HOKKAIDO UNIVERSITY

Title	Studies on DNA rearrangements in rice blast fungus
Author(s)	PHYO HAN THWIN
Degree Grantor	北海道大学
Degree Name	博士(農学)
Dissertation Number	甲第14652号
Issue Date	2021-09-24
DOI	https://doi.org/10.14943/doctoral.k14652
Doc URL	https://hdl.handle.net/2115/83150
Type	doctoral thesis
File Information	hyo_han_thwin.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Studies on DNA rearrangements in rice blast fungus

（イネいもち病菌における DNA 再編成に関する研究）

Hokkaido University Graduate School of Agriculture

Division of Biosystems Sustainability Science Doctoral Course

Phyo Han Thwin

TABLE OF CONTENTS

Table of contents..…….....i

List of tables…….....iv

List of figures……...vi

List of appendices……xi

CHAPTER ONE - General Overview

1.1. Rice blast disease 2

1.2. Gene-for-Gene theory 4

1.3. Cloning of *AVR-Pia* 8

1.4. *AVR-Pik* gene and the *Pik* gene 9

1.5. Development of molecular markers..... 10

1.6. Research objectives..... 13

CHAPTER TWO - Determination of *AVR-Pik* locus in Ina168 strain

2.1. Characterization of *AVR-Pik* locus in Ina168 strain 17

2.1.1. Materials and methods 19

2.1.2. Next generation sequencing analysis of cosmid clones of Ina168 strain..... 22

2.1.3. Extraction of genomic DNA from Ina168 and Ina168m95-5 strains..... 22

2.1.4. Confirmation of the presence of new sequences by PCR.....24

2.1.5. Results and Discussions 25

2.2. Analysis of the upstream of *AVR-Pik* in Ina168 strain 28

2.2.1. Cloning and analysis of *PvuI* fragment of Ina168 strain..... 28

2.2.2. Materials and methods 28

2.2.3. Analysis of *PvuI* fragment by sequencing analysis..... 31

2.2.4. Results and discussions 32

CHAPTER THREE - *AVR-Pik* deletion mechanism in Ina168m95-5

3.1. Analysis of cosmid clones containing low-copy regions..... 36

3.1.1 Materials and methods 36

3.1.2. Primary Screening 37

3.1.3. Secondary Screening.....40

3.1.4. Tertiary Screening.....41

3.1.5. Fourth Screening.....42

3.1.6. Analysis of cosmid clones by sequencing analysis.....45

3.1.7. Results and discussions.....48

3.2. Analysis of cosmid clones containing Inago2(short) and InagoI 54

3.2.1 Materials and methods 55

3.2.2 Results and discussions 61

3.3. Analysis of 5′ side of the *NheI* fragment of strain Ina168m95-5 66

3.3.1 Materials and methods 67

3.3.2 Results and discussions 70

CHAPTER FOUR - Identification of parasexual recombination in the field isolates of rice blast fungus

4.1. Microsatellite marker amplification around flanking sequences 73

4.1.1 PCR amplification 73

4.1.2. PCR purification and cell ligation 75

4.1.3. Results.....76

4.1.4. Discussions.....77

4.2. Plasmid DNA extraction and cloning of SSR markers flanking regions..... 77

4.2.1 Extraction of plasmid DNA..... 78

4.2.2 Plasmid DNA sequencing analysis 78

4.2.3 Probe preparation for hybridization 78

4.2.4 Digestion with restriction enzymes and purification..... 79

4.2.5 Results 80

4.3. Identification of chromosomal DNA 81

4.3.1 Population of rice blast fungus..... 82

4.3.2 Genotypic exchanging in populations 84

4.3.3 Chromosomal DNA extraction..... 86

4.3.4 Pulse-Field Gel Electrophoresis and Southern Hybridization..... 87

4.3.5 Results 89

4.3.6 Discussion 90

CHAPTER FIVE - General Discussion 92

REFERENCES.....98

APPENDIX.....108

ACKNOWLEDGEMENT 114

LIST OF TABLES

Table 2.1. Oligonucleotides for amplifying each PCR product in the novel region.....…….21

Table 2.2. Composition of reaction mixture for amplifying PCR products in the novel region …….25

Table 2.3. Cycle condition for amplifying PCR products in the novel region.....25

Table 2.4. Oligonucleotides for amplifying Ina168- *Pvu* I fragment..... 30

Table 2.5. Composition of reaction mixture for amplifying Ina168- *Pvu* I fragment....30

Table 2.6. Cycle condition for amplifying Ina168- *Pvu* I fragment.....30

Table 2.7. Oligonucleotides for sequence analysis of Ina168- *Pvu* I fragment.....31

Table 3.1. Oligonucleotides for 1st screening of cosmid clone of Ina168.....37

Table 3.2. Composition of reaction mixture for 1st screening of cosmid clone of Ina168.....38

Table 3.3. Cycle condition for 1st screening of cosmid clone of Ina168.....38

Table 3.4. Oligonucleotides for evaluation of cosmid clone of Ina168.....39

Table 3.5. Composition of reaction mixture for evaluation of cosmid clone of Ina168.....39

Table 3.6. Cycle condition for evaluation of cosmid clone of Ina168.....39

Table 3.7. Composition of reaction mixture for 2nd screening of cosmid clone of Ina168.....41

Table 3.8. Composition of reaction mixture for 3rd screening of cosmid clone of Ina168.....42

Table 3.9. Composition of reaction mixture for 4th screening of cosmid clone of Ina168.....44

Table 3.10. Cycle condition for 4th screening of cosmid clone of Ina168.....44

Table 3.11. Oligonucleotides for sequence analysis of cosmid 37H4.....46

Table 3.12 Oligonucleotides for 1st screening of cosmid clone of Ina168.....55

Table 3.13 Composition of reaction mixture for 1st screening of cosmid clone of Ina168.....55

Table 3.14 Cycle condition for 1st screening of cosmid clone of Ina168.....56

Table 3.15 Composition of reaction mixture for 2nd screening of cosmid clone of Ina168.....57

Table 3.16 Composition of reaction mixture for 4th screening of cosmid clone of Ina168.....59

Table 3.17 Cycle condition for 4th screening of cosmid clone of Ina168.....59

Table 3.18 Oligonucleotides for sequence analysis of cosmid 26F10.....60

Table 3.19. Oligonucleotides for amplifying each PCR product in the novel region..68

Table 3.20. Composition of reaction mixture for amplifying each PCR product in the novel region.....68

Table 3.21. Cycle condition for amplifying each PCR product in the novel region...68

Table 3.22. Oligonucleotides for sequence analysis of each PCR product in the novel region.....69

Table 4.1 Primers used for the amplification of SSR markers flanking regions.....74

Table 4.2. Genotypic characterization of rice blast fungus isolates using microsatellite markers.....83

LIST OF FIGURES

Fig 1.1 Blast lesions on leaf. (a) Rice leaf showing symptom of leaf blast (b) Rice stem nodes symptomatic of infection with rice blast pathogen.....4

Fig 1.2 Interaction between resistance (R) genes and avirulence (Avr) genes.....6

Fig 1.3 Effectors are detected by plant intracellular receptors, known as Resistance proteins (or R-proteins).....7

Figure 1.4 A proposed model of *AVR-Pia* deletion event in Ina168m95-1. *AVR-Pia* deletion event occurred through the homologous recombination between *Occan*^{9E12} and *Occan*^{3A3}.....9

Fig 1.5 Microsatellite agarose patterns of selected susceptible, segregant and resistant lines analyzed with the RM1233*I marker. Susceptible (C101A51) and Resistant (C101LAC) checks.....11

Fig 1.6 Chromosomal location of 13 QTLs for blast resistance in rice. SSR markers are indicated on the right of the chromosomes. Genetic distance (cM) is shown on the left of the chromosomes. Black bars in each chromosome are the location intervals of QTLs for blast resistance with their names on the right (Zhang et al, 2012).....12

Fig 2.1 The homology between *AVR-Pik* flanking region in Ina168 and DNA from Ina168m95-5. Overlapping of cosmids 20, 45 and 49 is indicated in the upper panel. Dotted region (gray) in Ina168 in the lower panel indicate unsequenced region. Shaded regions illustrate the homology

of DNA between Ina168 and Ina168m95-5. The u4 region was predicted as a terminal region of deletion mutation. Dotted lines (black) indicate the homologous region in opposite direction. The location of *Mfe* I site in Ina168 and Ina168m95-5 is indicated.....19

Fig 2.2 The structure of *AVR-Pik* flanking region in Ina168. Overlapping of cosmid 20, 45 and 49 is indicated in the upper panel. *AVR-Pik* gene is highlighted in yellow in the lower panel. The novel region found by Next-generation DNA sequencing is named 'n7 region' and is highlighted with red line.....27

Fig 2.3 Amplification of each PCR product in n7 region. Amplification of each PCR product in n7 region by GoTaq® Green Master Mix. The names of each product are indicated above lanes. All objective fragments were about 1 kb.....27

Fig 2.4 Amplification of Ina168- *Pvu* I fragment. Amplification of Inverse-PCR product by Platinum® PCR Super Mix High Fidelity. The fragment was about 4 kb.....32

Fig 2.5 The homology between *AVR-Pik* flanking region in Ina168 and DNA from Ina168m95-5. *AVR-Pik* flanking region in Ina168 is indicated in the upper panel and DNA from Ina168m95-5 is indicated in the lower panel. Shaded regions illustrate the homology of DNA between Ina168 and Ina168m95-5. Dotted lines indicate the homologous region in opposite direction. The location of *Pvu* I sites in Ina168 is indicated.....34

Fig 3.1 Amplification of the sequence containing *Inago1* and *Pyret* in 1st screening. Cosmid library plate number is indicated above lanes. The objective fragment was 627 bp and was amplified in cosmid library DNA pools No. 31, 37 and 50.....49

Fig 3.2 Amplification of the novel low copy sequence in 1st screening. Cosmid library plate number is indicated above lanes. The objective fragment was 535 bp and was amplified in cosmid library DNA pools No. 31, 37 and 50.....50
Fig 3.3 Amplification of the sequence containing *Inago1* and *Pyret* and the novel low copy sequence in 2nd screening. Cosmid library No. 31 and 37 were used for 2nd screening. The objective fragments were 627 bp and 535 bp and were amplified in 37F, 37G and 37H.....51
Fig 3.4 Amplification of the novel low copy sequence in 3rd screening. 37F, 37G and 37H were used for 3rd screening. The objective fragment was 535 bp and was amplified in 37F4, 37F6, 37G3, 37G4, 37G5, 37G6, 37G7, 37H3, 37H4 and 37H5.....52
Fig 3.5 Amplification of the novel low copy sequence in 4th screening. 37H4 was used for 4th screening. The objective fragment was 535 bp and was amplified in almost every colony.....53
Fig 3.6 Structures of cosmid 37 from Ina168 and DNA from Ina168m95-5. Cosmid 37containing the novel low copy region was obtained by screening Ina168 cosmid library. Dotted region in cosmid 37 indicates unsequenced region. Shaded regions illustrate the homology of DNA between cosmid 37 and Ina168m95-5. The location of *Nhe* I sites in cosmid 37 and Ina168m95-5 is indicated.....54
Fig 3.7 Amplification of the sequence containing *Inago2* (short) and *Inago1* in 1st screening. Cosmid library plate number is indicated above lanes. The objective fragment was 635 bp and was amplified in cosmid library DNA pools No. 26.....62
Fig 3.8 Amplification of the sequence containing *Inago2* (short) and *Inago1* in 2nd screening. Cosmid library No. 26 was used for 2nd screening. The objective fragment was 635 bp and was amplified in every sample.....62

Fig 3.9 Amplification of the sequence containing *Inago2* (short) and *Inago1* in 3rd screening. 26C and 26F were used for 3rd screening. The objective fragment was 635 bp and was amplified in almost every sample.....63
Fig 3.10 Amplification of the sequence containing *Inago2* (short) and *Inago1* in 4th screening. 26F10 was used for 4th screening. The objective fragment was 635 bp and was amplified in 10th colony.....64
Figure 3.11 Structures of cosmid 26 from Ina168 and DNA from Ina168m95-5. Cosmid 26 containing *Inago2* (short) and *Inago1* was obtained by screening Ina168 cosmid library. Dotted region in cosmid 26 indicates unsequenced region. Shaded regions illustrate the homology of DNA between cosmid 26 and Ina168m95-5. The location of *NheI* sites in Ina168m95-5 is indicated.....66
Fig 3.12 The location of PCR primers for amplifying each PCR product in the novel region. All primer pairs were used for amplifying each PCR product in the novel region.....68
Fig 3.13. Amplification of PCR product No. 143 and 144. Amplification of each PCR product by Platinum® PCR Super Mix High Fidelity. The names of each product are indicated above lanes. The objective fragments were about 3.5 kb in both cases.....70
Fig 3.14 The homology between cosmid 37 from Ina168 and DNA from Ina168m95-5. Cosmid 37 from Ina168 is indicated in the upper panel and DNA from Ina168m95-5 is indicated in the lower panel. Dotted region in cosmid 37 indicates unsequenced region. Shaded regions illustrate the homology of DNA between cosmid 37 and Ina168m95-5. The location of *Nhe* I sites in cosmid 37 and Ina168m95-5 is indicated.....71
Fig 4.1 PCR amplification for cloning of SSR marker flanking sequences.....76

Fig 4.2. SSR markers flanking DNA were cloned after plasmid DNA extraction from each markers.....81

Fig 4.3. One example a pair of set of genotypes for recombination candidates.....86

Fig 4.4. Chromosomal DNA separated in the contour-clamped homogeneous electric field (CHEF). The CHEF gel was stained with ethidium bromide.....90

LIST OF APPENDIX

Appendix I. Media and Buffer……109

Appendix II: DNA Damage agents, Antibiotics, and Fungicidal compounds used in this study……112

CHAPTER 1

General overview

CHAPTER 1
General overview

1.1 Rice blast disease

Pyricularia oryzae (teleomorph: *Magnaporthe oryzae*) is the causal agent of the rice blast, the most important disease that affects rice production worldwide (Wang Y et al. 2010). Rice blast has been causing epidemics in all rice-growing regions, and this disease is extremely difficult to control (Wilson and Talbot 2009). Since more than half of the global population depends on rice as a staple food crop, rice blast disease represents a significant factor that impacts upon global food security (Soanes et al. 2012). In Japan, blast disease accounted for 12% of the total area and amount of damage in rice produced in 2016, the highest percentage among all diseases. One of the most effective and economically viable ways of controlling this disease is the utilization of appropriate resistant cultivars that have major resistance genes against the rice blast. (Siluê D, Notteghem JL, Tharreau D *et al*., 1992). The Initial symptoms are white to gray-green lesions or spots with darker borders produced on all parts of the shoot, while older lesions are spindle-shaped (Fig 1a) and lesions can be seen on panicle neck node (Fig 1b) (Donald Groth, LSU AgCenter). It has been hypothesized that the ability to defeat rice *R*-genes may be due to the instability of *Avr*-genes in *M. oryzae*, including deletion and translocation of the genes, the insertion of transposons into the gene or promoter sequence, and point mutations. (Silva JC, Loreto EL, Clark JB et al. 2004).

The gene-for-gene hypothesis shows that a single plant *R*-gene product recognizes a unique avirulence protein. (Flor HH et al. 1971). Pathogenic variants may arise via sexual mating, mutation, or parasexual recombination. Within Japan, mutation and parasexual recombination seem to have been the two major causes of variation in the pathogenicity of *M. oryzae*, because until now, ascospore production from a cross between Japanese field isolates had never been reported. Using selected isolates from a well-characterized field population, low-copy probes were used to identify putative introgressions of DNA from isolates of one lineage into the genomes of isolates from other lineages. In recent years, use of chemical fertilizers has reduced to meet consumer demand for environmentally friendly agricultural products; however, this can lead to an increase in the occurrence of rice blast. Further, in 2016 and 2017, rice blast disease became a serious problem in Bangladesh and India. Moreover, blast disease affects not only rice but also wheat. (Kim et al., 2017). According to rice blast disease has the greatest impact as mentioned above. Thus, monitoring and precise prediction of the occurrence of this disease are important. Early prediction of the disease would be especially helpful for prevention this disease. Moreover, understanding to the disease mechanism is also necessary to sustainable control of rice blast fungus.

(a) (b)

Figure 1. Blast lesions on leaf. (a) Rice leaf showing symptom of leaf blast (b) Rice stem nodes symptomatic of infection with rice blast pathogen. (Donald Groth, LSU AgCenter)

1.2 Gene-for-gene theory

One type of important plant disease resistance, gene-for-gene resistance, is resulted from the interactions between products of the pathogen avirulence (AVR) genes and their matching plant resistance (R) genes. Plants producing a specific R gene product are resistant towards a pathogen that produces the corresponding AVR gene product. Gene-for-gene relationships are a widespread and very important aspect of plant disease resistance (Flor et al., 1971). There is no common structure between avirulence gene products. Because there would be no evolutionary advantage to a pathogen keeping a protein that only serves to have it recognised by the plant, it is believed that the products of AVR genes play an important role in virulence in genetically susceptible hosts. The majority of R gene works according to the gene-for-gene concept and race specific. However, resistance governed by major R genes generally breakdown after some years because of the frequent variation in the blast fungus population, and due to the ability of blast

fungus to adapt by mutating or deleting the corresponding avirulence genes (McDonald and Linde et al. 2002).

Race-specific plant resistance genes have evolved to detect the products of the corresponding avirulence genes and trigger effective resistance to plant pathogens. In nature, the effector molecules encoded by AVR genes were thought to facilitate disease development and were hypothesized to be under selection. It is theorized that new virulent races of the pathogen emerged by genetic modification of AVR genes using diverse mechanisms. The analysis of the AVR genes in naturally-occurring field isolates of a plant pathogen may yield valuable information for the deployment of R genes in field crops (Stukenbrock and McDonald et al. 2009). New molecular tools and available model plant-fungal interactions have improved the understanding of the fungal infection mechanisms.

The fungus *M. Oryzae* is highly variable and often can overcome the deployed resistant cultivars in a short period of time when resistance is dependent on one major R gene. The ability to defeat the R gene has been hypothesized to be due to the instability of AVR in *Magnaporthe oryzae* (Khang et al. 2008). The major R genes, *M. Oryzae* in rice are able to prevent the infection of races of *M. Oryzae* that contain a corresponding avirulence gene (Silue et al. 1992).

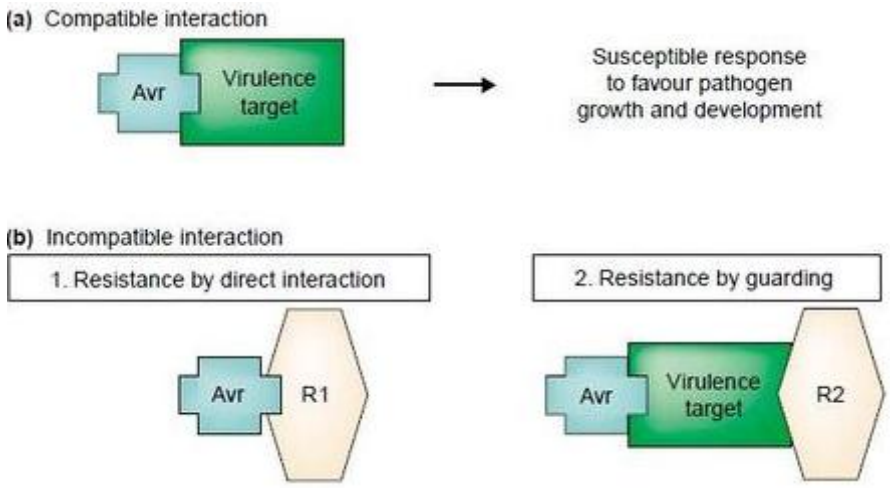


Fig 2. Interaction between resistance (R) genes and avirulence (Avr) genes

The AVR genes in *M. Oryzae* are highly diversified and arer predicted to be capable of rapid changes in nature (Jia et al. 2000). The insertion of retrotransposon in the last exon of ACE1 in a virulent isolate was hypothesized to be responsible for conversion from avirulence to virulence (Fudal et al. 2005). Transposon and telomeric sequences in the genomic regions neighboring AVR leads to the enhanced likelihood of gene loss (Silva et al.2004; Rehmeyer et al. 2006).

The breakdown of genetic resistance by plant pathogen populations is a major limit to the genetic control of crop disease. Increased research efforts have been made to propose breeding strategies, resistance gene or cultivar deployment strategies and cultivation methods that aim at controlling the pathogen evolution over time and its adaptation to the resistant cultivars (Kiyosawa et al. 1982; Finckh et al. 2000; Lindhout et al. 2002; Pink et al. 2002). The evolutionary potential of the pathogen population, when inferred from its population genetic and dynamic characteristics, aids prediction of the risk of pathogen evolution towards virulence and

of breakdown of resistance or other control methods (McDonald & Linde, 2002; Garcia-Arenal & McDonald, 2003).

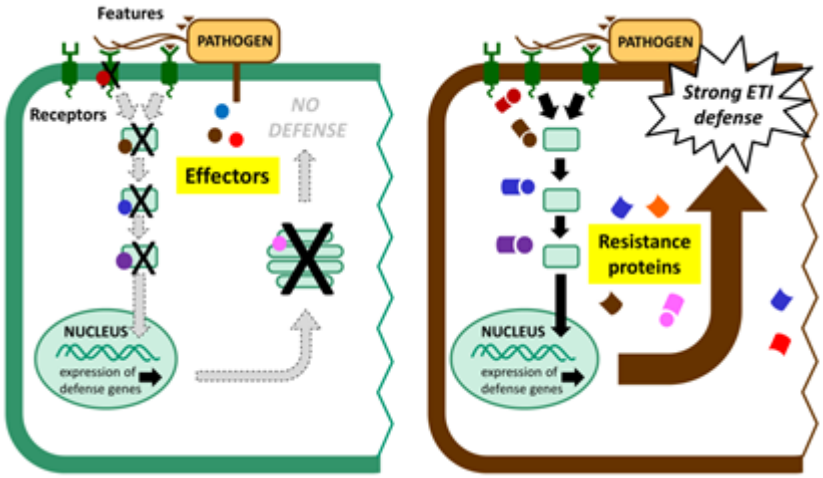


Fig 3. Effectors are detected by plant intracellular receptors, known as Resistance proteins (or R-proteins)

To date, about 100 quantitative blast R genes have been detected in rice, and about 22 R genes have been successfully cloned and characterized (Sharma et al., 2012, Ashkani et al., 2016). The gene-for-gene hypothesis shows that a single plant *R*-gene product recognizes a unique avirulence protein. A large number of studies have provided evidence that some AVR proteins can be recognized by plant R proteins. A strong and rapid immune response follows this recognition. Rice cultivars carrying single R gene for a specific pathogen race often become susceptible over time due to the appearance of new virulent races.

Indeed, AVR proteins produced by the pathogen directly or indirectly activate plant defence ststems upon recognition mediated by matching plant R proteins (Keen et al. 1990; Van Der

Biezen and Jones et al. 1998), such as a localized host-cell death termed the hypersensitive response. These highly specific interactions occur in a gene-for-gene gentic model, in which the absence or the lack of function of either or both these genes will result in disease instead of resistance (Flor et al. 1971). Cloning of R genes and AVR genes in different plant species and microbial pathogens, respectively, has significantly improved our knowledge of the molecular and biochemical bases of the gene-for-gene interactions (Hammond-Kosack and Parker et al. 2003). Understanding of a genetic identity of new M. *oryzae* race is important for accurate employment of rice cultivars with different R genes.

1.3 Cloning of *AVR-Pia*

In our laboratory, cloning of a non-pathogenic gene, AVR-Pia, from a Japanese rice blast fungus, Ina168, was carried out. A chromosome map was constructed from the cross between Ina168 and Guy11, but the discrepancy between the map and the actual genome structure indicated that cloning of the *AVR-Pia* gene by chromosome walking was difficult (Sone, 1997a, Fukiya, 2001a, Fukiya S. et al. 2001b). A host-specific mutant of rice blast fungus Ina168, which has acquired the ability to infect rice cultivar Aichi-Asahi (Pia), was isolated by cloning the Ina168m95-Pia gene. Based on the DNA sequence of cosmid clone 46F3, the putative region of the *AVR-Pia* gene was introduced into strain Ina168m95-1, and the complementation of the *AVR-Pia* gene was confirmed by confirming disease symptoms based on pathogenicity tests. The complementation of the *AVR-Pia* gene was confirmed by pathogenicity tests. The complementation of the *AVR-Pia* gene was confirmed by pathogenicity test. Since strain Ina168m95-1 was found to be lacking the *AVR-Pia* gene, the complementation of the *AVR-Pia*

gene with other clones, including the cosmid clone 46F3, was confirmed. The *AVR* gene *AVR-Pia* was cloned from the Japanese rice blast fungus Ina168 strain. The DNA sequences of Ina168 strain and Ina168m95-1, which lack the *AVR-Pia* gene, two cosmid clones 9E12 3A3 were analyzed based on the DNA sequences around *AVR-Pia* in Ina168. As a result, we could predict the mutation mechanism that transposons Occan^{9E12} and Occan^{3A3} caused homologous recombination, and the region between them were looped out, resulting in the deletion of 3 copies of *AVR-Pia*.

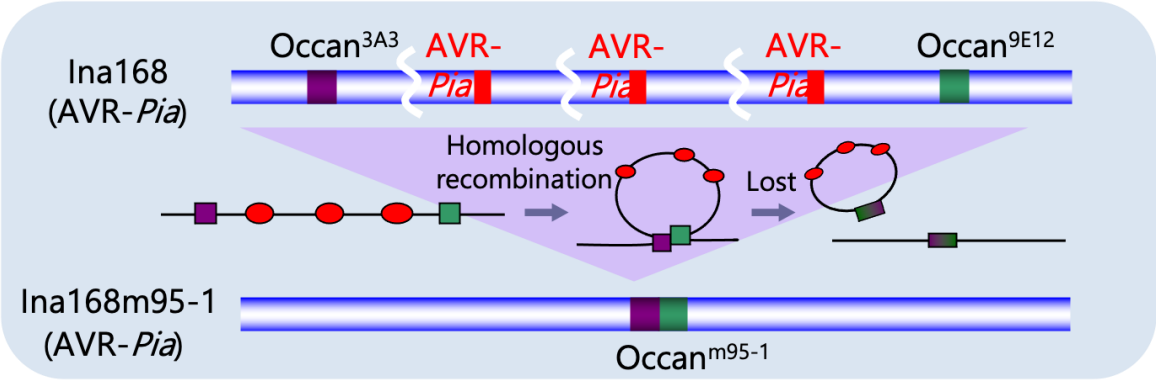


Figure 4. A proposed model of *AVR-Pia* deletion event in Ina168m95-1. *AVR-Pia* deletion event occurred through the homologous recombination between *Occan*^{9E12} and *Occan*^{3A3}.

1.4 *AVR-Pik* gene and the *Pik* gene

The AVR-Pik gene and its corresponding Pik gene have multiple alleles due to DNA substitutions caused by amino acid mutations. As many rice breeding programs have been carried out since the 1970s, it has become clear that rice breeding has become a major source of income. In the 1970s, many rice breeding programs were conducted, and various Pik alleles

introduced from China (Pik, Pik m, Pik s), India (Pik h), Pakistan (Pik p), etc. were utilized. On the other hand, from that time on, gradual mutations were found in the alleles of *AVR-Pik*. Furthermore, in 2009, the genomic information of the *AVR-Pik* gene (342 bp) was released, and it was found that it encodes 113 amino acids including the secretory signal (21 amino acids). Nowadays, 11 alleles of *AVR-Pik* have been found. Some of these were null allele by the insertion of transposon Pot3 or stop codons, but mainly by substitution of amino acids 46, 47, 48, and 78. The gene mutation in *AVR-Pik* is thought to be due to the selective pressure of alleles in *Pik*, and it can be said that the *AVR-Pik* and *Pik* genes have undergone co-evolution.

1.5 Development of molecular markers

The identification and isolation of additional host blast resistance (R) genes and pathogen avirulence gene are now required to deepen understanding of molecular mechanisms involved in the host-pathogen interaction (Valent et al. 1990). Generally R genes are identified in land races, cultivars and wild rice collections using differential physiological races of *Magnaporthe oryzae* (Tanksley et al. 1997). With fine mapping and cloning of many blast resistance genes, many PCR-based markers have been developed to screen and identify different blast resistance genes. DNA markers closely linked to a blast R gene that confers resistance to a particular race of the pathogen can be effectively employed for marker assisted selection, which is much faster than traditional pathogenicity assays. Accurate identification of a particular R gene in diverse elite germplasm using DNA markers and differential blast races is an essential step for ensuring the accuracy of R gene utilization

in using marker assisted selection for different rice bredding programs (Roy-Chowdhury et al. 2012).

Many rice varieties with complete resistance to *Magnaporthe* have been developed, but in many cases this resistance has been breakdown within a few years of the initial cultivation owing to the emergence of stronger virulent isolates of rice blast fungus (Han et al. 2001). Simple sequence repeat (SSR) or microsatellite is a PCR-based molecular marker, which has many advantages including generally codominant, highly reproducible, highly polymorphic and abundant in animal, plant and microbe genomes (Maroof et al. 1994). Microsatellite marker has become the most popular molecular marker system and has been extensively utilized in genetic mapping and gene tagging (Wang et al. 2005).

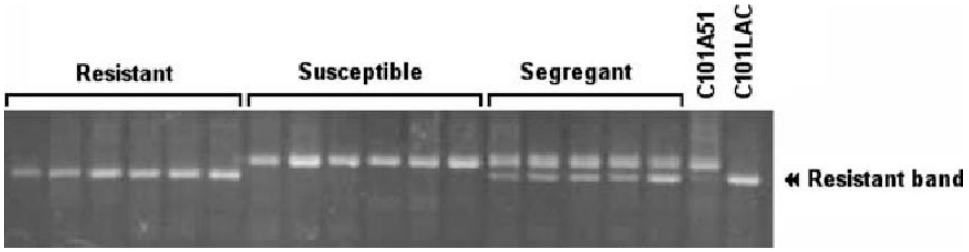


Fig 5. Microsatellite agarose patterns of selected susceptible, segregant and resistant lines analyzed with the RM1233*I marker. Susceptible (C101A51) and Resistant (C101LAC) checks

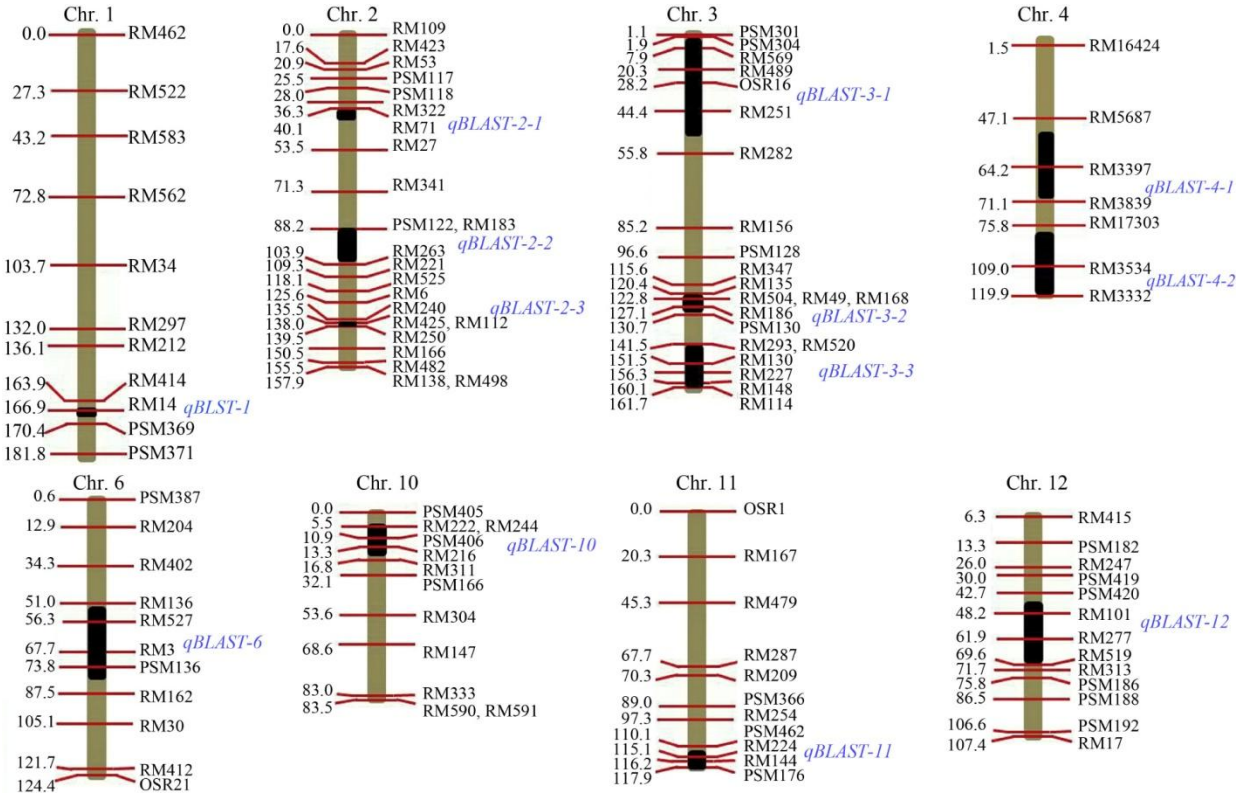


Fig 6. Chromosomal location of 13 QTLs for blast resistance in rice. SSR markers are indicated on the right of the chromosomes. Genetic distance (cM) is shown on the left of the chromosomes. Black bars in each chromosome are the location intervals of QTLs for blast resistance with their names on the right (Zhang et al, 2012).

In recent years, efforts have been made to develop SSR markers for constructing genetic maps in *Magnaporthe* (Brondani et al. 2000) first developed 24 SSR markers for genetic analysis (Kaye et al. 2003) integrated 23 SSR markers into the genetic map constructed by Nitta et al. 1997. More recently, Wang (et al. 2005) constructed a genetic map consisting of 121 SSR markers for mapping avirulence genes. Rice varieties with a number of alleles in common with any specific resistance might have a similar blast R gene, and understanding the natural diversity

at the specific gene is important for incorporation of specific R gene using DNA marker into rice breeding program (Jia et al. 2003). Conventional breeding is generally contingent upon the phenotype of artificial identification and performance of field resistance, therefore, newly emerged virulent race of pathogen cannot be easily recognized and the release of improved varieties cannot be certain (Zhang et al., 2006).

1.6 Research objectives

Rice blast is the most destructive disease of rice caused by the fungal pathogen *Pyricularia oryzae* (Cavara), and has been regarded as a principal model organism for the study of molecular mechanisms of fungal diseases and host-pathogen interactions in plants (Valent et al. 1990; Dean et al 1997). Although many fungal and bacterial plant pathogens are considered to be strictly asexual, a high degree of genetic variability is often observed in their populations (Ochman et al. 1984). A genetically recombining population in which each genotype is transient (depending on the time between recombination events) the entire population and future variants must be considered as the targets for control. Understanding the potential and occurrence of genetic recombination is vital in formulating effective strategies for managing pathogen populations. Development of resistant rice cultivars is the preferred management approach for blast.. Although the sexual stages of *Magnaporthe* can be produced between highly selected isolates in the laboratory, evidence of sexual mating has not been observed in nature (Notteghem et al.1992).

Resistance of rice plant to *P. oryzae* is known to follow gene-for-gene theory, where major resistance (R) gene is effective in preventing infection by a race of *P. oryzae* harboring the

corresponding avirulence (AVR) gene. Although cultivation of resistance cultivars with R genes has been successfully applied for management of rice blast diseases, the strong selection pressure by R genes and rapid pathogen evolution have caused the repetitive breakdown of the resistance. A loss function in an AVR gene allows the pathogen to avoid induction of resistance in the cultivar to gain pathogenicity to the cultivar, leading to an emergence of a new pathogenic race. AVR genes are responsible for production of effectors, which required for the effective infection, and it is suggested that AVR genes should be recovered after the deletion, from the analysis of genomic locus of AVR genes. One fact that the chromosomal rearrangements as a major mechanisms for host-specific adaptation. Parasexual recombination is considered to be the most plausible mechanism for AVR gene recovery, but no evidence was found for the parasexual exchange in the field conditions.

Therefore, the present study is trying to elucidate the mutation mechanism in the Ina168m95-5 strain, which is an *AVR-Pik* spontaneous mutant strain of the Ina168 strain owned by our laboratory. The previous study revealed that the Ina168 strain has one copy of *AVR-Pik* and that the mutant Ina168m95-5 lacks *AVR-Pik.* In addition, the previous study clarified both parent sequence and mutant sequence of *AVR-Pik* locus, therefore the mutation mechanism, which includes complicated rearrangement with at least 5 homologous recombinations. In this research, first we tried to confirm the structure of *AVR-Pik* locus in Ina 168 m95-5 because we determined them from short contigs. This is because there are many repetitive sequences around the *AVR* gene, therefore it is difficult to determine the sequence. It is also difficult to analyze with the next-generation sequencing.

On the other hand, we attempted the identification of the genetic recombination in rice blast field isolates by using simple sequence repeat (SSR) markers, which are widely distributed throughout the genome, and the repeat numbers are highly variable and these loci can be used as polymorphic markers. Therefore, exchange of markers might have been caused by the parasexual recombination within the population of rice blast fungus.

CHAPTER 2

Determination of *AVR-Pik* locus in Ina168 strain

CHAPTER 2

Determination of *AVR-Pik* locus in Ina168 strain

2.1 Characterization of *AVR-Pik* locus in Ina168 strain

The purpose of this study was to elucidate the mutation mechanism in Ina168m95-5, an avr-pik spontaneous mutant of Ina168 in our laboratory. In the previous study (Funabiki, 2015), we found that strain Ina168 has one copy of *AVR-Pik,* and strain Ina168m95-5 lacks *AVR-Pik*. We also found that Ina168 has one copy of *AVR-Pik* and that Ina168m95-5 lacks *AVR-Pik*. Next, we screened three cosmid libraries of Ina168 from our own cosmid library. Next, we sequenced three cosmid clones screened from the cosmid library of strain Ina168, and determined the nucleotide sequence of about 39 kb around the *AVR-Pik*. The sequence of the Ina168m95-5 strain was then analyzed based on the sequence information. The deletion of *AVR-Pik* involved a complex reorganization of the surrounding region.

However, the details of the deletion mutation mechanism of Ina168 strain were not clarified due to the presence of a region that is difficult to sequence, so we attempted to solve this problem by carefully determining the nucleotide sequences of both strains. Figure 2.1 shows the nucleotide sequences of Ina168 and Ina168m95-5 strains determined in previous studies. It is important to note that the area around the AVR gene contains many repetitive sequences, making sequencing difficult. The mutation mechanism of *AVR-Pia* was elucidated by careful sequencing

of the cosmid clones. As described above, we also determined the sequence of approximately 39 kb around *AVR-Pik* using cosmid clones. In addition, since the next-generation sequencing analysis of strain Ina168m95-5 is equally difficult, previous studies have used self-renewal of restriction enzyme-digested fragments of genomic DNA followed by Inverse-PCR to clone unknown sequences and determine their sequences. In the previous study, we used self-ligation of restriction enzyme-digested fragments of genomic DNA and subsequent Inverse-PCR to clone unknown sequences and analyze their sequences.

However, in order to clarify the mutation mechanism, we need pathogenic mutant strains with a clear origin. In this study, we used Ina168 strains. The strain Ina168m95-5 used in this study is a mutant of Ina168 strain that has acquired the ability to infect the rice variety *Pik*-m. The mutation mechanism is thought to be similar to that of field mutations. As the collapse of resistance due to mutation of non-pathogenic genes has become a problem, clarification of one of the mutation mechanisms at the molecular level is important for the future control of blast. In this study, we investigated the mutation mechanism of a non-pathogenic gene in the field.

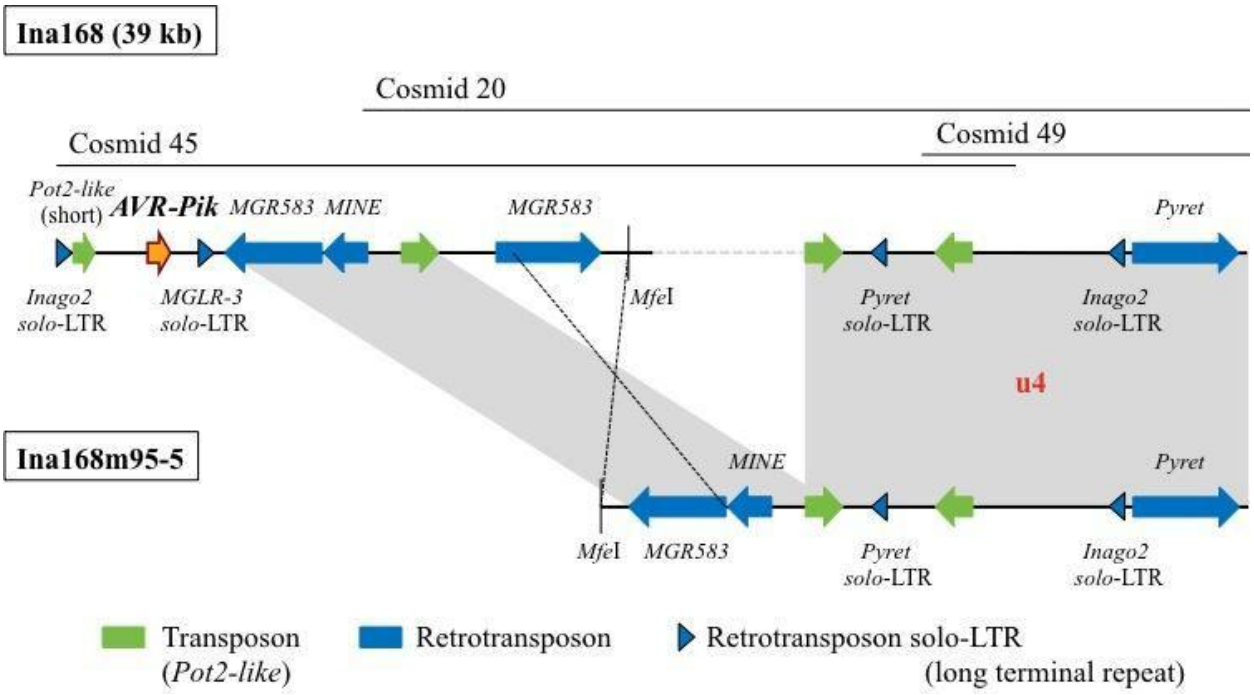


Fig 2.1 The homology between *AVR-Pik* flanking region in Ina168 and DNA from Ina168m95-5

Overlapping of cosmids 20, 45 and 49 is indicated in the upper panel. Dotted region (gray) in Ina168 in the lower panel indicate unsequenced region. Shaded regions illustrate the homology of DNA between Ina168 and Ina168m95-5. The u4 region was predicted as a terminal region of deletion mutation. Dotted lines (black) indicate the homologous region in opposite direction. The location of *Mfe* I site in Ina168 and Ina168m95-5 is indicated.

2.1.1 Materials and methods

(1) Fungus and bacterial strain

Rice blast fungus Ina168 strain is race number 101.1 and contains non-pathogenic genes such as AVR-Pii, Pia, Piz, Pita 2, Pib, and Pit in addition to *AVR-Pik*. Additional spontaneous

mutants Ina168m95-2~5 were isolated from lesions formed on the rice variety Tsuyuake, which possesses the R gene *Pi-k*^{m}. Ina168m95-6 was isolated from rice cultivar Kanto 51, which possesses the R gene *Pi-k* by spray inoculation of strain Ina168. Ina168m95-2~6 were therefore considered as spontaneous mutants of *AVR-Pik*, which corresponds to the R gene *Pi-k*.

2YEG liquid medium was sterilized by autoclaving (121 ºC, 20 minutes) after volume reduction with distilled water. The samples were prepared at each pH using Tris-HCl (pH 7.5, 8.0, 8.5) and distilled water, and then sterilized by autoclaving (121ºC, 20 min). Prepared to each pH using NaOH, volume fixed using distilled water, and sterilized by autoclaving (121ºC, 20 min). The volume was determined using distilled water, and then sterilized by autoclaving (121ºC, 20 min). Phenol (phenol, crystal, for nucleic acid extraction, Wako Pure Chemical Co. 0.1 % 8-Quinolinol was added to the solution, and the mixture was well stirred and suspended. The mixture was suspended. After suspension, the mixture was allowed to stand, the aqueous layer was removed, and Tris-HCl solution was added again. After suspension, the mixture was allowed to stand, and the aqueous layer was removed. This procedure was repeated several times, and finally the aqueous layer was replaced with TE solution (pH 8.0) and stored at 4ºC in the dark. Ribonuclease A Type I-A (Ribonuclease I; EC3.1.27.5) (SIGMA) was dissolved in distilled water to a concentration of 20 mg/mL. The DNase was inactivated in boiling water (5 min) and stored at -20ºC. The volume was determined using distilled water, and then sterilized by autoclaving (121ºC, 20 minutes). The above composition was determined using distilled water to make 50 × TAE. The solution was diluted 50 times with distilled water to obtain 50 × TAE. Ethidium bromide (Wako Pure Chemicals) was dissolved in distilled water to 0.5 μg/mL.

(2) Cosmid clones 20, 45, 49 of Ina168 strain

In our previous study, we screened cosmid clones around *AVR-Pik* from cosmid libraries No. 1 to 50 (96 samples each) of Ina168 strains owned by our laboratory (Kito, 2003). The cosmid library No. 1 to 50 was constructed by transforming each cloned cosmid clone and incubating it at -80°C. The cosmid library No. 1 ~ 50 consists of 4,800 Escherichia coli DH10B strains (96-well plates × 50) that were transformed from each cloned cosmid clone and stored as glycerol stocks at -80°C. The 96-well plate consists of 8 rows (A to H) and 12 columns (1 to 12). For example, cosmid clone 9E12 represents the clone contained in the ninth plate, row E, column 3 of the cosmid library. For example, cosmid clone 9E12 represents the clone in the ninth plate, row E, column 3 of the cosmid library. Since the plate for cosmid 45 was lost, the solution of the DNA pool of the 45th plate was transferred to the *E.coli* TOP10 strain (Invitrogen) and screened by colony PCR, which specifically amplifies *AVR-Pik*.

(3) Primers Construction

Primers for the preparation of each DNA region are shown in Table 2.1.

Table 2.1. Oligonucleotides for amplifying each PCR product in the novel region

No.	Primer name	Sequence (5′→3′)
133	7001F	CCGATGCTCTCAACCGACAT
	INAGO-F5	CTGCTGAACAAAACTATCCGATTCA
134	7001F	CCGATGCTCTCAACCGACAT
	INAGO-F5-F	TAACGGTACCCGAGACTACCCA
135	7001F	CCGATGCTCTCAACCGACAT

	INAGO-F8	AGACGTTGCAATATCAGGTATCGTG
136	6488F	GTAACCATGCCCAGGTGGC
	INAGO-F5-F	TAACGGTACCCGAGACTACCCA
137	6488F	GTAACCATGCCCAGGTGGC
	INAGO-F8	AGACGTTGCAATATCAGGTATCGTG
138	5993F	CAGTTCGTGCGAATCAATTTACTC
	INAGO-F5-F	TAACGGTACCCGAGACTACCCA
139	5993F	CAGTTCGTGCGAATCAATTTACTC
	INAGO-F8	AGACGTTGCAATATCAGGTATCGTG

2.1.2 Next generation sequencing analysis of cosmid clones of Ina168 strain

Next-generation sequencing analysis of cosmids 20, 45, and 49, which are cosmid clones around AVR-Pik screened from the cosmid library of Ina168 strains owned by our laboratory (PacBio RS II, De novo assembly, Macrogen Japan). Genetyx Ver.12 (Zenetics) was used to confirm the homology of the sequences with those determined in previous studies. We also performed BLAST searches. BLAST search was also conducted.

2.1.3 Extraction of genomic DNA from Ina168 and Ina168m95-5 strains

(a) Culture of the strains

The strains were inoculated into 2YEG liquid medium (100 mL) in a shaking flask (500 mL volume) and incubated at 27°C for 5 to 10 days (100 rpm, BIO-SHAKER BR-15, TAITEC).

(b) DNA extraction

The fungus was filtered under reduced pressure using a Buchner funnel on filter paper (No.1, ADVANTEC) and washed several times with distilled water. The fungus was then washed several times with distilled water. The fungus was wrapped in aluminum foil, frozen at -80°C, and lyophilized overnight (VD-500F, TAITEC). After drying, 40 mg of the dried fungus was placed in a 2 mL tube for a multibead shocker with a metal cone. After drying, 40 mg of dried bacteria were placed in a 2-mL tube with a metal cone for a multibead shocker (MB455U (S), Yasui Instrument) at 2,500 rpm for 30 s (ON Time 30 s, OFF Time 0 s) at room temperature. After grinding, 500 μL of DNA extraction buffer solution was added, and the mixture was stirred well using a vortex mixer. Then, 350 μL of phenol was added. After grinding, 500 μL of DNA extraction buffer solution was added, and the mixture was stirred well using a vortex mixer. In addition, 150 μL of chloroform was added. After mixing, 15,000 μL of chloroform was added. After mixing, the mixture was centrifuged at 15,000 rpm for 1 hour at 4˚C (TX-160, Tomy Seiko). The upper layer was obtained by centrifugation (TX-160, Tomy Seiko). The supernatant was removed and 50 μL of RNase A solution (20 mg/mL) was added. The supernatant was removed, and 50 μL of RNaseA solution (20 mg/mL) was added and kept at 37°C for 60 min using a heat block. The supernatant was removed and 50 μL of RNaseA solution (20 mg/mL) was added. The supernatant was separated by centrifugation under the same conditions for 5 minutes. The supernatant was removed. The supernatant was removed, and 500 μL of phenol was added, stirred well using a vortex mixer, and centrifuged in the same manner. The supernatant was removed and 250 μL of phenol was added. The supernatant was removed and 250 μL of phenol and chloroform were added. The supernatant was removed, and 250 μL of phenol and chloroform were added, stirred well using a vortex mixer, and centrifuged in the

same manner to obtain the upper layer. The supernatant was removed. The supernatant was removed, and 500 μL of chloroform was added, stirred well using a vortex mixer, and centrifuged in the same manner. The supernatant was removed and the upper layer was obtained by centrifugation. The supernatant was removed and 0.54 times the volume of isopropanol (2-propanol) was added. The supernatant was removed and 0.54 times the volume of isopropanol (2-propanol) was added, mixed well, and centrifuged under the same conditions for 10 minutes. The supernatant was discarded, and the resulting pellet was rinsed with 70 % ethanol and centrifuged in the same manner to obtain a pellet. The pellet was then dried at reduced pressure using a concentration centrifuge. After drying, 50 μL of TE solution was added to resuspend the DNA, and the mixture was allowed to stand overnight. After that, the concentration was measured by A 260 (NanoDrop™ Lite UV-Vis Spectrophotometer).

2.1.4 Confirmation of the presence of new sequences by PCR

PCR was performed using genomic DNA of Ina168 and Ina168m95-5 strains or cosmids 20 and 45 as templates. The reaction was performed in GoTaq® Green. GoTaq® Green Master Mix (Promega) was used for the reaction. The primers (Table 2.1) correspond to each DNA region when the novel sequence identified by the next-generation sequencing analysis was divided into approximately 1 kb each. The primer (Table 2.1) was used to amplify the PCR products No. 1 to 8 corresponding to each DNA region of the new sequence identified by the next-generation sequencing analysis. The reaction composition and conditions are shown in Table 2.2 and Table 2.3. The reaction was performed using the Gene Amp® PCR system 9700 (Applied Biosystems). After the reaction, 5 μL of the PCR product was used for electrophoresis

on a 1 % agarose gel (H14, TAKARA, dissolved in 1 × TAE). Electrophoresis with (1 × TAE, 135 V, i -MyRun.N, Cosmo Bio), and then immersed in ethidium bromide solution and stained to confirm whether DNA fragments were amplified or not under UV irradiation.

Table 2.2. Composition of reaction mixture for amplifying PCR products in the novel region

Component	Volume (μL)
GoTaq ® Green Master Mix (2 ×)	10.0
primers 10 μM *	0.2
10 μM *	0.2
template DNA **	1.0
SDW	8.6
Total	20

Table 2.3. Cycle condition for amplifying PCR products in the novel region

Step	Reaction	Temperature	Time	Number of cycle
1st	Predenaturaion	95ºC	2:00	1
2nd	Denaturation	95ºC	0:30	35
	Annealing	55ºC	0:30	
	Polymerization	72ºC	1:00	
3rd	Polymerization	72ºC	5:00	1

2.1.5 Results and discussions

(1) Next generation sequencing analysis of cosmid clones of Ina168 strain

The results of next generation sequencing analysis showed that 5, 214, and 21 contigs were generated from cosmids 20, 45, and 49, respectively. Genetyx Ver.12 (Genetics) and BLAST search were used. As a result of assembly based on Genetyx Ver.12 (Genetix) and BLAST search, one contig was found, and the contig was assigned to Ina168. This leads to a single contig, and the sequence of 48,910 bp around *AVR-Pik* of Ina168 strain was determined (Figure 2.2, Ina168 sequence (48,910 bp)). The length of the region, which was difficult to sequence in previous studies, is 5,960 bp. The n7 region contains 5,619 bp from the MGR583 side and 5,619 bp from the Pot2-like side. The n7 region includes a low-copy region of 5,619 bp from the MGR583 side (Ina168 sequence (48,910 bp), 20,698 ~ 26,316 bp). In this study, we found that a part of the reverse transcriptase gene (Appendix Ina168 sequence (48,910 bp), 26,317 ~ 28,656 bp), Inago1 solo- LTR (Ina168 sequence (48,910 bp), 28,658 ~ 28,829 bp). In addition, we found that there was no evidence for the presence of the nago1 solo- LTR in cosmid 45. In addition, we identified 4,106 bp (Ina168 sequence (48,910 bp), 44,805 ~ 48,910 bp) of Pyret in cosmid 45.

(2) Confirmation of the presence of novel sequences by PCR

Genomic DNA of Ina168 and Ina168m95-5 strains or cosmids 20 and 45 were used as templates, and PCR was performed using primers that amplify PCR products No. 1 to 8 corresponding to each DNA region when the n7 region is divided into approximately 1 kb each. The PCR was performed using primers that amplified PCR products No. 1 to 8 corresponding to each DNA region when the n7 region was divided into approximately 1 kb.

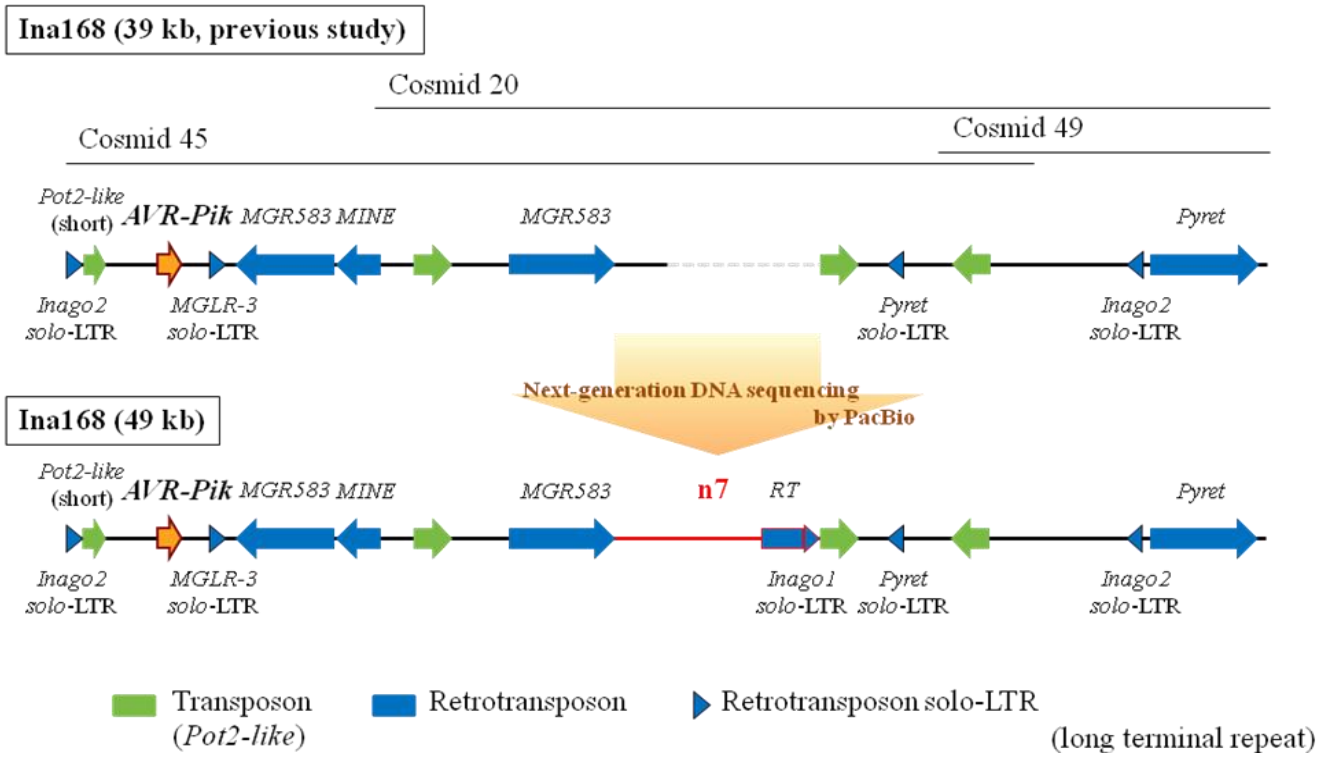


Fig 2.2 The structure of *AVR-Pik* flanking region in Ina168

Overlapping of cosmid 20, 45 and 49 is indicated in the upper panel. *AVR-Pik* gene is highlighted in yellow in the lower panel. The novel region found by Next-generation DNA sequencing is named 'n7 region' and is highlighted with red line.

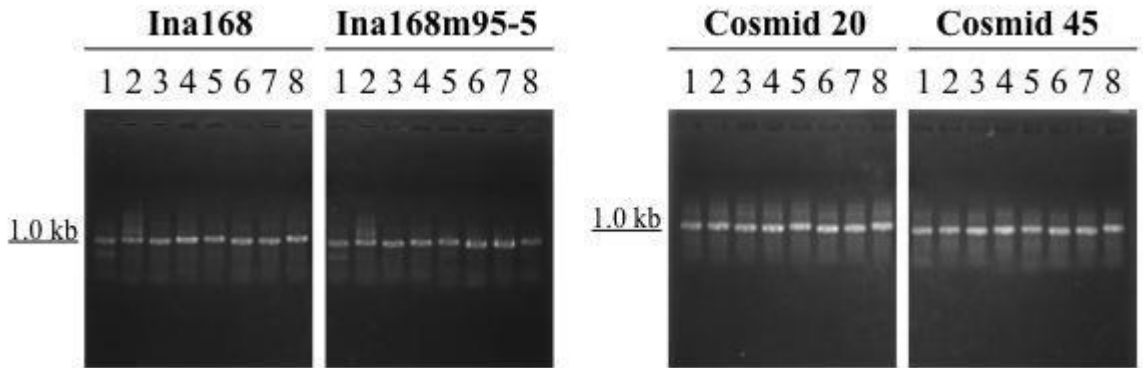


Fig 2.3 Amplification of each PCR product in n7 region

Amplification of each PCR product in n7 region by GoTaq® Green Master Mix. The names of each product are indicated above lanes. All objective fragments were about 1 kb.

As a result, a band of approximately 1 kb was detected in each DNA template (Figure 2.3). This suggests that the n7 region, including the 5,960 bp region identified by next-generation sequencing, is indeed present in strain Ina168 and is also conserved in strain Ina168m95-5. Therefore, if the n7 region is also conserved in Ina168m95-5, it is likely that it is also conserved in Ina168 as well. We predicted that the n7 region would be adjacent to the downstream of MGR583 as in Ina168.

2.2 Analysis of the upstream of AVR-Pik in Ina168 strain

2.2.1 Cloning and analysis of *Pvu I* fragment of Ina168 strain

Based on the sequence information, we screened and analyzed new cosmid clones from the cosmid library of Ina168 and identified homologous regions. However, there was no homology with the approximately 49 kb sequence of determined-Ina168 strain, and therefore, it could not be linked to a single contig. We further analyzed the upstream sequence of *AVR-Pik* in strain Ina168 using Inverse-PCR and PCR to identify the homologous region between these two sequences.

2.2.2 Materials and methods

(1) Extraction of genomic DNA from Ina168

The strains were inoculated into 2YEG liquid medium (100 mL) in a shaking flask (500 mL volume) and incubated at 27°C for 5 to 10 days (100 rpm, BIO-SHAKER BR-15, TAITEC). The reagents and culture media were prepared as in the previous section.

(2) Primers construction

Primers for the preparation of each DNA region and for sequencing analysis are shown in Tables 2.5 and 2.6.

(3) Cloning of *Pvu I* fragment by Inverse-PCR

Restriction enzyme digestion of DNA

In order to analyze the sequence upstream of Inago2 of Ina168, Inverse-PCR must be performed using restriction enzymes that do not cut Inago2. Therefore, we prepared 10 μg of genomic DNA of strain Ina168 and used *Pvu I*. This was done in the same way as described in previous section.

Purification by phenol/chloroform extraction and ethanol precipitation

The same procedure was performed as in the previous section.

Self-ligation

Self-ligation was performed as in previous section.

Purification by phenol/chloroform extraction and ethanol precipitation

The same procedure was performed as in the previous section.

Inverse-PCR

The 10 ng/μL mixture contains the total genomic DNA of strain Ina168. Therefore, Inverse-PCR was performed to recover only the target restriction enzyme fragments. The reaction was performed using Platinum® PCR. Super Mix High Fidelity (Invitrogen) and the primers shown in Table 2.4 were used in the reaction. The reaction composition and conditions

are shown in Table 2.5 and 2.6. The reaction was performed as in the previous section. The amplification of the DNA fragment (about 4 kb) was confirmed.

Table 2.4. Oligonucleotides for amplifying Ina168- *Pvu* I fragment

Target	Primer name	Sequence (5′→3′)
Ina168- *Pvu* I fragment	avr-pik-F	ATGCGTGTTACCACTTTTAACACATTC
	Inago-R5	CGGTCTCACTCTTTTCCCGA

Table 2.5. Composition of reaction mixture for amplifying Ina168- *Pvu* I fragment

Component	Volume (μL)
Platinum ® PCR Super Mix High Fidelity	47.0
primers 10 μM *	1.0
10 μM *	1.0
template DNA **	1.0
Total Volume	50

* described in Table 2.4

Table 2.6. Cycle condition for amplifying Ina168- *Pvu* I fragment

Step	Reaction	Temperature	Time	Number of cycle
1st	Predenaturaion	94ºC	2:00	1
2nd	Denaturation	94ºC	0:15	35
	Annealing	62ºC	0:30	
	Polymerization	68ºC	4:30	
3rd	Polymerization	68ºC	7:00	1

2.2.3 Analysis of *Pvu I* fragment by sequencing analysis

The PCR products in the previous section were subjected to gel filtration (MicroSpin™ S-300HR Columns, GE healthcare) and sequenced. The PCR products were gel filtered (MicroSpin™ S-300HR Columns, GE healthcare) and sequenced. The results are shown in Table 2.7. The results were checked for homology using Genetyx Ver.12 (Zenetics). BLAST search was also performed.

Table 2.7. Oligonucleotides for sequence analysis of Ina168- *Pvu* I fragment

Primer name	Sequence (5′→3′)
avr-pik-F	ATGCGTGTTACCACTTTTAACACATTC
PIK-RR1	GGTTGACGGCTCATAAGATAATGC
avr-pik-R	TTAAAAGCCGGGCCTTTTTTT
PIK-FF2	GGGGGCAAACAAGCATAAATTC
PIK-FR2	GAATTTATGCTTGTTTGCCCCC
PIK-FF3	AATCGTCCGGCTGAAAAGGT
PIK-FR3	ACCTTTTCAGCCGGACGATT
PIK-RR5	ATGAAGCGCGCATTGTATTTG
PIK-RF5	CAAATACAATGCGCGCTTCAT
RF20-F	GCCAACCGTTGGAATATGGA
RF20-R	ATATTCCAACGGTTGGCCG
Inago-F	GCTATGCTTACCCTGGCCGT
Inago-R	CCAGGGTAAGCATAGCACGG
Inago-F2	ACAGACTCTCGCCCAGGCAC
Inago-R2	GTGCCTGGGCGAGAGTCTGT
Inago-F3	CTGGCTGATAAAGGTGGCCG
Inago-R3	GTCGGCCACCTTTATCAGCC
Inago-F4	TCCACGAGTTTATTGCCACGTT
Inago-R4	AACGTGGCAATAAACTCGTGGA
Inago-R5	CGGTCTCACTCTTTTCCCGA

2.2.4 Results and discussions

(1) Cloning of Pvu I fragment by Inverse-PCR

The genomic DNA of strain Ina168 was digested with the restriction enzyme Pvu I. The *Pvu I* fragment was then cloned into a cell fly. The *Pvu I* fragment was then self-ligated to the known region. The *Pvu I* fragment was self-ligated and the region containing the unknown sequence was cloned by Inverse-PCR using primers prepared for the known region (Table 2.4). The *Pvu I* fragment was self-ligated and the region containing the unknown sequence was amplified by Inverse-PCR using primers (Table 2.4) prepared for the known region. As a result, a band of about 4 kb was observed in all the mixtures used as templates. As a result, a band of approximately 4 kb was detected in each mixture (Figure 2.4).

Fig 2.4 Amplification of Ina168- *Pvu* I fragment

Amplification of Inverse-PCR product by Platinum® PCR Super Mix High Fidelity. The fragment was about 4 kb.

(2) Analysis of the *Pvu I* fragment by sequencing analysis

The DNA fragment (approximately 4 kb) described in the previous section was subjected to sequencing analysis. As a result, the total length of the *Pvu I* fragment of Ina168 was 11.8 kb. (5,786 bp, 1 ~ 5,866 bp portion of retrotransposon Inago2) contained in the *Pvu I* fragment is the full length of retrotransposons. The 751 to 770 bp and 829 to 848 bp portions of the full-length (5,866 bp) Inago2 overlap, and the 771 to 829 ~ 848 bp of the full-length transposon Inago2 (5,866 bp) overlapped, and the 771 ~ 828 bp portion was absent. In addition, Pot2-like (short) (666 bp) was found upstream of Inago2. However, only 262 (*Pvu I* site) to 927 bp of the full-length transposon Pot2-like (1,858 bp) was conserved. This characteristic structure, in which Pot2-like and Inago2 are adjacent, was also present in Ina168m95-5 strain. The nucleotide sequence of Ina168 revealed by these results is shown in Figure 2.5 and Ina168 sequence (55,449 bp).

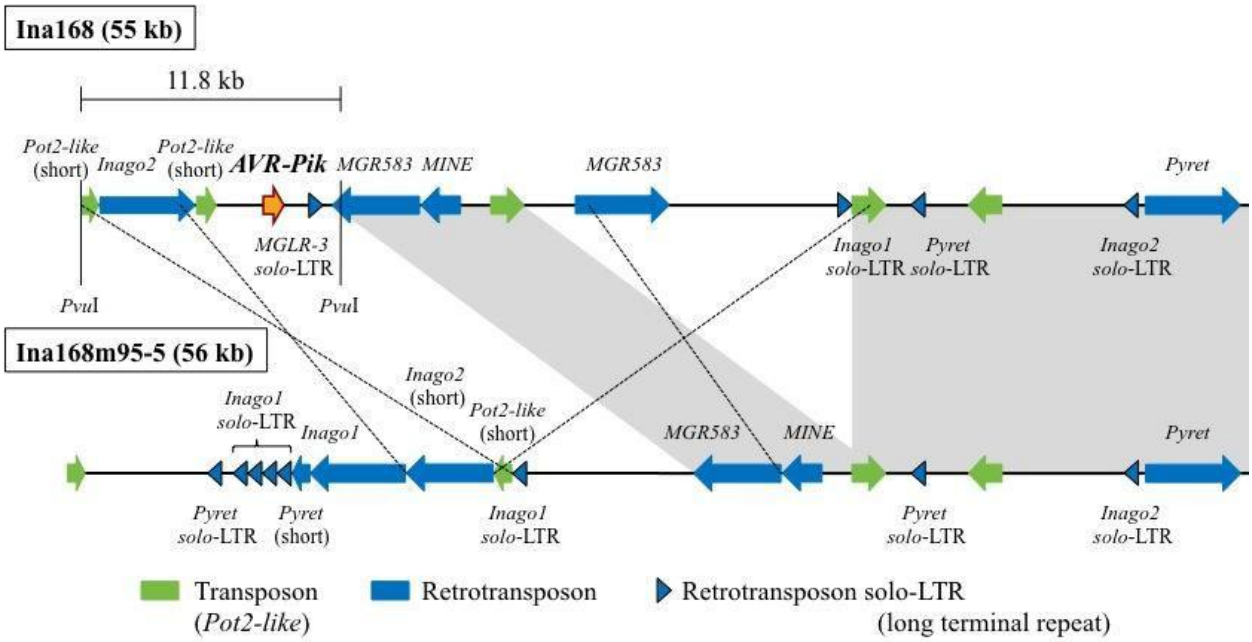


Fig 2.5 The homology between *AVR-Pik* flanking region in Ina168 and DNA from Ina168m95-5

AVR-Pik flanking region in Ina168 is indicated in the upper panel and DNA from Ina168m95-5 is indicated in the lower panel. Shaded regions illustrate the homology of DNA between Ina168 and Ina168m95-5. Dotted lines indicate the homologous region in opposite direction. The location of *Pvu* I sites in Ina168 is indicated.

CHAPTER 3

AVR-Pik deletion mechanism in Ina168m95-5

CHAPTER 3

AVR-Pik deletion mechanism in Ina168m95-5

3.1 Analysis of cosmid clones containing low-copy regions

We cloned the *Nhe I* fragment of strain Ina168m95-5 and analyzed its sequence. As a result, retrotransposon groups and low-copy regions that do not exist in the 49-kb sequence of Ina168 determined appeared. In order to identify the gene locus of Ina168 from which these sequences originated, we used the following method. In order to identify the gene locus of Ina168 from which these sequences were derived, we screened for new cosmid clones using the cosmid library of Ina168 and analyzed their sequences.

3.1.1 Materials and methods

Rice blast fungus Ina168 strain and additional spontaneous mutant strain were used. DNA solution of cosmid extracted from 96 samples of each plate into one sample. Primers were constructed for each DNA region are shown in Table 3.1, 3.4, and 3.11. Ampicillin sodium (Wako Junyaku) was prepared to 100 mg/mL using sterile water, filter sterilized (0.45 μm, ADVANTEC), and stored at -20ºC as a stock solution. After volume adjustment using distilled water (except Agar for liquid medium), the medium was sterilized by autoclaving (121°C, 20 min). Selection of transformed *E.coli* and extraction of cosmid DNA, ampicillin (final

concentration 60 μg/ml or 100 μg/ml) were added. 7.35 g/l of 50 mM calcium chloride solution CaCl2. 2H2O was sterilized by autoclaving (121°C, 20 min) after volume adjustment with distilled water, and 1 ml was dispensed into a conical tube (15 ml capacity, Falcon 2096, Becton Dickinson).

3.1.2 Primary screening

(a) PCR

We searched for a library pool containing a cosmid clone with a low-copy region that newly appeared in the Nhe I fragment of strain Ina168m95-5. The library pools containing the newly emerged low-copy region in the Nhe I fragment of PCR Ina168m95-5 strain were searched. PCR was performed using DNA pools No. 1 ~ 50 of each cosmid library as templates. The reaction was performed using GoTaq® Green Master Mix (Promega) and primers shown in Table 3.1. The reaction composition and conditions are shown in Table 3.2 and 3.3. After the reaction, 5 μL of the PCR product was run on a 1 % agarose gel (H14, TAKARA, dissolved in 1 × TAE) for electrophoresis (1× TAE, 135 V, i -MyRun.N, Cosmo Bio), immersed in ethidium bromide solution and stained. And then it was checked whether the target DNA fragment (627 bp) was amplified or not under UV irradiation.

Table 3.1. Oligonucleotides for 1st screening of cosmid clone of Ina168

Target	Primer name	Sequence (5′→3′)
The sequence containing *Inago1* and *Pyret*	INAGO-F8	AGACGTTGCAATATCAGGTATCGTG
	Out-R1	GAACCAAAACAAAATTCGTTTTCG

Table 3.2. Composition of reaction mixture for 1st screening of cosmid clone of Ina168

Component	Volume (μl)
GoTaq ® Green Master Mix (2 ×)	10.0
primers 10 μM *	0.5
10 μM *	0.5
template DNA **	2.0
SDW	7.0
Total Volume	50

* described in Table 3.1
** for positive control: Ina168m95-5 genomic DNA (100 ng/μl)

Table 3.3. Cycle condition for 1st screening of cosmid clone of Ina168

Step	Reaction	Temperature	Time	Number of cycle
1st	Predenaturaion	95ºC	2:00	1
	Denaturation	95ºC	0:30	
2nd	Annealing	60ºC	0:30	35
	Polymerization	72ºC	0:40	
3rd	Polymerization	72ºC	5:00	1

(b) Evaluation of PCR results

The vicinity of the region amplified in the previous section was amplified in the same manner to confirm whether the identified DNA pool indeed contained the target cosmid clone. The reaction was performed using GoTaq® Green Master Mix. The reaction was performed using GoTaq® Green Master Mix (Promega) and the primers shown in Table 3.4 were used in the reaction. The reaction composition and conditions are shown in Table 3.5 and 3.6. The

reaction composition and conditions are shown in Table 3.5 and 3.6. After the reaction, 5 μl of the PCR product was used for electrophoresis on a 1 % agarose gel (H14, TAKARA, dissolved in 1 × TAE) for electrophoresis (1× TAE, 135 V, i -MyRun.N, Cosmo Bio), immersed in ethidium bromide solution and stained to check whether the target DNA fragment (535 bp) was amplified under UV irradiation. The amplification of the target DNA fragment (535 bp) was confirmed under UV light.

Table 3.4. Oligonucleotides for evaluation of cosmid clone of Ina168

Target	Primer name	Sequence (5′→3′)
The novel low copy sequence	Nhe-F1	GTTTTGGGCAAACCTACCTCAC
	Nhe-F2	GGAGCTTCCCATCGGTGG

Table 3.5. Composition of reaction mixture for evaluation of cosmid clone of Ina168

Component	Volume (μL)
GoTaq ® Green Master Mix (2 ×)	10.0
primers 10 μM *	0.5
10 μM *	0.5
template DNA **	x ***
SDW	9-x
Total volume	20.0

* described in Table 3.4
** for positive control: Ina168 genomic DNA (100 ng/μl)
*** Cosmid DNA: 2.0 μL; Ina168 genomic DNA: 1.0 μl

Table 3.6. Cycle condition for evaluation of cosmid clone of Ina168

Step	Reaction	Temperature	Time	Number of cycle
1st	Predenaturaion	95ºC	2:00	1
2nd	Denaturation	95ºC	0:30	35
	Annealing	60ºC	0:30	
	Polymerization	72ºC	0:45	
3rd	Polymerization	72ºC	5:00	1

3.1.3 Secondary screening

(a) Preparation of glycerol stock

Since the plate of DNA pool No. 50 was lost, secondary screening was conducted for No. 31 and No. 37. The glycerol stock stored at -80°C was dissolved by standing at 37°C for 30 minutes. After dissolution, 10 μl of each glycerol stock was inoculated into 96-well plates (Nunc) containing LB solution with ampicillin (1 ml, final concentration 60 μg/ml), covered with a 96-well plate lid (Nunc), and incubated for 15 hours at 37°C with shaking. After incubation, 100 μm of culture medium was added to the plate and incubated for 15 hours at 37°C with shaking (1550 r/min, Mix-EVR, TAITEC). After incubation, 100 μl of the culture medium was transferred to a 96-well plate (Nunc), and the same volume of 40% glycerol solution was added to suspend the cells.

(b) Cosmid extraction

12 samples (500 μl each) were collected in 15-ml conical tubes (Falcon 2096) to make a total of 8 samples (6 ml each) in columns A to H. Then, cosmid DNA was extracted using NucleoSpin® Plasmid QuickPure (MACHEREY-NAGEL) according to the manual supplied

with the kit. After extraction, the concentration was measured by A 260 (NanoDrop™ Lite UV-Vis Spectrophotometer, Thermo Fisher Scientific) and stored at -20°C.

(c) PCR

PCR was carried out using the cosmid DNA solution extracted for each column in the previous section as a template. Reaction GoTaq® Green Master Mix (Promega) and primers shown in Table 3.1 and 3.4 were used for PCR. The reaction composition and conditions are shown in Tables 3.7 and 3.6. The reaction was carried out as in the previous section. We checked that whether the DNA fragments (627 bp and 535 bp) were amplified or not.

Table 3.7. Composition of reaction mixture for 2nd screening of cosmid clone of Ina168

Component	Volume (μl)
GoTaq ® Green Master Mix (2 ×)	10.0
primers 10 μM *	1.0
10 μM *	1.0
template DNA **	x ***
SDW	8-x
Total volume	20.0

* described in Table 3.1 and 3.4
** for positive control: Ina168m95-5 genomic DNA (100 ng/μl)
*** 100 ng

3.1.4 Tertiary screening

(a) Cosmid extraction

After lysis, inoculate 10 μl of each glycerol stock with the target DNA fragment into 5 ml of LB liquid medium supplemented with ampicillin (60 μg/ml final concentration) in a

medium test tube (18 mm diameter) and incubate at 37°C for 16 hours. The cells were incubated at 37°C for 16 hours with shaking (140 rpm, BIO-SHAKER BR-15, TAITEC). After incubation, 1.5 ml of the culture medium was used to extract cosmid DNA as in previous section. After extraction, the concentration was measured by A 260 (NanoDrop™ Lite UV-Vis Spectrophotometer, Thermo Fisher Scientific) and stored at -20°C.

(b) PCR

PCR reaction was performed using GoTaq® Green Master Mix (Promega) and primers shown in Table 3.4. The reaction composition and conditions are shown in Tables 3.8 and 3.6. The reaction was performed as in previous section. We checked that whether the DNA fragment (535 bp) was amplified or not.

Table 3.8. Composition of reaction mixture for 3rd screening of cosmid clone of Ina168

Component	Volume (μl)
GoTaq ® Green Master Mix (2 ×)	10.0
primers 10 μM *	0.5
10 μM *	0.5
template DNA **	x ***
SDW	9-x
Total volume	20.0

* described in Table 3.4
** for positive control: Ina168m95-5 genomic DNA (100 ng/μl)
*** Cosmid DNA: 2 ng; Ina168m95-5 genomic DNA: 100 ng

3.1.5 Fourth screening

(a) Preparation of competent cells and heat shock

We decided to transform cosmid clone 37H4, whose band was detected most clearly in the PCR in the previous section, into a competent cell Escherichia coli Top10 strain by heat shock. The E. coli Top10 strain was inoculated into LB plates and incubated at 37°C for one night. After incubation, a single colony was grown in LB liquid medium (5 ml) in a medium test tube (18 mm diameter) using an autoclaved (121ºC, 20 min) toothpick, and incubated for 15 h at 37ºC with shaking (130 rpm, BIO-SHAKER BR-15, TAITEC). After cultivation, 0.5 ml of the culture was inoculated into LB liquid medium (50 ml) in a 500-mL Sakaguchi flask (120 rpm, BIO-SHAKER BR-300LF, TAITEC) and incubated for 3.5 hours at 37°C with shaking. The culture medium was discarded by centrifugation (TX-160, Tommy Seiko) at 8,000 rpm for 2 min at 0˚C. 1 ml of ice-cold 50 mM calcium chloride solution was added and the cells were suspended using a pipet man, and then centrifuged under the same conditions for 2 min to obtain the upper layer.

The cells were kept at 0˚C. The supernatant was discarded using a pipetman, and 0.5 ml of ice-cold 50 mM calcium chloride solution was added, suspended using a pipetman, and kept ice-cold for 30 min. After ice cooling, the upper layer was obtained by centrifugation under the same conditions for 2 minutes. The supernatant was discarded using a pipetman, and 100 μl of ice-cold 50 mM calcium chloride solution was added and suspended using a pipetman until transformation by heat shock. To the prepared microtubes, 100 ng of cosmid DNA solution was added, tapped, and placed back in ice. After ice-cooling, the microtubes were held at 42˚C for 2 minutes, then immediately returned to ice and ice-cooled for 2 minutes. After ice cooling, the entire volume was added to SOC medium (1 ml) in a conical tube (15 ml volume, Falcon2096, BECTON DICKINSON) and incubated for 30 minutes at 37ºC for recovery (130 rpm, BIO-

SHAKER BR-15, TAITEC). After incubation, 200 μL of the culture was smeared onto ampicillin-loaded LB plates and incubated at 37ºC for 1 night.

(b) Colony PCR

Colony PCR was performed using autoclaved toothpicks. For the reaction, KOD FX Neo (TOYOBO) and primers shown in Table 3.4 were used. The reaction composition and conditions are shown in Table 6-9 and 6-10. The positive control is shown in previous section. After the reaction, 5 μl of the PCR product was subjected to electrophoresis (1 × TAE, 135 V, i-MyRun.N, Cosmo Bio) on a 1 % agarose gel (H14, TAKARA, dissolved in 1 × TAE), and then immersed in ethidium bromide solution and stained to determine if the target DNA fragment (535 bp) was amplified under UV irradiation.

Table 3.9. Composition of reaction mixture for 4th screening of cosmid clone of Ina168

Component	Volume (μl)
KOD FX Neo	0.5
primers 10 μM *	0.6
10 μM *	0.6
2 x PCR Buffer for KOD FX Neo	10.0
2mM dNTPs	4.0
SDW	4.0
Total Volume	19.7

* described in Figure 3.4

Table 3.10. Cycle condition for 4th screening of cosmid clone of Ina168

Step	Reaction	Temperature	Time	Number of cycle
1st	Predenaturaion	94ºC	2:00	1
2nd	Denaturation	98ºC	0:10	35
	Annealing	60ºC	0:30	
	Polymerization	68ºC	0:20	
3rd	Polymerization	68ºC	5:00	1

(c) Cosmid extraction

Using an autoclaved toothpick, inoculate the colonies into LB liquid medium (100 ml, final concentration 100 μg/ml) containing ampicillin in a 500-mL Sakaguchi flask, and incubate for 15 hours at 37°C with shaking (120 rpm). After incubation, 100 μg/ml of the culture medium was incubated at 37°C for 15 hours with shaking (120 rpm, BIO-SHAKER BR-300LF, TAITEC). After incubation, 100 μl of the culture solution was transferred to a microcentrifuge tube, suspended in the same volume of 40% glycerol solution, and stored at -80°C. The remaining culture medium was used to extract cosmid DNA as in previous section. After extraction, the concentrations were measured using A 260 (NanoDrop™ Lite UV-Vis Spectrophotometer, Thermo Fisher Scientific), and stored at 20°C.

3.1.6 Analysis of cosmid clones by sequencing analysis

The cosmid DNA solution extracted in the previous section was subjected to gel filtration (MicroSpin™ S-300HR Columns, GE healthcare) and sequenced. The sequencing was performed in the same way as in previous section, and the primers shown in Table 3.11 were

used. The results were checked for homology using Genetyx Ver.12 (Genetics). BLAST search was also performed.

Table 3.11. Oligonucleotides for sequence analysis of cosmid 37H4

Primer name	Sequence (5′→3′)
vec-F	CGTTTCCGTTCTTCTTCGTCAT
v37-F	GCCAGAGGCTGACTAGTAGCCC
v37-R	GGGCTACTAGTCAGCCTCTGGC
1R	AGAATTTTCGTCGATAGCGCCTAATA
1F	TATTAGGCGCTATCGACGAAAATTCT
INAGO-F3	AGTGAGCCCAGAGCCAGTAGAG
INAGO-R3	CTCTACTGGCTCTGGGCTCACT
INAGO-F2	CCGGCATGTTTGATGGTAATAAG
INAGO-R2	CCTTATTACCATCAAACATGCCG
INAGO-F	CCGAACGCAAACAGCGTC
INAGO-R	GACGCTGTTTGCGTTCGG
INAGO-F-F	TTATCCATCCGACCGAGCC
INAGO-F-R	GGCTCGGTCGGATGGATAA
INAGO-F-F2	CTCTGGACCTTGAGGCCCA
INAGO-F-R2	TGGGCCTCAAGGTCCAGAG
INAGO-F-F3	GAGGATCGATTTCATTGAACGG
INAGO-F-R3	TCCATTGTCCGTTCAATGAAATC
INAGO-F4	CCGGACTCCAAGGCGATATC
INAGO-R4	TTTTGATATCGCCTTGGAGTCC
INAGO-F5	CTGCTGAACAAAACTATCCGATTCA
INAGO-R5	TGAATCGGATAGTTTTGTTCAGCAG
INAGO-F5-F	TAACGGTACCCGAGACTACCCA
INAGO-F6	CAAGATCTTCGGCCATGGAG
INAGO-R6	CATGGCCGAAGATCTTGGG
INAGO-F7	GTAGACAAATACCAAACGAATTGGC
INAGO-R7	GCCAATTCGTTTGGTATTTGTCTAC
INAGO-F8	AGACGTTGCAATATCAGGTATCGTG
INAGO-R8	CACGATACCTGATATTGCAACGTCT
out-F1	CGAAAACGAATTTTGTTTTGGTTC
out-R1	GAACCAAAACAAAATTCGTTTTCG

out-F2	GGATTTACGAACGCCGATAGG
out-R2	CCTATCGGCGTTCGTAAATCC
Nhe-R7	TTGGGGTAATTACCGCTAGGTTT
Nhe-F7	AAACCTAGCGGTAATTACCCCAA
Nhe-R6-F	CGTGTCGGTACCAATCCTGG
Nhe-R6	CATTTAAAATTTCTTACCCCCGC
Nhe-F6	CGTGGTCCTTGGGGGTTTT
Nhe-F5-F	TATTTCCCCTTGGTAATCGGG
Nhe-R5	CATTTAAAATTTCTTACCCCCGC
Nhe-F5	GCGGGGGTAAGAAATTTTAAATG
Nhe-R4	CATCCAGCACCTCCAACACC
Nhe-F4	GGTGTTGGAGGTGCTGGATG
Nhe-R3	GGGAGCTTGGCCATAATACCA
Nhe-F3	TGGTATTATGGCCAAGCTCCC
Nhe-R2	GGAGCTTCCCATCGGTGG
Nhe-F2	CCACCGATGGGAAGCTCC
Nhe-R1	GTGAGGTAGGTTTGCCCAAAAC
Nhe-F1	GTTTTGGGCAAACCTACCTCAC
cos37H4-F1	TTGCCCCCTATAAGGAAATGC
cos37H4-R1	GCATTTCCTTATAGGGGGCAA
cos37H4-F2	GTCCACGGTTTGGGTTTCC
cos37H4-R2	GGAAACCCAAACCGTGGAC
cos37H4-F3	TGAGAAAGTGGCGGTCTCG
cos37H4-R3	CGAGACCGCCACTTTCTCA
cos37H4-F4	CGTTAAAGATGCCGGTGCC
cos37H4-R4	GGCACCGGCATCTTTAACG
cos37H4-F5	CTCCTACCAAAGAGACCGTCTTGA
cos37H4-R5	TCAAGACGGTCTCTTTGGTAGGAG
cos37H4-F6	GTCCATGGAGTGGTGGTGCT
cos37H4-R6	AGCACCACCACTCCATGGAC
cos37H4-F7	GCCAACCAGGCCTATCAGC
cos37H4-R7	GCTGATAGGCCTGGTTGGC
cos37H4-F8	TGGCCCTGTTCGCTACTGA
cos37H4-R8	TCAGTAGCGAACAGGGCCA
cos37H4-F9	GAGGCCGAGTAAGCTCGCT
cos37H4-R9	AGCGAGCTTACTCGGCCTC

cos37H4-F10	ATCGTTATTGTCAGGGAGCCAC
cos37H4-R10	GTGGCTCCCTGACAATAACGAT
RF19	ACCCCCCTAAAGTCCGGC
RR19	GCCGGACTTTAGGGGGGT
FR5	CGTTCAAAGCTTCTAAACAGTCCCTA
FF5	TAGGGACTGTTTAGAAGCTTTGAACG
RR21	TTGCTGACCAAAAGCTCGTTG
RF21	CCCAACGAGCTTTTGGTCAG
FR2	CCGTACCGCCTCCAATTTT
FF2	AAAATTGGAGGCGGTACGG
RR21-R	CGTCGTACGCCAACCAGATT
RR21-F	AATCTGGTTGGCGTACGACG
t7-u	GGAATAAGGGCGACACGGA

3.1.7 Results and discussions

(1) Primary screening

DNA pools 1 to 50 of each cosmid library were used as templates, and PCR was performed using primers (Table 3.1) that amplified the newly appeared region in the *Nhe I* fragment of strain Ina168m95-5. As a result, a band of 627 bp was detected using DNA pools No. 31, 37 and 50 as templates (Table 3.1). Next, DNA pools No. 31, 37 and 50 were used as templates, and PCR was performed using primers (Table 3.4) that amplify the vicinity of the regions amplified in the previous section. As a result, the expected band of 535 bp was detected in each of the DNA pools (Figure 3.2). Therefore, it was suggested that the identified DNA pools 31, 37, and 50 indeed contained the target cosmid clones.

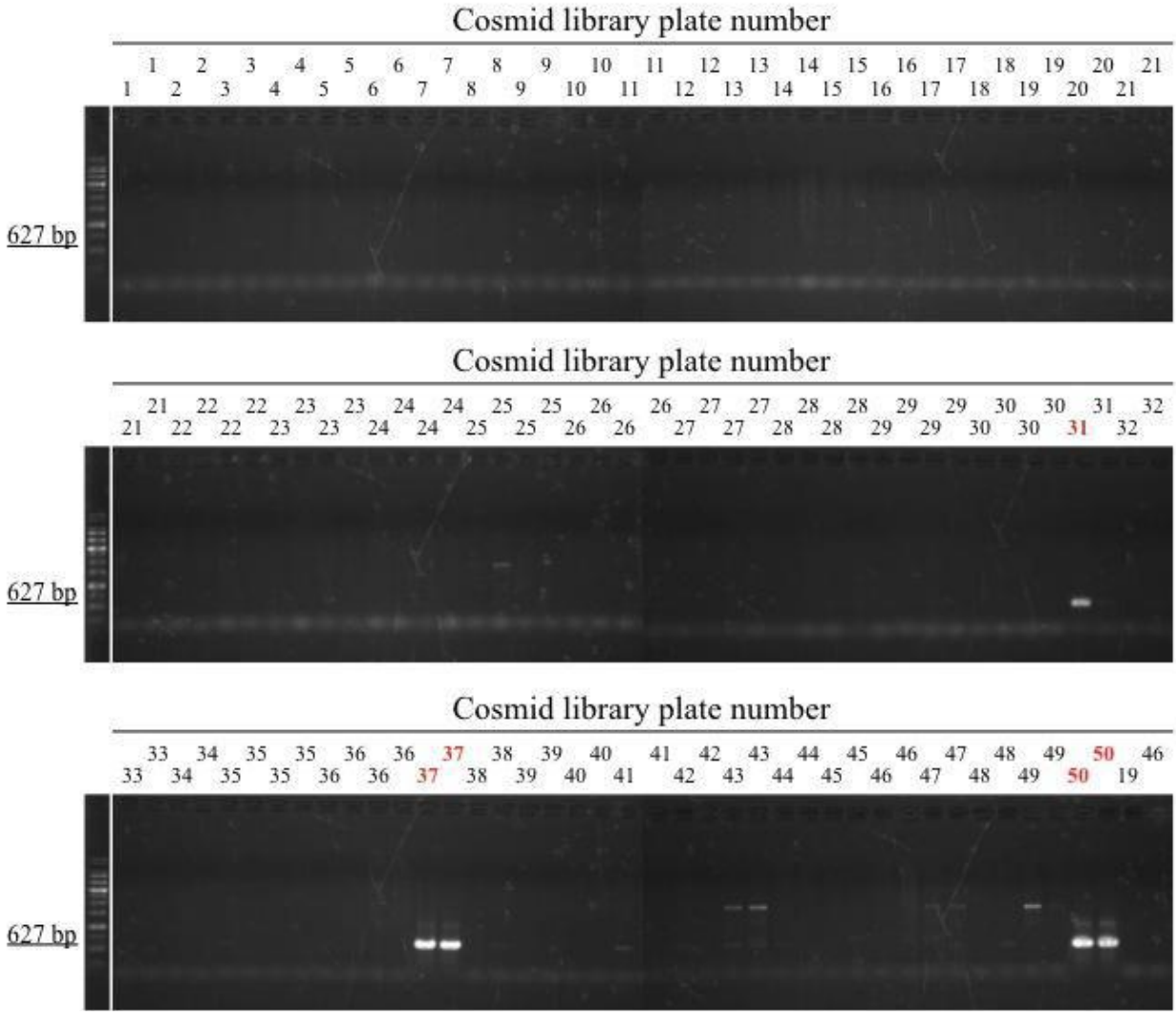


Fig 3.1 Amplification of the sequence containing *Inago1* and *Pyret* in 1st screening

Cosmid library plate number is indicated above lanes. The objective fragment was 627 bp and was amplified in cosmid library DNA pools No. 31, 37 and 50.

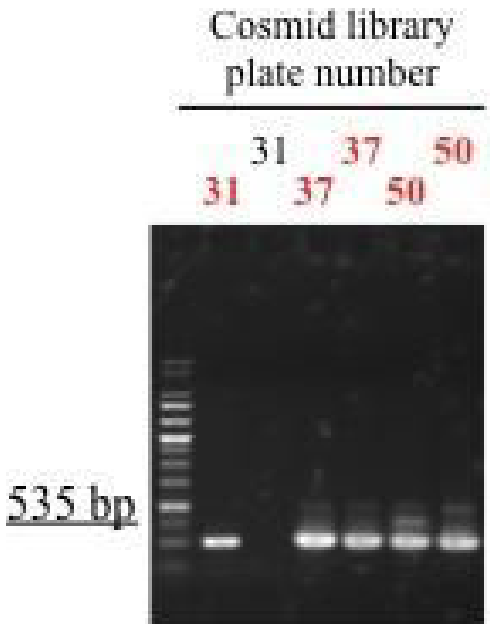


Fig 3.2 Amplification of the novel low copy sequence in 1st screening

Cosmid library plate number is indicated above lanes. The objective fragment was 535 bp and was amplified in cosmid library DNA pools No. 31, 37 and 50.

(2) Secondary screening

We extracted cosmid DNA from 12 samples in each row of each plate, and obtained a total of 16 samples (31A ~ H and 37A ~ H). These samples were used as templates and the primers shown in Table 3.1 and 3.4 were used. PCR was performed using the primers shown in Table 3.1 and 3.4. As a result, the expected bands of 627 bp and 535 bp were detected when 37F, 37G and 37H were used as templates, respectively (Figure 3.3).

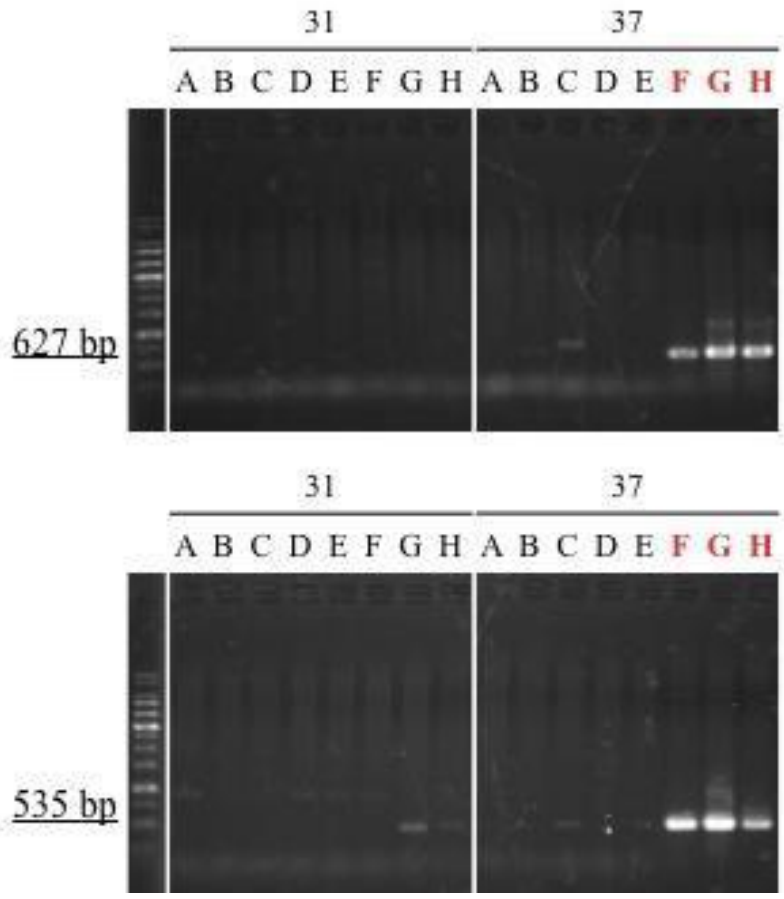


Fig 3.3 Amplification of the sequence containing *Inago1* and *Pyret* and the novel low copy sequence in 2nd screening

Cosmid library No. 31 and 37 were used for 2nd screening. The objective fragments were 627 bp and 535 bp and were amplified in 37F, 37G and 37H.

(3) Tertiary screening

We extracted cosmid DNA from 12 samples in these columns to obtain a total of 36 samples (37F1~12, 37G1~12, and 37H1~12). These samples were used as templates for PCR using the primers shown in Table 3.4. PCR was performed using the primers shown in Table 3.4.

The results showed that 37F4, 37F6, 37G3, 37G4, 37G5, 37G6, 37G7, 37H3, 37H4, 37H5, and 37H6 respectively (Figure 3.4).

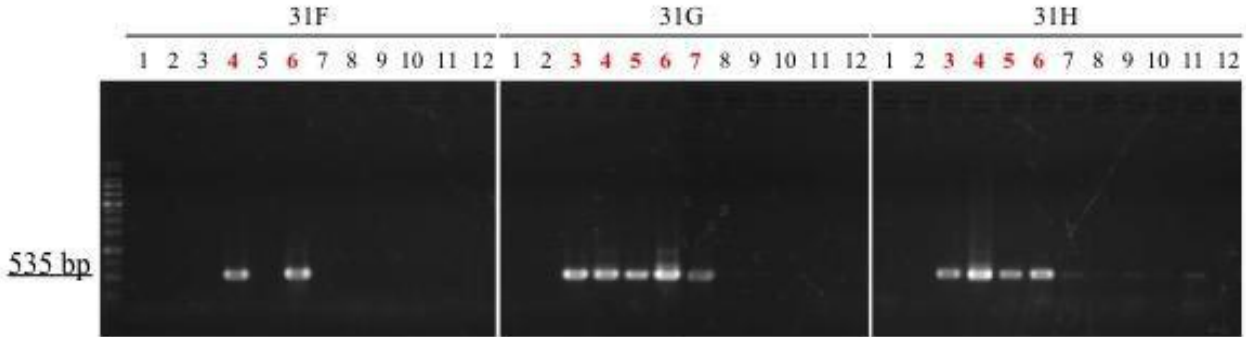


Fig 3.4 Amplification of the novel low copy sequence in 3rd screening

37F, 37G and 37H were used for 3rd screening. The objective fragment was 535 bp and was amplified in 37F4, 37F6, 37G3, 37G4, 37G5, 37G6, 37G7, 37H3, 37H4 and 37H5.

(4) Fourth screening

In consideration of the possibility of contamination, we introduced 37H4, which was detected as the darkest and clearest band in the PCR in the previous section, into the competent cell *Escherichia coli* TOP10 strain by heat shock. A single colony was obtained and purified. Colony PCR was performed using the primers shown in Table 3.4. The results showed that the expected 535 bp band was detected in almost all colonies (Figure 3.5). As a result, a 535 bp band was detected in almost all colonies (Figure 3.5). The 12th colony, from which the band was most densely detected, was mass-cultured to extract cosmid DNA, which we called cosmid 37.

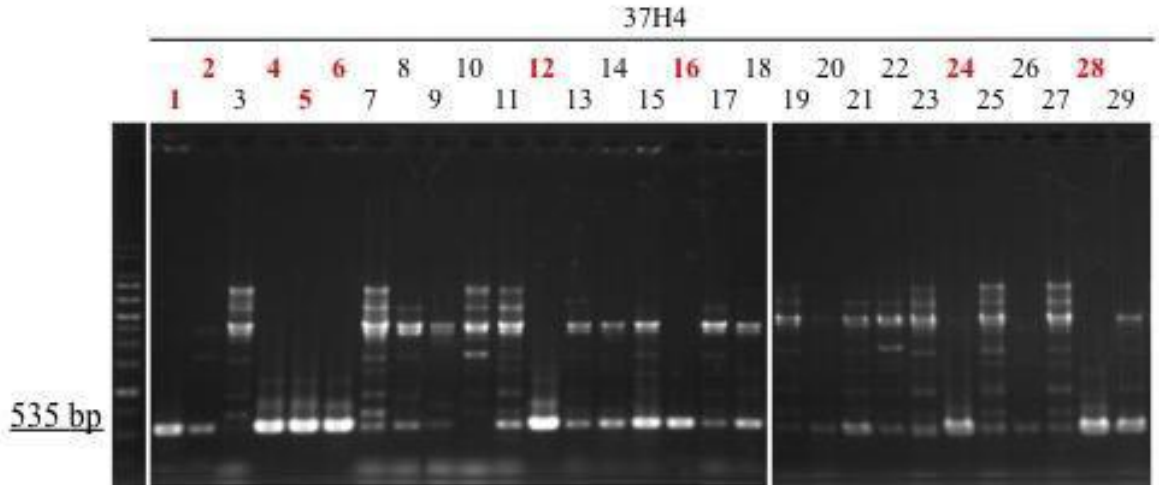


Fig 3.5 Amplification of the novel low copy sequence in 4th screening

37H4 was used for 4th screening. The objective fragment was 535 bp and was amplified in almost every colony.

3.1.8 Analysis of cosmid clones by sequence analysis

Sequencing analysis of cosmid 37 extracted in the previous section was performed. The results are shown in Figure 3.6. Cosmid 37 does indeed contain *Inago1*, *Pyret (short)*, and four copies of *Inago1 solo -LTR*, *Pyret solo -LTR*, and *Pyret solo* in the *Nhe I* fragment of strain Ina168m95-5, and indicating homology. However, *Inago2 (short)*, which is present in Ina168m95-5, was absent upstream of *Inago1*. *Pyret solo-LTR* (107 bp) and *Inago2 solo-LTR* (61 bp). On the other hand, the sequences of the low-copy region (4,809 bp), Pot2-like (662 bp, transposon 1,197 to 1,858 bp portion of Pot2-like), and the vector sequence were found. These sequences are shown in Cosmid 37 sequence 5´ side 1 (9,169 bp), Cosmid 37 sequence 5´ side 2 (9,169 bp), Cosmid 37 sequence 5´ side (2) (6,915 bp), Cosmid 37 sequence 5´ side (3) (7,053 bp), and Cosmid 37 sequence 3´ side (1,487 bp).

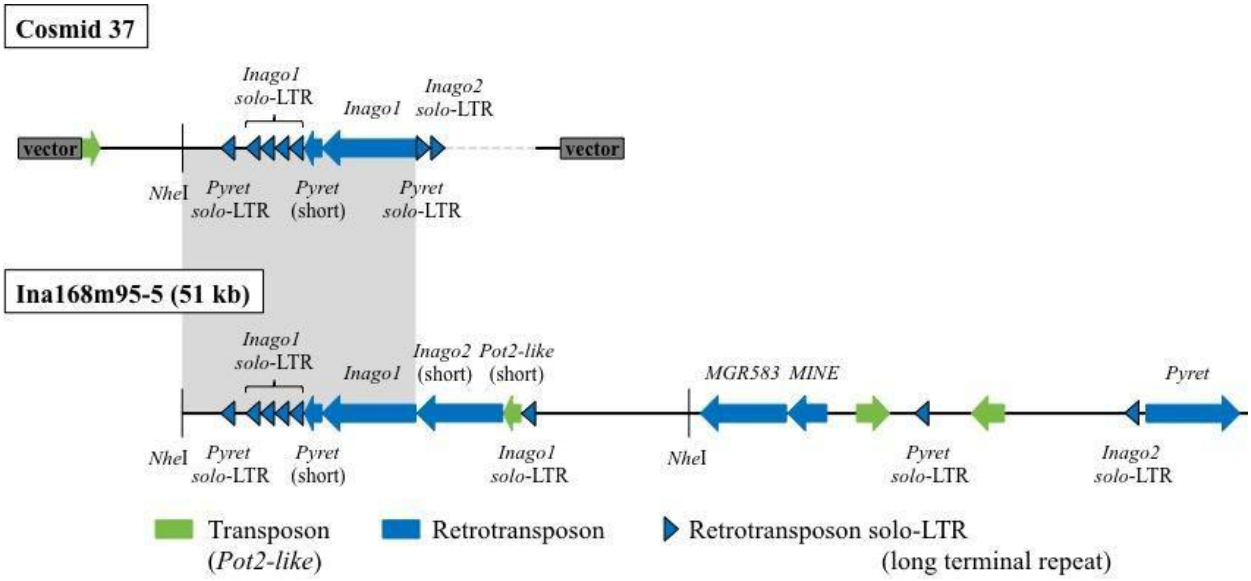


Fig 3.6 Structures of cosmid 37 from Ina168 and DNA from Ina168m95-5

Cosmid 37 containing the novel low copy region was obtained by screening Ina168 cosmid library. Dotted region in cosmid 37 indicates unsequenced region. Shaded regions illustrate the homology of DNA between cosmid 37 and Ina168m95-5. The location of *Nhe* I sites in cosmid 37 and Ina168m95-5 is indicated.

3.2 Analysis of cosmid clones containing Inago2 (short) and Inago1

We screened cosmid clones of Ina168 strains containing retrotransposons and low-copy regions in the *Nhe I* fragment of Ina168m95-5 strains and analyzed their sequences. The low copy region from Inago1 to the downstream *Nhe I* site of Ina168m95-5 strain was present, indicating homology. However, the origin of Inago2 (short) of Ina168m95-5 was unknown. The structure of Inago2 (short) and Inago1 adjacent to each other in Ina168m95-5 was not determined. In order for Inago2 (short) and Inago1 to be adjacent in strain Ina168m95-5, a

structure must exist at a locus other than cosmid 37 in the genome of strain Ina168. In order to identify the loci of Ina168 containing Inago2 (short) and Inago1, we screened for new cosmid clones using the cosmid library of Ina168 and analyzed their sequences.

3.2.1 Materials and methods

Rice blast fungus Ina168m95-5 was used. DNA solution of cosmid extracted from 96 samples of each plate into one sample. Primers were constructed for each DNA region are shown in Table 3.12 and 3.18. The reagents and media were prepared as in the previous section.

(1) Primary screening

We searched for a library pool containing cosmid clones with sequences adjacent to *Inago2 (short)* and *Inago1*. PCR was performed using DNA pools No. 1 to 50 of each cosmid library as templates. GoTaq® Green Master Mix (Promega) and the primers shown in Table 3.12 were used in the reaction. The reaction composition and conditions are shown in Tables 3.13 and 3.14. The reaction was performed in the same way as in previous section. We confirmed whether the target DNA fragment (635 bp) was amplified or not.

Table 3.12 Oligonucleotides for 1st screening of cosmid clone of Ina168

Target	Primer name	Sequence (5′→3′)
The sequence containing *Inago2* (short) and *Inago1*	Inago-F10	AAGCTCACGAGCCCAAAATATTC
	INAGO-R3	CTCTACTGGCTCTGGGCTCACT

Table 3.13 Composition of reaction mixture for 1st screening of cosmid clone of Ina168

Component	Volume (μL)

GoTaq ® Green Master Mix (2 ×)	10.0
primers 10 μM *	0.5
10 μM *	0.5
template DNA **	1.0
SDW	8.0
Total volume	20.0

* described in Table 3.12
** for positive control: Ina168m95-5 genomic DNA (100 ng/μl)

Table 3.14 Cycle condition for 1st screening of cosmid clone of Ina168

Step	Reaction	Temperature	Time	Number of cycle
1st	Predenaturaion	95ºC	2:00	1
2nd	Denaturation	95ºC	0:30	35
	Annealing	55ºC	0:30	
	Polymerization	72ºC	0:35	
3rd	Polymerization	72ºC	5:00	1

(2) Secondary screening

Preparation of glycerol stock

Secondary screening of DNA pool No. 26 was performed in the same way as in previous section.

Cosmid extraction

Cosmid extraction was performed as in previous section.

PCR

PCR was performed using the cosmid DNA solution extracted for each column in the previous section as a template. GoTaq® Green Master Mix (Promega) and the primers shown in Table 3.12 were used for the reaction. The composition and conditions are shown in Table 3.15 and 3.14. The reaction was performed in the same way as in previous section. We checked that whether the DNA fragment (635 bp) was amplified or not.

Table 3.15 Composition of reaction mixture for 2nd screening of cosmid clone of Ina168

Component	Volume (μL)
GoTaq ® Green Master Mix (2 ×)	10.0
primers 10 μM *	1.0
10 μM *	1.0
template DNA **	x ***
SDW	8-x
Total volume	20.0

* described in Table 3.12
** for positive control: Ina168m95-5 genomic DNA (100 ng/μl)
*** 100 ng

(3) Tertiary screening

Cosmid extraction

Cosmid extraction was performed as in previous section.

PCR

PCR was performed using each cosmid DNA solution extracted in the previous section as a template. The reaction was performed using GoTaq® Green Master Mix (Promega). Green Master Mix (Promega) and the primers shown in Table 3.12 were used. The reaction

composition and conditions are shown in Tables 3.15 and 3.15. The reaction was performed in the same way as in previous section. It was checked that whether the DNA fragment (635 bp) was amplified or not.

(4) Fourth screening

Electroporation method

We decided to transform the cosmid clone 26F10, whose band was detected most clearly in the PCR in the previous section, into the competent cell *Escherichia coli* Top10 strain by the electroporation method. The microtubes of the competent cell were filled with the cosmid clone 26F10. 100 ng of cosmid DNA solution was added to a microtube of the competent cell and placed in a cuvette after tapping. The microtubes were then tapped and placed in a cuvette. The cuvette was then mounted on a Gene Pulser (Bio-Rad) and electroporated at 2.5 V, 200 Ω, and 25 μF. Then, the entire volume was added to 1 ml of SOC medium in a carbon tube (15 ml volume, Falcon 2096, BECTON DICKINSON). The cells were then incubated for 30 minutes at 37ºC for recovery (130 rpm, BIO-SHAKER BR-15, TAITEC). After incubation, 50 ~ 200 μl were smeared onto ampicillin-loaded LB plates and incubated at 37ºC for 1 night.

Colony PCR

Colonies grown in the previous section were subjected to colony PCR using a toothpick sterilized by autoclaving (121ºC, 20 minutes). Colony PCR was performed using autoclaved toothpicks. For the reaction, KOD FX Neo (TOYOBO) and primers shown in Table 3.12 were used. The reaction composition and conditions are shown in Table 3.12. The reaction

was performed in the same way as in previous section. It was checked that whether the target DNA fragment (635 bp) was amplified or not.

Cosmid extraction

Cosmid extraction was performed as in previous section.

Table 3.16 Composition of reaction mixture for 4th screening of cosmid clone of Ina168

Component	Volume (μl)
KOD FX Neo	0.5
primers 10 μM *	0.6
10 μM *	0.6
2 x PCR Buffer for KOD FX Neo	10.0
2mM dNTPs	4.0
SDW	4.0
Total Volume	19.7

* described in Figure 3.12

Table 3.17 Cycle condition for 4th screening of cosmid clone of Ina168

Step	Reaction	Temperature	Time	Number of cycle
1st	Predenaturaion	94ºC	2:00	1
2nd	Denaturation	98ºC	0:10	35
	Annealing	55ºC	0:30	
	Polymerization	68ºC	0:17	
3rd	Polymerization	72ºC	5:00	1

Analysis of cosmid clones by sequencing analysis

The cosmid DNA solution extracted in the previous section was subjected to gel filtration (MicroSpin™ S-300HR Columns, GE healthcare) and sequenced. The sequencing was performed in the same way as in previous section, and the primers shown in Table 3.18 were used. The results were checked for homology using Genetyx Ver.12 (Genetics). BLAST search was also performed.

Table 3.18 Oligonucleotides for sequence analysis of cosmid 26F10

Primer name	Sequence (5′→3′)
vec-F	CGTTTCCGTTCTTCTTCGTCAT
26-2R-F	GAAGAATTCGCAAATTTGGTGG
Inago-F5-F	CGAACTCAGATACAGGTTAATGGC
Inago-F5-R	GCCATTAACCTGTATCTGAGTTCG
Inago-F6	GTTGGACAAACCCGATCCG
Inago-R6	CGGATCGGGTTTGTCCAAC
Inago-F7	CCTGCCGCTCGAAAAGG
Inago-R7	CCTTTTCGAGCGGCAGG
Inago-F8	CCGAGTCGACCTCCAGGG
Inago-R8	CCCTGGAGGTCGACTCGG
Inago-F9	CATACCCGATCCAAGACCGA
Inago-R9	TCGGTCTTGGATCGGGTATG
Inago-F10	AAGCTCACGAGCCCAAAATATTC
Inago-R10	GAATATTTTGGGCTCGTGAGCTT
1R	AGAATTTTCGTCGATAGCGCCTAATA
1F	TATTAGGCGCTATCGACGAAAATTCT
INAGO-F3	AGTGAGCCCAGAGCCAGTAGAG
INAGO-R3	CTCTACTGGCTCTGGGCTCACT
26-1F-F	CTACGGAAATGGCCAAATTAGC
26-1F-R	GCTAATTTGGCCATTTCCGTAG
26-1F-F2	GAACACCCAGCTTTTCGACG
26-1F-R2	GTCGTCGAAAAGCTGGGTG

INAGO-F-F	TTATCCATCCGACCGAGCC
INAGO-F-R	GGCTCGGTCGGATGGATAA
INAGO-F-F2	CTCTGGACCTTGAGGCCCA
INAGO-F-R2	TGGGCCTCAAGGTCCAGAG
INAGO-F-F3	GAGGATCGATTTCATTGAACGG
INAGO-F-R3	TCCATTGTCCGTTCAATGAAATC
INAGO-F4	CCGGACTCCAAGGCGATATC
INAGO-R4	TTTTGATATCGCCTTGGAGTCC
INAGO-F5	CTGCTGAACAAAACTATCCGATTCA
INAGO-R5	TGAATCGGATAGTTTTGTTCAGCAG
INAGO-F5-F	TAACGGTACCCGAGACTACCCA
INAGO-F6	CAAGATCTTCGGCCATGGAG
INAGO-R6	CATGGCCGAAGATCTTGGG
INAGO-F7	GTAGACAAATACCAAACGAATTGGC
INAGO-R7	GCCAATTCGTTTGGTATTTGTCTAC
INAGO-F8	AGACGTTGCAATATCAGGTATCGTG
INAGO-R8	CACGATACCTGATATTGCAACGTCT
Inago-F11	CAACCAGGAACTGGACCAAAAG
Inago-R11	CTTTTGGTCCAGTTCCTGGTTG
Inago-F12	CGCAATGGACCCATTACCAC
Inago-R12	GTGGTAATGGGTCCATTGCG
t7-u	GGAATAAGGGCGACACGGA

3.2.1 Results and discussions

(1) Primary screening

The DNA pools No. 1 to 50 of each cosmid library were used as templates, and PCR was performed using primers (Table 3.12) that amplified the sequences adjacent to *Inago2 (short)* and *Inago1*. As a result, a band of 635 bp was detected when using DNA pool No. 26 as a template (Figure 3.7).

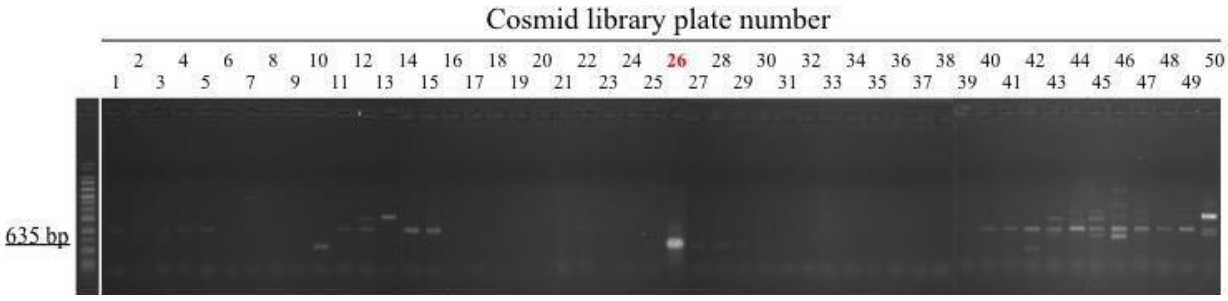


Fig 3.7 Amplification of the sequence containing *Inago2* (short) and *Inago1* in 1st screening

Cosmid library plate number is indicated above lanes. The objective fragment was 635 bp and was amplified in cosmid library DNA pools No. 26.

(2) Secondary screening

Cosmid DNA was extracted from 12 samples per row of each plate to obtain a total of 8 samples (26A ~ H). These samples were used as templates for PCR using the primers shown in Table 3.12. As a result, the predicted 635 bp band was detected in each of the cosmid DNA solutions (Figure 3.8).

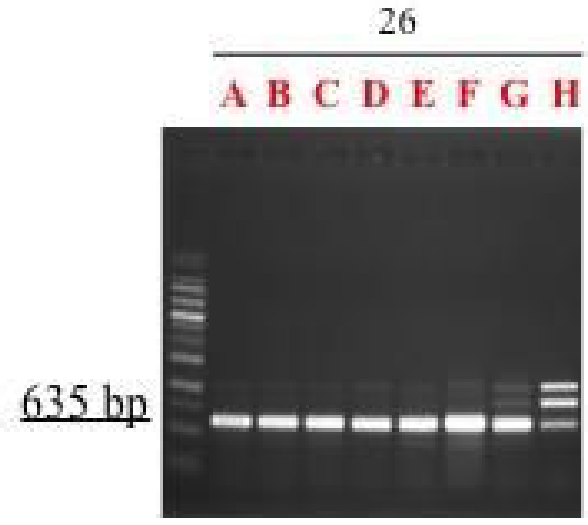


Fig 3.8 Amplification of the sequence containing *Inago2* (short) and *Inago1* in 2nd screening

Cosmid library No. 26 was used for 2nd screening. The objective fragment was 635 bp and was amplified in every sample.

(3) Tertiary screening

We extracted cosmid DNA from 12 samples of these columns, and obtained a total of 24 samples of 26C1~12 and 26F1~12. These samples were used as templates, and PCR was performed using the primers shown in Table 3.12. As a result, when almost all of the cosmid DNA solutions were used as templates, the expected band of 635 bp was detected (Figure 3.9).

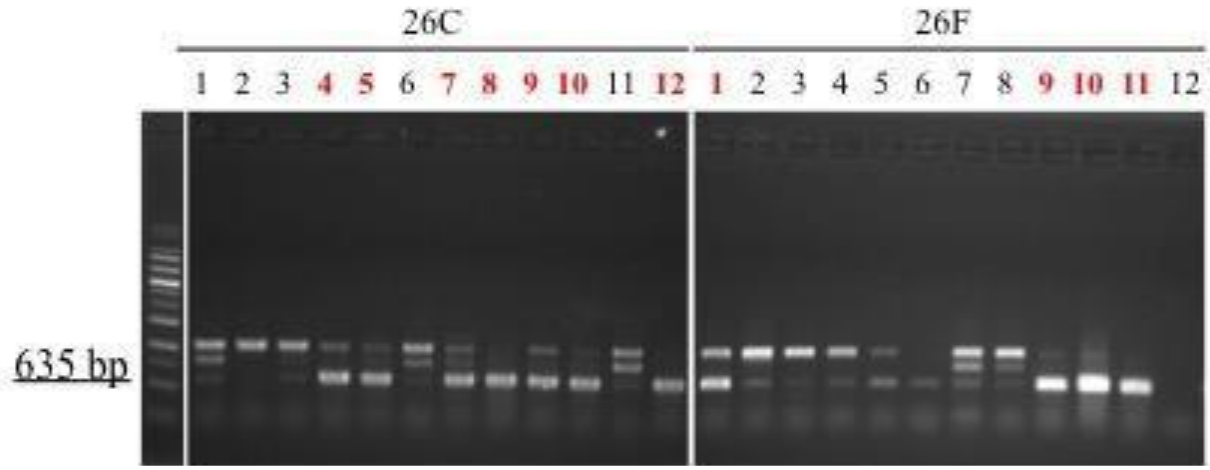


Fig 3.9 Amplification of the sequence containing *Inago2* (short) and *Inago1* in 3rd screening

26C and 26F were used for 3rd screening. The objective fragment was 635 bp and was amplified in almost every sample.

(4) Fourth screening

In consideration of the possibility of contamination, 26F10, which was detected as the darkest and clearest band in the PCR in the previous section, was introduced into the competent

cell Escherichia coli TOP10 strain by electroporation. Then, a single colony was obtained and purified. Colony PCR was performed on the grown colonies using the primers shown in Table 6-12. As a result, a band of 635 bp, which was expected from the tenth colony, was detected (Figure 3.10). After that, the 10th colony was mass cultured and the cosmid DNA was extracted and called cosmid 26.

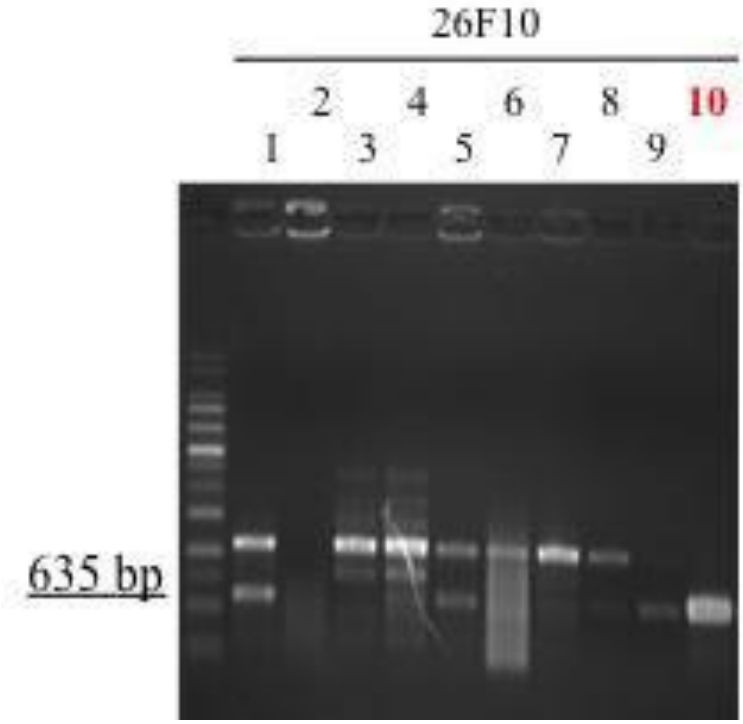


Fig 3.10 Amplification of the sequence containing *Inago2* (short) and *Inago1* in 4th screening

26F10 was used for 4th screening. The objective fragment was 635 bp and was amplified in 10th colony.

(5) Analysis of cosmid clones by sequence analysis

Sequencing analysis of the 26 cosmids extracted in the previous section was performed. The results are shown in Figure 3.11. On the 3´ side, there are indeed two cosmid clones, *Inago2 (short)* (2,652 bp, 1,687 ~ 4,341 bp portion of retrotransposon *Inago2*) and *Inago1 (short)*. The structure of Inago2 (short) was found. Upstream of Inago2 (short), there is a low-copy region (632 bp) and vector arrangement, and downstream of Inago1 (short), there is a low-copy region (928 bp), Inago1 (short) (4,231 bp, 1,868 ~ 6,104 bp portion of retrotransposon Inago1) and Inago2 (short) (300 bp, 1,868 ~ 6,104 bp portion of retrotransposon Inago2). On the 5´ side, Inago2 (short) (577 bp, 2,100 ~ 2,679 bp portion of the retrotransposon Inago2) was present, but as mentioned above, Inago2 was found in multiple copies. However, as mentioned above, multiple copies of Inago2 exist, and the downstream of Inago2 is overlapped by multiple signals. These sequences are Cosmid 26 sequence 5´ side (794 bp) and Cosmid 26 sequence 3´ side (9,873 bp).

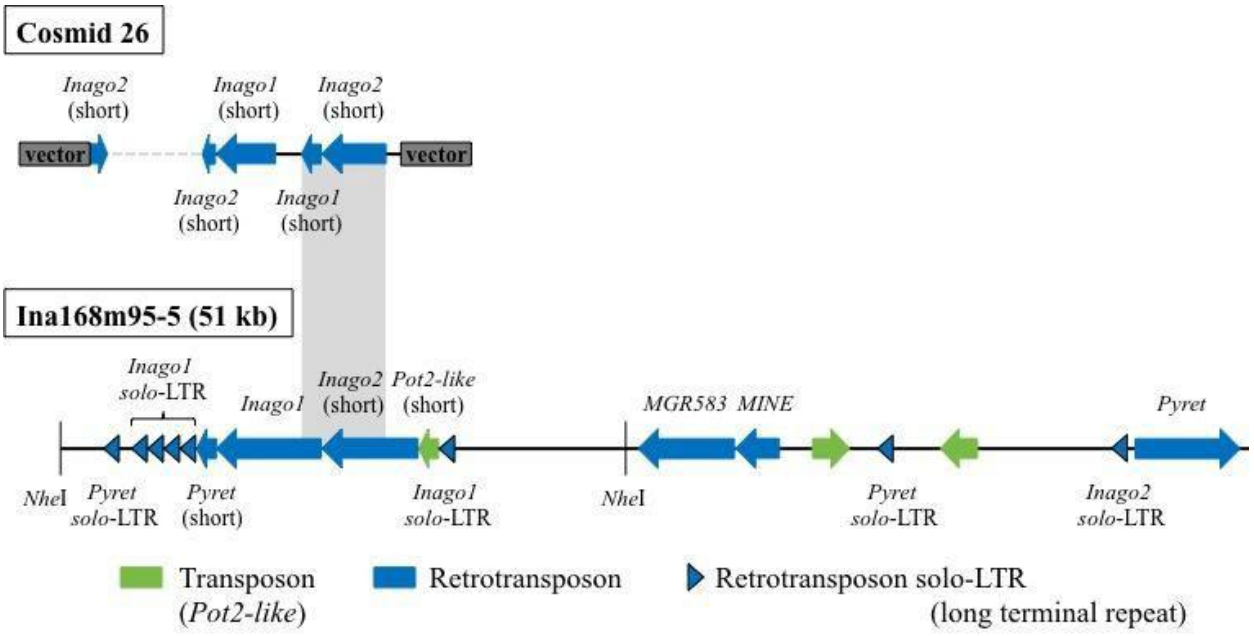


Figure 3.11 Structures of cosmid 26 from Ina168 and DNA from Ina168m95-5

Cosmid 26 containing *Inago2* (short) and *Inago1* was obtained by screening Ina168 cosmid library. Dotted region in cosmid 26 indicates unsequenced region. Shaded regions illustrate the homology of DNA between cosmid 26 and Ina168m95-5. The location of *NheI* sites in Ina168m95-5 is indicated.

3.3 Analysis of the 5´ side of the *NheI* fragment of strain Ina168m95-5

In the previous section, we screened cosmid clones of Ina168 strains containing retrotransposons and low-copy regions in the *NheI* fragment of Ina168m95-5 strains and analyzed their sequences. The low-copy region from Inago1 to the downstream *NheI* site of

Ina168m95-5 strain was present and showed homology. In addition, a low-copy region and a Pot2-like region were found. We performed PCR and sequencing analysis of this new region.

3.3.1 Materials and methods

Rice blast fungus Ina168m95-5 strain was used. Primers were constructed for each DNA region are shown in Figure 3.12 and Tables 3.19 and 3.22. The reagents and media were prepared as in the previous section.

(1) Confirmation of the presence of novel regions by PCR

PCR was performed using the genomic DNA of Ina168m95-5 strain as a template. The reaction was performed using Platinum® PCR Super Mix High Fidelity (Invitrogen) and the primers shown in Figure 3.12 and Table 3.19. The reaction composition and conditions are shown in Table 3.20 and 3.21. After the reaction, 5 μL of the PCR product was run on a 0.7 % agarose gel (H14, TAKARA, dissolved in 1 × TAE) for electrophoresis (1× TAE, 135 V, i - MyRun.N, Cosmo Bio), immersed in ethidium bromide solution and stained. And then it was checked whether the target DNA fragment (627 bp) was amplified or not under UV irradiation.

(2) Analysis of new regions by sequencing analysis

The PCR products were gel filtered (MicroSpin™ S-300HR Columns, GE healthcare) and sequenced in the same way as in previous section, using the primers shown in Table 3.22. The results are shown in Table 3.22. The results were checked for homology using Genetyx Ver.12 (Zenetics). BLAST search was also performed.

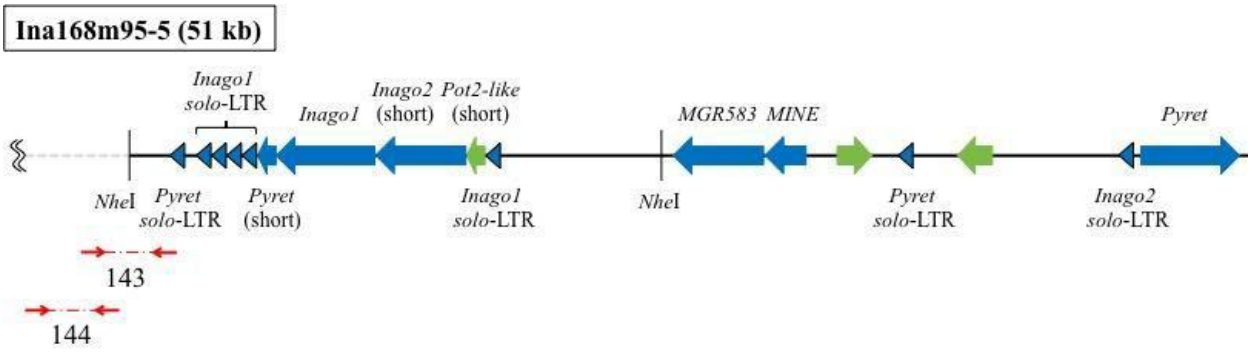


Fig 3.12 The location of PCR primers for amplifying each PCR product in the novel region

All primer pairs were used for amplifying each PCR product in the novel region.

Table 3.19. Oligonucleotides for amplifying each PCR product in the novel region

No.	Primer name	Sequence (5′→3′)
143	Nhe-R2	GGAGCTTCCCATCGGTGG
	Cos37H4-R6	AGCACCACCACTCCATGGAC
144	Cos37H4-F5	CTCCTACCAAAGAGACCGTCTTGA
	RR21-F	AATCTGGTTGGCGTACGACG

Table 3.20. Composition of reaction mixture for amplifying each PCR product in the novel region

Component	Volume (μL)
Platinum ® PCR Super Mix High Fidelity	47.0
primers 10 μM *	1.0
10 μM *	1.0
template DNA **	1.0
Total volume	50.0

* described in Figure 3.12 and Table 3.19
** Ina168m95-5 genomic DNA (100 ng/μL)

Table 3.21. Cycle condition for amplifying each PCR product in the novel region

Step	Reaction	Temperature	Time	Number of cycle
1st	Predenaturaion	94ºC	2:00	1
2nd	Denaturation	94ºC	0:15	35
	Annealing	62ºC	0:30	
	Polymerization	68ºC	3:30	
3rd	Polymerization	68ºC	7:00	1

Table 3.22. Oligonucleotides for sequence analysis of each PCR product in the novel region

Primer name	Sequence (5′→3′)
Nhe-R2	GGAGCTTCCCATCGGTGG
Nhe-R1	GTGAGGTAGGTTTGCCCAAAAC
Nhe-F1	GTTTTGGGCAAACCTACCTCAC
cos37H4-F1	TTGCCCCCTATAAGGAAATGC
cos37H4-R1	GCATTTCCTTATAGGGGGCAA
cos37H4-F2	GTCCACGGTTTGGGTTTCC
cos37H4-R2	GGAAACCCAAACCGTGGAC
cos37H4-F3	TGAGAAAGTGGCGGTCTCG
cos37H4-R3	CGAGACCGCCACTTTCTCA
cos37H4-F4	CGTTAAAGATGCCGGTGCC
cos37H4-R4	GGCACCGGCATCTTTAACG
cos37H4-F5	CTCCTACCAAAGAGACCGTCTTGA
cos37H4-R5	TCAAGACGGTCTCTTTGGTAGGAG
cos37H4-F6	GTCCATGGAGTGGTGGTGCT
cos37H4-R6	AGCACCACCACTCCATGGAC
cos37H4-F7	GCCAACCAGGCCTATCAGC
cos37H4-R7	GCTGATAGGCCTGGTTGGC
cos37H4-F8	TGGCCCTGTTCGCTACTGA
cos37H4-R8	TCAGTAGCGAACAGGGCCA
cos37H4-F9	GAGGCCGAGTAAGCTCGCT
cos37H4-R9	AGCGAGCTTACTCGGCCTC

cos37H4-F10	ATCGTTATTGTCAGGGAGCCAC
cos37H4-R10	GTGGCTCCCTGACAATAACGAT
FR5	CGTTCAAAGCTTCTAAACAGTCCCTA
FF5	TAGGGACTGTTTAGAAGCTTTGAACG
RR21-F	AATCTGGTTGGCGTACGACG

3.3.2 Results and discussions

(1) Confirmation of the presence of novel regions by PCR

Genomic DNA of Ina168m95-5 strain was used as a template, and PCR was performed using primers that amplified PCR products No.143 and 144. As a result, a 3.5 kb band, which is expected from the results of sequencing analysis of cosmid 37, was detected (Figure 3.13).

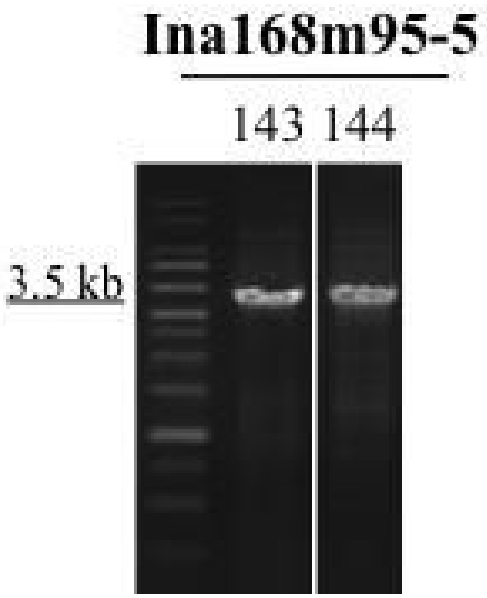


Fig 3.13. Amplification of PCR product No. 143 and 144

Amplification of each PCR product by Platinum® PCR Super Mix High Fidelity. The names of each product are indicated above lanes. The objective fragments were about 3.5 kb in both cases.

(2) Analysis of new regions by sequencing analysis

We performed sequence analysis of PCR products No.143 and 144 in the previous section. As a result, these sequences were identical to the sequences from the low-copy region near the *NheI* site to Pot2-like of cosmid 37. This indicates that these regions are conserved in Ina168m95-5 strain. The nucleotide sequence of Ina168m95-5 (56,169 bp) was shown in Figure 3.14.

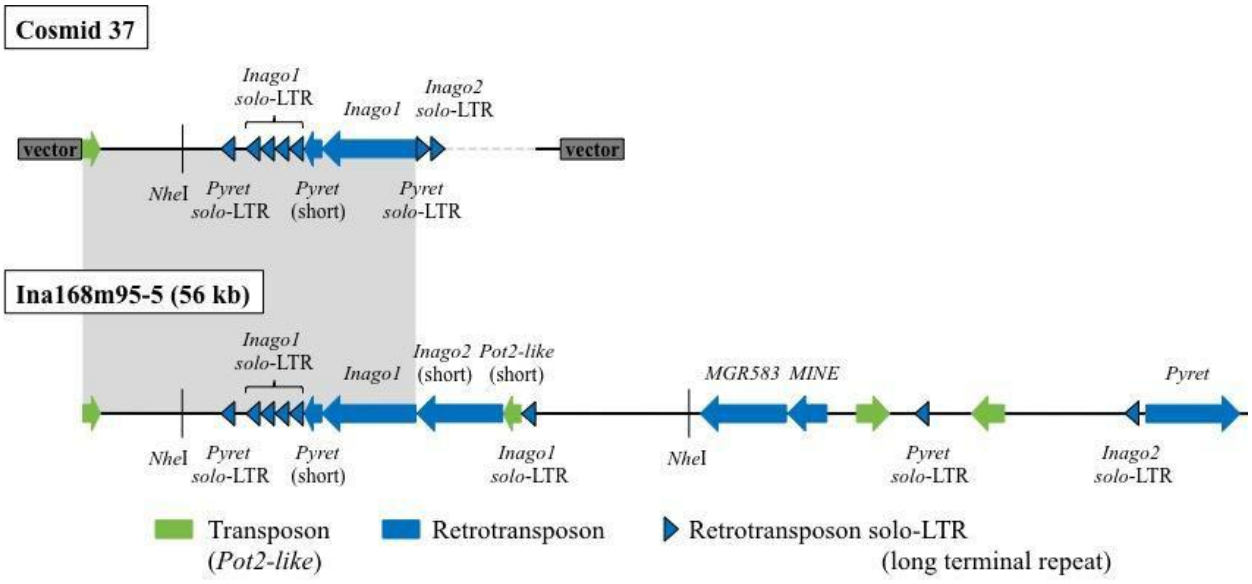


Fig 3.14 The homology between cosmid 37 from Ina168 and DNA from Ina168m95-5

Cosmid 37 from Ina168 is indicated in the upper panel and DNA from Ina168m95-5 is indicated in the lower panel. Dotted region in cosmid 37 indicates unsequenced region. Shaded regions illustrate the homology of DNA between cosmid 37 and Ina168m95-5. The location of *Nhe* I sites in cosmid 37 and Ina168m95-5 is indicated.

CHAPTER 4

Identification of parasexual recombination in the field isolates of rice blast fungus

CHAPTER 4

Identification of parasexual recombination in the field isolates of rice blast fungus

4.1 Microsatellite marker amplification around flanking sequences

To identify chromosomal locations in rice blast field isolates, amplify around flanking sequences of SSR marker DNA by using PCR to see the specific places in the genome or specific locations on the chromosomes. It is not easy to use for identification of the locus. Using SSR place for probing in the DNA, it would be much copied. It is not suitable to see the specific place in the genome or specific locations on the chromosomes. Therefore, design primers and amplified around the flanking regions of each SSR markers site and look from the genomic database to check it has to be a single sequence and low copy number.

Materials and methods

4.1.1 PCR amplification

Genomic sequence data of *Pyricularia oryzae* were downloaded from BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Ten pairs of forward and reverse primers from flanking regions of SSR markers were designed and synthesized based on the genomic DNA sequence

(Table 4.1). Ina 86-137 was used as DNA template for PCR reactions. All PCR reactions were performed using Taq PCR Master Mix (Qiagen Inc., Valencia, CA, USA). Each PCR reaction consisted of the following components: 25 µl of GoTaq Master Mix (contains 25U of Taq DNA polymerase, 10X Qiagen PCR buffer, 15mM $MgCl_2$, and 200 µM of each dNTP), 1µl of each forward and reverse primer, 1µl of Ina 86-137 genomic DNA and distilled water in total 50µl. Reactions were performed in Thermal Cycler with the following PCR program: 1 cycle at 95°C for 2 min for initial denaturation, followed by 35 cycles at 95°C for 30s, 55°C for 30s, extension of 72°C for 30s, and a final extension of 72°C for 5 min. The size of the amplified fragments was estimated by Stable DNA Ladder (Sigma Inc.) run in H14 gel electrophoresis for 20 min.

Table 4.1 Primers used for the amplification of SSR markers flanking regions

Primer name	**Sequence (5'-3')**	**Locus**	**Expected Fragment Length (bp)**	**Application**
P1P2F	TGCACTAAACACCGTGCAAGG	*Mgms01*	219	PCR
P1P2R	GCGTATTCGATCGTGTGCGT			
P9P10F	ATTCGCACGCATCATTCTGAC	Mgms04	245	PCR
P9P10R	TCGACCGACTACATCGTGCA			
P19P20F2	AGTGCTTGAGTGCGCCAAAC	Mgms06	229	PCR
P19P20R2	CCATGATGCCATTTGTGCTTTAT			
P21P22F	CTTCACCACTTTCGCAGCAAG	ms99-100	244	PCR
P21P22R	TCCTATCTGTACGGGCTTTGTCA			
P27P28F	TGTGAGACACGGCGGATGT	*Mgms08*	273	PCR
P27P28R	ATTGCTCTTGGCGATGACGA			
P29P30F	TGTGCGACTTCACCAAGAACTACTAC	*Mgms09*	220	PCR
P29P30R	AATCACACAGAGGACAGTGGCC			

P49P50F	CCTCTGGCCCTTGTCCATTT	*Mgms13*	248	PCR
P49P50R	GCTTGCTCTAGAGCTGCCCC			
P51P52F	ACAGGACATCAAACCGTTGAACA	*Mgms14*	225	PCR
P51P52R	CGAGGTTTGGTCTGGGACTGT			
P55P56F	CACAAAGCTGCGGCAAGC	*Mgms15*	213	PCR
P55P56R	TAGCCTTGGATCCAGCCATG			
pyrms8788F	TCAACAGATTCAAGCTTGTTTCCAC	ms87-88	203	PCR
pyrms8788R	TCGTACGCCAAAGCCTCAAC			

4.1.2 PCR purification and cell ligation

After DNA flanking sequences were amplified with PCR, the DNA products were purified using with Nucleospin Gel and PCR cleanup, (Macherey-Nagel, USA) followed by the manufacturer's instruction. Then the amplified DNA will remain purified for the insertion of pGEM-Easy Vector System (Promega). Rapid ligation buffer and DNA ligase enzyme were added together with vector into PCR product under 16°C for less than 24 hour for ligation. The restriction enzyme cut the DNA and open up for vector for insertion to ligase DNA sequences together to produce recombinant DNA. A carrier which behaves like DNA, the Ethachimate is added into the ligased DNA including sodium acetate for precipitation. The DNA were precipitated with concentrated ethanol and rinsed with 70% ethanol for purification. For transformation of recombinant DNA, the Top 10 competent *Escherchia coli* cells, kept under -80°C were thawed on ice and recombinant DNA was added. In order to increase the permeability of the cell membrane, allowing DNA to be introduced into the cell, the electroporation was

performed with Gene Pulser. To obtain maximal transformation efficiency of *E.coli,* incubated in SOC liquid medium for one hour at 35°C with properly shaking rpm. LB medium containing IPTG and X-Gal was prepared for inoculation including Ampicillin. Once plating with LB medium, pipetting the DNA samples onto medium surface for incubation at 37°C for 24 hour. After incubation for one day, the colonies were sub-cultured with Ampicillin containing LB medium for stock culture.

Results

An amplification of SSR flanking sequences was performed by PCR using Ina 86-137 DNA template with ten pairs of constructed primers. After amplification of DNA sequences, the expected size of DNA was analyzed by DNA ladder. Each marker expected sizes were around 200 bp (Fig 4.1).

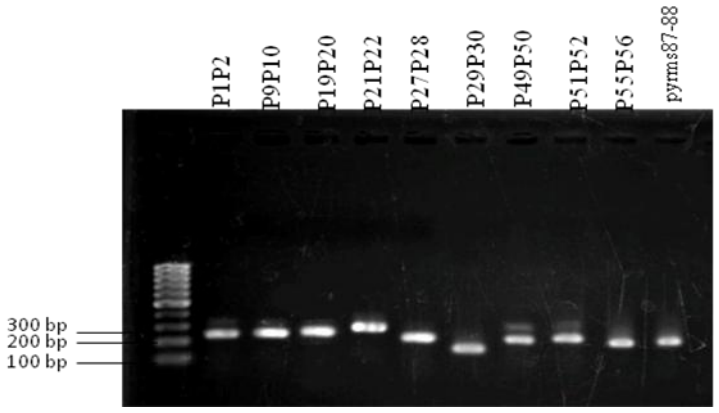


Fig 4.1 PCR amplification for cloning of SSR marker flanking sequences

Discussion

After cloning of SSR flanking sequences and amplification with PCR, it is showing the possibilities that these markers can be used for probing onto chromosomal DNA size differences between populations, if those markers were somehow related to that chromosomal size difference. Although parasexual recombination has been described in fungi, its role in shaping natural populations is poorly understood. Thus far, investigations of parasexual recombination have been limited to the use of complementing selectable auxotroph markers, and imposing forced heterokaryons (J. Kumar and R. J. Nelson et al. 1996).

4.2 Plasmid DNA extraction and cloning of SSR markers flanking regions

To analyze the sequencing of SSR markers flanking regions which has been cloned by PCR amplification, we extracted plasmid DNA from every marker we cloned by using Plasmid DNA Purification (Machnery-Nagel), and conducted DNA concentration check to run gel electrophoresis to confirm plasmid containing in the DNA. The sequencing analysis is relevant in order to find similarity, to infer it they are related in homologous. To precise, identification of sequence differences and variations such as point mutation and single nucleotide polymorphism (SNP) in order to get the genetic marker and to reveal the genetic diversity of sequences.

Materials and methods

4.2.1. Extraction of plasmid DNA

To extract plasmid DNA, the templates were incubated in the LB broth medium in 100 ml Erlenmeyer flask and shaking at 37°C for 24 hour at 150 rpm. After incubation, take out 1.5 ml from cultured medium to centrifuge with 11,000 × g for 1 min. After centrifugation, plasmid purification was performed by Nucleospin Plasmid EasyPure (MACHEREY-NAGEL, Germany). The plasmid DNA was resuspend with buffer inverting upside down to lyses and centrifuge again for 3-5 min at 12,000 × g. The supernatant was added by Buffer AQ and the flow-through was discarded. When plasmid DNA was recovered from column, checking DNA concentration was followed up with NanoDrop Lite Spectrophotometer. To confirm the plasmid containing in the markers we cloned, run gel electrophoresis for 20 min with H14 gel using 100bp DNA ladder.

4.2.2 Plasmid DNA sequencing analysis

After confirmation of plasmid DNA containing, sequencing analysis was performed. Dilute the primer M13 with SDW to 100 pmol/µl for both Primer M13 forward and reverse. Take 2 µl Big Dye terminator whcih was stored under -20° from stock and added with 3 µl Buffer to dilute. 1 µl of M13 forward and reverse primers were added into each forward and reverse primers tubes and then plasmid DNAs were added together with distilled water. After PCR was performed, purify the plasmid DNA with Big Dye Edge Bio (Applied Bio Sciences) for plasmid DNA sequencing analysis.

4.2.3 Probe preparation for hybridization

The recombinant DNA that incubated on the LB medium as colonies were inoculated in LB broth supplemented with ampicillin in a 100 ml Erlenmeyer flask and incubated at 37°C for one day with 150 rpm. The next day, transfer the incubated DNA broth medium into 50 ml Falcon tube for centrifugation at 6000 × g at 4°C for 15 min. The supernatant was completely discarded. The pellets were resuspend with 8 ml Buffer RES containing RNaseA with vortex to mix thoroughly. The Buffer Lys 8ml was added to suspend again and mix gently by inverting the tube more than 5 times and then incubated at room temperature for 5 minutes. After applying Equilibrium Buffer onto the rim of column and filter (Midi Preps), added 8ml of Buffer NEU to suspension and immediately mix the lysate inverting until turns into white from blue color. Then invert the tube three times before loading the lysate onto the column filter to avoid clogging the filter. The column filter was washed with 5ml of buffer EQU onto the rim of column filter. After that remove the filter and wash again with 8ml of Wash Buffer to column only. Transfer into 50ml Falcon tube and elute the plasmid DNA with 5 ml of Elution Buffer ELU. Added 3.5ml Isopropanol and vortex well enough and incubate for 5 minutes. After incubation for awhile, remove the plunger from the syringe and attach the Finalizer to the outlet. For washing, 70% ethanol was added and discarded the flow-through. Finally, to finalize the purification, add 800 µl of Buffer TRIS and concentration checked was performed by NanoDrop Lite Spectrophotometer.

4.2.4. Digestion with restriction enzymes and purification

The purified plasmid DNA was diluted to 10µg concentration and using EcoRI HF® restriction enzyme (New England Biolabs, Ipswich-Massachusetts, United States) with buffer

CutSmart 5µl each together with SDW for digestion at 37°C for 2 hour. The DNA sample was mixed with 5µl of 3M solution and 99.9% ethanol was used for precipitation. Then, the plasmid DNA was stored at -20°C for 15 minutes and centrifuged at 150,000 rpm for 20 minutes. The supernatant was discarded and 70% of ethanol was mixed thoroughly to centrifuge again. After all, let the DNA sample to dry with vacuum dryer with 10 minutes. To soluble the dried DNA, added 20 µl of distilled water. The electrophoresis was performed with 0.7% Seakam-GTG agarose gel and the plasmid DNA was mixed thoroughly with SIGMA 6x loading dye to check the plasmid under UV visible light.

The super-helical form of restriction endonuclease-treated plasmid was visualized under UV light and cut out the expected size 0.2kb band and treated with 100mg/0.1g gel purification buffer and incubated at 50°C for 10 minutes. During incubation vortex vigorously the plasmid DNA containing gel to dissolve in the buffer solution until gel goes off. The DNA recovery from gel was performed with Nucleospin Gel and PCR clean-up (MACHNERY-NAGEL, Germany). The purified DNA was eluted with 15µl elution buffer NE in centrifuge tube. The pure DNA was stored at -20°C and the concentration check with NanoDrop Lite UV Spectrophotometer was performed in next day.

Results

After extraction plasmid DNA, the DNA were successfully cloned using PCR from the flanking sequences around SSR markers, the amplified fragment expecting size 0.2kb band (Fig 4.2) was estimated with 1kb DNA Ladder. All markers present the same expected fragment size. In order to find similarity of expected sequences from genomic data, the sequencing analysis was

performed with DNA analyzer. Then, the PCR products were sequenced using the same primers (Table 4.1) for PCR amplification. The recombinant DNA which was grown on LB medium was prepared for Southern hybridization, which was digested with EcoRI HF® restriction enzymes (New England Biolabs, Ipswich-Massachusetts, United States). The enzyme was targeted the flanking regions of the marker that was cloned from SSR markers. The concentration of the expected fragment was eluted to get 10ng/µl for the determination of southern hybridization which was recovered from gel extraction.

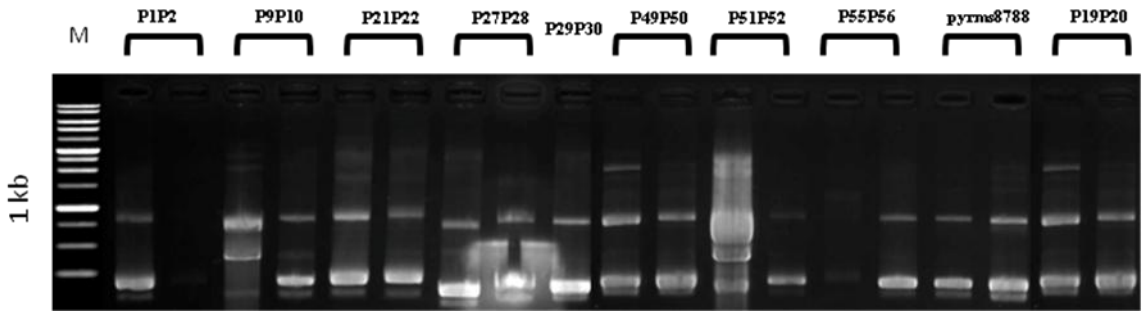


Fig 4.2. SSR markers flanking DNA were cloned after plasmid DNA extraction from each markers

4.3 Identification of chromosomal DNA in filed isolates of rice blast fungus

Genotypic marker selection

The development of resistant rice cultivars is the preferred management approach for blast. Under conditions conducive to the disease, however, recurrent blast epidermics have

occurred, characterized by lapse of host resistance and emergence of new virulent pathotypes of the fungus (Lee, E., and Cho, S. Y. et al. 1990). Although the sexual stage of *Magnaporthe* can be produced between highly selected isolates in the laboratory (Leung, H., and Williams, P. H. et al. 1987), evidence of sexual mating has not been observed in nature (Notteghem, J. L., and Silue, D. et al. 1992).

Segregation of morphological and nutritional markers in pairings between complementing auxotrophs has been demonstrated in forced heterokaryons, but the role of parasexuality in the absence of artificial selection remains obscure (Crawford, M. S., Chumley, F. G., Weaver, C. G., and Valent, B. et al. 1986). Therefore, to show the evidence for parasexual recombination in field isolates, we used the population of rice blast fungus isolates with microsatellite genotypes for screening of chromosomal size changes, from closely-located paddy fields in Daisen, Akita.

Materials and methods

4.3.1. Population of rice blast fungus

Initially, the populations of rice blast fungus strains were obtained from the characterization with microsatellite markers in Tohoku Agricultural Research Center. We differentiated 27 strains from the total 346 rice blast strains which were characterized with 12 SSR markers in 36 genotypes (Table 4.2) according to their different of alleles. These strains are isolated from very limited area but genotype is very variable. It is showing that there is a difference in the DNA between these strains. According to that difference between their lengths,

we grouped the recombination candidates from the possibility of chromosomal gene exchanging from these strains.

Table 4.2. Genotypic characterization of rice blast fungus isolates using microsatellite markers

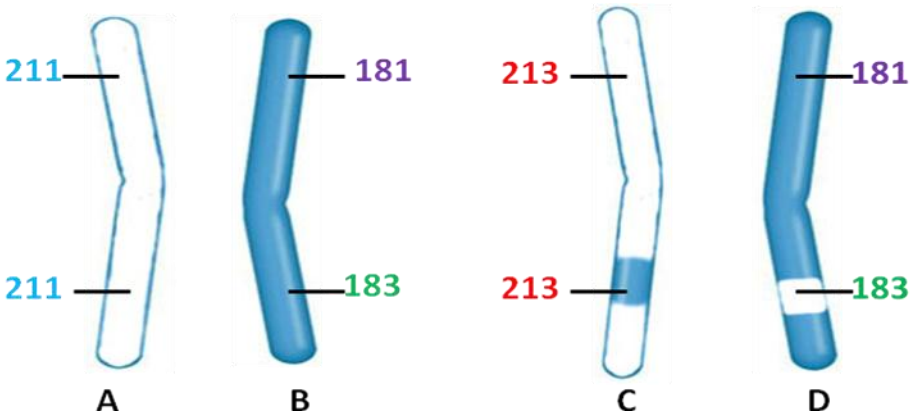
	Microsatellite markers										
Isolate Names	pgms5-6	pgms9-10	pgms21-pgms23-pgms27-pgms 29	pgms 51-pgms 49	pgms 55	pyms87-88	12SSR-genotype
13D-L813-9	273	254	263	213	229	219	260	211	175	173	1A
13D-P823-1	273	254	263	213	229	219	264	211	181	273	2D
13D-P807-2-5	273	254	263	213	229	219	260	211	173	286	3A
13D-P816-4	273	254	263	213	229	219	260	209	177	255	4A
13D-P816-7	273	254	263	213	229	219	260	209	175	286	5D
13D-P807-1-1	257	257	245	213	247	216	260	215	130	212	6A
13D-L823-4	257	257	255	213	247	216	260	219	130	229	7A
13D-P824-2	257	257	245	213	247	216	264	215	130	210	8A
13D-P816-6	257	260	245	213	247	216	260	215	130	212	9A
13D-P825-2	265	254	245	213	232	216	268	215	167	270	10A
13D-P818-4	265	254	245	213	232	216	268	217	167	270	11A
13D-P816-10	273	239	263	213	229	219	260	211	175	286	12A
13D-L823-8	273	254	263	213	229	216	264	211	181	249	13A
13D-P807-1-6	273	254	263	213	229	219	260	211	177	286	14B
13D-P815-5	273	254	263	213	229	219	260	211	179	286	15A
13D-L807-1-2	273	254	263	213	229	219	260	213	173	298	16A
13D-P807-2-8	273	254	263	213	229	219	260	213	175	286	17A
13D-P825-1	273	254	263	213	229	219	264	209	177	255	18A

13D-P826-7	273	254	263	213	229	219	264	209	181	249	19A
13D-L813-15	273	254	263	213	229	219	264	211	177	286	20A
13D-P822-7	273	254	263	213	229	219	264	211	179	249	21A
13D-P822-2	273	254	263	213	229	219	264	211	183	249	22A
13D-L819-2	273	254	263	213	229	219	264	213	181	249	23A
13D-L823-3	273	254	263	213	229	219	264	213	183	252	24A
13D-P822-8	273	254	263	213	229	219	264	225	181	249	25A
13D-P807-2-7	273	254	263	213	229	219	268	211	175	286	26A
13D-L807-2-1(	273	254	263	213	229	219	301	211	175	298	27A
13D-L806-15	273	254	263	213	229	228	260	211	175	286	28A
13D-P816-5	273	254	263	213	232	219	260	211	175	286	29A
13D-P818-6	273	254	263	216	229	219	260	211	175	286	30A
13D-L819-1	273	254	266	213	229	219	260	211	175	286	31A
13D-P828-4	273	254	263	213	241	216	260	209	167	221	32A
13D-P822-3	277	254	254	213	229	219	260	203	146	218	33A
13D-P812-1	277	254	260	213	229	216	260	213	163	218	34A
13D-P827-5	277	254	263	213	226	216	260	209	173	246	35A
13D-L807-2-6	277	254	263	213	229	219	260	211	175	286	36A

4.3.2. Genotypic exchanging in populations

First of all, we tried to figure out in some strains from that data showing four types of two genotypes of each two locus and each of every combination in there. The hypothesis of genotypic marker exchanging was proposed to find the possible recombination within the

populations. To precise, the set of four genotypes for two markers with each of two alleles is considered as a candidate (Fig 4.3). We have been identified 27 candidate genotype sets from the population of rice blast fungus. The size of chromosome is changing between these four strains and we assumed that that could be a strong evidence for recombination in these field isolates. According to these four strains set, it is showing that the possibility of parasexual recombination may occur.



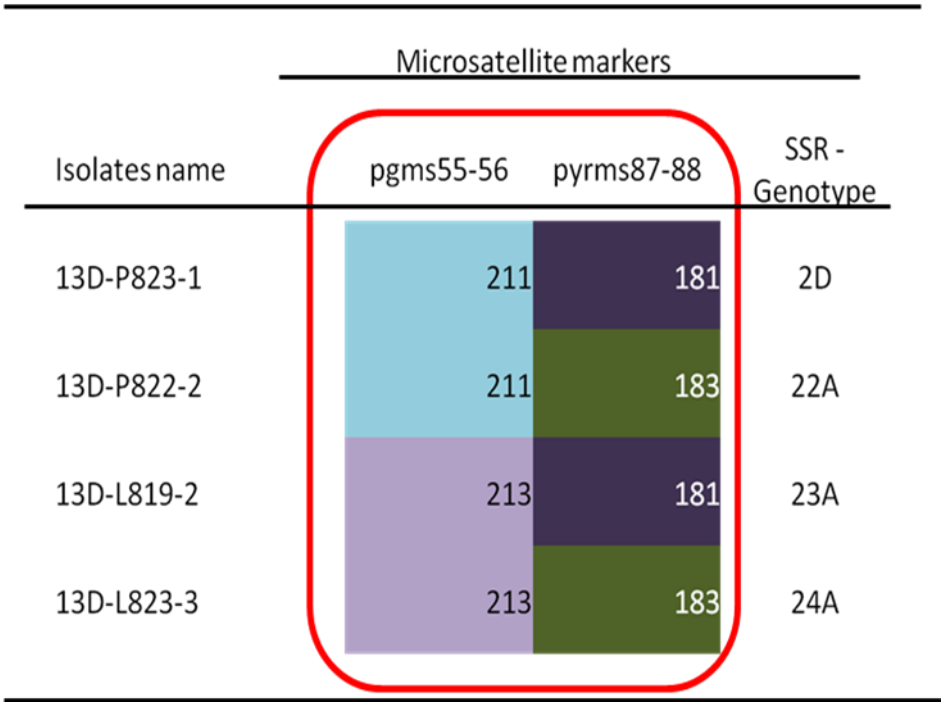


Fig 4.3. One example a pair of set of genotypes for recombination candidates

4.3.3. Chromosomal DNA extraction

The strains were grown on prune agar after culturing at 27°C for two weeks and then transferred into dry tubes which are covered with cotton wool in the dessicator for another two weeks. After this period, the transferred fungi to filter paper were inoculated on Oat meal agar at 25°C for about two weeks. When mycelia growth, scraped with cell scraper and stored at 25°C again for 3 days. Then, the fungi were inoculated into 200ml of 2YEG in Erlenmeyer flasks and incubated at 27°C for 3 days. After inoculation, around 2 grams of mycelia were separated from the media by filtration using a miracloth-1R (CALBIOCHEM, EMD, GERMANY) and digested by adding 5 ml of digestion buffer and incubating at 37°C for 1 hour with shaking at 15 rpm.

After digestion, the protoplasts were collected by filtration with a new miracloth-1R and the filtrate was washed with very few amount of STC buffer and was centrifuged at 3500 × g for 15 minutes at 4°C. The supernatant was discarded. The protoplasts were finally resuspended in 100 µl of isotonic buffer and their concentration was checked by counting using a Neubauer hemocytometer. The protoplast concentration was adjusted to 10^8 cells/ml, and transferred into 1.5 ml microcentrifuge tube and incubated on ice for awhile. After that, the protoplasts were mixed with Low Melt Preparative Grade Agar and then the solidified gels were incubated in the solution containing Proteinase K at 50°C with shaking for 2 days. After 2 days incubation, stored at -4°C for one day.

4.3.3. Pulse-Field Gel Electrophoresis and southern hybridization

The pulse-field gel electrophoresis was performed using the protoplasts which were solidified with agarose gel. About 2 liter of TAE buffer was added in the pulse-field machine and 120 hours run for 5 days with 1.1 voltage to electrified in 120 Included Angel for Block 1 and 48 hours run for 2 days with same voltage and same included Angel conditions for Block 2. The TAE buffer was changed before every 12 hours. After the 7 days run in pulse-field gel electrophoresis, the gel was stained in 10 mg/ml Ethidium bromide solution for 30 minutes and destained in distilled water for 15 minutes then after that photographed by UV detection. The gel was then soaked in fragmentation solution (see Appendix-I for composition), denaturation solution (see Appendix-I for composition) for 30 minutes each solution. And the gel was stained in neutralization solution (see Appendix-I for composition) for 15 minutes before blotting.

Blotting was performed according to Sambrook and Russel (2001) using a Hybond-N^+ nylon membrane attached to the gel and soaked in 20 x SSC (see Appendix-I for composition) as the transfer solvent. After overnight blotting, the membrane was removed, soaked in 6 x SSC for 5 minutes and the DNA was submitted to cross-link using a UV spectrolinker (Spectronics Corp., Japan) applying 1200 x 100 μJ/cm². The amplified DNA fragment which was cloned around SSR markers was used as a probe. The probe was boiled for 5 minutes and chilled in the ice. Then the probe was mixed together with 10 μl of reaction buffer and 2.2 μl of labeling reagent. After that 10 μl cross linker solution which is mixed with water was added into probe and centrifuged to mix thoroughly. The probe was then finally incubated at 37°C for 30 minutes. The DNA attached membrane was incubated with hybridization buffer (see Appendix-I for composition) in a plastic bag for 1 hour with shaking at 55°C in hybridization incubator. After incubation, the probe was pipetting with hybridization buffer solution onto the membrane and incubated again in hybridization incubator with shaking for overnight.

The gene images Alkphos Direct Labelling and Detection System (GE Healthcare) procedure was used to label the probe, hybridization, post-hybridization stringency washes, and detection. The overnight incubated membrane was washed with primary wash buffer (see Appendix-I for composition) in a small container for 10 minutes with shaking at 55°C. And then the membrane was washed again with new primary wash buffer for 10 minutes with shaking at 55°C. The membrane was continuously washed with secondary wash buffer (see Appendix-I for composition) with 1 minute with shaking. Before the secondary wash was performed, the membrane was rinsed with few amount of secondary wash buffer with 2 times. Then changed the secondary buffer with new into the container and the membrane was washed again for 1 minute

with shaking. The membrane was detected with detection reagent on a flat surface for 10 minutes. The bands on the membrane were visualized with imagequant LAS 4000 (Fujifilm Life Science, Roche Diagnostics), after 6 hour of exposure.

Results

After genotypic marker selection, we have been identified the possibility of genetic recombination 27 candidate genotype sets from the population of rice blast fungus according to the set of four genotypes for two markers with each of two alleles (Table 3). The chromosomal DNA was extracted from the fungi culture and the protoplasts were used to observe the occurrence of marker exchange in those candidate isolates using the microsatellite-probes for PFGE (pulsed-field gel electrophoresis)-separated chromosomal DNA (Figure 4.4). Additionally, the hybridization pattern changes may suggest that some exchange occurs between field isolates which can be proved as an evidence for parasexual recombination.

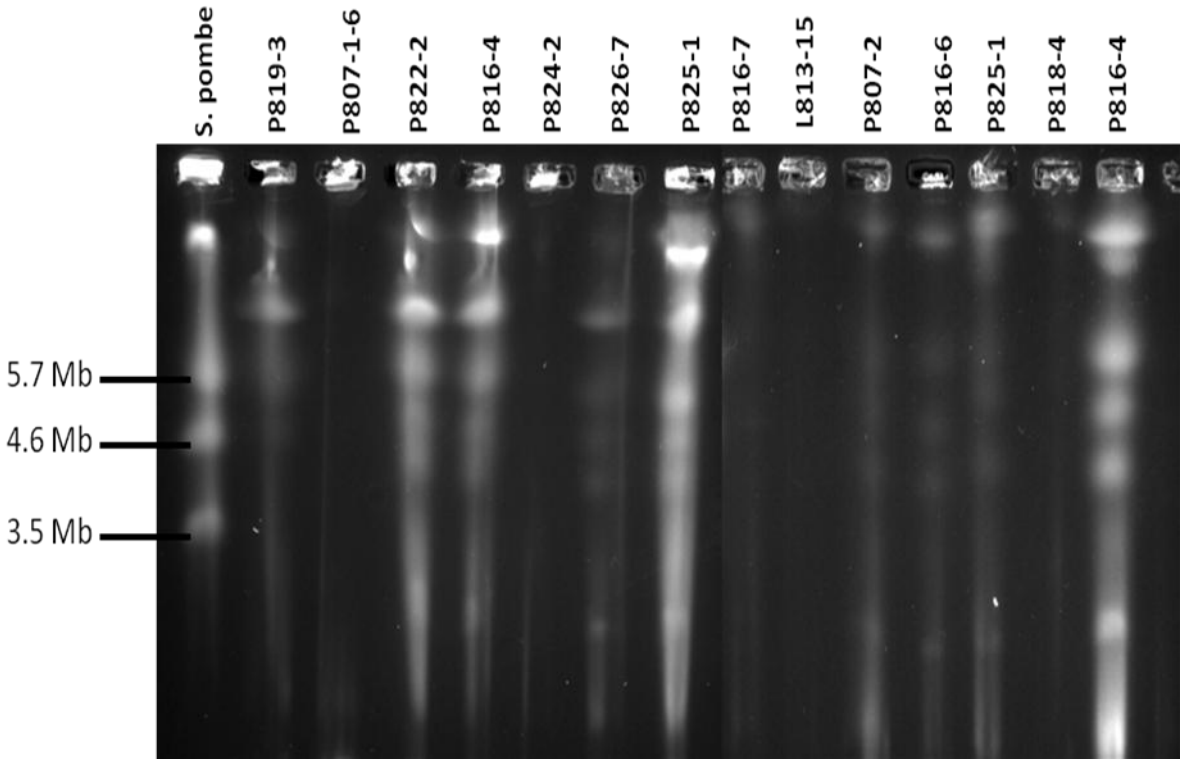


Fig 4.4. Chromosomal DNA separated in the contour-clamped homogeneous electric field (CHEF). The CHEF gel was stained with ethidium bromide.

Discussion

The evidence for parasexual exchange in the wild is scanty. Naturally occurring vegetative diploids of *A. nidulans* have been described (Upshall et al. 1981), but in a sexual species with extensive heterokaryon incompatibility, a sexual origin for natural variation seems more likely (Geiser et al. 1994). On the other hand, in a deliberate attempt to detect parasexual recombination in the wild for *Rhynchosporium secalis*, the agent of barley scald disease, no recombination for isozyme variants were recovered among 1240 isolates after two asexual generations (Goodwin et al. 1994). Similarly, there is no evidence was found for parasexual

exchange between isolates of *Colletotrichum gloeosporioides* strains in cocolonized lesions of the host plant (Chacko et al. 1994).

Initially, we inferred that there might have been such genetic exchanging in field isolates of rice blast fungus in natural conditions. Strong evidence has been needed to prove that chromosomal size exchange in 27 genotype strains set that we separated in PFGE with chromosomal DNA and analyzed with southern hybridization to find out the occurrence of marker exchange between these isolated strains.

Parasesexual exchange or genetic recombination within and among lineages may provide the means to purge deleterious mutations, reconstitute the genome, and generate novel variants (Kang, S., Sweigard, J. A., and Valent, B et al. 1995). Because isolates from different lineages can colonize tissue of the same rice cultivar (Nelson, R. J. et al. 1995, Genovesi, A. D., et al 1976), there is ample opportunity in nature for isolates of distinct lineages to undergo parasexual exchange of DNA. Such genetic exchanges within and between lineages may contribute significantly to pathotype evolution in nature. A possibility of recombination occur in natural condition was proved in this study using field isolates of rice blast fungus and exchange of microsatellite markers showing that the chromosomal size difference is occurred in closely located paddy fields.

CHAPTER 5

SUMMARY

CHAPTER 5

SUMMARY

Pyricularia oryzae (teleomorph: *Magnaporthe oryzae*) is the fungal plant pathogen responsible for blast disease, one of the most damaging diseases that affects rice production worldwide. Additionally, the grain yield losses caused by rice blast are worldwide, whereas in Japan the yield losses were estimated to vary between 20 to 100% (Kush and Jena 2009). Race-specific plant resistance (R) genes have evolved to detect the products of the corresponding avirulence (AVR) genes and trigger effective resistance to plant pathogens. In nature, the effector molecules encoded by AVR genes were thought to facilitate disease development and were hypothesized to be under selection. It is theorized that new virulent races of the pathogen emerged by genetic modification of AVR genes using diverse mechanisms. Although cultivation of resistant cultivars with R genes has been successfully applied for management of rice blast diseases, the strong selection pressure by R genes and rapid pathogen evolution have caused the repetitive breakdown of the resistance. A loss of function in an AVR gene allows the pathogen to avoid induction of resistance in the cultivar to gain pathogenicity to the cultivar, leading to an emergence of a new pathogenic race. AVR genes are responsible for production of effectors, and it is suggested that AVR genes should be recovered after the deletion, to regain fitness. However,

in this research, we attempted the elucidation of deletion mechanism of AVR-Pik gene and investigation of parasexual recombination in the field condition.

We found that strain Ina168 had one copy of *AVR-Pik* and the spontaneous mutant strain Ina168m95-5 lacked *AVR-Pik*. We analyzed the sequences of three cosmid clones 20, 45, and 49, which were screened from the cosmid library of Ina168 in our laboratory, and determined the nucleotide sequence of approximately 39 kb around the *AVR-Pik*. It was suggested that the deletion of *AVR-Pik* involved a complex reorganization of the surrounding region. In this study, we attempted to elucidate the mechanism of the deletion mutation of *AVR-Pik* by further sequencing of both strains. We performed next-generation sequencing analysis of cosmids 20, 45, and 49 to identify the n7 region and by combination of primer walking strategy and PacBio sequencing, sequence of approx. 49 kb DNA region covering *AVR-Pik* was determined. Additionally, 3´ side of *AVR-Pik* was obtained and sequenced with inverse PCR strategy. Finally, the sequence contigs of 55,449bp was determined. On the other hand, the sequence of Ina168m95-5 was completely conserved in the u4 region, which is downstream of AVR-Pik in Ina168, and was predicted to be the 3´ deletion end. We predicted that the 5´ side of the gene contains a deletion region containing AVR-Pik and a partially conserved region, suggesting that the surrounding region undergoes a complex reorganization as described above. However, there is not enough sequence information to elucidate the mechanism of the deletion mutation in *AVR-Pik*. In the vicinity of *AVR-Pik* of Ina168, we found many repetitive sequences such as five copies of transposon *Pot2-like*, two copies of retrotransposons *Pyret*, and two copies of retrotransposons *MGR583*, one copy of retrotransposons *Inago2*, and the retrotransposons solo-LTR sequences. The repetitive sequences are not suitable for the analysis of deletion mutations

because they are scattered over the genome in multiple copies. We focused on low-copy regions that do not contain such sequences for the identification of conserved *AVR-Pik* flanking DNA region in spontaneous *AVR-Pik* deletion mutants.

We focused on Ina168m95-5 mutant for the further analysis of deletion mechanisms of *AVR-Pik*. The sequence of Ina168m95-5 *AVR-Pik* locus was then revealed by repetitive inverse PCR and sequencing, and finally identified as sequence contigs of 56,183 bp. The n7 region was also conserved in Ina168m95-5, but a retrotransposon group was newly identified on its 5´ side, which was not present in the 55 kb region of Ina168. In the upstream of *AVR-Pik*, there were Inago2 and Pot2-like (short), which were fragmented by Inago2. In contrast, in Ina168m95-5 strains, the MINE, MGR583, and n7 regions existed adjacent to the u4 region and showed partial homology to the sequence downstream of *AVR-Pik* in Ina168 strains. However, Pot2-like (short) adjacent to the n7 region is shorter than the corresponding Pot2-like of Ina168, and its downstream sequences include I nago2 (short), I nago1, Pyret (short), four copies of Inago2 (short), Inago1, Pyret (short), and four copies of Inago1 solo-LTR, Pyret solo-LTR, and Pot2-like were found downstream. Therefore, we screened cosmid clones of Ina168 strains containing these novel regions and obtained cosmids 26 and 37, which were subjected to sequencing analysis. This indicates that the determined Ina1 solo -LTR, Pyret solo -LTR and Pot2-like sequences are homologous to those of Ina168m95-5. Thus, the origin of all the determined nucleotide sequences of Ina168m95-5 strains was clarified. Thus, we proposed the mechanism of the deletion of *AVR-Pik* in Ina168m95-5. We hypothesized that the occurrence of a double strand break caused the deletion event which was found at the 5´end of region u4. The multiple

homologous recombination which was triggered by a double strand break resulting the deletion of *AVR-Pik* and the structure of *AVR-Pik* locus in Ina168m95-5.

Parasexual recombination is a cellular event specific for fungi, which involves hyphal fusion of two individuals, followed by nuclear fusion and haploidization, resulting exchange of their DNA fragments. The hypothesis of genotypic marker exchanging was proposed to find the possible recombination within the populations. Those populations were isolated from very limited area and their genotypes were characterized by microsatellite markers. There will be very different genotypes were found in the populations. To figure out the recombination within these populations, we were looking for four genotypes with two markers which have different alleles in two loci. And if the two genotypes recombine at some allele positions, we can infer that those four genotypes set can be a candidate for parasexual marker exchange and 15 recombination candidates set were found. According to these recombination candidates sets, there would be highly possible recombination between individuals.

In order to identify the chromosomal locations of microsatellite markers, we constructed 10 pairs of primers. The cloning of flanking sequences of SSR markers were carried out to obtain DNA probes for identification of chromosomal location of microsatellite markers. The occurrence of marker exchange in those candidate isolates was analyzed with Southern hybridization using the microsatellite-probes for PFGE (pulsed field gel electrophoresis)-separated chromosomal DNA. The exchanging of chromosomal location of microsatellite markers which related to chromosomal size difference between closely-limited area isolates of populations can be proved as the evidence for parasexual recombination. In addition, this research first proposed the chromosomal separation of field isolates using microsatellite markers

exchanging linked to chromosomal size difference. That should be useful to further understanding of rice blast disease controlling in the field range with the molecular level analysis.

References

1. **A. John. Clutterbuck** (1996) Parasexual recombination in Fungi. Journals of Genetics. Springer Link

2. **A. K. Singh, P. K. Singh, Madhuri Arya, N. K. Singh and U. S. Singh** (2014) Molecular Screening of Blast Resistance Genes in Rice using SSR markers. Department of Genetics and Plant Breeding, Institute of Agricultural Research Sciences, Varanasi-221005

3. **Alina Ott, Brian Trautschold, Devinder Sandhu** (2011) Using Microsatellites to Understand the Physical Distribution of Recombination on Soybean Chromosomes. PLos ONE 6(7): e22306

4. **Baker B., Zambryski P., Staskawisz B. and Dinesh-Kumar S. P** (1997) Signaling in Plant-Microbe Interactions. *Science* 276: 726-733

5. **Barbara Valent and Forrest G. Chumley** (1991) Molecular genetic analysis of the rice blast fungus, *Magnaporthe grisea*. Annu. Rev. Phytopathol. 1991. 29:443-67

6. **Böhnert H. U., Fudal I., Dioh W., Tharreau D., Notteghem J. L. and Lebrun M. H** (*2*004) A putative polyketide synthase/peptide synthetase from *Magnaporthe grisea* signals pathogen attack to resistant rice. *The Plant Cell* 16: 2499-2513

7. **Cesari S., Thilliez G., Ribot C., Chalvon V., Michel C., Jauneau A., Rivas S., Alaux L., Kanzaki H., Okuyama Y., Morel JB., Fournier E., Tharreau D., Terauchi R. and Kroja T** (2013) The Rice Resistance Protein Pair RGA4/RGA5 Recognizes the

Magnaporthe oryzae Effectors AVR-Pia and AVR-CO39 by Direct Binding. The *Plant Cell* 25: 1463-1481

8. **Claudia Kaye, Joelle Milazzo, Sophie Rozenfeld, Marc-Henri Lebrun, and Didier Tharreau** (2003) The development of simple sequence repeat markers for *Magnaporthe grisea* and thteir integration into an established genetic linkage map.

9. **Couch B. C. and Kohn L. M** (2002) A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from *M. grisea*. *Mycologia* 94: 683-693

10. **Daboussi M. J. and Capy P** (2003) Transposable elements in filamentous fungi. *Annual review of microbiology* 57: 275-299

11. **Dean R. A., Talbot N. J., Ebbole D. J., Farman M. L., Mitchell T. K., Orbach M. J., Thon M., Kulkarni R., Xu J. R., Pan H., Read N. D., Lee Y. H., Carbone I., Brown D., Oh Y. Y., Donofrio N., Jeong J. S., Soanes D. M., Djonovic S., Kolomiets E., Rehmeyer C., Li W., Harding M., Kim S., Lebrun M. H., Bohnert H., Coughlan S., Butler J., Calvo S., Ma L. J., Nicol R., Purcell S., Nusbaum C., Galagan J. E. and Birren B. W** (2005) The genome sequence of the rice blast fungus *Magnaporthe grisea*. *Nature* 434: 980-986

12. **Dioh W., Tharreau D., Notteghem J. L., Orbach M. and Lebrun M** (2000) Mapping of avirulence genes in the rice blast fungus, *Magnaporthe grisea*, with RFLP and RAPD markers. *Molecular Plant-Microbe Interactions* 13: 217-227

13. **Farman M. L., Taura S. and Leong S. A** (1996a) The *Magnaporthe grisea* DNA fingerprinting probe MGR586 contains the 3' end of an inverted repeat transposon. *Molecular Genetics and Genomics* 251: 675-681

14. **Farman M. L., Tosa Y., Nitta N. and Leong S. A** (1996b) *MAGGY*, a retrotransposon in the genome of the rice blast fungus *Magnaporthe grisea*. *Molecular Genetics and Genomics* 251: 665-674

15. **Flor H. H** (1971) Current status of the gene-for-gene concept. *Annual Review of Phytopathology* 9: 275-296

16. **Fudal I., Böhnert H. U., Tharreau D. and Lebrun M. H** (2005) Transposition of MINE, a composite retrotransposon, in the avirulence gene *ACE1* of the rice blast fungus *Magnaporthe grisea*. *Fungal Genetics and Biology* 42: 761-772

17. **Fukiya S., Kodama M., Kito H., Sone T. and Tomita F** (2001b) Establishment of a new cross of the rice blast fungus derived from Japanese differential strain Ina168 and hermaphroditie rice pathogen Guy11. *Bioscience Biotechnology Biochemistry* 65: 1464-1473

18. **Fukuoka S., Saka N., Koga H., Ono K., Shimizu T., Ebana K., Hayashi N., Takahashi A., Hirochika H., Okuno K. and Yano M.** (2009) Loss of Function of a Proline-Containing Protein Confers Durable Disease Resistance in Rice. *Science* 325: 998-1001

19. **Gous Miah, Mohd Y. Rafii, Mohd R. Ismail, Adam B. Puteh, Harun A. Rahim, Kh. Nurul Islam, and Mohammad Abdul Latif** (2013) A review of Microsatellite Markers

and Their Applications in Rice Breeding Programs to Improve Blast Disease Resistance. International Journal of Molecular Sciences ISSN 1422-0067

20. **Hamer J. E., Farrall L., Orbach M. J., Valent B. and Chumley F. G.** (1989) Host species-specific conservation of a family of repeated DNA sequences in the genome of a fungal plant pathogen. *Proceeding of the National Academy of Sciences of the United States of America* 86: 9981-9985

21. **Hebert T. T** (1971) The Perfect Stage of *Pyricularia grisea*. *Phytopathology* 61: 83-87

22. **Hua L., Wu J., Chen C., Wu W., He X., Lin F., Wang L., Ashikawa I., Matsumoto T., Wang L. and Pan Q** (2012) The isolation of *Pi1*, an allele at the *Pik* locus which confers broad spectrum resistance to rice blast. *Theor. Appl. Genet.* 125: 1047-1055

23. **Jeon J., Park S. Y., Chi M. H., Choi J., Park J., Rho H. S., Kim S., Goh J., Yoo S., Choi J., Park J. Y., Yi M., Yang S., Kwon M. J., Han S. S., Kim B. R., Khang C. H., Park B., Lim S. E., Jung K., Kong S., Karunakaran M., Oh H. S., Kim H., Kim S., Park J., Kang S., Choi W. B., Kang S. and Lee Y. H** (2007) Genome-wide functional analysis of pathogenicity genes in the rice blast fungus. *Nature genetics* 39: 561-565

24. **Jia Y, McAdams S. A., Bryan G. T., Hershey H. P. and Valent B** (2000) Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. *The EMBO Journal* 19: 4004-4014

25. **Jose Romao and John E. Hamer** (1992) Genetic organization of a repeated DNA sequence family in the rice blast fungus. Department of Biological Sciences, Purdue University, IN 47907

26. **Ju Huang, Weina Si, Qiming Deng, Ping Li and Sihai Yang** (2014) Rapid evolution of avirulence genes in rice blast fungus *Magnaporthe oryzae*. Huang et al. BMC Genetics 2014, 15:45

27. **Kang S., Lebrun M. H., Farrall L. and Valent B** (2001b) Gain of virulence caused by insertion of a *Pot3* transposon in *Magnaporthe grisea* avirulence gene. *Molecular Plant-Microbe Interactions* 14: 671-674

28. **Kaye C., Milazzo J., Rozenfeld S., Lebrun M. H. and Tharreau D** (2003) The development of simple sequence repeat markers for *Magnaporthe grisea* and their integration into an established genetic linkage map. *Fungal Genetics and Biology* 40: 207–214

29. **Khang C. H., Park S .Y., Lee Y. H., Valent B. and Kang S** (2008) Genome organization and evolution of the *AVR-Pita* avirulence gene family in the *Magnaporthe grisea* species complex. *Molecular Plant-Microbe Interactions* 21: 658-670

30. **Kiyosawa S** (1982) Genetics and epidemiological modeling of breakdown of plant disease resistance. *Annu. Rev. Phytopathol.* 20: 93-117

31. **Lesley A. Boyd, Christopher Ridout, Donal M. O'Sullivan, Jan E. Leach, and Hei Leung** (2013) Plant-pathogen interactions: disease resistance in modern agriculture. National Institute for Agricultural Botany. Cambridge. Trends in Genetics, April 2013, Vol. 29, N0.4

32. **Liu J., Wang X., Mitchell T., Hu Y., Liu X., Dai L. and Wang G. L** (2010) Recent progress and understanding of the molecular mechanisms of the rice-*Magnaporthe oryzae* interaction *Molecular Plant Pathology* 11: 419-427

33. **Luo C. X., Fujita Y., Yasuda N., Hirayae K., Nakajima T., Hayashi N., Kusaba M. and Yaegashi H** (2004) Identification of *Magnaporthe oryzae* avirulence genes to three rice blast resistance genes *Plant Disease* 88: 265-270

34. **Luo C. X., Yin L. F., Koyanagi S., Farman M. L., Kusaba M. and Yaegashi H** (2005) Genetic mapping and chromosomal assignment of *Magnaporthe oryzae* avirulence genes *AvrPik*, *AvrPiz*, and *AvrPiz-t* controlling cultivar specificity on rice. *Phytopathology* 95: 640–647

35. **Mark. L. Farman, Satoru Taura, Sally A. Leong** (1996) The *Magnaporthe grisea* DNA fingerprinting probe MGR586 contains the 3´ end of an inverted repeat transposon. Department of Plant Pathology, University of Wisconsin

36. **Miki S., Matsui K., Kito H., Otsuka K., Ashizawa T., Yasuda N., Fukiya S., Sato J., Hirayae K., Fujita Y., Nakajima T., Tomita F. and Sone T** (2009) Molecular cloning and characterization of the *AVR-Pia* locus from a Japanese field isolate of *Magnaporthe oryzae*. *Molecular Plant Pathology* 10: 361-374

37. **Orbach M. J., Farrall L., Sweigard J. A., Chumley F. G. and Valent B** (2000) A telomeric avirulence gene determines efficacy for the rice blast resistance gene *Pi-ta*. *The Plant Cell* 12: 2019-2032

38. **Robert S. Zeigler** (1998) Recombination in *Magnaporthe grisea*. Rev. Phytopathol. 1998. 36:249-75

39. **Sone T., Abe T., Yoshida N., Suto M. and Tomita F** (1997b) DNA fingerprinting and electrophoretic karyotype of Japanese isolate of rice blast fungus. *Annals of the Phytopathological Society of Japan* 63: 155-163

40. **Sone T., Takeuchi S., Miki S., Satoh Y., Ohtsuka K., Abe A., and Asano K** (2013) Homologous recombination causes the spontaneous deletion of *AVR-Pia* in *Magnaporthe oryzae*. *FEMS Microbiology Letters*, 339 (2): 102-109. doi:10.1111/1574-6968.12058

41. **Talbot N. J** (2003) On the trail of a cereal killer: Exploring the biology of *Magnaporthe grisea*. *Annual Review of Microbiology* 57: 177-202

42. **Thiago M P, Leila G, Valacia L, Anne P and Marta F** (2012) Tagging microsatellite marker to a blast resistance gene in the irrigated rice culivar Cica-8. Crop Breeding and Applied Biotechnology 12: 164-170

42. **Valent B., Farrall L. and Chumley F. G** (1991) *Magnaporthe grisea* genes for pathogenicity and virulence identified through a series of backcrosses. *Genetics* 127: 87-101

43. **Wang B., Daniel J. and Wang Z** (2017) The arms race between *Magnaporthe oryzae* and rice: Diversity and interaction of *AVR* and *R* genes. *Journal of Integrative Agriculture* 16 (12): 2746-2760

44. **Weihuai Wu, Ling Wang, Shu Zhang, Zekang Li, Yu Zhang, Fei Lin, and Qinghua Pan** (2014) Stepwise Arms Race Between *AvrPik and Pik* Alleles in the Rice Blast Pathosystem. MPMI Vol.27, No.8, 2014, pp.759-769. DOI:10.1094/MPMI-02-14-0046-R

45. **Wei-Li, Yanxia Feng, Haiyan Sun, Yuanyu Deng, Hanshou Yu, Huaigu Chen** (2014) Analysis of simple sequence repeats in the *Gaeumannomyces graminis* var. *tritici* genome and the development of microsatellite markers. Curr Genet (2014) 60:237-245

46. **Yamada M., Kiyosawa S., Yamaguchi T., Hirano T., Kobayashi T., Kushibuchi K. and Watanabe S** (1976) Proposal of a new method for differentiating races of *Pyricularia oryzae* Carava in Japan. *Annals of the Phytopathological Society of Japan* 42: 216-219

47. **Yoshida K., Saitoh H., Fujisawa S. Kanzaki H., Matsumura H., Yoshida K., Tosa Y., Chuma I., Takano Y., Win J., Kamoun S. and Terauchi R** (2009) Association genetics reveals three novel avirulence genes from the rice blast fungal pathogen *Magnaporthe oryzae*. *The Plant Cell* 21: 1573-1591

48. **Zhai C., Lin F., Dong Z., He X., Yuan B., Zeng X., Wang L. and Pan Q** (2011) The isolation and characterization of *Pik*, a rice blast resistance gene which emerged after rice domestication. *New Phytol*. 189: 321-334

49. **Zhang S., Wang L., Wu W., He L., Yang X. and Pan Q** (2015) Function and evolution of *Magnaporthe oryzae* avirulence gene *AvrPib* responding to the rice blast resistance gene *Pib*. *Scientific Reports* 5, 11642

50. **Zhou E., Jia Y., Singh P., Correll J. C. and Lee F. N** (2007) Instability of the *Magnaporthe oryzae* avirulence gene *AVR-Pita* alters virulence. *Fungal genetics and biology* 44: 1024-1034

APPENDICES

Appendix I. Media and Buffer

LB-Amplicillin

Polypeptone 10 g/L
Yeast Extract 5 g/L
Sodium Chloride 10 g/L
Fill up with autoclaved distilled water 1L
Adjust pH to 7.0
Autoclave at 121°C for 15 min, allow to cool down to 60°C, add ampicillin to a final concentration of 100 μg/mL.

LB-Agar/Ampicillin

Polypeptone 10 g/L
Yeast Extract 5 g/L
Sodium Chloride 10 g/L
Fill up with autoclaved distilled water 1L
Adjust pH to 7.0
Agar 15 g/L
Autoclave at 121°C for 15 min, allow to cool down to 60°C, add ampicillin to a final concentration of 100 μg/mL.

Oatmeal Agar

Oatmeal 50 g/L
Sucrose 20 g/L
Agar 35 g/L

Add oatmeal to water, boil at 95°C for 30min and filtrate using a gauze and funnel. Mix the filtrate, sucrose, agar and dissolve in water filling up to a final volume of 1 liter. Autoclave at 121°C for 20 min and allow to cool down to 60°C.

2YEG (Yeast Extract and Glucose)

Yeast Extract 2 g/L
Glucose 10 g/L
Autoclave at 121°C for 15 min.

Prune Agar

Prune 4g/L
Yeast Extract 1 g/L
Lactose 5 g/L
Agar 17 g/L
Autoclave at 121°C for 20 min.

Buffers

DNA Extraction Buffer

1 M Tris-HCl (pH 8.5) 200 mL/L
0.5 M EDTA (pH 8.0) 50 mL/L
NaCl 15 g/L
Sodium dodecyl sulfate 5 g/L
After volume determination using distilled water, the samples were sterilized by autoclaving (121ºC, 20 min).

Sterile Distill Water (SDW)

Autoclave distilled water at 121°C or 15 min and allow to cool down at room temperature.

50X Tris Acetic acid EDTA (TAE) Buffer

Tris 242 g
EDTA 18.6 g
Acetic acid 57 mL

Electrophoresis buffer

1 X TAE (200 mL of 50X TAE in 9.8 L of DH_2O)

TE buffer

10mM Tris-Cl, pH 7.5
1 EDTA
Make from 1 M stick of Tris-Cl (pH 7.5) and 500mM stock of EDTA (pH8.0).
10 mL 1 M Tris-Cl pH 7.5 per liter.
2mL 500mM EDTA pH 8.0 per liter.

1M Tris (crystallized free base)

Tris (hydroxymethyl) aminomethane
FW 121.4 g/mol
60.57 g in 0.5 L mq water
pH to 7.5 using HCl

0.5M EDTA

Diaminoethane tetraacetic acid
FW 372.2 g/mol
18.6 g in 100 ml mq water
pH to 8.0 using NaOH
• EDTA Will not be soluble until pH reaches 8.0
• Use vigorous stirring, moderate heat and time

STC Buffer

Sucrose 20%
1M Tris-HCL (1 mL per 100 mL), 10 mM (pH 7.3)
2.5 M CaCl2 (2 mL per 100 mL) Mix and autoclave at 121 ° C

Digestion Buffer Stock

Sucrose (MW: 342) 0.6M (205.5 g/L)
Maleic acid monosodium salt (MMS) (C4H304Na.3H20), 50 mM (9.65 g/L)
Dissolve MMS in DH20 and adjust to pH 5.5 before adding sucrose

Working Digestion Buffer (5 mL)

Yatalase 0.1 g
Cellulase 0.025 g
Dissolve in 5 mL of Digestion Buffer Stock.

Southern Hybridization buffers

Depurination solution (P-Solution)
12NHCl 20 mL
SDW 860 mL

Denaturation solution (D-Solution)
NaCl (An. Grade) 43.83 g
NaOH 10.00 g
SDW fill up to 1 Liter

Neutralization Solution (N-solution)
NaCl (An. Grade) 43.83 g

Tris (Mwt, 121.14) 30.28 g
SDW 1 Liter
Adjust to pH 7.5 with 12NHCl

20x SSC
Trisodium citrate Dihydrate 88.23 g
NaCl (An. Gnd) 175.3 g
SDW 1 Liter
Adjust to pH 7.0 with I2NHCI

Pre-hybridization buffer

NaCl 0.5M 5.8 g
Blocking Reagent 4% (w/v) 8 g
Stirring with magnetic stirrer for 1 hour. (Adding Blocking reagent slowly)

Primary Wash Buffer

Urea 2M 120g

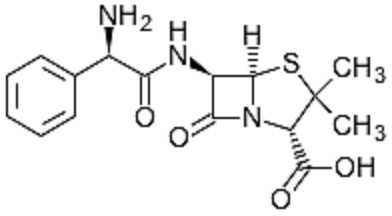
SDS 0.1%(w/v) 1 g

0.5M Na Phosphate pH7.0 100 ml
NaCl 150mM 8.7g
1.0M $MgCl_2$ 1ml
Blocking Reagent 0.2%(w/v) 2g
Stirring with magnetic stirrer for overnight.

Secondary Wash Buffer

20 x Stock Seconday Wash buffer 50 ml
1 M $MgCl_2$ 2 ml
Sterilized Water 948 ml

Appendix II: DNA Damage agents, Antibiotics, and Fungicidal compounds used in this study

Name	Ampicillin; Anhydrous ampicillin
Formula	C16H19N3O4S
Exact mass	349.1096
Mol weight	349.4048
Structure	

Acknowledgements

Acknowledgements

It is of great pleasure to express my sincere and deep gratitude to my advisor and guide Prof. Dr. Teruo Sone, whose expertise, understanding, and generous support made it possible for me to work on a challenging and new topic that was of great interested for me. His enthusiasm for the rice blast kept me continuously engaged with the research, and his personal generously helped make my time at Hokkaido University enjoyable. My special thanks go to and Prof. Dr. Yoshitomo Kikuchi for reading and making the corrections that improved the quality of this thesis.

I owe a deep sense of gratitude to Dr. Abe Ayumi who provided me the best technical guidance during all my experiments. Her prompt guidance, timely suggestions with kindness, and wisdom have enable me to complete my thesis.

My sincere thanks to Prof. Dr. Asano Kozo, Prof. Dr. Yoshitomo Kikuchi, Dr. Souichirou Kato, Dr. Wataru Kitagawa and Prof. Dr. Taichi Takasuka for all comments and suggestions during our weekly seminars. Their advices and scientific approach have helped me to accomplish my tasks.

My gratitude also goes to all students in the Applied Molecular Microbiology laboratory, for their kind help and co-operation.

My appreciation also extends to the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) for supporting my studies at Hokkaido University.

I would like to acknowledge all my friends from several different countries I have made in Sapporo. Our friendship is one of the best things that has happened to me in Japan.

Above ground, I indebted to my family and fiancé, who filled me with a life of joy in the hours of difficulties. To all of you I say Thank you very much.