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Molecules and Cells

Isolation of TC/AG Repeat Microsatellite Sequences for Fingerprinting Rice Blast Fungus and Their Possible Horizontal Transfer to Plant Species

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Genome fingerprinting has been a major role in characterization of population structure and analysis of the variability in phytopathogenic fungi. In order to characterize Korean rice blast fungal isolates, the genomic DNAs were digested with *Alu*I endonuclease and subsequent PCR amplifications using random decamer primers with combinations of microsatellite primers had been carried out. This Alu-Inter SSR technique revealed high polymorphism among the Korean blast fungal isolates. Then, fragments from the Alu-Inter SSR analysis were isolated to be used as probes in Southern hybridization, which also revealed high polymorphism between isolates to distinguish individuals. The sequences of the isolated fragments contained TC/AG tandem repeats interspersed with a 30 bp direct repeat. In gel blot analysis, the isolated TC/AG repeat microsatellite sequences were proved to be useful for characterizing the isolates in blast fungi in addition to the conventional MGR (*Magnaporthe grisea* repeat) probes. One interesting point was that the rice blast fungus derived TC/AG repeat microsatellite sequences were abundant in non-rice blast fungi and plant species, but not in other fungi and yeasts. A discussion on the possible horizontal gene transfer between phytopathogenic fungi and host plants is presented.

Keywords: Alu-Inter SSR; Blast Fungus; Fingerprinting; Horizontal Gene Transfer; Microsatellite.

Introduction

Rice blast disease, caused by *Magnaporthe grisea*, is one of the most damaging crop disease worldwide. Effective breeding for blast resistant rice cultivars has not been successful by the frequent turnover of its pathogenecity. Variations in repeated DNA sequences were demonstrated to cause the genetic variations in several phytopathogens (Farman *et al*., 1996; Levy *et al*., 1991; Roumen *et al*., 1997; Vera Cruz *et al*., 1996). Numerous kinds of repetitive DNA sequences are known to be present in the genome of *M. grisea* (Dobinson *et al*., 1993; Farman *et al*., 1996; Hamer *et al*., 1989; Kachroo *et al*., 1994). Among these, the MGR586 element would be the most extensively used repetitive element for characterizing the population and tagging morphological traits in the blast pathogen (Chen *et al*., 1995; Hamer and Givan, 1990; Levy *et al*., 1991; Xia *et al*., 1993). The host-specific conservation of MGR related repetitive sequences was demonstrated (Hamer *et al*., 1989). Other kinds of transposable elements such as MAGGY and POT2 were also isolated in this fungus (Farman *et al*., 1996; Kachroo *et al*., 1994; 1995). The MAGGY element is scattered throughout the genome and involved in the genomic rearrangement in the blast fungus. Therefore, the high pathogenic variability in the blast fungus might be induced by the frequent transposition of these transposable elements.

DNA fingerprinting has been a central role in the analysis of population structure in *M. grisea* and provided valuable information for understanding the resistance (George *et al*., 1998; Ziegler *et al*., 1995). MGR586 was known to be present abundantly all over the chromosomes

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Abbreviations: MGR, *Magnaporthe grisea* repeat; rep-PCR, repetitive element based polymerase chain reaction; SSR, simple sequence repeats.

in haploid *M. grisea* genome. Since the MGR586 could generate more than 50 resolvable bands in gel blot analysis, it was used to identify the distribution of clonal lineages within and among the pathotypes of blast fungus (Levy *et al*., 1991; Rouman *et al*., 1997). More recently, George *et al*. (1998) showed good correlation between two lineages derived from MGR586 and repetitive element based polymerase chain reaction (rep-PCR).

Since the amount of useful genetic information in a given organism is conditioned by variability, much effort has been focused on the detection of variability at the DNA level. Highly variable regions, consisting of tandemly repeating arrays of short oligonucleotide sequences, have been characterized in diverse eukaryotic genomes. Depending on the sizes, they have been described as minisatellite and microsatellite sequences (Hamada *et al*., 1982; Jeffreys *et al*., 1985). Microsatellites or simple sequence repeats (SSRs) are the stretches of DNA consisting of tandemly repeated mono-, di-, tri-, tetra-, or penta-nucleotide units which are highly variable in the number of tandem repeat between individual in a species (Hamada *et al*., 1982; Powell *et al*., 1996). In a survey of DNA sequences in EMBL and GenBank, Wang *et al*. (1994) demonstrated that all kinds of SSRs are present in every 23.3 kb in plant species. The abundance and high variability of the microsatellites made them highly preferred markers in a variety of different genetic studies in plant and animal species (Goldstein and Pollock, 1997; Gupta *et al*., 1994). However, the reports on the microsatellites in fungal species are scanty. Therefore, this study aimed to isolate microsatellite sequences for genomic fingerprinting the phytopathogenic fungus, *M. grisea* and their homologous sequences in other fungi, yeasts, and plant species.

Materials and Methods

Fungal strains and DNA extraction Rice blast fungi were collected for several years from rice field in Korea and race was determined by the infecting profiles in a set of rice cultivars as shown in Table 1. The fungi were cultured in the Fries medium (30 g sucrose, 5 g ammonium tartrate, 1 g NH_4NO_3, 1 g H_2PO_4, 0.5 g $MgSO_4$, 0.1 g $CaCl_2$, 0.5 g casein hydrolysate in a liter of distilled water) with shaking at 90 rpm for 5 d at room temperature. Then, the fungi containing medium was filtered through Whatman No. 1 filter paper and lyophilized. One gram of the lyophilized mycelium was powdered in a mortar and pestle in liquid nitrogen. The rest of the DNA extraction protocol was the same as that of Scott *et al*. (1993).

Alu-Inter SSR Fungal genomic DNA (1 μg) was digested with *Alu*I and diluted with 500 μl of TE buffer (pH 7.4, 10 mM Tris, 1 mM EDTA). Five microliters of the diluted DNA was mixed to make 25 μl of PCR reaction mixture containing 0.2 μM random primer and 0.4 μM microsatellite primer, 200 μM dNTP, 1 unit of *Taq* polymerase and 2.5 μl of 10× reaction buffer. The random primer sequence was GGGCCCGAGG and microsatellite primer sequences were either one of the $(AG)_8G$, $(AG)_8A$, $(AG)_8C$ or $(TC)_8G$, respectively. After 15 μl of mineral oil was added on top of the reaction mix, PCR reaction was conducted with the following profile; 1 cycle of 90 s at 94°C for full denaturation, 40 cycles of 94°C for 30 s, 42°C for 30 s, 72°C for 90 s, and 1 cycle of 10 min at 72°C for full extension. After PCR reaction, 7 μl of the amplified products were mixed with gel loading buffer (98% formamide, 10 mM EDTA, 0.05% BPB, 0.05% Xylen Cyanol FF), denatured for 5 min at boiled water bath, and separated in a pre-warmed 4.5% denaturing acrylamide gel (acrylamide/bisacrylamide 19:1, 7.5 M urea in 1× TBE) for 3 h at 80 W. Then, the separated DNA fragments were stained with silver staining with Silver Sequenase Kit with supplier's manual (Promega, USA).

DNA cloning and sequencing The amplified and separated fragments were cut out from the gel and mixed with the same reaction mixture as the Alu-Inter SSR reaction to re-amplify the DNA fragments. The re-amplified fragments were cloned into the pGEM-T easy vector (Promega, USA) and transformed into the XL-1 Blue bacterial strain. The nucleotide sequences of the cloned fragments were determined with the LKB automatic DNA sequencer.

Southern hybridization Ten μg of the genomic DNAs were digested with *Eco*RI, *Hae*III, *Alu*I, respectively. Then, the digested DNA was electrophoresed in a 0.8% agarose gel and

Table 1. Infection profiles of the rice blast isolates in a set of rice sultivars.

Cultivar/Rice	KJ101	KJ201	KJ301	KJ401	KI101	KI201	KI315	KI401
Tetep	R	R	R	R	S	R	R	R
Taebaegbyeo	R	R	R	R	S	S	R	R
Tongil	R	R	R	R	S	S	S	R
Yushin	R	R	R	R	S	S	S	S
Kanto 51	S	R	R	R	S	S	R	S
Nongbaeg	S	S	R	R	S	S	R	S
Jinheang	S	S	S	R	S	S	R	S
Nagdongbyeo	S	S	S	S	S	S	S	S

The isolates of KJ and KI types were designated with the infection of japonica type and indica type rices, respectively. R, resistance; S, susceptible.

transferred to Hybond N^+ nylon membrane by the alkaline transfer method. Probes were made either with non-radio labeling system with Gene Image (Amersham International) or ^{32}P-dATP with the random labeling method. Southern hybridization was conducted for 16 h at 65°C with hybridization buffer (0.25 M disodium phosphate, pH 7.2, 1 mM EDTA, 7% SDS). The membranes were then washed with 2× SSC, 1% SDS once at room temperature and twice with the same solution at 65°C (20 min/each). The membranes were further washed at 65°C and room temperature for 10 min each with 0.2× SSC, 1% SDS. Hybridization signals were then detected, either with Southern-Light (TROPIX, USA) for non-radio labeled probes according to the manufacturer's instructions, or directly visualized onto autoradiography of X-ray film for radio labeled probes.

Statistical analysis The presence or absence of specific bands across the isolates in Alu-Inter SSR and gel blots were recorded as 1 or 0, respectively. Dendrogram was constructed on the basis of Nei and Li's calculation (1979); $S_{xy} = 2N_{xy} / (N_x+N_y)$, where N_{xy} refers to the number of bands in common between isolate X and Y. In addition, N_x and N_y denote the total number of bands for isolates x and y, respectively. All the calculations were done using an arithmetic average option in the Numerical Taxonomy System for PC (NTSYS-PC) (Roholf, 1992).

Results

Alu-Inter SSR amplification for blast fungal genomes In order to check the amplified products, they were separated in agarose gels, where the amplified products showed whole smearing patterns which did not permit band recording. However, distinct banding patterns were generated after separation in denaturing PAGE in the size range of 200 to 700 bp and there were many polymorphic bands between fungal isolates (Fig. 1). The number of scorable bands were between 30 to 50 depending on the primer combination and about 15% of the scored bands were polymorphic between isolates, from which 60 polymorphic bands were obtained from a total of 350 bands.

Gel blot analysis We isolated and cloned two fragments from the gel and re-amplified with the same primer combination of $(AG)_8C$ with random primer (GGGCCCGAGG) in order to check whether these bands represent true absence or presence in each isolates. Since the approximate sizes of the isolated fragments were 300 and 660 (fragments designated by arrows in Fig. 1), they were

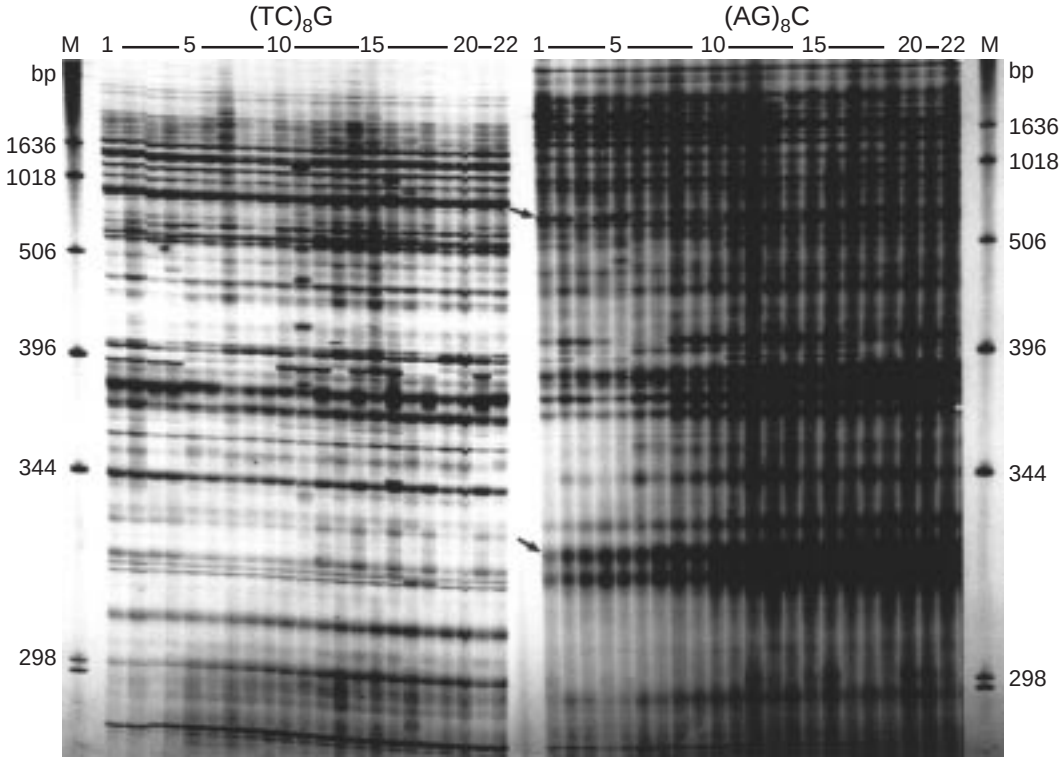

Fig. 1. DNA fingerprinting patterns of the 22 blast fungal races by *Alu*I Inter-SSR analysis in denaturing PAGE gels. The blast fungus isolate in each lane was KJ101 (lane 1), KJ101-91-33 (lane 2), KJ201-89-08 (lane 3), KJ201-89-25 (lane 4), KJ201-90-91 (lane 5), KJ301 (lane 6), KJ301-87-25 (lane 7), KJ301-89 (lane 8), KJ301-90-03 (lane 9), KJ301-92-13 (lane 10), KJ401-89-10 (lane 11), KJ401-89-44 (lane 12), KJ401-89-56 (lane 13), KJ401-7Nak-04 (lane 14), KI101-5-21 (lane 15), KI101-90-12 (lane 16), KI201-86-08 (lane 17), KI315-87-153 (lane 18), KI315b-84-419 (lane 19), KI401-89-12 (lane 20), KI401-90-52 (lane 21), and KI401-93-41 (lane 22), respectively. The arrows indicate the fragments that were isolated for Southern and sequence analyses.

named pKFJ300 and pKFJ660, respectively. While the 660 band was polymorphic among isolates, the 300 band was present in all isolates in the Alu-Inter SSR amplification. Both fragments showed hybridization in all the isolates in gel blot hybridization, indicating that they are highly repetitive in the genome and not specific to any isolates (Fig. 2). Moreover, the hybridization patterns were very similar between two probes, which was anticipated since they were derived from $(AG)_8C$ anchored PCR amplification (data not shown). The distinct hybridization patterns between isolates prompted us to use them in fingerprinting the fungal isolates according to their hybridization patterns. Next, we checked that they were present in other non-rice pathogens of *M. grisea*, which in turn showed that they were present in other *M. grisea* (Fig. 3). The blast fungi derived from other grasses such as common millet, foxtail millet, *Digitaria*, *Setaria* species, and west world grass carried high identity copies of the homologous sequences to the pKFJ660, and the hybridization patterns were distinct from each other. However, the pKFJ660 homologous sequences were either not present or in very small quantities in *Aspergillus*, *Botrytis*, and *Chaetomyum* (Fig. 3). Comparison of the copy numbers and hybridization profiles with those of MGR586 (Hamer *et al*., 1989) revealed that the copy number of the isolated fragments was almost the same as that of MGR586 (Fig. 4).

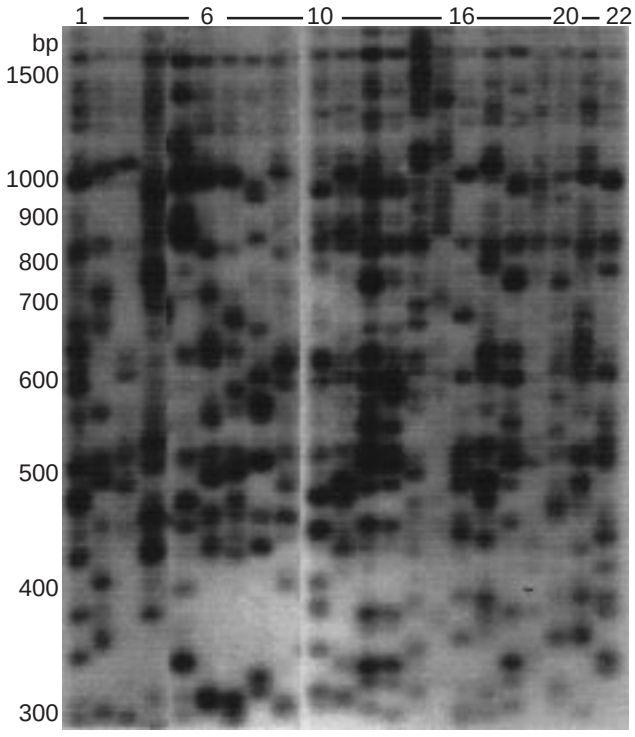

Fig. 2. Sourthern hybridization pattern of the pKJF660 on the 22 *Alu*I digested blast fungal race genomic DNAs. The blast isolate in each lane is the same as in Fig. 1. Note the high polymorphism between isolates.

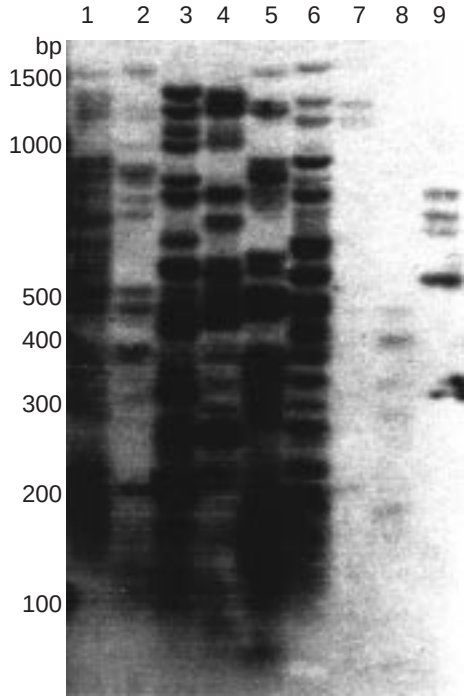

Fig. 3. Southern hybridization pattern of the pKFJ660 on the *Alu*I digested genomic DNAs of several blast fungi, *Aspergillus* (lane 7), *Botrytis* (lane 8), and *Chaetomyum* (lane 9). The host of the blast fungi were common millet (lane 1), foxtail millet (lane 2), *Digitaria sanguinali* (lane 3), KJ301 of the rice blast (lane 4), *Setaria viridis* (lane 5), and west world grass (lane 6), respectively.

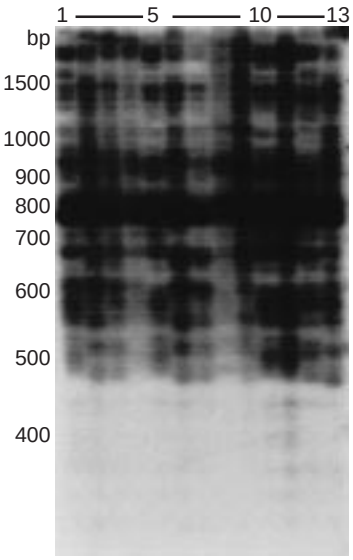

Fig. 4. Comparison of the gel blots hybridized with MGR586. The genomic DNAs were digested with *Alu*I. The blast fungus isolates in each lane was KJ301-92-13 (lane 1), KJ401-89-10 (lane 2), KJ401-89-44 (lane 3), KJ401-89-56 (lane 4), KJ401-7Nak-04 (lane 5), KI101-5-21 (lane 6), KI101-90-12 (lane 7), KI201-86-08 (lane 8), KI315-87-153 (lane 9), KI315b-84-419 (lane 10), KI401-89-12 (lane 11), KI401-90-52 (lane 12), and KI401-93-41 (lane 13), respectively. Note the high identity with polymorphism.

Fingerprinting analysis The pathotype profiles of the blast fungal isolates in a set of differential cultivars is shown in Table 1. The numbers after KJ401-89-56 means that this was one of the isolates collected in the rice field at 1989 and resistant to all the different cultivars except Nagdongbyeo. The numbers designating other isolates are the same as KJ401-89-56. Although the origins of KI101-5-21 and KJ401-7Nak-04 were not clear, they were numbered by the infection profiles on the different cultivars. There were two big lineages at 66% of similar levels in the clustering dendrogram based on profiles of the Alu-Inter SSR and RFLP with pKFJ660 (Fig. 5). The KI typed and KJ typed isolates were not clustered together. In addition, the sub-isolates in each group (e.g. KJ401-89-10, KJ401-89-56, KJ401-89-44, KJ401-7Nak-04, etc.) were not closely clustered. The similarity index ranged from 0.65 between KI315b-84-419 and KJ101 to 0.95 between KJ201-89-25 and KI101-5-21. The average population diversity was 0.75 among the 22 isolates.

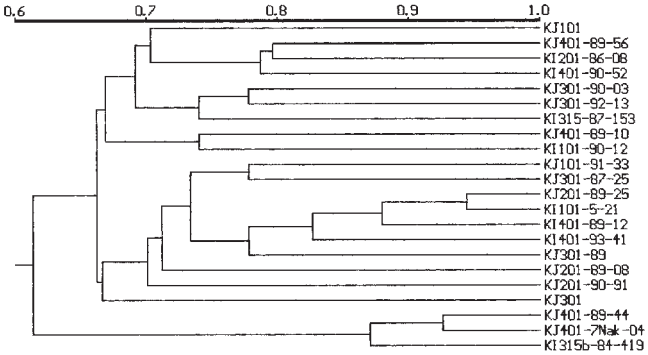

Fig. 5. The clustering dendrogram based on the Alu-Inter SSR fingerprinting and Southern hybridization pattern of the pKJF660 on the 22 blast fungal race genomic DNAs.

Sequences of the isolated fragments In sequence analysis, the actual sizes of the 300 bp and 660 bp bands were revealed to be 312 bp and 660 bp, respectively (Fig. 6). Both fragments consisted of high TC/AG rich microsatellite sequences which were separated by 30 bp direct repeats. Interestingly, the random primer sequence (GGGCCCGAGG), which was used in the original Alu-Inter SSR PCR amplification, was on the border of the 30 bp direct repeat sequence. In the 660 bp fragment, the 30 bp direct repeats were separated with spacers which were highly saturated with TC/AG tandem repeat units. The spacers I, II, III, IV were numbered to indicate the spacer from random decamer primer to the first 30 bp direct repeat sequence, from the first 30 bp direct repeat to the second 30 bp direct repeat, from the second 30 bp direct repeat to third 30 bp direct repeat, and from the third 30 bp direct repeat to $(TC)_8G$ SSR primer site, respectively. The sizes of the spacer I, II, III, IV were 190 bp, 140 bp, 73 bp, and 139 bp, respectively, and these differences were derived from the differences of the number of TC/AG repeat units.

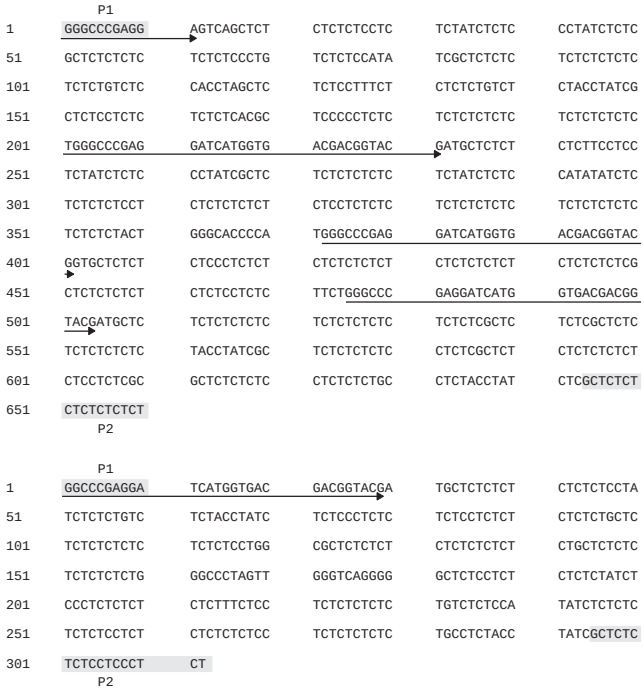

Fig. 6. Nucleotide sequences of pKFJ660 (GenBank Accession No. AF218625) and pKFJ312 (GenBank Accession No. AF218626). P1 and P2 are the sites for primer binding in Alu-Inter SSR. The direct repeat sequences were marked with arrow heads underneath.

The 312 bp fragment also contained the 30 bp direct repeat at one end. Fourteen bp in the flanking region of the $(TC)_8G$ SSR primer in the 312 bp fragment and 660 bp fragment were identical. However, there was an extra TC/AG repeat unit just before the SSR primer in 660 bp fragment. Therefore, the 312 bp and 660 bp fragments might be related ones, causing similar hybridization patterns in gel blot analysis.

Southern blot analysis in plant species Since the pKFJ660 carried TC/AG repeat microsatellite sequences and 30 bp direct repeats, the presence of homologous sequences in plant species was checked by gel blot analysis. Surprisingly, there were very high hybridization signals in various plants including monocots and dicots (Fig. 7). In the survey of short tandem repeats in 54 different plant species, Wang *et al*. (1994) demonstrated that AG/TC repeats were present in about every 302 kb in plant genomes. Screening of the homologous sequences from the genomic libraries in rice and oats is currently being carried out to check the levels of homology between them. Since rice hybridization showed many scorable bands, hybridization patterns in several rice varieties were studied, from which the rice varieties were able to be

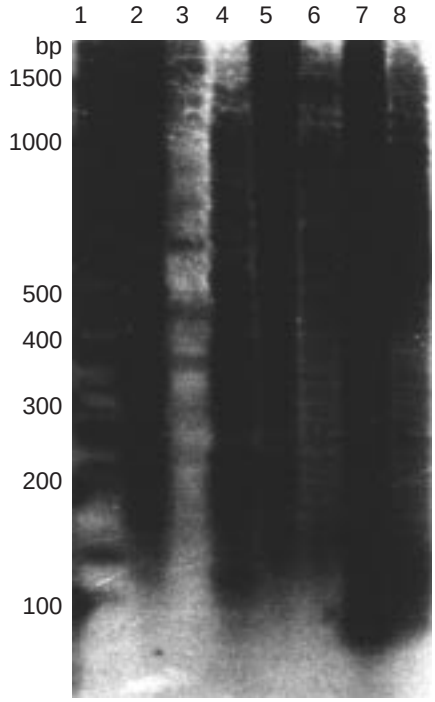

Fig. 7. Southern hybridization pattern of the *Alu*I digested genomic DNAs from several plant species with pKFJ660 as a probe. The plant species were *Arabidopsis* (lane 1), cabbage (lane 2), corn (lane 3), rice (lane 4), barley (lane 5), oats (lane 6), wheat (lane 7), and rye (lane 8), respectively.

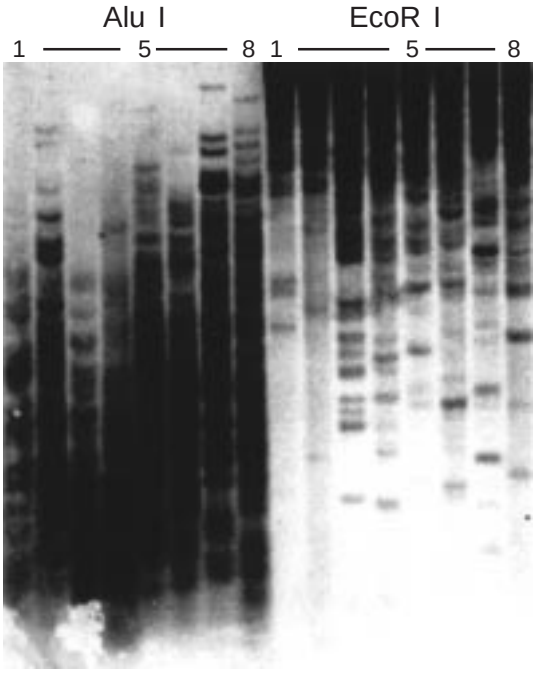

Fig. 8. Southern hybridization pattern of the pKJF660 on *Alu*I and *Eco*RI digested rice genomic DNAs. The rice varieties were Suwon426 (lane 1), Hwaseongbyeo (lane 2), Ilpumbyeo (lane 3), Olbyeo (lane 4), Sobackbyeo (lane 5), Odaebyeo (lane 6), Jinbuolbyeo (lane 7), and Hapkang 21 (lane 8), respectively.

distinguished by the hybridization pattern (Fig. 8). Oligonucleotide sequences of the core repeat of M13 and human minisatellite as probes have been successful for identifying different genotypes in rice cultivars (Ramakrishna *et al*., 1995; Zhou *et al*., 1997). The pKFJ660 will be a very useful probe in identifying different genotypes in rice cultivars.

Discussion

RFLP fingerprinting with MGR586 has been utilized for understanding the population structure and genetic changes in blast fungus in various populations (Borromeo *et al*., 1993; Levy *et al*., 1991; Xia *et al*., 1993). However, the technical complexities associated with RFLP analysis can limit the use of MGR586 fingerprinting analysis in large scaled studies. Recently, George *et al*. (1998) demonstrated a rapid method for population analysis in the blast fungus by rep-PCR (repetitive element-based polymerase chain reaction). Although this method can provide an efficient way to analyze the population structure in fungal species, sequence information is required to ensure the amplification of the repetitive DNA sequences. In our study, we used microsatellite-anchored primer and RAPD decamer to amplify the blast genomic DNA. Prior to the amplification, genomic DNA was digested with *Alu*I endonuclease to increase the efficiency. This Alu-Inter SSR technique produced more than 30 amplified fragments per reaction, which were highly polymorphic between fungal isolates, making them suitable for genome fingerprinting. The Alu-Inter SSR technique is simple and efficient for detecting changes in the genomes of the blast fungus without sequence information. In Southern blot analysis using an amplified fragment, pKFJ660, as a probe, the polymorphism was so high that there were no isolates sharing all the bands. MGR (*Magnaporthe grisea* repeated DNA) sequences have been frequently used to map pathogenicity genes and construct a genetic map for *M. grisea* (Hamer and Givan, 1990; Valent and Chumley, 1991). Therefore, in addition to the MGR sequences, the isolated fragment pKFJ660 could also be a good probe for blast fingerprinting analysis .

Dobinson *et al*. (1993) demonstrated a consequence of the clonal organization of the fungal populations by the amplification pattern of different retro-elements within populations of *M. grisea*. However, there was a discrepancy between the lineages produced by the Alu-Inter SSR with the infection profiles on a set of differential rice cultivars in the current study. One possible reason for this could be that the amplified fragments were mainly composed of microsatellite sequences which were genetically inert. The rice blast isolates in this study were collected for several years in Korea and sub-cultured for several generations in the laboratory. Therefore, the minor changes at DNA level during sub-cultures should not be

ruled out. Hong *et al*. (1996) showed that there was some correlation in the lineages of the KJ type isolates and KI type isolates by RAPD analysis in Korean rice blast isolates. However, they also noted some discrepancies in the lineages produced by MGR586 fingerprinting with the infection profiles with a set of differential cultivars.

Any kinds of microsatellite sequences were known to be present 5×10^3 to 3×10^5 copies per plant genome in a variety of plant species (Wang *et al*., 1994). The sequence of the pKFJ660 showed very high content of TC/AG repeat microsatellite sequences with 30 bp direct repeats. To our knowledge, there is no report on the microsatellite sequences in blast fungus. Therefore, this sequence could be the novel microsatellite sequence in blast fungus. Wierdle *et al*. (1997) demonstrated the genetic instability of GT microsatellite sequences in yeast by the polymerase slippage during replication to ensure the polymorphism in the number of tandem repeat of the microsatellite motifs. Another interesting phenomenon in the sequence of the pKFJ660 would be the 30 bp direct repeats in the flanking regions of the TC/AG repeat microsatellite sequences. Many different retro-element like sequences were known in the blast fungal genome. Valent and Chumley (1991) showed that MGR583, which is related with MGR586, was a LINE-like retro-element. With the discovery of *grasshopper*, a gypsy class retrotransposon in the genome of *M. grisea*, Dobinson *et al*. (1992) argued that transposition and subsequent genetic rearrangement by the retroelement sequences were the main sources of the genetic polymorphism in *M. grisea* since the predominant mode of reproduction of this blast fungus was asexual in nature. Another class of retro-element, MAGGY, was found in the genomes of *M. grisea* by Farman *et al*. (1996). MAGGY was also present in the blast isolates infecting other plant species Graminae with different copy numbers, giving rise to the argument that this sequence was horizontally transmitted to other sub-specific groups.

Horizontal gene transfer was known in various cases between phytopathogenic microbes and plants. Hanekamp *et al*. (1997) showed evidence that the *avrD* in *Pseudomonas syringae* pv. tomato was transferred to *Erwinia carotovora*. Holst-Jensen *et al*. (1999) also noted the possible horizontal gene transfer of group I introns in rDNA between closely related plant pathogenic fungi. P element horizontal transfer between species in the same genus is well known to be the cause of horizontal gene transfer (Quesneville and Ansolabehere, 1997). In animal species, the numerous LINEs were also known to be transferred between species by RNA intermediate (Kordis and Gubensek, 1998). The pKFJ660 sequence in our study might be a case of horizontal gene transfer between related fungi and host species. The reason is not clear why the pKFJ660 homologous sequences are abundant in some plant species, but less abundant or non-existant in other fungi and yeasts. If the transfer was made by the RNA intermediate or transposition, the gene transfer between phytopathogens and host plants could be more frequent than the transfer between different fungi or yeasts in different genera. Gene transfer between plants and micro-organisms was also demonstrated by Bertolla and Simonet (1999), in which they argued that the horizontal gene transfer might promote genome plasticity to permit the micro-organisms to adapt efficiently to any change in their environment.

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