextrose broth without shaking for 5 days. Mycelium was filtered through several layers of cheese cloth, placed in microcentrifuge tubes, and lyophilized at −80°C. Lyophilized mycelium was ground to powder with a sterile wooden stick and 500 μl of extraction buffer (100 mM Tris pH 8.0, 50 mM EDTA, 100 mM NaCl, 10 mM β-mercaptoethanol, and 1% SDS) was added. Tubes were incubated at 65°C for 10 min and cooled on ice. 250 μl of cold 5 M potassium acetate was added and incubated on ice for 20 min. The samples were then centrifuged for 10 min at 4°C and the supernatant was treated with 5 μl of 10 mg ml^{-1} RNase for 30 min at 37°C. The samples were then treated with 10 μl of 10mg ml^{-1} Proteinase K for 10 min at 37°C. Samples were extracted once with 0.5 ml phenol : chloroform : isoamylalcohol (25 : 24 : 1) and once with 0.5 ml chloroform : isoamylalcohol (24 : 1). To the 0.5 ml aqueous layers, 1 ml of 100% ethanol was added and incubated at room temperature for 5 min. Samples were then centrifuged at 6,000×g for 15 min at room temperature. The pellet was washed with 70% ethanol, and dried under vacuum. DNA was dissolved with 50 μl of TE (10 mM Tris pH 8.0 and 0.1 mM EDTA, pH 8.0) and stored at −20°C.

Table 1. Host plants and races of isolates of *Magnaporthe grisea* used in this study

Common name	Scientific name	Race
Rice	*Oryza sativa*	KJ101
	Oryza sativa	KJ201
	Oryza sativa	KI315a
	Oryza sativa	KI315b
Common millet	*Panicum miliaceum*	−
Foxtail millet	*Setaria italica*	−
Reed canary grass	*Phalaris arundinacea*	−
Green foxtail	*Setaria viridis*	−
Crab grass	*Digitaria sanguinalis*	−
Barnyard grass	*Echinochloa crus-galli* var. *frumentacea*	−

Southern hybridization. DNA blotting to nylon membrane was by capillary action essentially as described previously(17). Probes of plasmids and genomic DNAs were prepared by priming the DNA with random hexanucleotides in presence of deoxynucleotides plus digoxigenin-conjugated dUTP (dig-dUTP) using Klenow fragment of DNA polymerase I. Hybridization and immunological detection of the dig-dUTP were as described by manufacturer (Boehringer-Mannheim).

Phylogenetic analysis. Banding patterns of hybridization were used to compare the relationship among *M. grisea* strains. Each band with a different electrophoretic mobility was assigned a position number. If the DNA from a strain contained the specific band it was assigned the value "1" for this position. If the DNA lacked the band it was assigned the value "0" for this position. The values for each position were compared for various strains of the fungus. The relationship of strains was estimated from these values by Wagner parsimony with PAUP program (19). Tree was rooted by using a data set containing the value "0" for all numbers.

RESULTS

Genomic library was constructed from *Hin*dIII-digested *M. grisea* DNA fragment into the vector pUC118. Twenty-three recombinant clones were randomly selected. Plasmids were isolated and digested with *Pvu*II

which cut the plasmid at positions flanking the polylinker cloning site, and then labelled.

Among six endonucleases on genomic DNA of this fungus, *Eco*RI gave distinct bands of around 2.5~2.8 kb after staining with ethidium bromide (data not shown). Moreover the number and size of bands varied within isolates from the different hosts, polymorphism was revealed among them even before the southern hybridization analysis using a RFLP probe.

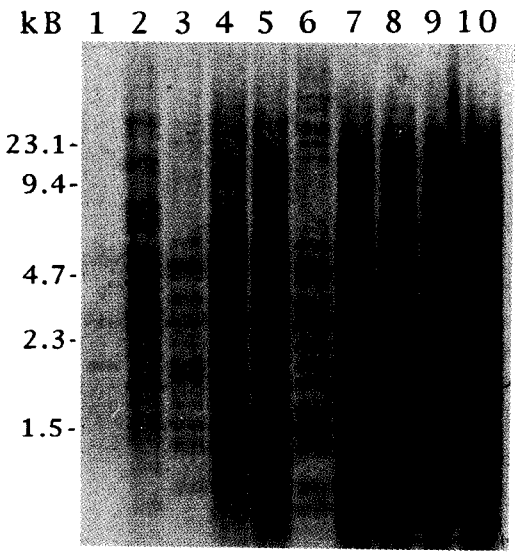

Fig. 1. Survey of repetitive sequence in isolates of *Magnaporthe grisea*. Genomic DNA was digested with *Hin*dIII, fractionated on a 0.7% agarose gel, transferred to nylon membrane and hybridized to pMJ6. The lanes contained DNA from isolates of rice and grasses as follows: 1 : Barnyard grass, 2 : Foxtail millet, 3 : Crab grass, 4 : Reed canary grass, 5 : Common millet, 6 : Green foxtail, 7 : Rice race KI315b, 8 : Rice race KI315a, 9 : Rice race KJ201, 10 : Rice race KJ101.

To choose repetitive sequence twenty clones were selected for southern blot analysis on KJ201 isolate which infects on Japonica type rice varieties as described by Hamer *et al.* (13). A clone named pMJ6 was found as having the repetitive DNA sequences. This method was also successfully used to select repetitive DNA sequences in this fungus. Southern blot analysis employing *Pvu*II-digested pMJ6 as a probe showed around 30 distinct bands upon hybridization to *Hin*dIII-digested genomic DNA of rice-attacking isolates, but only two bands were shown in DNA from the isolate of barnyard grass (Fig. 1). The band number is varied between the isolates obtained from different hosts. As shown in the southern blot depicted in Fig. 1, probe pMJ6 hybridized to show many bands, demonstrating the potential of this clone, for genetic analysis polymorphism. Rice infecting isolates and other isolates were distinct in showing 30 bands compared to only two bands of barnyard grass isolate. This probe also hybridized to show several bands of *Eco*RI-digested genomic DNA similar to *Hin*dIII-digested DNA, revealing polymorphism among isolates (data not shown).

MGR(3) which was already used for detecting DNA characteristics of *M. grisea* was used for analysis polymorphism of this fungus (Table 2). Using MGR as a probe only one band were shown in *Eco*RI-digested of barnyard grass isolate similar to the band pattern obtained by using pMJ6 as a probe. Although banding pattern for each isolate was different, it revealed about 30 bands and the patterns of bands on rice isolates were similar to those of clone pMJ6. When digested with *Hin*dIII, relatively few bands appeared, making more dif-

Table 2. Data set showing presence (1) or absence (0) of restriction fragments at 85 positions hybridizing to genomic clone pMGR

Isolate	Restriction fragments
Rice KJ101	00000000001000010000001000010000101000010010001000100101001010001000001011001001000 11
Rice KJ201	00000001001100010000011000010000101000010010001000100101001010001100001001001001000 11
Rice KI315a	00000010010100001000000100001000010010101000101000010110010100101100101000100100010 01
Rice KI315b	00000000000000001000100100001000010010101000101000010110011100101100101100100100000
Green foxtail	00100101000101010000010000010000000100000001001000100010100000000000000000000100000000
Common millet	01000001000100000001000010000100000000100010000100100001000001000000000000000000010000
Reed canary grass	00011000100100100010100100010001001000100101010001001000000011000111001010010010001 01
Crab grass	00100000000101010000000000100000010000101000000010001001000000000000000000010000000000
Foxtail millet	10010000000000010010000000100001001000010000001010000100010000000100100101000000010 1000
Barnyard grass	00000000000000000000000000000000000000000000000000000000000010000000000000000000000000000000
NUL	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000

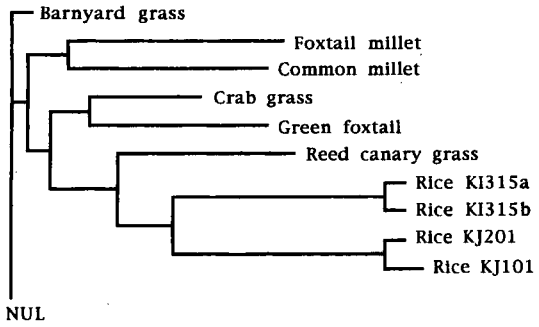

Fig. 2. A computer-generated dendrogram of 10 isolates of *Magnaporthe grisea* was analyzed by PAUP program derived from the data given in Fig. 1 and Table 2.

ficult to detect the differences among isolates.

The distinct polymorphisms resulted from hybridization of pMJ6 and MGR were used to create a dendrogram of the relationships among isolates from different hosts. Phylogenetic analysis using by PAUP program was performed based on the data set which derived from the presence or absence of hybridizing bands at certain sites showed Fig. 1 and Table 2. The null value used at all position in Table 2, was assigned as a designated ancestor. The tree distinguished the isolates from the different hosts. The barnyard grass isolate was distinct from all others. Isolates from common millet were clusted into a different group with barnyard grass isolate and they also differed from rice-infecting isolates (Fig. 2).

While the low genetic relationship was revealed among rice-isolates and reed canary grass, foxtail millet, and barnyard grass one another, rice-infecting isolates were rather closely related each other, especially so within the same race, like KI or KJ race.

No close genetic relationship showed among all other isolates except rice-infecting isolates. In addition, relatively low relatedness was also shown between isolates belong to KI and KJ race group each other.

DISCUSSION

Magnaporthe grisea causes blast of gramineous plants, and it is the most destructive pathogen to economically important crops. This fungus is found throughout the world and causes severe damage to the rice under suitable conditions (15). Due to the active genetic variation of *M. grisea*, numerous races have been identified from isolates infecting rice plants (8, 15). Since the pathogen can alter its pathogenic characteristics very rapidly, even it is known to exist the variation in disease causing ability within a pathogen population, a breeding scheme for disease-resistant rice cannot assure complete resistance. Futhermore, it is still unclear whether the isolates infecting weeds or cultivated grass can also infect rice plants (5). The exact relationship among the isolates from different hosts has been studied. The information concerning genetic variation of this pathogen may be valuable to plant pathologists and breeders, if this could be an useful reference for the pathological differences between rice and other grasses infecting pathogen.

Variation in DNA level can be detected by restriction fragment length polymorphism (RFLP) analysis which can identify minor variations that may not be expressed at the protein level. Repetitive element provides a powerful tool to determine genetic variation and relationship (2, 3, 9, 12). The variable nature of the repetitive element helps us for identifying genetic differences between closely related fungal isolates. Repetitive probe, pMJ6, identified in this paper revealed the high, variability among different fungal isolates, and thus detect many polymorphisms among isolates. pMJ6 was successfully used to infer genetic relationship and phylogeny among isolates of *M. grisea*. Distinct polymorphism was apparent among the isolates from different hosts. Some isolates including barnyard grass pathogen showed a low copy number, whereas more than 30 bands were shown on DNAs from isolates of rice plant and common millet. The existence of distinct polymorphism between isolates is quite remarkable considering although they belong to the same species, *M. grisea*.

pMJ6 gave very similar band number and patterns compared to MGR probe which was discovered by Hamer *et al.* (3) and has been used to investigate genetic variation of this fungus. It was reported that *M. grisea* isolates that do not infect rice contain relatively few copies of MGR sequence. Both probes hybridized to only two sites of DNA from barnyard grass isolate. Han and Nelson (6) also reported that isolates from some of the non-rice hosts make only a few bands in the case of using MGR probe. The phylogenetic tree was constructed using PAUP program to analyze genetic relationship among isolates. Barnyard grass isolate was the most distant from rice-infecting isolates as expected. Foxtail millet isolate and common millet isolate also showed to be relatively remote to barnyard grass isolate. There was no close relationship between isolates

from different hosts except those from rice. Isolates from the same race group of rice are very closely related at DNA level, however considerable genetic differences were evident between race groups of KI and KJ.

The concerted analysis of RFLP relationship and pathological data among various isolates having different hosts will be necessary to verify the latter point and for RFLP data to be used in practical breeding scheme.

요 약

도열병균, *Magnaporthe grisea*, 균주간의 유전적 유연관계를 분석하고 그들의 유전에 관한 기본 정보를 얻고자 DNA polymorphism 분석을 실시하였다. 기주가 다른 도열병 균주들이 공시되었고 cloning에 의해 벼 도열병균 KJ201레이스 균주로부터 생성된 임의 선발 genomic clone들이 공시균주들간의 polymorphism을 밝히기 위해 사용되었던 바 그 중 repetitive sequence를 보유한 repeated copy clone 하나가 선발되었다. Clone pMJ6에 의해 밝혀진 repetitive sequence는 Southern hybridization시 벼 분리균주에는 약 30개, 다른 기주 분리균에도 20~33개의 밴드를 형성하였다. 반면 피 분리균주에는 단지 두 개의 밴드만을 나타내 분리기주가 다른 균주간에 뚜렷한 polymorphism이 존재하였으며 parsimony 분석에서도 역시 아주 먼 cluster를 형성하여 피 분리균은 다른 기주 분리균과 유전적으로 상당히 먼 것으로 추정되었다. 공시균의 genomic DNA를 *Hin*dIII로 처리했을 때 pMJ6에 의한 밴드 양상은 공시균을 *Eco*RI으로 처리했을 때의 MGR probe의 밴드 양상과 유사하여 이 repeated copy clone이 도열병균주간의 유전적 유연관계를 분석하는데 MGR 못지 않게 유용할 것으로 보인다.

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