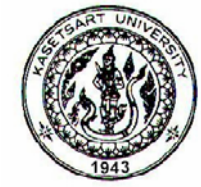

THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Doctor of Philosophy (Genetic Engineering)

DEGREE

Genetics Engineering | Interdisciplinary Graduate Program

FIELD | **PROGRAM**

TITLE: Identification of *Magnaporthe grisea* Avirulence Genes Specific to Rice Blast Resistance Genes

NAME: Mr. Tanee Sreewongchai

THIS THESIS HAS BEEN ACCEPTED BY

(Mr. Theerayut Toojinda, Ph.D.) **THESIS ADVISOR**

(Associate Professor Apichart Vanavichit, Ph.D.) **COMMITTEE MEMBER**

(Ms. Pattama Sirithunya, Ph.D.) **COMMITTEE MEMBER**

(Mr. Didier Tharreau, Ph.D.) **COMMITTEE MEMBER**

(Assistant Professor Siriwan Prapong, Ph.D.) **GRADUATE COMMITTEE CHAIRMAN**

APPROVED BY THE GRADUATE SCHOOL ON _____

(Associate Professor Gunjana Theeragool, D.Agr.) **DEAN**

THESIS

IDENTIFICATION OF *MAGNAPORTHE GRISEA* AVIRULENCE GENES SPECIFIC TO RICE BLAST RESISTANCE GENES

TANEE SREEWONGCHAI

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Genetic Engineering)
Graduate School, Kasetsart University
2008

Tanee Sreewongchai 2008: Identification of *Magnaporthe grisea* Avirulence Genes Specific to Rice Blast Resistance Genes. Doctor of Philosophy (Genetic Engineering), Major Field: Genetic Engineering, Interdisciplinary Graduate Program. Thesis Advisor: Mr. Theerayut Toojinda, Ph.D. 110 pages.

Avirulence genes of blast fungus *Magnaporthe grisea* were studied using Quantitative trait loci (QTL) approach. Two haploid mapping populations derived from THL1000xTHL16 and B1-2xTH16 crosses were developed by artificial crossing in the laboratory. THL1000 was isolated from a wild rice *Oryza rufipogon*. B1-2 was isolated from cultivated rice, *Oryza sativa*, and TH16 was isolated from barley, *Hordium vulgare*. Eighty eight and two hundred eighty eight haploid progenies were successfully developed from THL1000xTHL16 and B1-2x TH16 crosses respectively. These populations were used to study the arising of new virulence pathotype from the genetic recombination and to identify the genomic location of avirulence (*Avr*) gene that specific to the resistance gene in 10 rice cultivars such as Sariceltik, Jao Hom Nin (JHN), C101TTP, C101PKT, C104PKT, C105TTP, Azucena, CNT1, IR64 and IR57514.

Transgressive segregation for virulence and avirulence characteristics were observed in rice cultivars Azucena, C101TTP, IR64 and IR57514. Two *Avr* loci that are specific to the resistance genes in Sariceltik were mapped on *M. grisea* chromosomes 2 and 4 in the THL1000xTHL16 haploid population. THL1000 contributed both alleles for the avirulence characteristic. Ten *Avr* loci that are specific to the resistance genes in rice cultivars JHN, C101TTP, C101PKT, C104PKT, C105TTP, Azucena, CNT1, IR64 and IR57514 were mapped on chromosomes 1, 2, 4, 6 and 7 in the B1-2xTH16 haploid population. THL16 contributed *Avr* alleles specific to the resistance genes carried by JHN, C101PKT, C104PKT, C105TTP, CNT1, IR64. B1-2 contributed *Avr* alleles specific to the resistance genes carried by Azucena, C101TTP and IR57514.

_____ _____ ___ / ___ / ___

Student's signature Thesis Advisor's signature

ACKNOWLEDGEMENTS

First of all, I would like to grateful thank and deeply indebted to my thesis advisor Dr.Theerayut Toojinda and co-advisors Dr.Pattama Sirithunya, Asso.Prof.Dr.Apichart Vanavichit, and Dr.Didier Therreau for providing an opportunity to work with them. They have advice, encouragement and valuable suggestion for completely doing and writing of thesis. They maintained an open mind towards this thesis. They allowed me the freedom to develop and pursue this research topic. By allowing me to work independently, they shown much confidence in my abilities which I not only greatly appreciate, but also believe it has helped me to grow as a researcher. I take this opportunity to extend my sincere gratitude to Assoc. Prof. Dr. Nawarat Udomprasert from the Graduate School for her valuable comments and suggestions to write this thesis.

I would like to express my sincere to the National Center for Genetic Engineering and Biotechnology (BIOTEC), Ministry of Science and Environment and the Rockefeller Foundation for the financial support during this program of study.

The completion of this thesis would not have been possible without the support of the Rice Gene Discovery unit, National Center for Genetic Engineering and Biotechnology (BIOTEC), Kasetsart University, Kamphangsaeng, Nakornpathom, Thailand. Thank you so much to the staff members in this unit for their technical assistants in molecular biology that I never done before. To my colleagues and workers in the Rice Blast Project and the Hybrid Rice Project, I thank you all for what you have done for me. Without their help, I can not success in this research. I have leaned many useful techniques from them.

Finally, I would like to express my appreciation and deep gratitude for my family for their supports and encouragement throughout my study.

Tanee Sreewongchai

Octuber, 2008

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF ABBREVIATIONS

bp	=	base pairs
^{o}C	=	degree Celsius
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleotied triphosphate
ETH	=	ethylene
g	=	gram
h	=	hour
HR	=	hypersensitive respond
ISR	=	induced systemic resistance
JA	=	jasmonic acid
kb	=	kilobasepair (1,000 base pair)
LRRs	=	leucinerich repeats
l	=	litter
mg	=	microgram
µl	=	microliter
MgCl2	=	magnesium chloride
M	=	molar
Mb	=	mega base pair
NBS	=	nucleotide binding site
ng	=	nanogram
PAGE	=	polyacrylamide gel electrophoresis
PCR	=	polymerase chain reaction
RFA	=	rice flour agar
RL	=	resistant loci
SA	=	salicylic acid
SAR	=	systemic acquired resistance

IDENTIFICATION OF *MAGNAPORTHE GRISEA* AVIRULENCE GENES SPECIFIC TO RICE BLAST RESISTANCE GENES

INTRODUCTION

Rice is the staple food for nearly one-half of the world's population and it can be consider as the most important crop on the earth. More than 90% of world's rice is grown and consumed in developing countries. One of the most serious factors to reduce rice production is blast disease. This disease is widespread and damages rice growing area which has been reported from almost 70 rice growing countries of the world. Outbreaks of rice blast disease cause serious problem in all rice-growing regions of the world. It causes significant crop losse causing between 11% and 30% of grain yield losse annually. This represents a loss of 157 million tones of rice, which can be feed to 60 million people. Losses due to blast may range up to 90%, depending upon the part of the plant infected such as on leaf and panicle (Zeigler *et al*., 1994).

In Thailand, rice is the most important crop and rice is the main food for Thai people. Rice plants can grow in every parts of Thailand. Currently Thailand is the world's no.1 rice exporter. In 2003, about 26.5 % of the total global rice export was made by Thailand. Although Thailand is one of the largest producer and consumers, the grain yield production per area is almost lowest in the rice production countries (Meyer and Prasertsri, 2007). The low grain yield per production is caused by many factors such as abiotic and biotic stress. Rice blast disease is one of biotic stresses for rice in Thailand. It has been reported year and it always parting a serious problem in rice production (Sriboonjit and Viboonpong, 2000).

The suitable strategy to control this disease is to use blast resistance rice variety and many researchers have been concerning to improve new blast resistance varieties to solve this problem. JHN rice variety was determined to have broad spectrum blast resistance in Thai blast pathogen population. It showed 0.99 broad

spectrum resistance index mening it has resistance to 99 isolates from 100 tested isolates. Two QTLs affecting blast resistance were located on chromosome 1 and 11. They were determined as having quantitative genetic control and the QTL on chromosome 11 has a major effect (Sirithunya *et al*., 2004). Recently, these blast resistance loci are widely used in many breeding programs to improve blast resistance in target or new varieties. Since blast pathogen is diverse in Thailand, the new resistance varieties can be overcome by new virulence blast isolates in the few years of releasing them.

Nowadays, rice - blast pathogen pathosystem has been used as a model organism to study a model to understand the nature of pathogen population structure and the breakdown of plant resistance genes. This has led to efforts to understand the genome of the fungus in order to determine the types of variation that allows inferion in newly deployed resistance varieties. These studies helped to formulate strategies for breeding durable resistance in rice (Ebbole, 2007). The molecular genetic of rice blast pathogen and its correlated pathotypes have been extensively studied by various approaches (Babujee and Gnanamanickam, 2000). Since blast pathogen genome sequence are available showing microsatellite or simple sequence repeat (SSR) distribution throughout the genome, many SSR markers have been developed and used as one of the most informative markers for genome mapping, DNA fingerprint and population studies (Brondani *et al*., 2000a; Kaye *et al*., 2003; Ma *et al*., 2006b). Moreover, integrative analyses between useful segregations of SSR alleles and phenotypes of interested traits could help identify regions containing gene(s) that affects phenotype. Quantitative Trait Loci (QTL) mapping is one of the most powerful methods which it widely use in rice to identify blast resistance genes (Ramalingam *et al*., 2003; Sallaud *et al*., 2003; Sirithunya *et al*., 2002). However, it has been rarely used to identify genes in blast fungus.

In order to evaluate potential sexual genetic recombination in developing new virulence blast progenies, crossing rice infected isolates between two differente host plants; wild rice (*O. rufipogon* Griff.) and barley (*Hordeum vugarle* L.) are needed. There are 2 *M. grisea* crosses using in this thesis; the first is the identification of avirulence gene specific to wild rice (*O. rufipogon*) in THL1000 and THL16 cross,

and the second is the identification of avirulence gene specific to Jao Hom Nin rice variety in B1-2 and TH16 cross. The two F1 haploid populations developed from these crosses can reveal the phenomenon of sexual recombination that might occur in new virulence isolates in nature.

In B1-2xTH16 cross, a fungal population generated from this cross of blast pathogen isolates that showed differential disease reactions on JHN rice variety at leaf and panicle blast symptoms were used for the genetic characterization of avirulence genes in this pathogen. A genetic linkage map of blast pathogen population was developed using microsatellite markers. The relationships between the disease phenotypes and the genetic map were analyzed to identify genes associated with virulence/avirulence characteristics in blast pathogen by using quantitative trait loci approach. The close link of molecular markers to these loci will provide a foundation for map based cloning. Cloning and characterization of these genes will enable an extensive understanding of the interaction between resistance gene in rice and avirulence gene in blast pathogen in future.

OBJECTIVES

1. To study the sexual genetic recombination of blast pathogens developed from the crosses of two isolates derived from different host plants such as cultivated rice (*Oryza sativa* L.), wild rice (*O. rufipogon* Griff.) and barley (*Hordeum vugarle* L.).

2. To observe new virulence phenotype arising from sexual genetic recombination

3. To develop linkage maps of blast pathogen population using molecular markers.

4. To identify number, location and effect of avirulence genes through QTL analysis.

5. To saturate the linkage map within the region flanking Avr gene that corresponds to blast resistance gene in JHN rice variety.

LITERATURE REVIEW

Blast disease

Causal organism

The causal organism of blast disease is a heterothallic ascomycetous fungus *Magnaporthe grisea* (Hebert) Barr (anamorph *Pyricularia grisea* Sacc). This hermaphroditic heterothallic ascomycete has wide host range and vareity specificity. More than 50 gramineous hosts, including economically important crop as rice (*Oryza sativa* L.), wheat (*Triticum aestivam*) and millets (*Eleusine* spp. *Echinochoa* spp. *Panicum* spp., and *Setaria* spp) can be infected by this pathogen. The name of this pathogen was previous called as *Pyricularia grisea*. Then the perfect stage of *P. grisea* was first discovered from crossing crabgrass isolate (Hebert, 1971) and described it as *Ceratophaeria grisea* and this name was change to the genus *Magnaporthe* by Barr. Although, the telemorph was rarely encountered in nature, but the sexual stage was known. The sexual stage, *Magnaporthe grisea* is heterothallic ascomycetes and is controlled by bipolar mating type locus (*MAT*). The name *Magnaporthe grisea* (Hebert) Barr and *Pyricularia grisea* Sacc were accepted to refer to the blast fungus under the rules of the International Code of Botanical Nomenclature.

The taxonomic definition of the anamorph state of blast fungus is as follows:

Division: Eumycota
Subdivision: Ascomycotina
Class: Pyronomycetes
Order: Diaporthales
Family: Magnaporthaceae
Genus: Magnaporthe

(Alexopolos *et al*., 1996)

Blast symptoms

Rice pathogenic isolate of the blast pathogen produces lesions on all parts of the rice plant; leaves, neck node, and panicles (Figure 1). When lesions appear on leaves, they are often white to gray-green with dark green or brown border. Their shape varies but lesions are characteristically spindle shape or eye shape. There are several types of lesions based on the resistance and plant age. Leaf reactions vary from pinpoint infections to large elliptical lesions up to 1.5 cm long and 0.3-0.5 cm broad. Large lesions without signs of plant resistance are referred to sometimes as acute lesions, while small lesions indicate a degree of resistance. Blast infection in early plant stages can cause stunting or death, depending on the severity of the attack and on the humidity and temperature during the infection period. Neck or panicle usually causes more severe damage to the crop than leaf blast. Neck blast is seen as a gray brown lesion around the panicle nodes just below the panicle, causing the panicle to fall over ("rotten neck"). Early attack at this point will result in no or very few grain filling and high harvesting loss. A later attack can be less damaging, although the grains will not completely develop (Kato, 2001; Merhrotra and Aggarwal, 2003; Ou, 1980; Tenjo and Hamer, 2002). In addition, (Sesma and Osbourn, 2004) reported a new facet of the *M. grisea* life cycle, where the fungus can undergo a different and previously uncharacterized set of programmed developmental events that are typical of root-infecting pathogens. They also show that root colonization can lead to systemic invasion and the development of typical disease symptoms on the above ground parts of the plant.

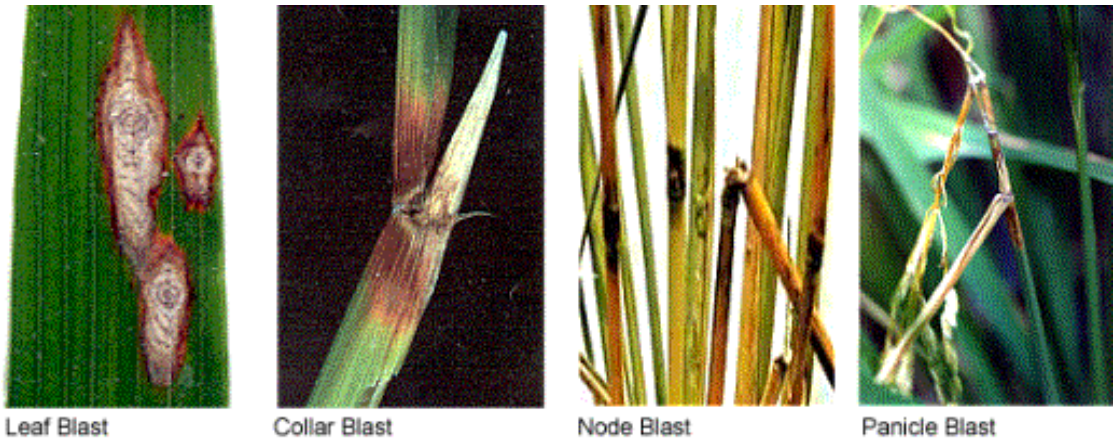

Figure 1 Typical disease symptoms caused by the rice blast fungus *M. grisea*.
Source: http://www.knowledgebank.irri.org

The life cycles

There are two life cycles of blast pathogen. The first is asexual or infection life cycle. The infection by rice blast fungus starts when the conidia attach on a host leaf and anchors itself to the leaf cuticle with spore-tip mucilage. Germination proceeds with the extension of a germ tube, which undergoes hooking and swelling at its tip and then differentiates into an infection structure called the appressorium. During maturation, the appressorium becomes melanized, except for a well-defined pore between the appressorium and the rice leaf (Howard and Valent, 1996). The formation of this infection structure on the host surface marks the onset of the disease. A penetration peg is then driven through the host surface and the infection hypha invades and grows through the rice leaf (Talbot, 2003). At this stage, the symptoms become evident and small oval lesions begin to appear, accompanied by local chlorosis. Eventually, the growing lesions become necrotic and may coalesce. Conidia are produced again on lesions on the rice plant about 5 to 7 days after infection. The production of conidia increases with increase in the relative humidity. Most of the conidia are produced and released during the night. The conidia are carried by air to neighboring plants for spreading the blast disease (Figure 2).

The second one is sexual life cycle. The blast fungus can be cultured on defined media and grow under *In vitro* condition. The sexual life cycle of blast fungus have been found in 1971 (Hebert, 1971). Mating in this pathogen is hypothesized to

occur between two haploid cells of opposite mating types, MAT1-1 and MAT1-2, through the secretion and binding of peptide pheromones. The role of pheromone signaling in mating is thought to involve recognition of cells of the opposite mating type (Shen *et al*., 1999). Three weeks after crossing, they will form sexual fruiting bodies called perithecia. Perithecia are flask-shaped bodies that carry asci—bags containing ascospores, the products of meiosis in abundance. Asci can be dissected to liberate the ascospores, which are arranged as unordered octads (four pairs of spores representing the products of meiosis that have undergone a subsequent mitotic division) or as larger populations of randomly selected ascospores. The ascosopres can germinate and grow as conidia in culture medium or in nature (Talbot, 2003)

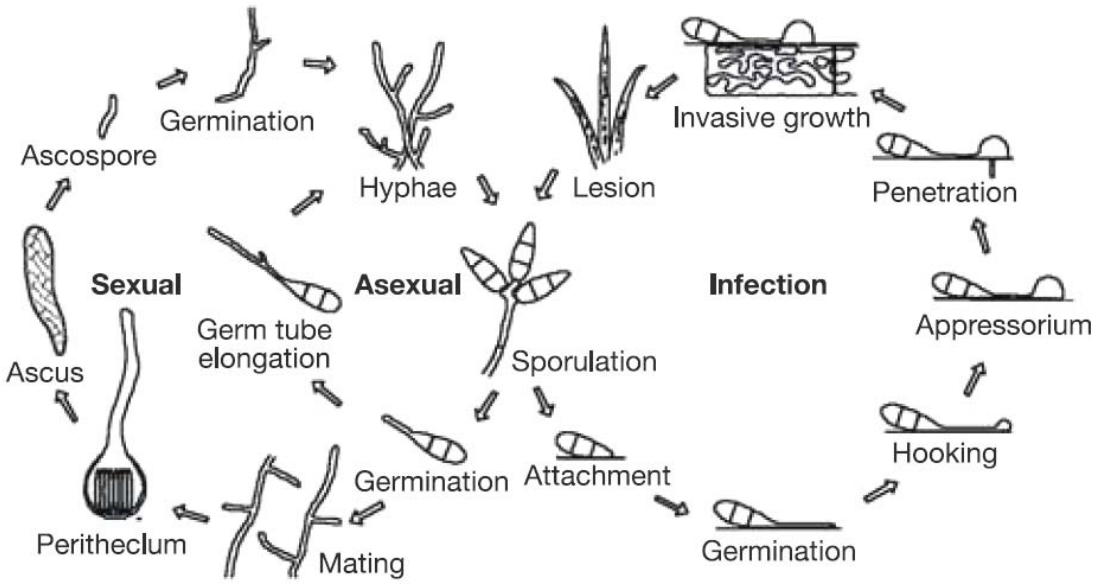

Figure 2 Life history of *M. grisea*.
Source: Dean *et al*., (2005)

Genetic diversity of blast pathogen

Breakdowns of blast resistant varieties were common in many rice-growing areas. Diversity of pathogen populations are cited as main cause of the breakdown in resistance varieties. Blast pathogen is known for its high capacity in asexual reproduction and high variation of pathogenicity in the fields (Zeigler *et al*., 1994). In

nature, the heterokaryosis and parasexual cycle cause the variation of *M. grisea* (Crawford *et al*., 1986; Genovesi and Magill, 1976). Although parasexual recombination has been described in blast fungus, its role in natural population is poorly understood (Zeigler *et al*., 1997).

Characterization of pathogen population was used in terms of the amount of genetic variation among individual in a population and phylogenic relationship of individual within and between sub populations (Leung *et al*., 1993). Population for genetic analysis requires the use of different markers. In plant pathogens, virulence has been widely used as primary character to assess the pathotypic structure of pathogen populations. More recently pathogen populations have been widely characterized into different lineages. Lineage here is defined as statistically similar group of individuals inferred to be related via descent from a common ancestor. The nature clone of many plant pathogen, including *P. grisea*, in nature that consist of sets of sub-clones (Zeigler *et al*., 1997). The set of 18 microsatellite markers were developed for population studies. Preliminary results on six populations from different geographical origins show that this set covers a wide range of variation for the number and frequency of the detected alleles. This set of markers can be adapted to the study of *M. grisea* population with narrow and broad genetic basis, but also in the measurement of parameters relying either on rare or on frequent alleles (Santoso *et al*., 2007).

Genetic analysis was done in 779 isolates from different kinds of host such as rice, weed, and barley. All of these samples were randomly collected from farmer's field and some in rice research centers of Thailand. Data of amplified fragments length polymorphism (AFLP) fragments generated by using 6 primer combinations were used in the analysis. The dendrogram of 779 isolates showed wide diversity. The result shows that blast pathogen isolates can be differentiated from host into 2 lineages of rice, 1 lineage for barley, 1 lineage for wild rice and 1 lineage for weeds in rice fields. Moreover, at the similarity level of 0.9, the biggest group derived from rice cultivar, had 16 main lineages (Hutamekalin *et al*., 2001). The higher diversity of blast pathogen isolates were observed in northern, north-eastern and central Thailand while eastern and southern parts were rather low. Genetic diversity indices elucidated an

abundance of pathogen lineages existing in northern Thailand suggesting that it should be the centre of diversity (Sirithunya *et al*., 2007). Five hundred twenty-seven blast pathogen isolates were collected across three seasons at five sites in Thailand. Significant differences in pathotype diversity were detected across sites, seasons, and among isolates collected from exotic versus indigenous hosts. Isolates and pathotypes with greater numbers of virulence genes (as inferred from compatibility with NILs) were less common than those with fewer virulence genes. Analysis of virulence distributions among isolates grouped according to their MGR586 DNA-fingerprint similarities also showed that, for the most commonly represented lineages, isolates with fewer virulence genes predominated. Lineages represented by one or a few isolates had greater numbers of virulence genes (Mekwatanakarn *et al*., 2000).

Magnaporthe grisea genome

Since there are many pathogens to be investigated and given our limited pool of resources, the most reasonable research approach is to fully understand a 'model' of pathogenic organism and apply that knowledge to design control strategies for other related pathogens. A candidate 'model' organism should have the following characteristics (Dean *et al*., 2005; Xu *et al*., 2006):

1. It should use an infection strategy that is evolutionarily related to other pathogens.

2. It should have a relatively small genome size.

3. It should be economically important.

4. It should be relatively well studied and amenable to molecular and classical genetic experimentation.

Magnaporthe grisea is an excellent model organism for studying fungal phytopathogenicity and host-parasite interactions. *M. grisea* is a haploid, filamentous Ascomycete with a relatively small genome of ~40 Mb contained in 7 chromosomes (Dean *et al*., 2005). The majority of fungal pathogens belong to this taxonomic class or exist as related asexual forms (Agrios, 1997). *M. grisea* is also closely related to the non-pathogen *Neurospora crassa*, a leading model organism for the study of eukaryotic genetics and biology. Unlike many phytopathogenic fungi such as mildews and rusts, the rice blast fungus can be cultured on defined media, facilitating biochemical and molecular analyses. Significantly, early stages of the infection process including germination, appressorium formation and penetration can be studied ex planta (Dean *et al*., 2005; Talbot, 2003; Xu *et al*., 2006; Zeigler, 1998).

The International Rice Blast Genome Consortium has agreed to use the rice-infecting strain 70-15 as the seminal isolate for genome sequencing. This isolate was developed by numerous back crosses to the wild isolate Guy 11 (Chao and Ellingboe, 1991; Leung *et al*., 1988). Strain 70-15 (Mat1-1) is pathogenic on rice and is fully fertile (acts as both male and female). Crosses can be made with its sib strain 70-6 and many other strains carrying Mat1-2. Numerous resources have been generated for this isolate, including cDNA, cosmid and BAC libraries, many of which are distributed among the *Magnaporthe* community. Approximately 7X coverage of M. oryzae strain 70–15 was sequenced. In addition, 38 BACs of chromosome 7, the smallest chromosome, were individually sequenced. The genome assembly consists of 2,273 sequence contigs longer than 2 kb, ordered and oriented within 159 supercontigs. The total length of all sequence contigs is 38.8 Mb. Thirty-three scaffolds representing 32.8 Mb or 85% of the draft assembly were ordered on the genetic map, indicating that the assembly is reasonably good despite the abundance of repetitive sequences. The assembly also displays considerable long-range continuity. The scaffold N50 of 1.6 Mb (=over 50% of all bases in scaffolds larger than 1.6 Mb) is similar to that of the *N. crassa* assembly (Dean *et al*., 2005). Recently, approximately 2X coverage each of two *M. oryzae* field isolates, Y34 and P131, have been sequenced. Preliminary analysis indicated that Y34 and P131 had additional 5.7 Mb and 1.5 Mb sequences, respectively, that were absent in the laboratory strain 70–15 (Xu *et al*., 2006). There are some available databases for genome sequence of *M. grisea* on NCBI

(http://www.ncbi.nlm.nih.gov/ projects/ mapview/ map_search.cgi?taxid=148305), *Magnaporthe Grisea* Database (http://www.broad. mit.edu/ annotation/ genome/magnaporthe_grisea/Home.html) and MGOS (http://www.mgosdb.org/).

Rice and blast pathogen interaction

Plants are challenged continuously by many different potential pathogens but they are very successful in resisting the vast majority of them. Thus, plants have evolved sophisticated defence systems to combat these potential pathogens, which make use of very diverse infection strategies. Plants employ series of defence barrier. A first barrier of passive defence includes the waxy cuticle and the plant cell wall. Already at this stage, many potential pathogens are prevented from entering the plant. When specific pathogens are able to evade or break this barrier, either through wounds or stomata, by producing cuticle or cell wall dissolving enzymes or by mechanical disruption, plants contain as a second barrier of defence large amounts of so-called preformed antimicrobial compounds aimed at directly inhibiting pathogen growth. As a third barrier of defence, plants have developed the ability to activate defence. Some inducible defence mechanisms are mediated by or activated through the plant signaling molecules, salicylic acid, jasmonic acid and ethylene. One of the most effective inducible defence mechanisms is based on the gene-for-gene interaction resulting in a rapid localized cell death (the hypersensitive response, HR) and activation of local and systemic defences.

Constitutive defences

The initial defence layer that potential pathogens encounter is permanently present and consists of mechanical and chemical barriers. The outer layer of most plant organs, the cuticle, is composed of layers of fatty acid-like compounds also known as wax. The main purpose of this waxy layer is to protect the plant from desiccation and pathogen entry. Most viral, bacterial and fungal pathogens are unable to disrupt this layer and can only enter the plant through wounds or natural openings like stomata and hydathodes. Some pathogens, like *Magnaporthe grisea* and

Colletotrichum spp., have developed mechanisms to enter the plant through the cuticle. After surface attachment, these fungi develop appressoria and penetration pegs that can build an enormous turgor pressure to mechanically disrupt the plant cuticle (Bechinger *et al*., 1999). Other fungal pathogens like Fusarium ssp. produce cutinase to dissolve the waxy layer. The underlying cell wall is a barrier that can also stop pathogens from entering the cell. The cell wall mainly consists of (hemi-) cellulose, a polymer of ß-1,4-glucans and pectin. Many different bacterial, fungal and oomycete pathogens produce cell wall-dissolving enzymes like cellulases, polygalacturonases and xylanases (Lisker *et al*., 1975; Sexton *et al*., 2000; Shi *et al*., 2000). These enzymes are predominantly expressed during infection and are often required for full virulence. When pathogens succeed in breaching these mechanical barriers, most plants still contain significant amounts of antimicrobial compounds, phenols, phenolic glycosides, unsaturated lactones, sulphur compounds, saponins, cyanogenic glycosides and glucosinolates. These compounds are released from the plant by lysis of vacuoles. In some cases precursors are activated by de novo synthesized plant enzymes, the so-called phytoanticipins (Osbourne, 1996).

Induce resistances

Specific pathogens are able to circumvent various constitutive defence layers, whereas plants can respond by switching on induced defence mechanisms that can provide resistance to viruses, bacteria, fungi, oomycetes, nematodes and insects. Until now, three pathways have been identified that are dependent on salicylic acid (SA), jasmonic acid (JA) and ethylene (ETH), respectively.

Salicylic acid-dependent resistance pathway Salicylic acid (SA) signaling is essential for systemic acquired resistance (SAR) and is important for the initiation of local defence responses and for some gene-for-gene interactions. SAR is initiated when plants are challenged with pathogens that induce local necrosis. SAR is completely dependent on SA since plants unable to accumulate SA caused by the expression of a bacterial salicylate hydroxylase (*NahG*) are no longer able to develop SAR. The role of SA is probably restricted to local signaling since SA is not the

mobile signal for SAR development. SAR can also be induced by the SA analogs INA (2,6-dichloroisonicotinic acid) and BTH (benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester). SA can be synthesized in plants through the conversion of phenylalanine to trans-cinnamic acid mediated by the enzyme phenylalanine ammonia lyase (PAL) and subsequent chain shortening to benzoic acid, which can be hydroxylated to SA. SA can be found in two forms in the plant. The first from is free SA that probably has a signaling function and second is the main storage form, ß-O-D-glucosalicylic acid (SAG), which is probably inactive in signaling (Hammond-Kosack and Jones, 2000).

Jasmonic acid (JA) and ethylene-dependent (ETH) resistance pathway provides resistance against a number of necrotrophic fungal pathogens and insects. Jasmonic acid (JA) and ethylene (ETH) are often simultaneously required for resistance responses against specific pathogens. This is demonstrated by the fact that expression of some defence genes requires both plant hormones. For example, the pathogen-dependent induction of the plant defensin gene Pdf1.2 requires both signaling pathways. JA and JA-relatedcompounds are also involved in developmental processes like pollen development and wound responses. ETH is, next to a role in pathogen defence, also involved in several physiological processes like fruit ripening and senescence. Both hormones are required for the development of Induced Systemic Resistance (ISR) and play a role in resistance to insects and necrotrophic fungal pathogens. JA is produced through the octadecanoid pathway from the fatty acid metabolite α-linolenic acid, which is oxygenated to hydroperoxy-linolenic acid by lipoxygenases. Further processing by allene oxide synthase and allene oxide cyclase results in 12-oxophytodienoic acid and further reduction to 12-oxophytoenoic acid. Subsequent carboxyl chain shortening by β-oxidation results in the formation of JA. ETH is synthesized from the amino acid methionine via S-adenosyl methionine and 1-aminocyclopropane-α-carboxylic acid (ACC) mediated by ACC synthase and ACC oxidase respectively (Berger, 2002; Bleeker and Kende, 2000; Hammond-Kosack and Jones, 2000).

Induced Systemic Resistance (ISR), a third pathway, is also dependent on JA and ETH and can be induced by some non-pathogenic rhizobacteria. ISR in plants

resembles the SAR phenomenon as in both cases activation results in protection of distal parts of the plant. ISR can be triggered by non-pathogenic rhizobacteria that are commonly found on plant roots. Many plant species are able to develop rhizobacteria-mediated ISR resulting in increased pathogen resistance. Most research on ISR has been performed on Arabidopsis with the ISR-inducing bacterium *Pseudomonas fluorescens* WCS417r. In Arabidopsis, resistance can be induced against the bacteria *P. syringae* pv tomato DC3000 and *Xanthomonas campestris* pv. amoracia, the fungal pathogens *Fusarium oxysporum* and *Alternaria brassisicola* and partially against the oomycete *Hyaloperonospora parasitica*. ISR can be distinguished from SAR by the fact that ISR is independent of SA but requires functional JA/ETH signaling pathways. A remarkable similarity between ISR and SAR in Arabidopsis is their requirement for a functional *Npr1* gene. Since the induction of ISR is dependent on JA and ETH, it comes as no suprise that the Arabidopsis mutants *jar1* and *coi1* and the Arabidopsis ETH mutants *ein2* and *etr1* fail to develop ISR, respectively. Expression of *NahG* has no effect on the development of ISR, indicating that ISR is independent of SA. The establishment of SAR is recognized by accumulation of specific sets of pathogen related (PR) transcripts and proteins. Likewise, when resistance is induced in Arabidopsis by treatment with JA and/or ETH, a specific, other, set of PR proteins is induced. Interestingly, none of the genes that are either SA- or ETH/JA- responsive are upregulated during ISR. One JA-inducible gene, *Atvsp1*, was upregulated when ISR-induced plants were challenged with pathogens. ISR seems to be a reinforcement or enhancement of the JA/ETH-dependent basal resistance (Hammond-Kosack and Jones, 2000; Pieterse *et al*., 2001; Van Wees *et al*., 1999).

Specific resistance (gene-for-gene)

Gene-for-gene resistance has originally been described in the 1940s by Flor who studied the genetics of the interaction between flax and the rust fungus *Melampsora lini*. He observed that for each dominant resistance gene in the plant, one dominant avirulence gene in the rust fungus was present (Flor, 1946). The initial definition of pathogen avirulence genes implies that they have the ability to induce resistance in hosts carrying the corresponding resistance genes. At first, the proposed

working model for these gene-for-gene interactions implied a receptor-ligand model were the R-gene product (receptor) directly binds the avr gene product (elicitor or ligand) to trigger resistance. To date only in a few cases a direct interaction between the elicitor and the R protein has been shown (Deslandes *et al*., 2003; Jia *et al*., 2000). Now evidence accumulates that avirulence proteins possess virulence functions. Presumably, avirulence proteins bind to a plant target different from the R-protein. Resistance gene products might have evolved as guards of the virulence target, sensing its modification followed by initiation of plant defences. This hypothesis, known as the guard-model, has first been described by (Van der Biezen and Jones, 1998).

Host specificity of *M. grisea*

The basis of host-parasite specificity between plants and microorganisms has been discussed by many phytopathologists. There are two distinguished types of host–parasite interaction: one determines whether or not a plant is a host for a parasite, and the other determines the gene-for-gene specificity. The specificity determining host species range is plant species specificity, and the specificity determining cultivar range within a given host species is cultivar specificity. In the former, compatibility is specific, and the resistance involved (nonhost resistance) is genetically complex. In the latter, incompatibility is specific, and the resistance involved (cultivar resistance) is controlled by gene-for-gene interactions. However, data have been reported that may be interpreted as suggesting that gene-for-gene interactions underlie the plant species specificity (Heath, 1981; Heath, 1991).

Magnaporthe grisea (Hebert) Barr, the causal agent of blast disease, includes several subgroups that are pathogenic to a restricted range of plant species, i.e., Oryza pathotype pathogenic to cultivated rice (*Oryza sativa* L.) and wild relative rice, Setaria pathotype pathogenic to foxtail millet (*Setaria italica* Beauv.), Panicum pathotype pathogenic to common millet (*Panicum miliaceum* L.), Eleusine pathotype pathogenic to finger millet (*Eleusine coracana* (L.) Gaertn.), Digitaria pathotype pathogenic to crabgrass (*Digitaria sanguinalis* (L.) Scop.), Eragrostis pathotype to weeping

lovegrass (*Eragrostis curvula*), Triticum pathotype to wheat (*Triticum aestivum* (L.) Thell.), Hordeum pathotype to barley (*Hordeum vulgare* L.) (Kato *et al*., 2000; Murakami *et al*., 2003; Sirithunya *et al*., 2007; Sweigard *et al*., 1995). This specificity between the pathotypes and gramineous plant species appears to be plant species specificity according to the traditional usage. However, there are some reports suggesting that the plant species specificity of *M. grisea* is governed by rather simple mechanism.

There are many publications on identification of these host specific genes. Crossing between Eleusine isolates with weeping lovegrass isolates suggested that their pathogenicity to finger millet or weeping lovegrass is conditioned by a single gene (Yaegashi, 1978). The two unlinked major genes, *PWL1* and *PWL2*, which control species specificity to weeping lovegrass were demonstrated, and showed that they have characteristics similar to avirulence genes that condition cultivar specificity (Kang *et al*., 1995; Sweigard *et al*., 1995). Host species specificity of *M. grisea* toward foxtail millet was analyzed using F1 cultures derived from a cross between a Triticum isolate (pathogenic on wheat) and a Setaria isolate (pathogenic on foxtail millet). On foxtail millet cvs. Beni-awa and Oke-awa, avirulent and virulent cultures segregated in a 1:1 ratio, suggesting that a single locus is involved in the specificity. This locus was designated as *Pfm1*. On cv. Ki-awa, two loci were involved and one of them was *Pfm1*. The other locus was designated as *Pfm2*. Interestingly, *Pfm1* was not involved in the pathogenic specificity on cv. Kariwano-zairai. These results suggest that there is no “master gene” that determines the pathogenic specificity on all foxtail millet cultivars and that the species specificity of *M. grisea* toward foxtail millet is governed by cultivar-dependent genetic mechanisms that are similar to gene-for-gene interactions controlling race cultivar specificity (Murakami *et al*., 2003).

R-genes and their functions

Resistance genes are essential components for recognition of specific pathogens and the activation of plant defence pathways including HR. To date many resistance genes have been cloned providing resistance to pathogenic viruses, bacteria,

fungi, nematodes and insects. The more insight into the different classes of R proteins has been existed in these plants. R genes can be grouped into five classes.

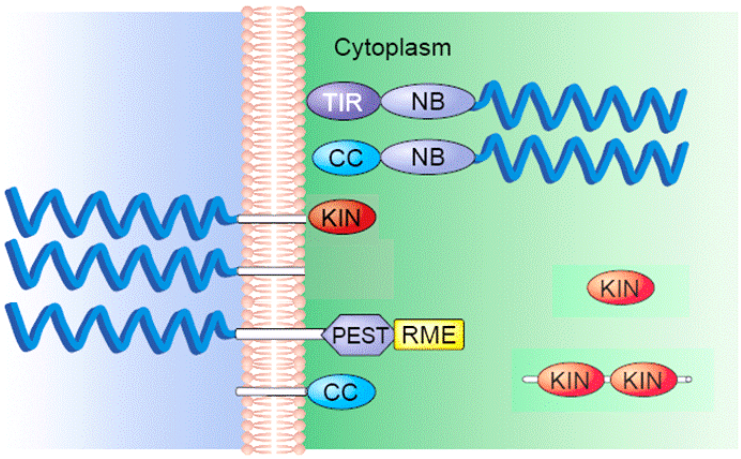

Figure 3 Major families of R proteins
Source: Mc Dowell and Woffenden (2003)

The first class encodes cytoplasmic receptor-like proteins that contain an LRR domain and a nucleotide binding site (NB). This class is the largest class of R-proteins. The LRRs (leucine rich repeats) are known in other proteins to be involved in protein-protein interactions, reception ligand binding and protein-carbohydrate interactions. The conserved NBS (nucleotide binding site) domain is involved in ATP or GTP binding (Goff *et al*., 2002). The family of genes encoding proteins with the LRR-NBS motif have been identified in many plant species such as Arabidopsis, tomato, tobacco, flax, barley and rice etc. (Baker *et al*., 1997; Hammond-Kosack and Jones, 2000; McDowell and Woffenden, 2003). This domain also designated as NB-ARC or Ap-ATPase domain might be involved in regulating cell death after avirulence determinant recognition (Hammond-Kosack and Jones, 2000).

The NBS-LRR proteins can be subdivided into two subclasses. One containing an amino terminal region with homology to drosophila Toll and mammalian Interleukin (IL)-1 receptors (TIR-NB-LRR proteins). The other subclass containing putative amino terminal coiled-coil domains (CC-NB-LRR proteins) (Hammond-Kosack and Jones, 2000). Rice contains about 600 CC-NBS-LRR genes but no

obvious TIR-NB-LRR domains were found, whereas in Arabidopsis the TIR-NB-LRR subclass is prevailing (Goff *et al*., 2002). NBS-LRR proteins are supposed to be localized in the cytosol. One of the best studied NBS-LRR proteins, *Pi-ta*, is localized to the plasma membrane, which is required for its function (Jia *et al*., 2000).

The second class called Receptor-Like Proteins (RLPs), which has extracellular LRR region and transmembrane domain. These genes are the *Cf*-genes and *Ve*-genes from tomato mediating Cladosporium and Verticillium resistance, respectively, contain extracellular LRRs, a transmembrane domain and a short cytoplasmic tail. The tomato Cf genes confer gene-for-gene resistance to certain races of the fungal pathogen Cladosporium fulvum. The cytoplasmic domain of the *Cf-9* protein interacts with a thioredoxin homolog in the cytoplasm, which appears a negative regulator of *Cf-9*-mediated cell death and defence in tomato and *N. benthamiana* (Rivas *et al*., 2004). The tomato Verticillium resistance proteins *Ve1* and *Ve2* contain, in addition to the extracellular LRR region and transmembrane domain, a cytoplasmic domain with similarity to endocytosis (ECS) domains. Furthermore, *Ve1* contains an N-terminal coiled-coil domain and *Ve2* a cytoplasmic PEST domain, found in proteins with a short half-life (Kawchuk *et al*., 2001). The Arabidopsis *RPW8* protein contains a membrane anchor, fused to a putative coiled-coil domain (CC) (McDowell and Woffenden, 2003).

The third class of R-genes found in a wide variety of plant species and other eukaryotes, contains extracellular LRRs, a transmembrane motif and a cytoplasmic serine/threonine kinase domain (LRR-KIN). These putative receptor kinases might transduce an extracellular signal directly through phosphorylation of other host cellular targets, but only a small portion of these genes is involved in disease resistance. Whereas *Xa21* and *Fls2* are LRR-KIN determining resistance to *Xanthomonas oryzae* bacteria, expressing *Avr-Xa21* in rice and a conserved domain in bacterial flagella in Arabidopsis, respectively (Song and Goodman, 2001). The rice genome contains about 450 genes with extracellular LRRs of which approximately half contains a cytoplasmic RLK domain (Goff *et al*., 2002).

The fourth class of genes includes *Pto*, which confers resistance to the bacterial pathogen *P. syringae* pv. tomato, containing *avrPto*. *Pto* encodes a serine-threonine kinase (KIN) without LRRs, and another (maize *Rpg1*) contains two kinase domains. These genes are homologous to mammalian *Raf*, *IRAK*, and Drosophila Pelle kinases (Baker *et al*., 1997; McDowell and Woffenden, 2003).

The fifth class includes the *HM1* gene, which confers resistance to the fungal pathogen *Cochliobolus carbonum* race 1. *HM1* encodes a reduced form of nicotinamide adenine dinucleotide phosphate (NADPH)–dependent reductase that inactivates toxin produced by *C. carbonum* race 1. *HM1* is distinct from the above-mentioned R genes because an Avr component is not involved in toxin degradation by *HM1* (Hammond-Kosack and Jones, 2000).

Rice blast resistance genes

The genetics of blast resistance in rice has been extensively studied. The first Pi gene in rice was named by (Kiyosawa, 1966). The approach used several varieties that carried different, single resistance genes as differential cultivars to characterize the specific virulence of different isolates of the pathogen (Flor, 1946). Using seven differential cultivars and several blast isolates described three resistance genes, *Pi-a*, *Pi-i* and *Pi-k*. Other investigators have used similar approaches, using different germplasm and blast isolates (Yamasaki and Kiyosawa, 1966). The relationships between the different genes are sometimes difficult to determine when different blast isolates are used to characterize them. Determining their position on the rice physical map would be very helpful in this case. To date, approximately 50 major blast resistance genes have been named and mapped on rice chromosomes. There are many techniques to identify these resistance genes such as chromosome walking, genetically mapped simple sequence repeat (SSR), restriction fragment length polymorphism (RFLP) and resistance gene analog (RGA) markers, and using rice genomic information (Hammond-Kosack and Jones, 2000; Vanavichit *et al*., 2005). Now a days, a total of 8 blast disease resistant genes have been cloned such as *Pib* (Wang *et al*., 1999), *Pita* (Bryan *et al*., 2000), *Pik*^{h} (Sharma *et al*., 2005), *Pi9* (Qu *et al*., 2006),

Pi2 (Zhou *et al*., 2006), *Pizt* (Zhou *et al*., 2006), *Pi36* (Liu *et al*., 2007) and *Pi37* (Lin *et al*., 2007) and all of them belonged to the NBS – LRR class of resistance proteins.

In Thailand, mapping of blast resistance genes in a RIL population from a cross between KDML105 x CT9993 by fifteen virulent blast isolates were located on chromosomes 7 and 9. In particular, the *QTL(ch9)* was mapped near the *Pi5(t)* locus (Sirithunya *et al*., 2002). Then two rice populations used for QTL analysis of blast resistance genes were double haploid derived from cross Azucena x IR64 and recombinant inbred lines derived from cross KDML105 x JHN. These QTL loci with tightly linked markers are useful for the development of marker aided selection (MAS) scheme for improving blast resistance in many rice varieties such as KDML105 and RD6 (Noenplab *et al*., 2006; Sirithunya *et al*., 2004). However, the resistance possessed by new breeding cultivars often breakdown in a short period of time after released. The breakdown of resistance may be cause by an increment of prevalence of previously rare pathotype or a development of novel pathotypes (Ou, 1980). Therefore breeding for resistance against high diversity and variability of the pathogen is most challenging for plant breeders. To effectively breed for blast resistance, genetic information about resistance genes, avirulence genes and interaction must be understood.

Avirulence genes and their functions

It is intuitive that a pathogen carries genes for pathogenicity that enable it to parasitize host plants. However, the idea that pathogens carry genes for avirulence, which prevent the pathogens from infecting certain hosts, is less so. The mechanisms of host resistance, pathogen variation, and coevolution between host and pathogen can be clearly explained by the pathogen avirulence (*Avr*) genes, which were first proposed in the gene-for-gene hypothesis proposed by H. H. Flor (Flor, 1946; Flor, 1971). The studies of Flor demonstrated that on varieties of flax containing a gene for resistance (*R* gene) to the avirulent parent race (morphologically indistinguishable pathogen variant within a pathogen species), the F2 progeny of the fungus segregated in a monofactorial ratio of 1 virulent: 3 avirulent. Thus, pathogen virulence was

recessive and pathogen avirulence was dominant. On the varieties that have more than one *R* gene to the avirulent parent race, the F2 progeny of the fungus segregated in ratios according to the corresponding numbers of *R* genes in host varieties. This suggests that for each gene conditioning resistance in the host, there is a corresponding gene conditioning virulence in the pathogen (gene-for-gene relationship). In the genetic studies of Flor, the recessive virulence gene was always emphasized as the active determinant that conditioned cultivar-specific virulence rather than the dominant *Avr* genes, probably because of the dilemma posed by the concept that pathogen avirulence was determined by its own dominant gene. Flor's genetic studies showing that *Avr* genes were genetically dominant led to the subsequent molecular cloning of the first *Avr* gene from a bacterial pathogen *Pseudomonas syringae*. Since then, over 40 *Avr* genes have been cloned from gram-negative bacterial pathogens of the genera *Pseudomonas*, *Xanthomonas*, *Erwinia*, and *Ralstonia* (Leach and White, 1996). About 18 *Avr* genes have been cloned from diverse fungi, including *Magnaporthe grisea*, *Cladosporium fulvum*, *Rhynchosporium secalis*, and *Melampsora lini*, and Oomycetes, including *Phytophthora infestans* and *P. parasitica (Lauge and Dewit, 1998)*. The cloned *Avr* genes determine cultivar specificity in that *Avr* genes, when transferred into a virulent pathogen race, render the recipient race avirulent to the host cultivars that carry corresponding *R* genes. This fits the original gene-for-gene hypothesis that predicted the dominance of the *Avr* gene. Disruption of an *Avr* gene makes an avirulent pathogen race virulent to the previously resistant cultivar, demonstrating that cultivar specific virulence is conditioned by the absence of the *Avr* gene. This contrasts with Flor's prediction that a recessive virulence gene conditions cultivar specific virulence. Thus, the historical term 'virulence genes' referring to hypothetical alternate allele of cultivar specific *Avr* genes appears to be a misnomer since they do not condition the cultivarspecific virulence (Hammond-Kosack and Jones, 2000; Lauge and Dewit, 1998; Ludere and Jooste, 2001).

Avr genes are often distinguished from pathogenicity genes. However, many *Avr* genes have been demonstrated to be required for full virulence on the host lacking the R gene (Van'T Slot and Knogge, 2002). This suggests that *Avr* genes have dual roles as being pathogenicity factors as well as avirulence factors. When pathogen genes trigger resistance responses on the host carrying *R* genes, they are termed *Avr*

genes. At the same time, they are required for virulence on the host lacking R genes. To overcome the confusing terminology for the same gene, the new term ‘effector’ gene has been adapted to designate pathogen genes that are involved in the plant-pathogen interaction, regardless of their function as avirulence or pathogenicity factors (McDowell and Woffenden, 2003). Because many pathogen genes have been first characterized as avirulence determinants, many of them are still called *Avr* genes. The term ‘*Avr* gene’ will be used here rather than ‘effector gene’.

Structure and properties of fungal *Avr* genes

To date 18 fungal *Avr* genes have been cloned and characterized. When their DNA and protein sequences are compared, there seems to be little similarity. However, some of them share common features such as cysteine-rich regions and signal peptides (Van'T Slot and Knogge, 2002). Cysteine pairs can form intra-molecular disulfide bonds that stabilize the proteins. Signal peptides are short discrete stretches of amino acid sequence, which are part of a protein and direct proteins to their proper cellular and extracellular locations. The *Avr* genes *Avr2*, *Avr4*, and *Avr9* from the tomato leaf mold *Cladosporium fulvum* and *NIP1*(Necrosis-Inducing Peptide) from the barley scald fungus Rhynchosporium secalis encode low-molecular weight proteins. These proteins contain an even number of cysteine residues for disulfide bonds and a signal peptide for extracellular targeting. It has been shown that the substitution of one single cysteine residue of *Avr4* and *Avr9* significantly compromised the HR-inducing activity of the proteins (Van'T Slot and Knogge, 2002). The cysteine residues of *Avr9* have been also shown to be involved in disulfide bonds. Thus, cysteine residues appear to be important for the stable protein structure and for the full avirulence activity. All of these *Avr* proteins are localized in the apoplastic space, indicating the role of their signal peptide sequences for secretion. Signal peptides are also found in other *Avr* gene products including *Avr-Pita* and *PWL* (Pathogenicity toward Weeping Lovegrass) genes from *M. grisea* and *AvrL567* from the flax rust fungus *Melampsora lini* (Lauge and Dewit, 1998; Orbach *et al*., 2000; Sweigard *et al*., 1995). Most of the fungal *Avr* genes encode proteins that have no similarity to the characterized proteins in the databases. Only two *Avr* genes, *AVR-Pita*

and *ACE1* (for Avirulence Conferring Enzyme1) from *M. grisea*, encode proteins that show similarity to zinc metalloprotease and polyketide synthase, respectively. These enzymatic activities appear to be responsible for their avirulence activity (Bohnert *et al*., 2004; Orbach *et al*., 2000).

The avirulence genes of *Magnaporthe grisea*

The filamentous ascomycete *Magnaporthe grisea* is the causal agent of blast disease on many species of the grass family, such as rice, wild relative rice, barley, wheat, foxtail millet, and weeping lovegrass. *M. grisea* initiates infection by a germinating conidium that quickly differentiates into a specialized cell, the appressorium. Once mature, the melanized appressorium generates enormous hydrostatic pressure that forces a narrow penetration peg through the plant cuticle and epidermal cell wall. After penetration, the fungus grows intracellularly and produces sporulating lesions within five to seven days.

Genotype-specific *Avr* genes of *M. grisea*

The *M. grisea* isolates that carry the *Avr-Pita* (*Avr2-YAMO*) gene are avirulent on rice cultivars that carry the corresponding resistant gene *Pi-ta* (Orbach *et al*., 2000). The *Avr-Pita* gene is located very close to the telomere of chromosome 3 and encodes a predictedpolypeptide of 223 amino acids. *Avr-Pita* exhibits substantial similarity to *NPII*, a neutral zinc metalloprotease from Aspergillus oryzae. Based on this homology, the N-terminus of *Avr-Pita* was predicted to be further processed to an active form of 176 amino acids. This *Avr-Pita176* protein, but not the intact *Avr-Pita223* protein and *Avr-Pita166* (which has an additional deletion at the N-terminus), triggers the *Pi-ta*-dependent HR when produced inside rice cells by transient expression (Jia *et al*., 2000). In the region that corresponds to the consensus zinc binding domain of neutral zinc metalloproteases, residue Glu-177 of *Avr-Pita223* (i.e. Glu-130 of *Avr-Pita176*) is predicted to be essential for metalloprotease activity. Interestingly, replacement of this Glu residue by Asp, as found in spontaneous gain of virulence mutants, abolishes the HR-inducing ability of *Avr-Pita176*. This implies that the protease activity of *Avr-Pita*, although not yet biochemically demonstrated, plays

an essential role in avirulence (Orbach *et al*., 2000). The majority of spontaneous virulent mutants of *M. grisea* that carry deletions ranging from 100 bp up to 10 Kb, which is consistent with the genetic instability observed for genes that are located at a telomere (Jia *et al*., 2000; Orbach *et al*., 2000). In addition to point mutations and deletions, gain of virulence on Pi-ta rice cultivars was also mediated by an insertion of a pot3 transposon into the promoter of *Avr-Pita* (Kang *et al*., 2001).

The *Avr1-CO39* gene of *M. grisea* has been identified as the minimal (1.05 kb) fragment that confers avirulence on rice cultivar CO39. Only a small number of rice-infecting *M. grisae* isolates from the Philippines, however, are avirulent on this cultivar. While most virulent isolates lack the entire *Avr1-CO39* locus, it appeared that in some cases complex genomic rearrangements have occurred at the *Avr1-CO39* locus, each of which resulting in non-functional alleles (Farman *et al*., 2002).

The Avirulence Conferring Enzyme1 (*ACE1*) gene was identified from *M. grisea* isolates. This gene was specifically recognized by rice (*Oryza sativa*) cultivars carrying the resistance gene *Pi33*. This recognition enabled resistant plants to activate a defense response. *ACE1* was isolated by map-based cloning and encodes a putative hybrid between a polyketide synthase and a nonribosomal peptide synthetase, enzymes involved in microbial secondary metabolism. *ACE1* was expressed exclusively during fungal penetration of host leaves, the time point at which plant defense reactions was triggered. *ACE1* appears to be localized in the cytoplasm of the appressorium. Mutation of the putative catalytic site of the b-ketoacyl synthase domain of *ACE1* abolishes recognition of the fungus by resistant rice. This suggests that Ace1 biosynthetic activity is required for avirulence. The results were consistent with the hypothesis that the fungal signal recognized by resistant rice plants is the secondary metabolite whose synthesis depends on *ACE1*. This recognition could be guard hypothesis (Babu *et al*., 2004).

Species-specificity conferred by *PWL* genes of *M. grisea*

The *PWL2* (for Pathogenicity toward Weeping Lovegrass) gene of *M. grisea* determines host-species specificity. Strains of the fungus expressing PWL2 are

avirulent on weeping lovegrass, but virulent on rice and barley. *PWL2* encodes a glycinerich protein of 145 amino acids with a putative signal peptide for extracellular targeting. Analysis of spontaneous virulent mutants on weeping lovegrass revealed that the PWL2 allele is genetically unstable, although it is not located at a telomere. As found for the *avr*-pita-deficient mutants, spontaneous deletion of *PWL2* had no apparent effect on virulence under laboratory conditions. Strains of *M. grisea* also evade *PWL2* recognition by a single base pair change that results in the creation of a putative N glycosylation site. This *PWL2* mutant protein exhibits reduced elicitor-activity either due to glycosylation or due to the amino acid change itself (Sweigard *et al*., 1995).

The *PWL2* gene is a member of a rapidly evolving gene family of which the homologue *PWL1* and the allelic *PWL3/PWL4* genes map at different chromosomal locations. The *PWL2* protein is 75 percent identical to the *PWL1* protein, and 51- and 57 percent identical to the *PWL3* and *PWL4* proteins, respectively. Opposed to *PWL1* and *PWL2*, the *PWL3* and *PWL4* genes are non-functional *Avr* genes, as they do not confer avirulence on weeping lovegrass. In contrast to *PWL3*, *PWL4* becomes functional in preventing infection of weeping lovegrass when its expression is driven by either the *PWL1* or the *PWL2* promoter. This indicates that *PWL4* encodes a functional *Avr* protein, which is not recognized by weeping lovegrass due to lack of expression of the gene (Kang *et al*., 1995).

Interaction of resistance and avirulence gene product

Several models have been proposed for the biochemical basis of *Avr* gene function. In the simplest elicitor-receptor model, the *Avr* gene product is an elicitor or ligand that is directly recognized by the receptor produced by the corresponding R gene, which is located outside or inside the host cell. This specific recognition activates a signal transduction pathway that leads to resistance responses, often involving HR. Two models have been proposed addressing the question how and where *Avr* proteins are recognized by resistant plant genotypes (Dangl and McDowell, 2006; Hammond-Kosack and Jones, 2000; McDowell and Woffenden, 2003). To

overcome the host plant, a pathogen has attached to a plant cell and is expressing a suite of virulence proteins. These proteins are translocated into plant cells via Type III secretion in bacteria or other unknown mechanisms in fungi and oomycetes. Once inside, they target host proteins controling defense responses, metabolism or other plant process that affect pathogen virulence. Disease results from the collective action of the virulence proteins. The plant cell does not express an R protein that is capable of recognizing any virulence protein. Thus, the plant cannot detect the pathogen efficiently and defenses are, at best, only weakly induced (Figure 4, panel a).

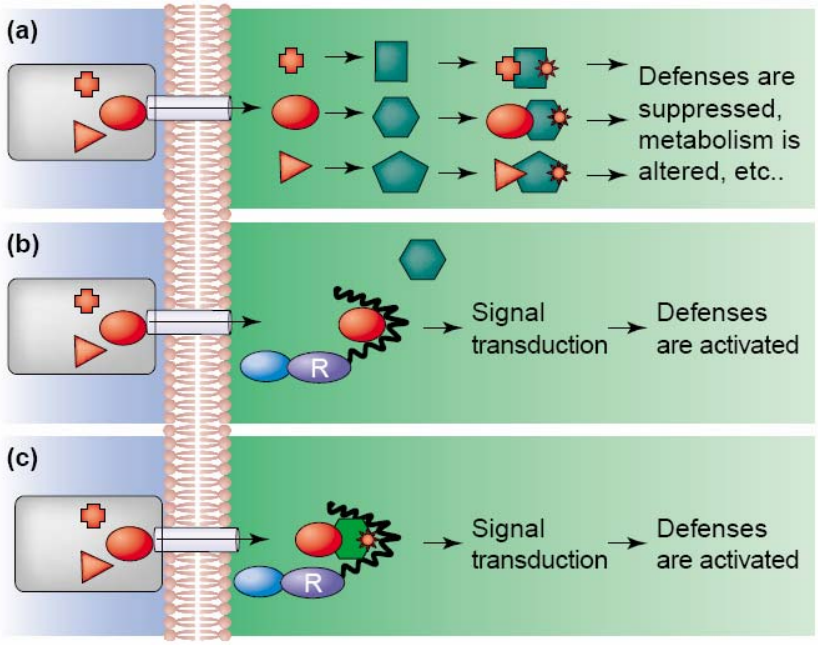

Figure 4 Interactions between pathogen *Avr* proteins and plant *R* proteins.
Source: Mc Dowell and Woffenden (2003)

Direct perception of *Avr* proteins

This model reflects the most simple interpretation of Flor's gene-for-gene hypothesis; a classical receptor-ligand model that predicts a direct interaction between *Avr* and *R* gene products (Figure 4, panel b). Thus far, direct physical interaction has only been demonstrated for *Pto* from tomato and *AvrPto* from *Pseudomonas syringae*, and for *Pi-ta* from rice and *AVR*-Pita from *M. grisae*. The cytoplasmic localization of both *Pto* and *Pi-ta* is consistent with the observation that *AvrPto* and *Avr-Pita* induce a

HR when expressed inside plant cells (Jia *et al*., 2000; Tang *et al*., 1996). *Pto* is a Ser/Thr kinase that interacts with and phosphorylates a second Ser/Thr kinase, *Pti1*, and several defense related transcription factors, such as *Pti4*, *Pti5*, and *Pti6*. The most straight forward explanation for induction of plant defense responses would be that binding of *AvrPto* to *Pto* leads to a conformational change of the kinase protein that in turn triggers downstream defense signaling pathways. *Pto* function, however, requires *Prf*, a NB-LRR-encoding gene. Therefore, the "guard" model was put forward to rationalize the mechanism of *AvrPto*-induced defense activation. *Avr*-Pita recognition, on the other hand, is mediated by direct interaction with the LRD of *Pi-ta* (Bryan *et al*., 2000). It appeared that the putative metalloprotease activity of *Avr-Pita* is required for its direct interaction with *Pi-ta* (Jia *et al*., 2000), suggesting a protease-dependent defense elicitation model. Possibly, *Pi-ta* contains protease cleavage sites, which upon proteolytic processing renders an active form either by a conformational change or by the release of elicitor peptide(s) that trigger(s) defense responses.

Indirect perception of *Avr* proteins or Guard hypothesis

In addition to direct perception of *Avr* proteins by *R* proteins, the guard hypothesis has been proposed that postulate an indirect interaction between *Avr* and *R* gene products to take place. A *R* protein which called 'guard' in this case detects a modified host protein which called 'guardee'. The guardee can be a complex with the 'attacking' virulence protein and some target protein in host plant (McDowell and Woffenden, 2003). The ongoing battle between plants and pathogens could in turn have led to development of strategies by which R proteins act as "guards" to monitor the behavior of molecules that are targets of *Avr* proteins. The function of *Avr-Pto* for *P. syringae* is to target Pto and suppress the non-specific defense pathway induced by this kinase (Van der Biezen and Jones, 1998). The *Avr-Pto-Pto* complex or *Avr-Pto*-activated *Pto* is recognized by *Prf*, which subsequently initiates defense responses. A variety of mutations has been identified that disrupt the avirulence function of *AvrPto* without affecting its virulence function. Moreover, these mutants failed to interact with *Pto*, which, in line with the "guard" hypothesis, implies that *Avr-Pto* interacts

with virulence targets other than *Pto* (Hammond-Kosack and Jones, 2000; McDowell and Woffenden, 2003).

The "guard" hypothesis could also explain why, in spite of the fact that extracellular perception of *Avr9* is consistent with the predicted extracellular location of *Cf-9*, no direct interaction between *Avr9* and *Cf-9* has been detected (Luderer *et al*., 2002). Moreover, an *Avr9*-specific high affinity-binding site (HABS) has been identified in plasma membranes of susceptible *Cf* as well as resistant *Cf-9* tomato plants, implying the involvement of a third component in *Cf-9*-mediated perception of *Avr9*. One possibility could be that the HABS represents the virulence target of *Avr9*. *Cf-9* may "guard" this virulence target, sense its modification by *AVR9* and trigger downstream defense responses leading to resistance (Hammond-Kosack and Jones, 2000; Van'T Slot and Knogge, 2002).

Identification of avirulence genes

Discovery of the perfect stage of blast fungus in *In vitr*o enabled possibility of genetic analysis. Crossing between isolates that are differentially pathogenic on various varieties or hosts revealed the existence of many gene-for-gene interactions between rice and this fungus (Murakami *et al*., 2003; Valent and Chumley, 1991). Using this strategy, research can develop new genetic recombination to study their interested trait such as pathogenic ability to specific rice varieties.

The use of molecular markers in genetic studies of *M. grisea* has raveled information to levels of precision not previously possible. Unlike traditional markers, molecular markers are direct manifestations of genetic content and can therefore serve as reliable indices of genetic or pathotypic variation. They are not influenced by environmental factors and hence are highly reproducible. Besides, these are cost-effective and less cumbersome. Restriction fragment-length polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphisms (AFLP) and Sequence Characterized Amplified Regions (SCAR) analyses have been used as valuable tool to characterize avirulence genes in this

pathogen (Luo *et al*., 2004; Pongam *et al*., 1998; Soubabere *et al*., 2001; Yasuda *et al*., 2005).

Molecular markers are needed as anchors for physical mapping, which is underway for *M. grisea*, for population studies, and for map based cloning. Map based or positional cloning, presently the most common method for cloning genes of interest in eukaryotes is dependent on the availability of markers closely linked to the gene of interest and, therefore, marker-dense maps are an invaluable tool. Microsatellites or simple sequence repeat (SSR) markers are tandem repeated DNA sequences that occur throughout the eukaryotic genome. Length polymorphism arises from variations in the number of repeated units, probably due to DNA polymerase slippage during replication of the SSR (Vanavichit *et al*., 2005). The development and mapping of SSR markers for the *M. grisea*, there are three different sources were used to obtain these. First, a Blast - based search was conducted using public databases which contain a significant and growing number of genomic and EST (expressed sequence tag) sequences of *M. grisea*. Second, a partial genomic library enriched for (GA)n and (GT)n sequences was constructed from the *M. grisea* strain GUY11 as a source of random SSR containing clones. Third, a search of a proprietary EST library was performed (Kaye *et al*., 2003). Polymorphism analysis at SSR loci is a simple and direct approach for estimating the genetic diversity of *M. grisea* isolates and a powerful tool for studying *M. grisea* genetics (Brondani *et al*., 2000b). Several SSR loci have been identified, characterized, and screened for their ability to detect polymorphism among several isolates of *M. grisea*. The map location of some of these SSR loci have been successfully determined on a reference genetic map derived from the cross between the isolates Guy 11 and 2539 (Kaye *et al*., 2003; Santoso *et al*., 2007).

Quantitative trait loci mapping

A trait refers to a genetically determined characteristic, which could be anything of phenotypes. Two kinds of traits, Mendelian and quantitative, are distinguished. A Mendelian trait is determined by a single gene (or few genes), following classical Mendelian inheritance patterns, such as 3:1 for a phenotypic ratio

from a trait controlled by a single dominant gene in an F2 family. In contrast, multiple genes could determine a quantitative trait and its value is continuous, such as plant height and human weight. Quantitative traits are very common and are important both in applied and theoretical studies. For example, increasing crop production or plant disease resistance all requires the manipulation of quantitative traits.

QTL mapping has been carried out for various traits in many species. The theory of QTL mapping was first described by (Sax, 1923), who noted that seed size in bean, a complex trait, was associated with seed coat color, a simple, monogenically-controlled trait. Modern QTL mapping is derived from this idea, with the key innovation being that defined sequences of DNA act as the linked monogenic markers. New interest was generated when studies with rice, maize and tomatoes demonstrated that some markers explained much of the phenotypic variance of complex characters (Tanksley, 1993).

Data for QTL mapping usually have two components: marker data and trait values. Marker data includes marker genetic map position and marker genotype. Trait values can be continuous, such as disease leaf area, or they may be categorical, such as leaf size denoted by large, medium and small. Sample size needs to be considered when planning the experimental design. With a greater sample size, detection of QTL with smaller effect is more efficient (Manly and Olson, 1999; Vanavichit *et al*., 2005; Young, 1996).

Genetic markers

In a broad sense, a genetic marker refers to any heritable character that can be used to distinguish one individual from another in a population. The distinction can be at different levels such as phenotype, protein or DNA. Phenotypic traits can be markers if the variation observed in the population of interest is entirely explained by a single Mendelian factor. At the protein level, allozymes can be used as markers. These are soluble proteins with different mobility on an electrophoresis gel. The mobility difference is a result of unequally charged protein due to amino acid

substitutions. In current QTL mapping practice, variation at the DNA level is typically used because it is the most abundant and easily scored type of variation due the rapid development of genome technology. Variation in DNA sequence is detected by hybridization and polymerase chain reaction (PCR) based methods. Commonly used DNA markers include restriction fragment length polymorphism (RFLP). The RFLP may result either from mutation in restriction endonuclease sites or from deletions or insertions of DNA between the sites. Polymorphisms detected by PCR result from insertion and deletions between, and mutation in primer binding sites. PCR based markers include sequence tagged sites (STS), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequenced repeats (SSR or microsatellites), variable number of tandem repeats (VNTR or minisatellites) and single nucleotide polymorphisms (SNP). Among these markers, RFLP, SSR and SNP are commonly used for mapping QTL. The term microsatellite refers to DNA sequences with repeating units of 1-6 nucleotides. For example (GA)n and (CTG)n are microsatellites, where n is the number of repeating units. They are often multi-allelic, are usually locus specific, and are evenly distributed along chromosomes and randomly distributed throughout the genome. (Vanavichit *et al*., 2005).

Maps and map construction

A genetic map describes orders and positions of identifiable landmarks on DNA. Theses landmarks might be genes or genetic markers. A genetic map provides information on marker order along the chromosomes. Estimating recombination frequency between two positions generates a genetic map. Software has also been developed to construct genetic maps and a popular one is MAPMAKER (Lander *et al*., 1987).

Molecular marker technologies permit researchers to construct high-density genetic maps for any species amenable to genetics and use them for detecting, mapping, and estimating the effects of QTL. The analysis involves testing DNA markers throughout a genome for the likelihood they are linked with a QTL. Individuals in an appropriate mapping population (F2, backcross, recombinant inbred)

are analyzed for DNA marker genotypes and the phenotype of interest. For each DNA marker, the individuals are split into classes according to marker genotype. Mean and variance parameters are calculated and compared among the classes. A significant difference between means suggests that there is a relationship between the DNA marker and the trait of interest. In other words, the DNA marker is probably linked to a QTL (Vanavichit *et al*., 2005).

Finally, QTL mapping, like any genetic study, is only as good as its phenotypic scoring method. In studies of disease resistance, factors all the way from a suitable inoculum to difficulties in quantitative estimation of resistance make QTL mapping more challenging. Fortunately, powerful computer software programs are now available to analyze QTL mapping results and better DNA marker systems have been developed to simplify the technique and increase marker density (Manly and Olson, 1999; Vanavichit *et al*., 2005; Young, 1996).

QTL mapping methods

Various statistical methods have been developed for QTL mapping. The most commonly used methods for QTL mapping are based on the maximum-likelihood method. From simple to more complicated, three approaches are commonly used: single marker analysis (SMA), simple interval mapping (SIM); and simple composite interval mapping (sCIM) (Broman, 2001; Manly and Olson, 1999; Vanavichit *et al*., 2005; Young, 1996).

Single marker analysis (SMA) SMA tests the association between marker genotypes and trait values using t-tests, ANOVA models or regression. In other words, it tests trait value differences among markers groups. SMA is the least informative of the analyses, because recombination, as well as the additive and the dominant effects of a QTL may be confounded. SMA often fails to give reliable estimates of numbers and positions of QTL and the magnitude of their effects.

Simple Interval mapping (SIM) Thoday, in 1961 introduced simple interval mapping and a mathematical treatment of this method was presented by Lander and

Botstein (1989). SIM uses two observable flanking markers to construct an interval within which to search for QTL along the chromosomes. A map function, either Haldane or Kosambi, is used to translate from recombination frequency to distance or vice visa. Then, a LOD score is calculated at each increment in the interval. Finally, the LOD score profile is calculated for the whole genome. When a peak has exceeded a threshold value, there is evidence that a QTL has been found at that location.

Simple Composite interval mapping (sCIM) Jansen and Stam (1994) and Zeng (1994) developed sCIM. This method is an extension of SIM that places certain markers into the model as cofactors. The sCIM fits parameters for a target QTL in one interval while simultaneously fitting partial regression coefficients for background markers to account for variance caused by non target QTL. In theory, sCIM gives more power and accuracy than simple SIM because the effects of other QTL are not present as residual variance.

MATERIALS AND METHODS

I. Development of mapping population

Fungal isolates

There are three groups of blast pathogen isolates to be used in this study. First group is parental isolates (THL1000 and THL16) and their 88 F_1 progenies developed by Ms. Suchada Primpisuthrawon, BIOTEC, Thailand, and which were kept in filter paper. The second group of blast isolates was used in pathogenicity assay in JHN rice variety. It was composed of 346 Thai blast pathogen isolates and 124 worldwide blast pathogen isolates. The third group is the known mating type with high fertile isolates, TH12 and THL59; and TH16 and THL32 are mating type Mat1-1 and Mat1-2 respectively. The stocks of each fungal isolates were stored as dried-mycelium on filter paper and they can be regrown when cultured on RFA medium plates.

Single conidia isolation

Blast isolates were obtained from collected samples. In each blast disease symptom sample, the location, the date, the plant's parts from which sample were obtained, and the host-cultivar were be noted. The samples were given the number, followed by the type of lesions that were sampled as leaf (L), collar (C), neck (N) or seed (S). The flow scheme of activities for single conidia isolation of each isolate are described as below.

Each sample with the blast lesions were placed in Petri dish on filter paper and the optimum amounts of moisture were applied to create high humidity. After incubating in 25 – 28^{o}C for 1 or 2 days in dark condition, conidia were raised and they were picked up using sealed-end Pasteur pipette and deposited them on a 4.0% water agar plate. Then using the glass needles, they were gently stroked onto the agar surface to separate each of conidium individually. The germinated conidia were observed after a few hours. The tungsten needle was gently cut out well-separated and

germinated conidium from the water agar and transferred to a Petri dish with RFA medium. After three or four days, the young colonies could be observed. Blast pathogen isolates are typically gray or white colonies. A single colony is transferred using a sterile scalpel to cut out a small piece from the colony edge. Using the new RFA medium Petri dish with filter paper, transfer the cut out pieces onto the filter paper. Then, growing them as the same condition as culturing and after the filter papers were covered with growing mycelium, each of them was removed to a new Petri dish and let it dried in the desiccator for 14 days. When they were completed dried, the paper was cut into small pieces, and were transferred into aluminum foil envelope. The aluminum envelopes were kept inside a plastic bag. The dried-mycelium in filter papers in the sealed bags were stored at –20^{o}C and can be kept for at least 10 years.

Mating type determination

The culturing method for mating type determinations were tested by culturing the mycelia plugs of unknown-mating type and known-mating type on RFA medium plates. The known mating type isolates were TH12 and THL59 for mating type 1-1 (Mat1-1) and TH16 and THL32 for mating type 1-2 (Mat1-2). All of known mating type isolates were used as mating type testers. The testers are hermaphrodites, highly fertile and produced a high number of perithecia which they can mate easy with isolates having the opposite mating type. The flow scheme of activities for mating type determination was descriped.

Stock of each known and unknown mating type isolates were re-grown on RFA plates. The plates were incubated at 25-28^{o}C with 12 hr. fluorescent light per day for 7 days. Then, the new RFA medium plates were divided to 4 quarters. The piece of each of known mating type 1-1 isolates, TH12 and THL59 were placed onto the opposite site of quarter part at the middle of each part and the piece of two unknown mating type were placed at the remain quarters. On the other hand, the same unknown mating type isolates were grown with Mat1-2 testers, TH16 and THL32 as the same format of culturing. Then, Petri dish with known and unknown were incubated at 19^{o}C with 12 hr. fluorescent light per day. After 2 weeks, the perithecia bulbs rise from the

zone of confluence between two colonies. The unknown-mating type isolate that could from perithecia with Mat 1-1 isolates, TH12 or THL59, were judged to be mating type Mat1-2 and the isolate that could from perithecia with Mat1-2 isolates, TH16 or THL32, were judged to be mating type Mat1-1. The sexual types of unknown isolates, male, female and hermaphrodite were designated in each fertile cross. Male fertile in unknown isolates were scored when perithecia were observed in only tester isolates at perithecia producing zones. Female fertile in unknown isolates were scored when perithecia were observed in only unknown isolates at zone of perithecia production. Hermaphrodite fertile in unknown isolates were scored when perithecia were observed in both of known and unknown isolates at zone of perithecia production.

Mapping population developments

The genetic recombinations from two blast fungal isolates were produced under laboratory condition. Since *M. grisea* is haploid organism then F1 progenies population from cross was used as mapping population. The two methods of mapping population developments are ascospore culture methods and ascus culture method. The details of each method were descriped.

Ascospore culture method: The ascus is composed of 8 ascospores representing to individual genotype. The flow scheme of activities for ascospore culture method is shown in ผิดพลาด! ไม่พบแหล่งการอ้างอิง. Two fungal isolates of opposite mating types were cultured with the same method of mating type determination described above. After 16-20 days, at the confluent zone of two colonies, the perithecia rise indicated presume of ascospores inside. With the aid of a stereomicroscope, a scalpel was used to excise perithecia bulbs from the zone of confluence. Bulbs were transferred to 4.0 % water agar plates and perithecia head was cut to release asci inside the bulb by using scalpel. Then, the individual ascus was separated across the agar surface using sealed-end Pasteur pipette. After 10 – 20 minutes, asci from individual ascospores were massaged gently with a sealed-end Pasteur pipette until the ascus sac broke up. Using a sealed-end Pasteur pipette, the eight ascospores were spareted out in a line. After 1-2 hrs, agar plugs containing the individual germinated ascospores were

excised with a scalpel and transferred to fresh RFA medium plates and were incubated at 25^{o}C with continuous light until colonies were formed. After 7 days, agar plugs containing mycelium of a single germinated ascospore was isolated and cultured on filter paper and were placed on individualy fresh RFA plates. After 7 days the filter paper with grown mycelia were dried in a desiccator for 14 days. The dried filter papers were cut into small pieces and were kept in aluminum foil sac, vacuum-sealed and stored at –20^{o}C.

Ascus culture method: The ascus in larger than ascospore and easier to deal with under stereomicroscopes. The flow schemes of activities for ascus culture method are shown in ผิดพลาด! ไม่พบแหล่งการอ้างอิง. Two fungal isolates of opposite mating type were cultured using the same method of mating type determination described above. After 16-20 days, Asci were isolated and separated across the surface of 4.0 % water agar plates as described above. Individual ascus was spread across the agar surface using sealed-end Pasteur pipette. After 16 hrs, germinated asci were excised individually and were transfered to fresh RFA plates. The asci were incubated at 25^{o}C with continuous light until colonies were formed after 3 – 5 days of culturing. The colonies were brushed lightly with sealed pasture pipette to pick up conidia. Each conidium was speared across the surface of water agar plates. After 16 hrs, a single germinated conidia from each ascus culture was isolated and cultured on filter paper and individually transfer into fresh RFA plates. After 7 days, the filter paper with grown mycelia was dried in desiccator for 14 days. The dried filter papers were cut into small pieces and were kept in aluminum foil sac, vacuum-sealed and stored at –20^{o}C.

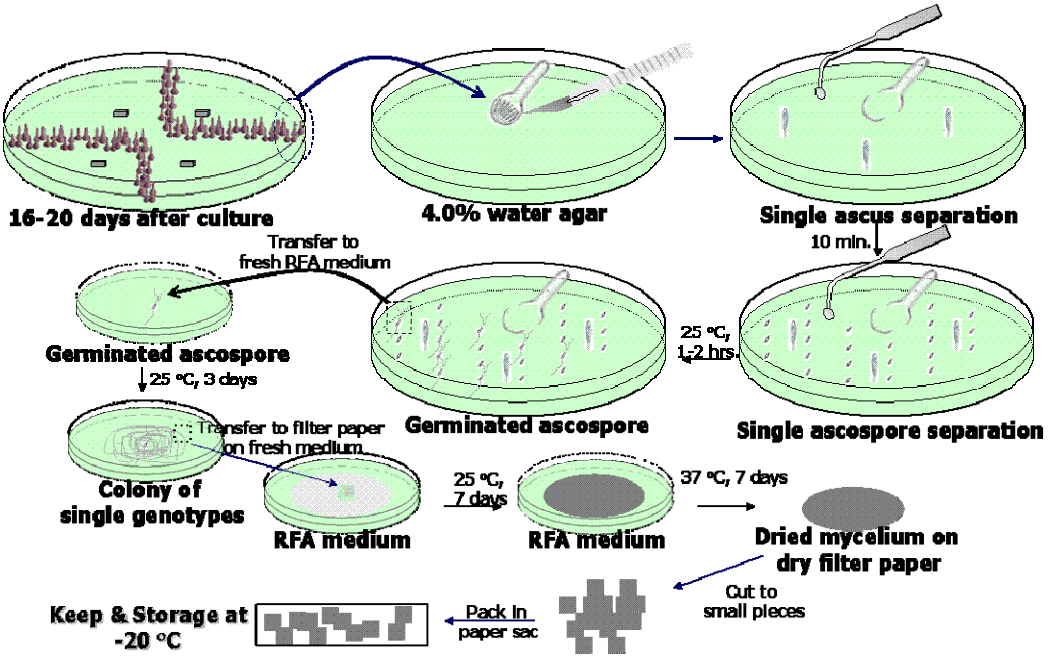

Figure 5 Ascospore culture methods for mapping population development.

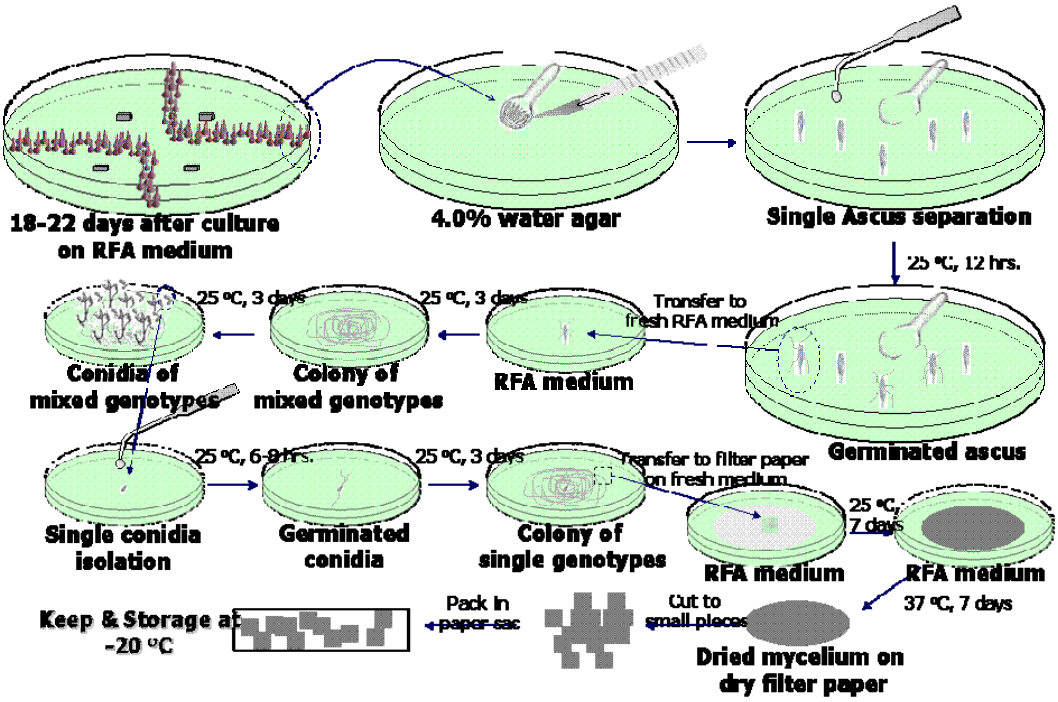

Figure 6 Ascus culture methods for mapping population development.

II. Determination of new virulence phenotype arising from genetic recombination

Rice variety

Rice variety for blast disease screening

Rice varieties used in this study were classified in to two groups as shown in Table 1. The First group was *O. sativa* group which composed of 54 rice varieties. Another group was wild rice species comprising 38 accessions of *O. rufipogon*, 14 accessions of *O. nivara* and 1 accession of *O. officinalis*.

Table 1 Two groups of rice varieties were used in this study.

Group	List
1. *Oryza sativa*	- 12 near isogenic lines; C101 A51, C101 LAC, C101 PKT, C101 TTP-6, C102 TTP, C103 TTP, C104 LAC, C104 PKT, C105 TTP-1, C105 TTP-2 (L23), C105 TTP-4 (L23) and CO39. - 39 varieties; Aichi asahi, Bl 1, Caloro, Chokoto, Dular, F145-2, Fujisaka5, Fukunishiki, IR1529, Jc92, Jc93, K1, K2, K3, K59, K60, Kanto51, Karkati87, Kusabue, Maratelli, Moroberekan, Nato, Nipponbare, Np125, Ou244, Pi No4, Ptb25, Residiv80, Rico1, Shin2, St1, Xuan1461, Usen, Zenith, Zhong156, Zhong201, 75-1-127, Hom Nin (JHN), IR64 and CT9993. - 3 susceptible variety; LTH, Sariceltik, and KDML105
2. Wild rice	- 38 accessions of *O. rufipogon* - 14 accessions of *O. nivara* - 1 accession of *O. officinalis*

Pathogenicity assays

Leaf blast inoculation

Fungal isolates to be used in pathogenicity test were grown on rice flour agar (RFA, 2.0% of rice flour, 2.0% of agar and 0.2% of yeast extract) media and incubated them at 25^{o}C with 12 hrs, fluorescent light per day. After 8-10 days, conidia were washed by steriled-distilled water and adjusted the concentration of conidia to 5x10^{4} conidia/ml. Twenty five milliliters of conidia suspension with 0.5 % gelatin were sprayed on leaf of 3 week old rice varieties in 2 replication that were planted in 15 x 24 x 5 cm. tray. Then, inoculated plants were incubated in high humidity chamber for 16 hrs, before continue to grown in a greenhouse. After 7 days, evaluations of blast symptoms were done according to a standard reference scale with 7 lesion type categories (Table 2 and Figure 7). In short, the most common as well as the most susceptible if the majority of the lesions developed were types 5 or 6. Where no sporulation developed, the disease severity index were types 0-2 and were considered as resistant and types 3-4 as intermediate (Roumen *et al*., 1997).

Table 2 The 7 lesion types scales for the assessment of symptoms induced by the blast pathogen on rice leaves.

Lesion type	Symptoms
0	No evidence of infection
1	Brown pinpoint smaller than 0.5 mm., without sporulation
2	Brown pinpoint smaller than 0.5-1mm., without sporulation
3	Small eyespots about 1-3 mm, lesion capable of sporulation gray center
4	Small eyespots about 3 mm or more longs, lesion capable of sporulation gray center and dark margin.
5	Susceptible sporulating type, coalescence lesion without dark margin
6	Susceptible sporulating type, lesion without dark margin

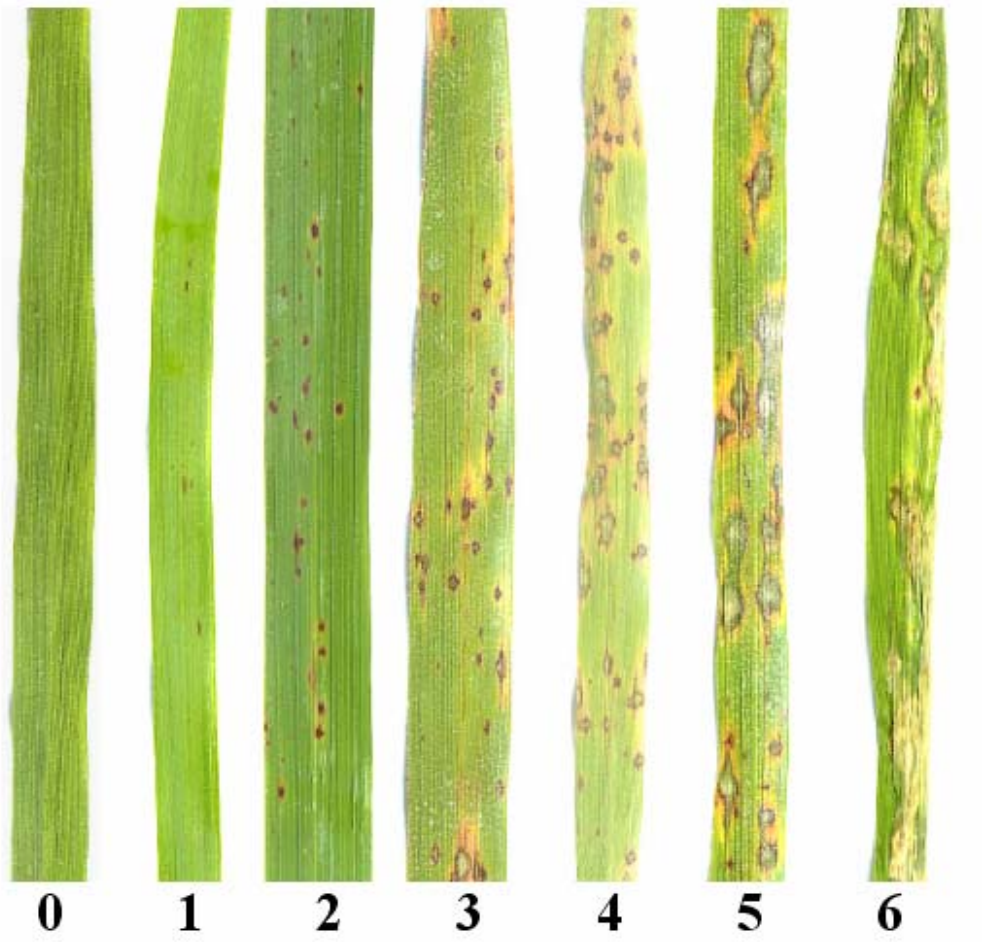

Figure 7 The 7 lesion type scales for the assessment of symptoms induced by the blast pathogen on rice leaves.

Panicle blast inoculation

Panicle blast evaluations were assayed at heading to flowering stage of rice plant. Rice seed were sowed and continues by grown in pot, 30 cm diameter and 20 cm deep. The first fertilizer application was commenced using 15-15-15 at 187.5 kg/ha., approximately 30-35 days after seeding. The second one was applied 55-60 days after seeding using (46-0-0) at 125 kg/ha. The panicle blast inoculums were adjusted to $5x10^4$ conidia/ml., and these were injected into the leaf sheath of flag leaf at heading or flowering stage. The plants were maintained under high humidity from automatic misty-spray sprinklers (Noenplab *et al*., 2006). The panicle blast symptoms

were recorded at 14 days after inoculation on incidence of percentage of infected by blast pathogen on panicle (IRRI, 2002).

III. Linkage map construction of blast pathogen population using molecular markers

Extraction of fungal genomic DNA

Sample preparation

Stock of each blast isolates on filter paper were cultured on RFA medium plates and kept at 25^{o}C under fluorescent light. After 5 - 7 days, agar plugs containing mycelium was isolated and cultured it in 0.25% Potato Dextrose Broth (PDB) liquid medium. The culturing plates were incubated at 25^{o}C in dark condition for 7 days. The mycelia were harvested from the liquid medium using forceps. The liquid medium must be removed as much as possible before keeping in 1.5 ml microtube. The samples of each isolates were freeze-dried for 12 hours and stored at -20^{o}C.

DNA extraction

The DNA extractions were performed by using DNA trap kit (http://dnatec.kps.ku.ac.th). This procedure is quick, use small amount of simple, low cost per sample and does not use dangerous chemicals. The bind mix solution contains guanidine and silica gel that can denature and precipitate protein instead of phenol and/or chloroform. Guanidine can break cell wall and cell membrane while silica gel is a positively charged that can form complex with DNA to precipitate. Using propanol, residual protein and polysaccharide were removed. This kit composed of 5 buffers such as extraction buffer, neutralizing buffer, bind mix buffer, washing buffer I, washing buffer II and elution buffer. The steps in DNA extraction can be followed in the instruction manual.

DNA quantity and quality

The quantity and qualitaty of extracted-DNA were performed in agarose gel electrophoresis. Each DNA sample was added with loading dye before depositing into a well of 1.0 % agarose gel. The standard DNA concentrations were also deposited. The electrophoresis were done using 100 volts for 60 min in 0.5 TBE buffer. After electrophoresis, the DNA bands were visualized by straining agarose gels with ethidium bromide. DNA concentrations were rated by comparing the intensity of DNA band with standard DNA concentration.

PCR (Polymerase chain reaction) amplification

Simple sequence length polymorphism (SSLP) analysis was performed by choosing the microsatellite markers from reference population, GUY11/2539, that were located throughout the 7 chromosomes of *M. grisea* (Tharreau et. al., unpublished data). The PCR reactions were done in a total volume of 10 µl containing 1X of PCR buffer, 0.25 ml of each dNTPs, 2.0 mM of $MgCl_2$, 0.16 U of Taq polymerase, 0.5 M each of forward and reverse primers, and 5 ng of genomic DNA template. DNA amplification was done in a DNA Thermal Cycle using program for initial denaturing step of 1 min at 95^{o}C followed by 25 cycles of 94^{o}C for 30 s, 55^{o}C for 30 s and 72^{o}C for 30 s, and final elongation of 72^{o}C for 5 m (Kaye *et al*., 2003).

Visualization of PCR amplification products

There are two methods to visualization of PCR product; agarose and acylamide gel electrophoresis.

Amplification product were separated on agarose by using protocol previously described (Kaye *et al*., 2003). In brief, PCR products in each reaction were deposited in each wells of 3.0% agarose gell in 0.5% of TBE buffer. The electrophoresises were performed with 100 volts for 120 minutes in 0.5% TBE buffer. Then, the gells were stained with 1 ppm of Edthriduim Bromide in distilled water for 30 minuites and

destained with distilled water for 20 minutes. The DNA bands were made visible in UV illuminator and photo of each gell was recored.

The polyacrylamide electrophoresis gels were prepared by wiping the chamber plate with absolute ethanol using Kimwipes for 3 times and wiping with clear view until dry. A 32 x 37 cm glass plate is wiped with absolute ethanol using Kimwipes for 3 times and allowed to dry up. The glass is applied with 1000 µl of Bind Silane solution, and then washed again with absolute ethanol. A 50 ml of 4.5 % acrylamide gel solution is prepared and is poured to the assembled gel plates and the gel is allowed to polymerize for 1 hr. An amount of 2.5 µl of amplied products with sequencing dye solution were denatured for 5 min at 94 °C in the thermocycler and placed on ice before loading to the well. The running conditions was, 60 W for 75 min, after a pre-run of the gels for 0.5 hr. in 1x TBE solution.

Gels were silver stained using two methods according to the steps described in ผิดพลาด! ไม่พบแหล่งการอ้างอิง the protocol of Panaud *et al*., (1996) (Method 1) was followed exactly as recommended. The improved staining method we developed by Benbouza *et al*., (2006) (Method 2) Gels were dried at room temperature and DNA bands were viewed directly with the aid of a white light box, then scanned.

Table 3 Steps of the two compared procedures for silver staining.

Step	Method 1 Panaud *et al*., (1996)		Method 2 Benbouza *et al*., (2006)	
	Solution	Time	Solution	Time
1. Fixation	10% acetic acid (Cold)	20 min.	10% ethanol, 0.5% acetic acid	3 min.
2. Rinse	dH2O	Quick wash, 3 times	-	-
3. Impregnation	Per liter: 1 g AgNO3, 1.5 ml of 37% HCOH	30 min.	Per liter: 2 g AgNO3	5 min.
4. Rinse	dH2O	Quick wash, 1 time	dH2O	Quick wash, 1 time
5. Development	Per liter: 30 g Na2CO3, 1.5 ml of 37% HCOH, 2 mg of Na2SO3.5H2O (Cold)	Until band are appear (2-5 min.)	Per liter: 15 g NaOH, 2.0 ml of 37% HCOH	Until band are appear (2-5 min.)
6. Stop	10% acetic acid (Cold)	5 min.	10% acetic acid	5 min.
7. Rinse	dH2O	5 min.	dH2O	5 min.
Total time		65 min.		23 min.

IV. Mapping of avirulence genes in two developed blast pathogen populations

Linkage map construction and QTL analysis

One hundred and twenty-six microsatellite markers were used in PCR amplification of genomic DNA of parental isolates, THL1000 and THL16, and B1-2 and TH16. The polymorphisms of DNA band between parental isolates were chosen for next step. These markers were applied in F1 progenies of mapping population in each crosses. The segregation data of each microsatellite in each progeny were scored and analyzed by MAPMAKER 3.0 software for linkage map construction (Lander *et al*., 1987). After that, combination of microsatellite data and pathotype data were performed by using nQTL software (Tinker and Martha, 1995).

The nQTL was performed to analyze QTL. The simple interval mapping (SIM) and the simplified composite interval mapping (sCIM) techniques were used to determine the association between phenotypes and marker genotypes. Each data set was analyzed with 1,000 permutations, a 5 cM walking speed and a Type I error rate of 5%. The significant threshold was used to declare the presence of a QTL. Associations of markers with traits were reconfirmed using simple regression, multiple regression and ANOVA analysis procedures in STATGRAPHIC3.0 (Manugistic, 1997).

V. Developing new microsatellite markes and narrow down avirulence locus

Narrow down of QTL region

The genome sequencing project of *M. grisea* is available on the *Magnaporthe grisea* Database website (http://www.broad.mit.edu/annotation/fungi /magnaporthe/index.html) and NCBI (http://www.ncbi.nlm.nih.gov/). The flanking markers of identified-QTL regions were used to BLAST search in NCBI website and they showed the locations on physical map of *M. grisea*. Then, DNA sequence between these markers was retrieved from the database. Along with this DNA sequence, microsatellite repeat and marker specific to each repeat were designed with the aid of Primer3 software (http://frodo.wi.mit.edu/) at parameters essentially described as follows: 18–25 nucleotides in length, devoid of secondary structure or consecutive run of a single nucleotide, a GC content around 50%, a annealing temp around 60^{o}C, and preference of G or C at the 3′-end. These new microsatellite markers were rechecked for the location of each marker on physical map by BLAST search against the same database, NCBI. The formation of primer dimer between forward and reverse primer and hair pin loop of each primers were tested.

The new microsatellite markers were screened for polymorphic alleles between parental isolates, B1-2 and TH16. Then, the markers showing polymorphic DNA band in parental isolates were applied in all mapping population. By the aid of Graphical

Genotype Program, GGT, the fine map of this QTL region was constructed. This data were compared with phenotype data of each individual blast isolates in QTL analysis. This analysis could narrow down QTL region to be a specific physical map region. Gene prediction of this narrow downed region was performed by using available database and bioinformatics tools.

RESULTS

I. Development of mapping population

To study the sexual recombination in the blast fungus two crosses of blast fungus were developed in laboratory condition. Mating type determination was performed to classify blast fungus isolates having the opposite mating type. The identified mating type isolates were crossed together in controlled environment to produce new genetic recombination. The crossing methods used in this study were ascospore and ascus culturing methods. The result of this part of study is below.

Mating type determination of virulence isolates

The highly fertile and hermaphrodite tester isolates composed of TH12 and THL59 identified as mating type 1-1; and TH16, THL16 and THL32 determining as mating type 1-2. Two blast pathogen isolates, THL1000 was isolated from wild rice, *O. rufipogon* and B1-2 was isolated from JHN rice and were used to determine mating type characteristic. After culturing blast isolates together with the tester isolates for 3 weeks, the perithecia rose from the zone of confluence between two colonies. The THL1000 can be crossed to the tester isolate, THL16 and it was identified to be of mating type 1-1. While, the B1-2 can be crossed to the tester isolate, TH16 was identified to be of mating type 1-1.

Crossing and ascospore isolation

The progenies from crossing parental isolates can be used in evaluating potential genetic recombination of new virulence isolates and used as mapping population. Two crosses were done, THL1000/THL16 and B1-2/TH16. The crossings were performed under laboratory condition. There are two crossing methods used in the isolation of progenies.

After growing 4 parental isolatates, THL1000, THL16, B1-2 and TH16 together in each cross for 18 – 20 days, the perithecia bulbs rose from the confluent zones of two colonies. Using the stereomicroscope, the healthy perithcium bulbs having large size and perithecium neck appeared in aerial part with dark perithecium appearing in medium indicated bundles of perfect forming of ascospore. Individual healthy perithecium was picked up and place in 4.0% water agar plate. Inside perithecia bulbs contained a lot of acsi and in each ascus has 8 ascospores. The individual ascospore indicated new genetic recombination of two parental isolates and it was isolated individually on water agar medium plate (Figure 8). After ascospores geminated and formed colonies, one or two ascospores representing to one ascus were picked up and grown in RFA medium plate. The stock of each ascospore was kept at -20^{o}C as a dried-mycelium on filter paper in aluminum sag which was vacuum sealed. This technique was designed as ascospore culturing method and a total of 88 random ascospore progenies were developed in THL1000/THL16 cross (ผิดพลาด! ไม่พบแหล่งการอ้างอิง).

Some of this part of research was done under the cooperation of the Rice Blast Project at Rice Gene Discovery Unit and CIRAD. The new technique of mapping population development method was learned from Dr. Didier Tharreau at CIRAD. This technique is called ascus culturing method. To deal with ascus is easier than ascospore when working under the stereomicroscope because ascus has larger size than ascospore (80 μm and 20 μm, respectively) (Figure 9). The ascus culturing method is more popular than another one. Following this method, a total of 304 blast progenies were developed and storied as the same method as ascospore culturing method for B1-2/TH16 cross (ผิดพลาด! ไม่พบแหล่งการอ้างอิง). These populations were used in the next step.

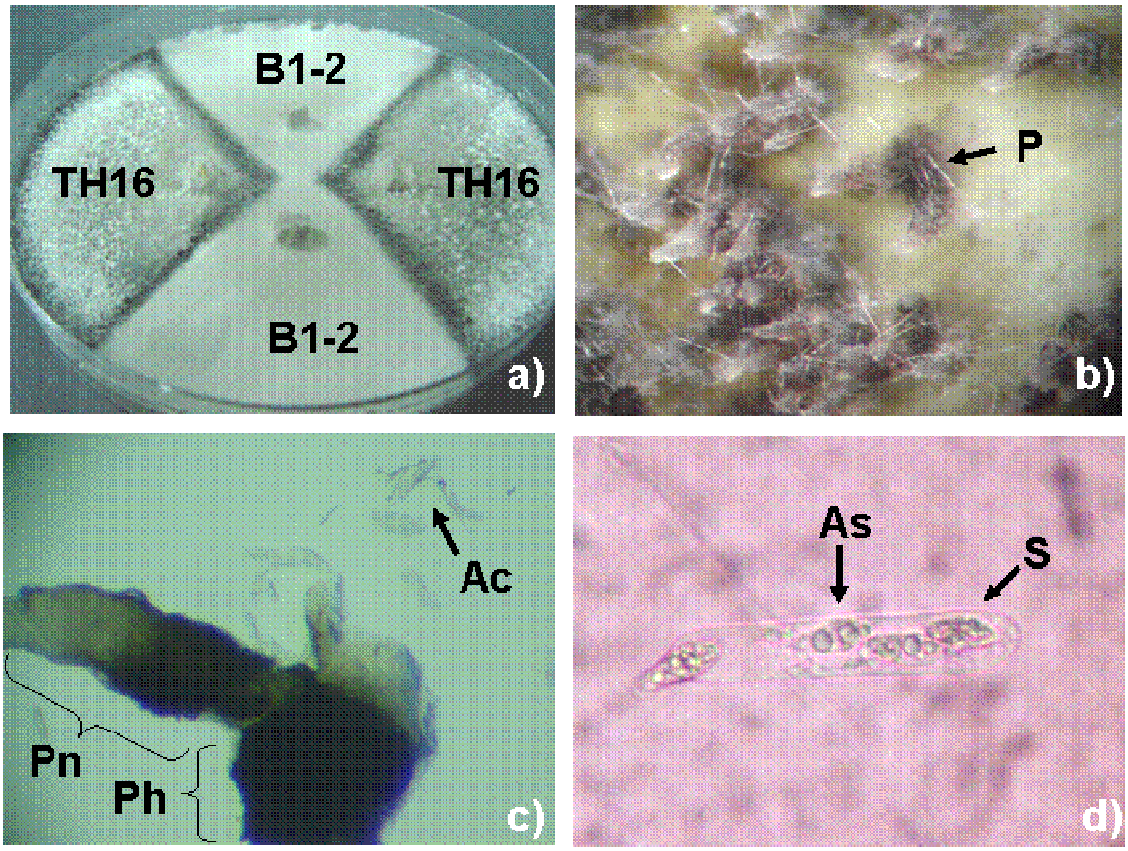

Figure 8 Crossing between B1-2 and TH16 isolates; a) the perithecia formation between the cofluent zones of two isolates; b) the perithecia (P) rise from the medium; c) two parts of perithecium, perithecium neck (Pn) and perithecium head (Ph) with asci (Ac) inside; d) the asci are usually compose of 8 ascospore (As) in ascus sac (S).

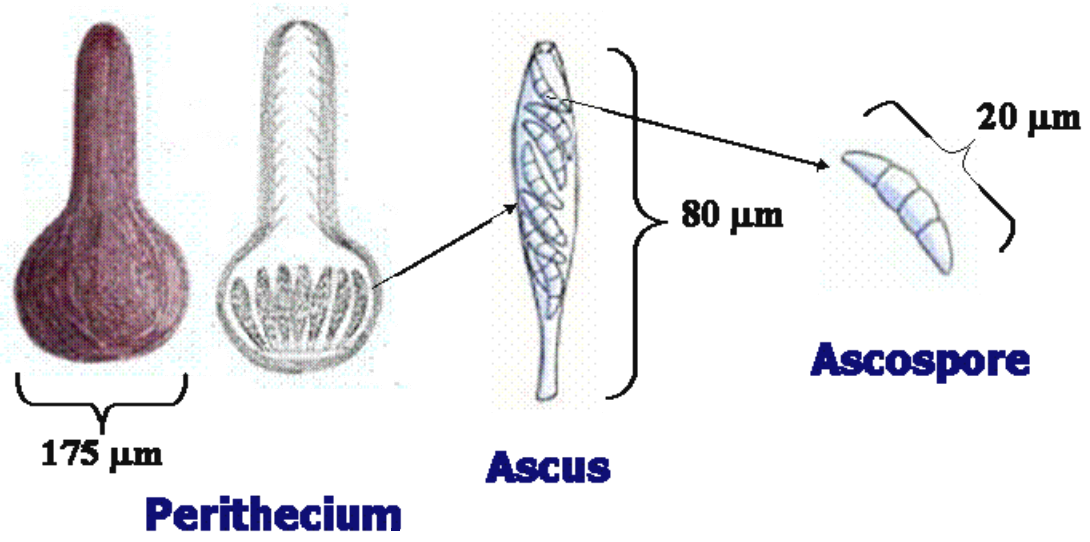

Figure 9 Morphology of perithecium, ascus and ascospore of *M. grisea*.
Source: Hanlin, (1998) and Uddin *et al*., (2003).

II. Determination of new virulence phenotype arising from genetic recombination

THL1000xTHL16 cross

Pathogenicity testing of parental isolates: Pathogenicity tests of THL1000 and THL16 were done on rice listed above (Table 1). THL16 was found to be pathogenic on 28 cultivars (scores ranging from 4 to 6) including 5 cultivars carrying blast resistance genes such as C105 TTP-4 (*Pi-4b*), K59 (*Pi-t*), C101 PKT (*Pi-4a*), Shin2 (*Pi-ks*), and K1 (*Pi-ta*). This isolate was not pathogenicity in all lists of wild rice accessions. In contrast, THL1000, isolated from *O. rufipogon*, failed to infect all tested cultivars and all wild rice accessions accepted 1 *O. rufipogon* accession. Hence,

sixteen varieties that showed pathogenicity difference between parental isolates were chosen for pathogenicity tests with all progenies from their cross.

Analysis of pathogenic segregation in *M. grisea* population: Eighty-eight progenies were randomly chosen to study pathogenic distribution on sixteen rice cultivars such as Aichi asahi, C105TTP-4, CO39, F145-2, Jc92, K1, K59, Karkaty87, Kassa Calh, Maratelli, Nato, Nipponbare, PTB25, Residiv80, Sariceltik, and Xuan1461. According to the results, it was found that only Sariceltik offered pathogenic segregation of the population while the rest did not. Moreover, physical observation revealed some progenies had white colony that related to an inactivation of melanin synthesis resulting in infecting failures. As a result, fifteen progenies (17 %) were discarded; only 73 progenies were employed in future processes. The pathogenic segregation in 73 progenies was similar to normal distribution. Confirmation of pathogenic ratio was performed by chi-square test, and the proportion of pathogenic: intermediate: non-pathogenic fitted to 1:2:1 ratio (Figure 10)

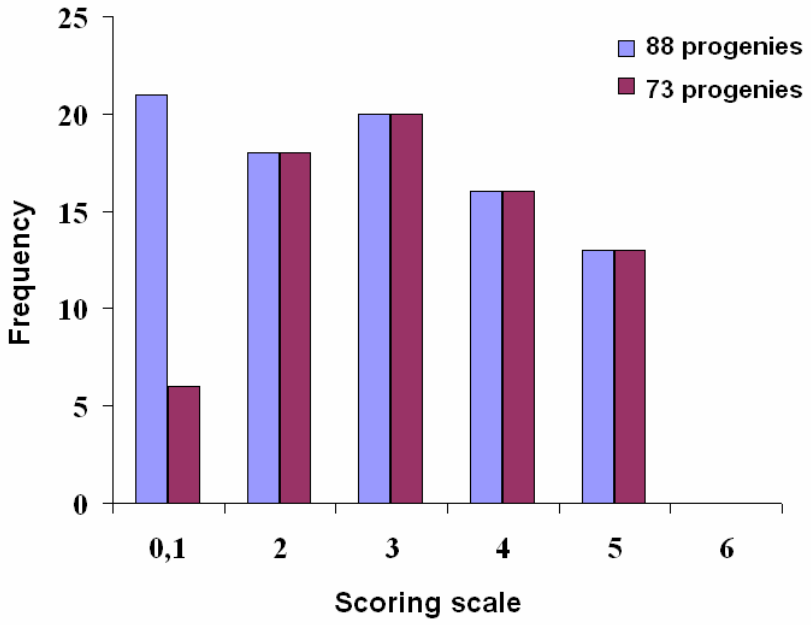

Figure 10 Frequency of pathogenicity score of progenies derived from THL1000xTHL16 cross on Sariceltik rice variety. From the total of 88 progenies tested only 73 progenies were used in pathogenicity test eliminating mutant isolates with white colonies.

B1-2xTH16 cross

Screening for virulence isolates on JHN rice variety: The Jao Hom Nin rice variety has been demonstrated broad spectrum resistance to Thai blast pathogen populations. It had been identified that the position of resistance genes were located on chromosome 1 and 11 (Noenplab *et al*., 2006). The resistance gene on chromosome 11 showed major effect while gene on chromosome 1 showed minor effect. The resistance characteristic in two stages such as seedling for leaf blast resistance and flowering stage for panicle blast resistance, could identify same region on these chromosomes. It could be the same gene that govern both leaf and panicle blast resistance. These blast resistance loci have been widely used in many rice breeding programs to improve blast resistance in target or new rice varieties (Noenplab *et al*., 2006; Sirithunya *et al*., 2004).

Broad spectrum resistance of JHN rice variety: The Thai blast pathogen isolates were grouped into two which was used in this study. The first group was composed of 96 blast isolates and they were used to evaluate blast resistance in JHN rice variety accompanied with 4 standard rice varieties, IR64, CT9993, KDML105 and Sariceltik. Second group of blast isolates composed of 250 isolates were used to screen blast resistance ability in JHN and KDML105 rice varieties. The blast isolates were selected from the stock of 1,228 isolates of Thai's blast pathogen population that were collected throughout rice growing areas in Thailand during 1998 – 2001. The blast pathogen isolates in the North of Thailand showed more diversity than from other parts. Then, the blast pathogen isolates in this region were selected in higher proportion than from other regions (Table 4)

The three candidate resistance rice varieties JHN, IR64 and CT9993 were been used for blast resistance loci identification using Quantitative Trait Loci analysis. The first set of blast isolates, 96 isolates, were inoculated in these rice varieties together with 2 susceptible rice varieties, KDML105 and Sariceltik. These varieties showed the variation of resistance reactions. The broad spectrum resistance (BSR) index was used to identify the percentage resistance ability on tested-blast isolates. The 100 % BSR

means that rice variety can resist to all tested-isolates. On the other hand, 0 % BSR means that rice variety can not resist to any tested blast isolates. JHN rice could resist all tested-isolates and showed 100 % broad spectrum resistance index. IR64 rice variety showed 93.8 % of broad spectrum resistance index while CT9993 rice variety showed 97.7 %. The two susceptible varieties, KDML105 and Sariceltik showed low broad spectrum resistance index which were 50.0 and 13.3 %, respectively. In this study, the broad spectrum resistance was not only identified in candidate resistance rice varieties but also identified virulence blast isolates towards JHN too. In this set of blast isolates we could not find any virulence isolates on JHN rice. Then new set of 250 blast isolates was setup to inoculate on JHN rice variety.

The second set of 250 blast isolates were used inoculation of two rice varieties, JHN and KDML105. In this result, none of the "THL" isolates could infect JHN. The most virulent isolates could express the reaction type on JHN rice with a score of 4 showing intermediate reaction, by THL308, THL309, THL310 and THL316. The KDML105 rice also showed broad spectrum resistance index similar to the fist set of blast isolates. Fortunately, the 10 virulence blast isolates provided by Dr. Poonsak Mekwatanakarn from Ubon Rice Research Center were included in this study. Two of these isolates could show fully pathogenic reaction types with a score of 5, on JHN rice while the rest isolates were shown as intermediate reaction type, score 3 – 4. These isolates are namely B1 and B2. To purify to virulence isolates, the infected leaves of JHN rice of each virulence isolates were use in single conidia isolation in laboratory. Each virulence isolate was kept as two sub samples to represent one as original isolates. The codes of new isolate were designed as B1-1 and B1-2, and B2-1 and B2-2 for the original isolates B1 and B2, respectively. The stock of each isolates was storied at -20^{o}C which was used in the next step of study.

Table 4 Number of blast pathogen isolates was used in screening for broad spectrum resistance of JHN rice variety.

Group of isolates	Original region						
	Central	East	North	Northeast	West	South	Total
1st group	12	6	47	18	2	13	98
2nd group	51	0	129	66	1	1	248
Total	63	6	176	84	3	14	346

For worldwide isolates, JHN rice variety was used to evaluate blast resistant reaction for worldwide blast pathogen isolates. This part of this research was done under the collaboration between Rice Blast Project at Rice Gene Discovery Unit and CIRAD, France. A total 124 isolates from worldwide collection were used to inoculate on JHN and resistant rice varieties, CT13432 carrying *Pi1*, *Pi2* and *Pi33*. The result showed JHN was very susceptible to 7 blast isolates composed of 2 isolates from Colombia and 5 isolates from China. For the intermediate blast reactions, score 3 – 4 on this variety, 7 isolates were observed which can be broken down into 1 isolate from Madagascar, 3 isolates from India, 1 isolates from Bangladesh and 2 isolates from China. These resultted indicated that JHN rice tends to be susceptible to blast pathogen from China. On the other hand, the CT13432 rice variety showed to resist to all tested-isolates and it can be a new resistant source to improve blast disease resistance in Thailand in the future.

Phenotypic segregation in mapping population: The JHN and 25 differential host set of rice varieties were used to screen blat disease resistance at seedling stage with parental isolates, B1-2 and TH16. JHN and 3 near isogenic lines of CO39 rice variety namely C101PKT, C104PKT and C105TPP demonstrated polymorphism on pathogenicity between parental isolates. These varieties were applied in screening all progenies derived from cross between B1-2 and Th16 isolates. JHN was inoculated on two stages of rice, seedling and flowering stages but the other 3 varieties were inoculated only on seedling stage.

Segregation of pathogenicity on JHN rice variety: Two stages of JHN rice variety, seedling and flowering stages were inoculated by parental isolates, B1-2 and TH16. The blast isolate B1-2 showed full pathogenicity on JHN at both stages while TH16 was showed non-pathogenic on this rice variety (Figure 11). The 140 random ascospores of *M. grisea* from a cross between B1-2 and TH16 were selected and used as mapping population. The segregation of avirulence vs. virulence characteristics were studied in all these progeny isolates using JHN rice cultivar at two stages, seedling and flowering stage. Chi-square values were used to test the hypothesis of segregation of avirulence versus virulence characteristic at the expected ratio of 1:1 for a single major gene control. The segregation ratios of avirulence to virulence characteristics fitted to 1:1 on both stages of this rice variety. The values of Chi-square were 3.919 and 0.591 for leaf blast and panicle blast respectively. These segregation ratios indicated that avirulence or virulence characteristics of this mapping population on this variety were controlled by single genetic locus. Moreover, the correlation of virulence and avirulence between leaf and panicle blast of all progenies was 0.791 which showed significant correlation. This suggests that avirulence gene for leaf blast and panicle blast might be the same locus or tightly link together on this population.

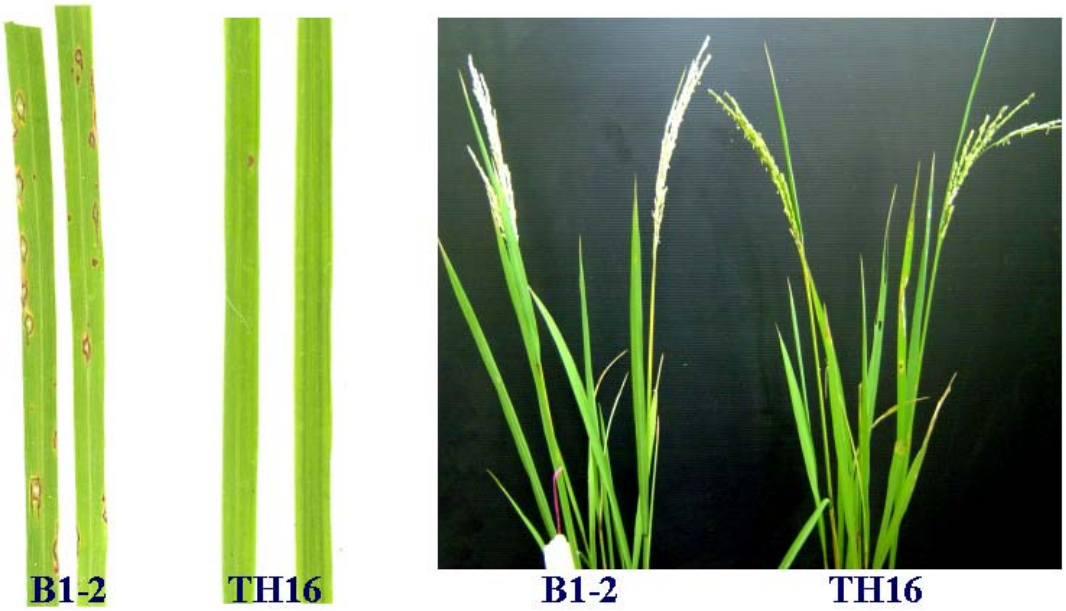

Figure 11 Pathogenicities of parental isolates, B1-2 and TH16, on JHN rice cultivar. Leaf blast inoculation was done at 3 week-old rice (left) and panicle blast inoculation was done at flowering stage of rice plant (right).

Segregation of pathogenicity on C101PKT, C104PKT and C105TTP rice varieties: The C101PKT and C104PKT rice vareities were derived from several back crosses of CO39 by PKT rice variety. Many near isogenic rice lines were developed from this cross such as C101PKT and C104PKT. These two varieties were identified carrying different blast disease resistance genes. The C101PKT and C104PKT varieties carried *Pi-4a* and *Pi-3* respectively. The segregation of virulence and avirulence charactersistics of progenies dereived from B1-2 and TH16 cross on C101PKT, C104PKT showed clearly separated group on virulence and avirulence characteristics in both varieties. These progenies of mapping population could show virulence on C104PKT than C101PKT (Table 5). The Chi-square analyses were performed in order to see if the segregation ratio 1:1 for virulence and avirulence was acceptable. The C101PKT showed Chi-square and P-value of Chi-square as 3.814 and 0.050, while C104 PKT had 0.794 and 0.373 for Chi-square and P-value, respectively (Table 5). These ananlyses could clearlyfy that avirulence character was controlled by single genetic locus in this mapping population. Moreover, the virulence and avirulence character in these varieties showed the same patterns and it could not find

any recombination of pathogenicity between these varieties. This indicated that the avirulence locus corresponding to these varieties could be the same loci.

Table 5 Chi-square analysis on pathogenicity of progenies derived from B1-2 and TH16 cross on three rice varieties.

Pathogenicity class	C101PKT	C104PKT	C105TTP
Avirulence	74	68	99
Virulence	52	58	27
Total	126	126	126
Chi-square value	3.841	0.794	41.143
P-value of Chi-square	0.050	0.373	0.000

The C105TTP rice vareities were derived from several backcrosses of CO39 by TTP-4-L23 rice variety. These varieties carried *Pi-4b* gene. The segregation of virulence and avirulence charactersistic data of progenies derived from B1-2 and TH16 cross on C105TTP showed clearly separated groups on virulence and avirulence in this variety. The Chi-square analyses were performed in these varieties to see wether the segregation ratio of 1:1 for virulence and avirulence is acceptable. The C105TTP showed Chi-square and P-value of Chi-square as 41.143 and 0.000, respectively meaning, the segregation ratio was not acceptable (Table 5).

Mating type segregation: Mating type determinations were performed using parental isolates as the testers because these parental isolates were highly fertile and hermaphroditic sexual. The blast isolates B1-2 and TH16 were determined as mating type 1-1 (Mat1-1) and 1-2 (Mat1-2) respectively. The data of mating type determination of 140 progenies was classified into three groups which was composed of Mat1-1, Mat1-2 and unclassified (incompatible). The segregation ratio of Mat1-1 and Mat1-2 fitted to 1:1 which showed 0.883 and 0.347 of Chi-square value and P-value. This finding indicated that the mating type could be governed by single genetic locus from the parental isolates.

Transgressive segregation

Transgressive segregation in the progenies derived from 2 crosses, THL1000xTHL16 and B1-2xTH16 were used in this study. The progenies derived from the THL1000/THL16 cross were screened for transgressive segregation of virulence chactersitic on 12 rice varieties including Sariceltik rice variety. All data demonstrated that none of progenies from this cross showed any virulence characteristic on tested varieties except Sariceltik. On the orther hand, the progenies dereived from B1-2xTH16 cross were inoculated on 11 rice varieties and they showed transgressive segregation (Figure 12 and Appendix Table 1).

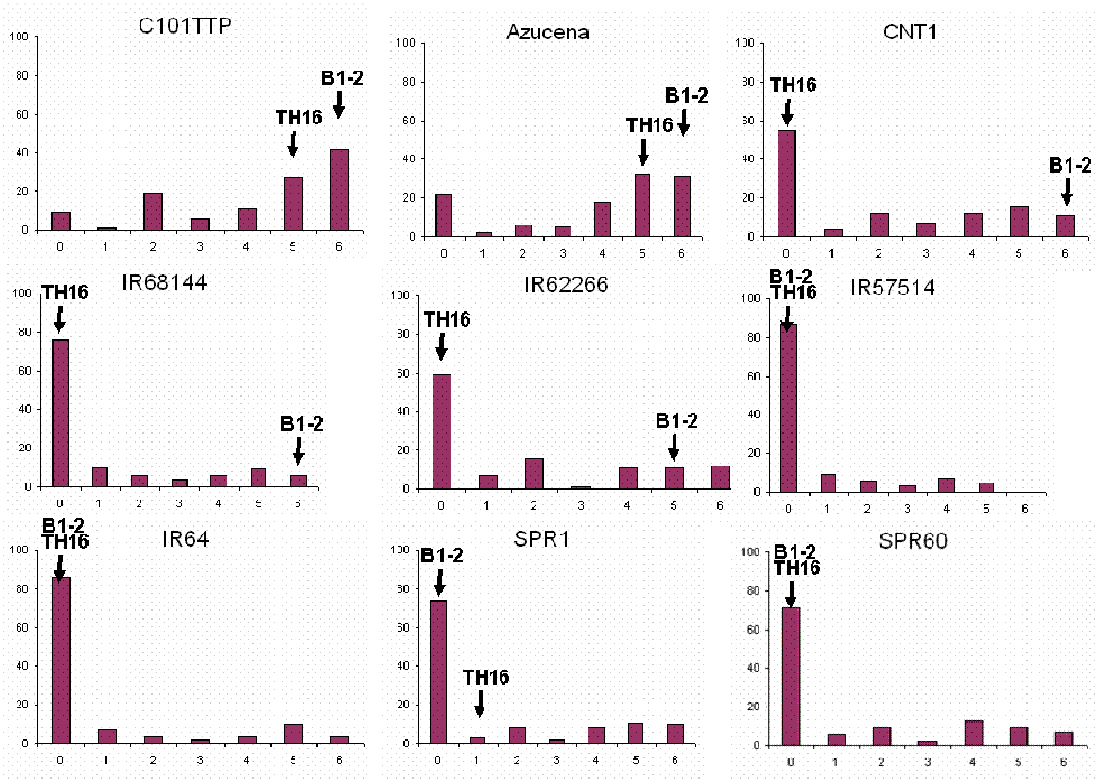

Figure 12 Pathogenicicty distribution of progenies derived from B1-2xTH16 cross on 9 rice varieties

III. Linkage map construction of blast pathogen population using molecular markers

Information about the genetic map of Guy11x2539 was used as the reference population to select microsatellite markers throught the collaborative project between Rice Blast Project and CIRAD (Figure 13). The sequences of each microsatellite markers were provide by Dr. Didier Tharreau.

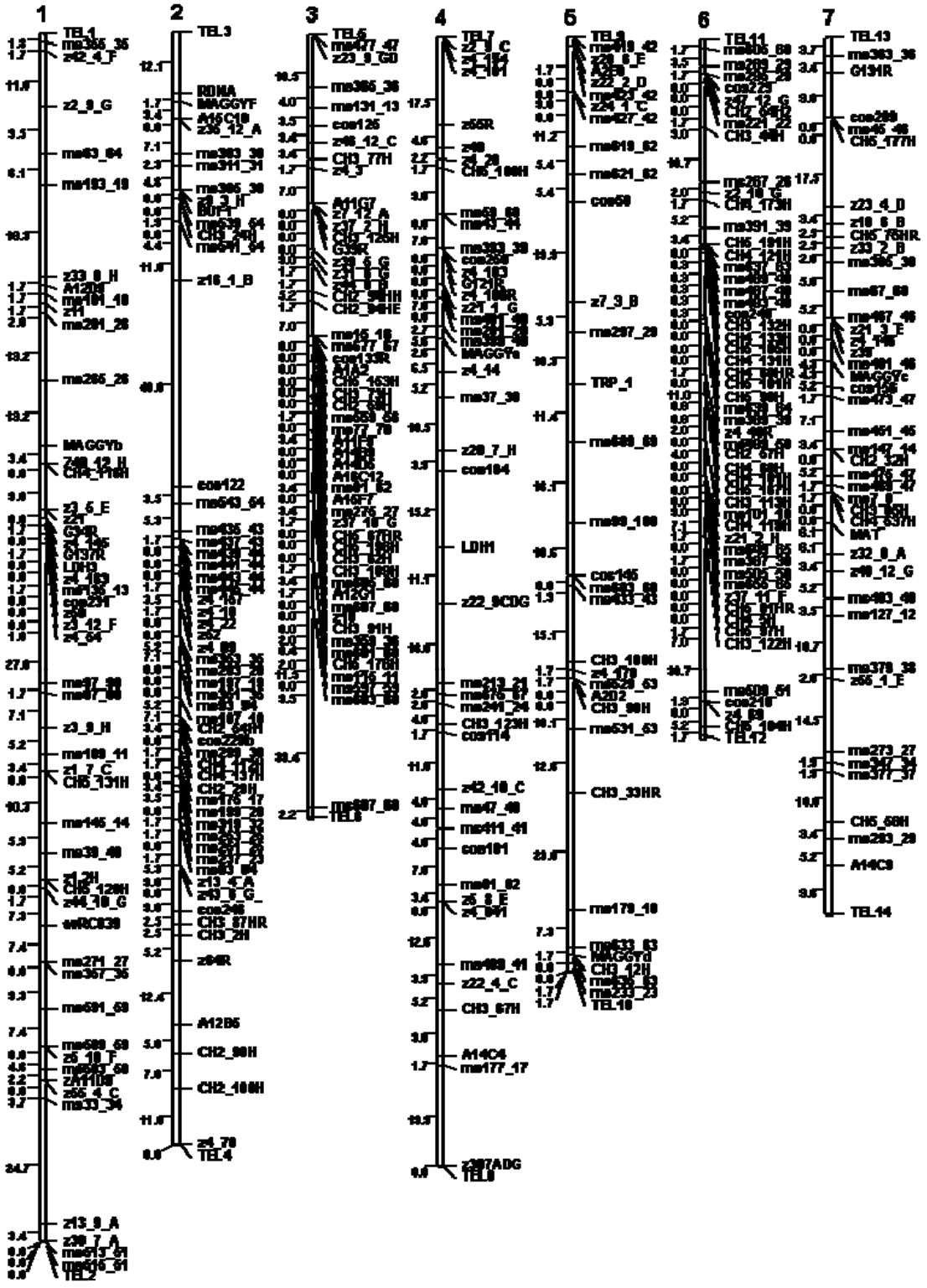

Figure 13 Genetic linkage map of reference population, Guy11x2539.

THL1000xTHL16 cross

A total of 106 molecular markers, specific to microsatellite repeat, were used to screen the distribution of THL1000 and THL16 alleles. Fifty-eight markers (54.7 %) demonstrated polymorphisms between two parental isolates. All of the polymorphic markers were chosen to differentiate the segregation of alleles among progenies. A total of 58 markers screened in 88 progenies were used to construct the linkage map by MapMaker software. Base on the locations of markers on reference population, Guy11x2539, 7-linkage maps were constructed from these data. The 7 linkage maps spanned 634 cM with an average density of one marker on every 17.74 cM (Figure 15).

B1-2xTH16 cross

The sequences of 126 microsatellite markers specific to microsatellite repeat were supported and provided by Dr. Didier Tharreau from CIRAD and were synthesized at the DNA Technology Laboratory, Khampaengsaen Campus, and Kasetsart University. All selected markers were chosen by location and the distribution throughout all regions of 7 chromosomes of *M. grisea* (Figure 13).

Parental survey with microsatellite markers: The simple sequence length polymorphism, SSLP, technique was applied to study the segregation of microsatellite alleles in each specific regions of each chromosome. The PCR products were separated in polyacrylamide gels and were visualized by using silver strain technique. The 126 microsatellite markers were screened in parental isolates, B1-2 and TH16. The 73 markers (57.9 %) demonstrated polymorphisms of PCR products between two parental isolates.

Table 6 Number of tested microsatellite markers and polymorphic markers on parental isolates, B1-2 and TH16

Primer	Chr. 1	Chr. 2	Chr. 3	Chr. 4	Chr. 5	Chr. 6	Chr. 7	Total
Tested primer	17	26	16	15	16	19	17	126
Polymorphic primer	10	14	11	6	10	11	11	73
% polymorphic primer	58.8	53.8	68.8	40.0	62.5	57.9	64.7	57.9

Genotyping of mapping population: The screening for polymorphic markers of PCR products on parental isolates can obtain 73 markers (57.9 %) demonstrating polymorphisms between two parental isolates. The selected 39 markers distributed throughout all 7 chromosomes of this pathogen were chosen based on the position of each marker on each chromosome of reference population, Guy11x2539. These markers differentiated the segregation of alleles among 140 progenies of the mapping population. The data showed allele segregation in each marker that fit to 1:1 ratio.

Linkage map construction: A linkage map was constructed using MAPMAKER/QTL software from the data of allele segregations in all markers. Distances between markers were computed using the Kosambi mapping function (Lander *et al*., 1987). The 7-linkage groups were constructed from these data. The linkage on chromosome 1, 2, 3, 4, 5, 6 and 7 consisted of 4, 6, 5, 5, 7, 6, and 6 markers and each linkage map spanned 63.3, 19.4, 86.0, 98.1, 120.0, 43.2, and 12.387 cM respectively. The 7 linkage maps spanned 483.1 cM with an average density of one marker on every 17.74 cM (Figure 16).

Intregrated map

Since, there are 2 mapping populations and some of markers in each linkage map are the same then 2 linkage maps were intregrated. The result showed that new genetic linkage map spanning 7 linkage groups composed of 12, 12, 10, 10, 10, 10 and

15 markers in each chromosome. The total genetic length was 832 cM with average on 118.86 cM per chromosome. The longest linkage map was chromosome 3 with 243.7 cM but the shortest was chromosome 6 with 48.3 cM (Table 7)

Table 7 Intregrated map of THL1000xTHL16 and B1-2xTH16 showing number of marker in each linkage group.

Chromosome	No. of marker	Length (cM)	Average marker/cM
1	12	80.4	6.7
2	12	58.9	4.9
3	10	243.7	24.4
4	10	114.3	11.4
5	10	157.1	15.7
6	10	48.3	4.8
7	15	129.3	8.6
Taotal	79	832	10.5

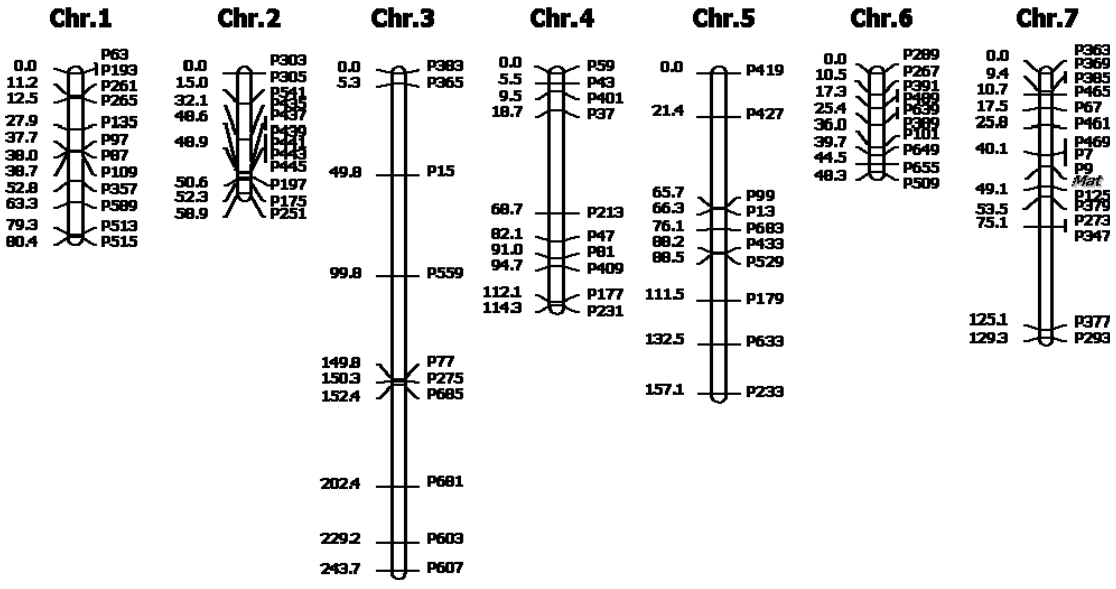

Figure 14 Intregrated map of THL1000xTHL16 and B1-2xTH16

IV. Mapping of avirulence genes in two developed blast pathogen populations

THL1000xTHL16 cross

Data of linkage map construction and pathogenicity of individual progenies were integrated and analyzed together by nQTL software. Two putative QTL regions were detected with LOD score higher than thresholds from each analysis suggesting these regions might contain genes involving pathogenic abilities toward Sariceltik. These QTLs were located on chromosome 2 between markers Pyms435 and Pyms443 with 1.0 cM distance between two markers and in chromosome 4 between Pyms213 and Pyms47 with 17.5 cM distance between two markers. LOD scores of two regions were detected as 3.32 and 3.27, on chromosomes 2 and 4, respectively. The coefficients of pathogenic determination (R^2) were 22.01 % and 15.49 % respectively with THL16 as sole contributor of all pathogenic alleles and the total coefficient of determination (R^2) was 33.24 %. Other phenotypes such as mating type and colony color were also mapped (Table 8 and Figure 15).

Moreover, QTL analyses of two phenotypes, mating type and white colony color (buff- colored colony) were also identified in this population. Mating type gene was located on chromosome 7 closed-linked to Pyms9 marker with LOD score of 11.89 and the coefficient of determination was 79.13 %. Moreover, buff-colored colony appeared as transgressive segregation since it could be mapped on chromosome 2 closely-linked to Pyms305 marker. This QTL had a LOD score of 7.99 and the coefficient of determination was 30.31 % (Table 8 and Figure 15).

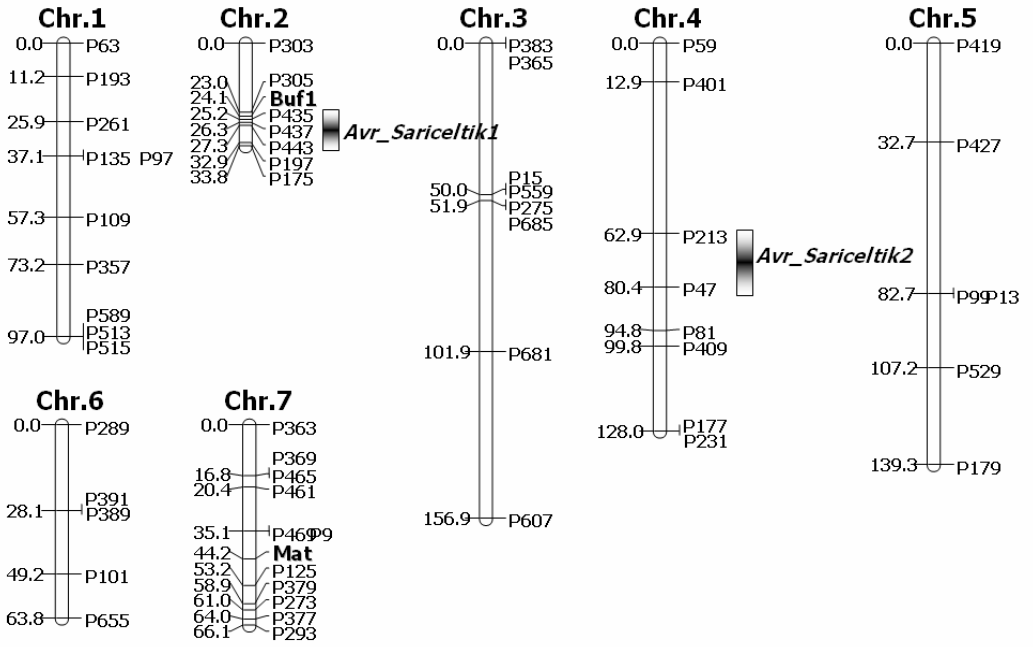

Figure 15 QTL regions of avirulence characteristic toward Sariceltik rice variety (*Avr_Sariceltik*) of blast pathogen from THL1000xTHL16 cross.

Table 8 Summary of the loci identified for pathogenicity towards Sariceltik rice variety by QTL analysis in THL1000xTHL16 cross.

Traits	Chromo-some	Peak QTL	LOD score	R2		Contributor
				Individual	Total	
Avr_Sariceltik	2	Pyms435-Pyms443	3.32	22.01	33.24	THL16
	4	Pyms213-Pyms47	3.27	15.49		THL16
Mating type	7	Pyms9-Pyms215	11.89	79.13		THL1000 (Mat1-1)
buff colored colony	2	Pyms305-Pyms435	7.99	30.31		-

B1-2xTH16 cross

Avirulence characteristic on JHN rice: The numbers, genomic locations and effects of QTL were resolved using nQTL software with a significant LOD threshold. The genomic location, LOD score of detected QTL and phenotypic variance explained (PVE) are shown in Table 9 and Figure 16. The QTL analysis of virulence and avirulence characteristics specific to seedling stage of JHN was a significant locus. This mapped QTL region was designed as *Avr-JHN(lb)* as gene controlling avirulence characteristic on leaf blast symptoms at seedling stage. It was significant to both Simple Interval Mapping and Simple Composite Interval Mapping analysis. The Pyms305 and Pyms437 located on chromosome 2 flanked a QTL with a LOD score and PVE values of 4.862 and 14.071, respectively. At flowering stage, the QTL analysis of virulence and avirulence characteristics of JHN rice showed a significant locus, too. It was designed as *Avr-JHN(pb)* for gene controlling avirulence characteristic on panicle at flowering stage. The linked flanking markers to this QTL locus were the same markers, Pyms305 and Pyms437, on chromosome 2 and it showed LOD score and PVE as 9.196 and 25.996, respectively. The simple interval mapping was performed in both stages of rice and it was showntht, the location of these QTLs could be located between markers Pyms305 and Pyms437. These two markers 9.4 cM genetic distance between them and QTLs were closely linked to Pyms437 than Pyms305 from this analysis in both leaf and panicle blast evaluations.

Avirulence characteristic on C101PKT, C104PKT and C105TTP rice: The 3 near isogenic lines, C101PKT, C104PKT and C105TTP, derived from several back crosses to CO39 rice variety were used in the identification of avirulence gene corresponding to individual varieties.

The C101PKT rice variety: the QTL analysis based on Simple Interval Mapping (SIM) and Simple Composite Interval Mapping (sCIM) analyses with 5 cM of walking speed, 5% of type I error and 1000 permutaion analysis were obtained in this analysis. The thresholds to accept QTL were 10.8 and 35.1 for SIM and sCIM respectively. The avirulence gene corresponding to this variety was mapped on

chromosome 6 between markers Pyme289 and Pyms267. This QTL was significant at SIM with a score of 38.2 score, but not significant at sCIM (26.5 score). The most tightly linked marker to this avirulence gene was Pyms289. The regression analysis showed PVE of this marker toward avirulence characteristic was 64.053 %, and had additive of 1.212. The contributor of avirulence charactesistic was TH16, while B1-2 was contributor for virulence characteristic (Table 9 and Figure 16). This avirulence gene was designed as *Avr-C101PKT*.

The C104PKT rice variety: the QTL analysis was performed using the same condition as in C101PKT. The thresholds to accept QTL region were 10.6 and 32.9 for SIM and sCIM respectively. The avirulence gene corresponding to this variety was mapped on chromosome 6 between markers Pyme289 and Pyms267. This QTL was significant at SIM with a score of 32.8, but not significant at sCIM with 22.6 score. The most tightly linked marker to this avirulence gene was Pyms289. The regression analysis showed that this marker had a PVE towards avirulence characteristic of 60.632 %, and with an additive effect of 1.192. The contributor of avirulence charactesistic was TH16, while B1-2 was contributor for virulence characteristic as in C101PKT (Table 9 and Figure 16). This avirulence gene was designated as *Avr-C104PKT*.

The C105TTP rice variety: the QTL analysis was performed ausing the same condition as in C101PKT. The thresholds to accept present of QTL were 10.5 and 36.1 for SIM and sCIM respectively. The avirulence gene corresponding to this variety was mapped on chromosome 4 between markers Pyme409 and Pyms177. This QTL was significant at SIM with 37.3 score, and significant at sCIM with 40.8 score. The most tightly linked marker to this avirulence gene was Pyms177. The regression analysis showed that PVE of this marker favored avirulence characteristic of 25.136 %, and with additive effect of 0.706. The contributor of avirulence charactesistic was TH16, while B1-2 was contributor for virulence characteristic in C101PKT (Table 9 and Figure 16). This avirulence gene was designed as *Avr-C105TTP*.

The Azucena rice variety: the QTL analysis was performed using the same condition. The thresholds to detect present of QTL region were 10.4 and 37.8 for SIM

and sCIM respectively. The avirulence gene corresponding to this variety was mapped on chromosome 1 and 2 between markers Pyme87 – Pyme515 and Pyms197 – Pyme175 respectively. The QTL on chromosome1 was significant only at SIM with a score of 22.5. The QTL on chromosome 2 was significant at SIM with a score of 23.9 score, and significant at sCIM with 42.7 score. The most tightly linked marker to this avirulence genes were Pyms515 and Pyms175. The regression analysis showed PVE of this marker towards avirulence characteristic was 25.136 %, and it showed -3.2 and -2.08 additive effect. The contributor of avirulence charactesistic was B1-2, while TH16 was contributor for virulence characteristic (Table 9 and Figure 16). This avirulence gene was designated as *Avr-Azucena*.

The C101TTP rice variety: the QTL analysis was performed using the same condition. The thresholds to accept QTL region were 9.9 and 37.3 for SIM and sCIM respectively. The avirulence gene corresponding to this variety was mapped on chromosome 1 between markers Pyme87 and Pyms515. This QTL was significant only at SIM with a score of 11.1. The most tightly linked marker to this avirulence gene was Pyms515. The regression analysis showed that the PVE of this marker towards avirulence characteristic of 25.136 % and with -1.56 additive effect. The contributor of avirulence charactesistic was B1-2, while Th16 was contributor for virulence characteristic (Table 9 and Figure 16). This avirulence gene was designated as *Avr-C101TTP*.

The CNT1 rice variety: The same QTL analysis condition was performed. The thresholds to accept present of QTL were 10.8 and 39.4 for SIM and sCIM respectively. The avirulence gene corresponding to this variety was mapped on chromosome 4 between markers Pyme409 and Pyms177. This QTL was significant only at SIM and with a score of 33.4. The most tightly linked marker to this avirulence gene was Pyms177. The regression analysis showed that the PVE of this marker towards avirulence characteristic was 25.136 % and had 2.27 additive effects. The contributor of avirulence charactesistic was TH16, while B1-2 was contributor for virulence characteristic (Table 9 and Figure 16). This avirulence gene was designated as *Avr-CNT1*.

The IR57514 rice variety: The same condition for QTL analysis was performed. The thresholds to accept present of QTL were 10.4 and 38.5 for SIM and sCIM respectively. The avirulence gene corresponding to this variety was mapped on chromosome 7 between markers Pyme67 and Pyms461. This QTL was significant with a score of 20.8 for SIM, and significant as well for sCIM with 41.8 score. The most tightly linked marker to this avirulence gene was Pyms461. Regression analysis showed that the PVE of this marker towards avirulence characteristic was 25.136 %, and it showed -1.23 in additive effects. The contributor of avirulence charactesistic was B1-2, while TH16 was contributor for virulence characteristic (Table 9 and Figure 16). This avirulence gene was designated as *Avr-IR57514*.

The IR64 rice variety: The same condition for QTL analysis was performed. The thresholds to accept present of QTL were 10.3 and 41.5 for SIM and sCIM respectively. The avirulence gene corresponding to this variety was mapped on chromosome 4 between markers Pyme409 and Pyms177. This QTL was significant only at SIM with 12.3 score. The most tightly linked marker to this avirulence gene was Pyms177. The regression analysis showed that the PVE of this marker towards avirulence characteristic was 25.136 %, with 1.3 additive effects. The contributor of avirulence charactesistic was TH16, while B1-2 was contributor for virulence characteristic (Table 9 and Figure 16). This avirulence gene was designated as *Avr-IR64*.

Mating type characteristic: The mating type characteristic was mapped on chromosome 7. Linked-markers Pyms7 and Pyms379 flanking this trait showed LOD score and PVE of 29.942 and 76.376 %, respectively. This characteristic was used as one of the markers in linkage map construction and was added to chromosome 7 between Pyms7 and Pyms379 (Figure 16). The genetic control for mating type characteristic was found in the same position in previous report (Kaye *et al*. 2003).

Table 9 Segregation of virulence/avirulence characteristic for leaf and panicle blast assay on JHN rice variety and 7 rice varieties and mating type segregation of progenies derived from the cross between B1-2 and TH16 isolates.

Varieties	Trait	Blast isolates		Ratio Vir:Avr	QTL name	Chr.	Flanking marker	LOD score	PVE (%)
		B1-2	TH16						
JHN	Leaf blast	Vir	Avr	1 : 1 ^{ns}	*Avr_JHN(lb)*	2	P303-P437	4.86	14.1
	Panicle blast	Vir	Avr	1 : 1 ^{ns}	*Avr_JHN(pb)*	2	P303-P437	9.19	26.0
C101PKT	Leaf blast	Vir	Avr	1 : 1 ^{ns}	*Avr_C101PKT*	6	P289-P267	8.40	64.1
C104PKT	Leaf blast	Vir	Avr	1 : 1 ^{ns}	*Avr_C104PKT*	6	P289-P267	7.22	60.1
C105TTP	Leaf blast	Vir	Avr	1 : 1 **	*Avr_C105TTP*	4	P409-P177	8.21	25.1
Azucena	Leaf blast	Vir	Vir	-	*Avr_Azucena*	1	P87-P515	4.95	22.0
						2	P197-P175	5.26	
C101TTP	Leaf blast	Vir	Vir	-	*Avr_C101TTP*	1	P87-P515	2.44	16.0
CNT1	Leaf blast	Vir	Avr	1 : 1 ^{ns}	*Avr_CNT1*	4	P409-P177	7.35	32.0
IR57514	Leaf blast	Avr	Avr	-	*Avr_IR57514*	7	P67-P461	4.58	25.2
IR64	Leaf blast	Avr	Avr	-	*Avr_IR64*	4	P409-P177	2.71	17.2
	Mating type	Mat1-1	Mat1-2	1 : 1 ^{ns}	*Mat*	7	*Mat*	29.94	76.4

Note Avr = avirulence reaction, Vir = virulence reaction.

^{ns} = non significant, ** = significant

Chr. = chromosome

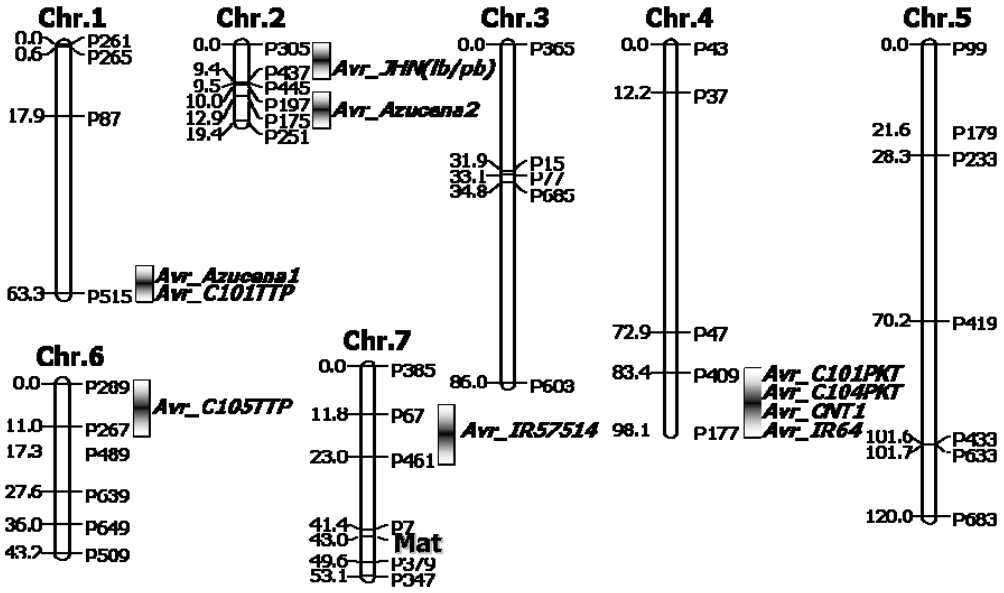

Figure 16 Seven linkage maps of 140 progenies derived from B1-2xTH16 cross. The location of QTL controlling avirulence characteristic toward JHN rice variety for leaf blast, *Avr_JHN(lb)*, avirulence characteristic on JHN for panicle blast, *Avr_JHN(pb)*, *Avr_101TTP*, *Avr_C101PKT*, *Avr_C104PKT*, *Avr_C105TTP*, *Avr_Azucena*, *Avr_CNT*, *Avr_IR64* and mating type (*Mat*) characteristic were shown in the linkage map.

V. Developing new microsatellite markes and narrow down avirulence locus

The mapping population derived from B1-2/TH16 mapped the avirulence locus specific to resistance gene in JHN rice. This mapping population has 304 progenies and additional 140 progenies were used in fine mapping of this location. The genome sequence database of *M. grisea* is available in the website. The location of flanking markers to *Avr* locus was located in the physical map and the DNA sequence in this region was used to find new micrisatellite markers. These markers were used to narrow down the QTL region.

Narrow down QTL region

The sequence of each marker was located on the physical map of this blast pathogen using the Basic Local Alignments Search Tools, BLAST, in NCBI website (http://www. ncbi. nlm. nih. gov/ genome/ seq/ BlastGen/ BlastGen. cgi? Taxid = 148305). The chromosome 2 of *M. grisea* is composed of 3 super-contigs which spanned 6734.0 kb. The locations of Pyms305 and Pyms437 on physical map are on chromosome 2 at 961.4 kb and 2138.5 kb, respectively. The sequence between flanking markers was taken from this database and the locations of microsatellite repeats were scaned. Specific markers to each microsatellite repeat were developed by using Primer 3 software. A total 23 microsatellite markers were developed and synthesized in this sequence, and were namely as *M. grisea* microsatellite repeat marker (MR). The 11 MR markers were mapped in the QTL region where 9 and 3 MR markers were added at the start and end of QTL region, respectively (Table 10 and Figure 17). These markers were tested for polymorphism on parental isolates, B1-2 and TH16. The condition for PCR performed was the same as the condition used in Pyms marker. The data showed that the 11 new MR markers were polymorphic between parental isolates.

To narrow down the QTL region of *Avr_JHN*, the mapping population was increased from 140 to 286 progenies. The high throughput techniques in polyacrylamide gel electrophoresis were applied. The first technique was a sample loading in 3 layers on the same gel. A total of 288 PCR product can be load in a gel. The second technique was a quick strainig technique. This could save time to perform the silver strain which used only 23 minutes. A total of 5 markers were performed in this mapping population namely Pyms305, Pyms437, MR4, MR14 and MR18.

Table 10 List of microsatellite markers, class of repeat, number of repeat, length, left primer sequence, right primer sequence and location on physical map on chromosome 2 of *M. grisea*

Marker name	Class of repeat (Motif)	No.of Repeats	Left primer	Right primer	Physical map position (kb)
			Start of chromosome		-
MR01	TATTA	3	CAACACCGTTTAGGAATCTC	ACAGAATTTAGGTCACCACG	286.1
MR02	AG	6	CGAATACTTTATCGCAATCC	AGTCAGTCATCTCCCACAAC	387.2
MR03	TGA	7	CAGCTTCCGGGTTATCCAT	GCGTGCTTGTACCACAAGGT	486.6
MR04	TTG	5	TAGGTTTCGGAATGTAATCG	ACGACGTCACTATGCTTTCT	489.4
MR05	CAG	6	CCGTTCTGATGATACACAAG	GGGATACAGTGCTAGGTGG	573.9
MR06	CTGT	8	ATGGATGCTTCTGCTGCTG	GGTCGATCCGGATAACAAGA	596.3
MR07	TTCT	6	AAATACGAAGGGTCAGGACT	CAAGTCAGAAGCAACTGTCA	691.3
MR08	ACAC	7	TTGGCTTTAATCCGTGAACC	GACGGTGAGGGTTGCTAGTC	709.4
MR09	TTTTC	3	ATTCCTTCTCCATAGGGTGT	GCATGGTCCAAACATAAGAT	739.4
Pyms303			TGCATGAAGCTGATTTGCTC	TTTGACTCTCGCTCCCTCTC	865.1
Pyms305			CGTGCCAGAAAGACCTGAAGC	CAGGGGATGAACTCCGATGG	961.4
			Gap start		963.2
			Gap end		1163.2
MR10	AGT	10	GTTCCATCAGGTGACCATCA	CCTTGTTTTCCCCCTGTGTA	1274.9
MR11	ACAC	13	AGACCTTCATGGCGACTGAG	AACGCGTATCGCTGGTAACT	1347.7
MR12	AGT	7	TGTCCATAGGTGAGGTGTGC	CCTTGTTTTCCCCCTGTGTA	1423.1
MR13	TC	15	GGCAGGTAGACACGACCAAG	ACTTCCGGATGAATCACCAA	1816.3
MR14	CA	11	ACCGTACTCGCAGTGTTTCA	AGACGGACATTTGGCATAGG	1848.9
MR15	GT	12	CCGTGCCTAAGACAGGTAGG	GGCGGATACCAGACCTGTAA	1895.4
MR16	GA	11	CGATCACGACCTTGGACAC	CCTTCGGTGAGTTGGCTTAG	1922.9
MR17	TC	30	CCAGTTGATTGCTCCACGAT	GGGGCTCTGAACAAAAGAGA	1952.0
MR18	ACAC	5	GCATGCAAGACCCTTTTCTT	AGTCGCGGAAGTAGTGGAAA	2034.5
MR19	CA	11	GCATGCAAGACCCTTTTCTT	AGTCGCGGAAGTAGTGGAAA	2034.6
MR20	TGTG	7	AAGGAAGGGCTGAACTTTCC	GGAAAATTCACGAGGATGGA	2050.2
Pyms437			GCCGCAACAATGACTTAACA	CCGCGGTAGGTAAATGAGAG	2138.5
MR21	TG	10	CGTAGAATTTCGCCCCTGTA	TACCAATGGACCCGATTGAC	2229.4
MR22	CTGT	6	CCGATCTGAGTTCGAGCAGT	AGCTTTGAAGCTTGGAGCAA	2348.8
MR23	TG	20	TGGACGAGACCAGTGAATGA	GCTGGACCCTGGTTTTGAT	2385.9
Pyms445			TGATGCTTTGCGTGTCTTTC	GGTATGCAACCCAATCCTTG	2629.5
Pyms197			GGCAGTGTTGGGAAGTCAGT	CACAGCTCATGAAGGCAAAG	3130.0
Pyms251			CGTTCCGTATTGTAAGCCGTA	CTGGACAACGAATGCTTGAA	4647.5
Pyms175			GTTTGACAGACACGCGAGAA	CGCTCGAGTCTACAGCACAG	4665.5
			Gap start		4865.0
			Gap end		5065.0
			End of chromosome		6734.0

The genotyping data of 5 microsatellite markers on 286 mapping population were analyzed to identify the location of genetic material controlling avirulence charactersistic for leaf and panicle blast evaluation in JHN. In avirulence charactersistic corresponding to leaf blast, MR18 marker showed the highest value of PVE (47.75 %). This PVE value was higher than Pyms437 that was identified as a tightly linked marker in previous QTL analysis. The same result in avirulence charactersistic corresponding to panicle blast, MR18 marker showed highest value of PVE (63.17 %). This PVE value was higher than Pyms437 identified as a tightly linked marker in previous QTL analysis (Table 11). This result demonstrated that the genetic material controlling avirulence charactersistic for leaf and panicle blast evaluation in JHN could be located between markers MR14 on 1848.9 kb and MR18 on 2034.5 kb in which MR18 marker were tightly linked to these traits (Figure 17).

Table 11 The phenotypic value explaned by 5 microsatellite markes of avirulence charactersistic on leaf and panicle blast evaluation of 288 mapping population derived from B1-2xTH16 cross.

Triats	**MR4**	**Pyms305**	**MR14**	**MR18**	**Pyms437**
Leaf blast	5.06	4.78	21.53	47.75	24.11
Panicle blast	11.74	15.23	32.14	63.17	29.51

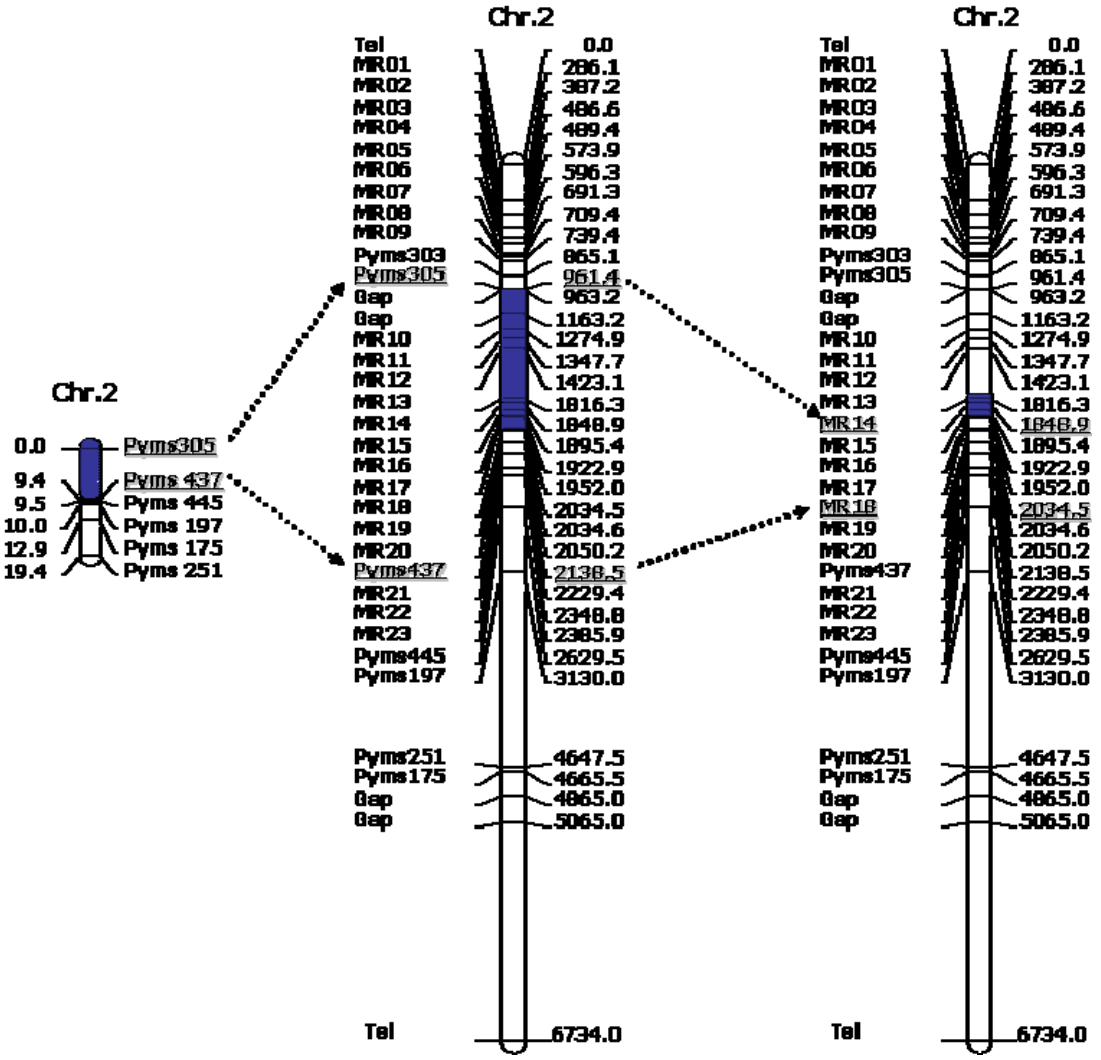

Figure 17 Integration between genetic map (left) and physical map (right), and location (kb) of 'Pyms' and 'MR' microsatellite markers on chromosome 2 of *M. grisea*. Blue box indicated to QTL gerion of *Avr*_JHN after narrow down (right).

Gene prediction in target region

The available data of DNA sequence of *M. grisea* in NCBI hompage could facilitate prediction of candidate genes in this region. The DNA in chromosome 2 of *M. grisea* could predict 1963 genes. The located of *Avr*_JHN locating on chromosome 2 between markers MR14 and MR18 spanned 185.6 kb. This region carried 90 predicted genes. These genes can be classified to 4 conserved proteins, 64 hypothetical proteins and 22 predicted proteins. There is 1 gene, MGG_02393, predicted as Cutinase enzyme activity that functions in extracellular region, but the rest were predicted to act intracellularly.

DISCUSSION

I. Development of mapping population

The sexual compatibility in the blast fungus is determined by a single gene with two allelic forms, *Mat1-1* and *Mat1-2*. The sexual stage of *M. grisea* can be induced under laboratory condition, and the new genetic recombination can be produced by crossing between opposite mating type isolates (Hebert, 1971). In this study, the 2 crossing methods, ascus and ascospore culturing were applied to generate new genetic recombinations. The perithecia bulbs were raised from the confluent zones of two colonies. Inside there bulbs, there are many acsi, each ascus usually containing 8 ascospores. The individual ascospore is a new genetic recombinace. Since the size of ascus (80 μm) is larger than ascospore (20 μm), working under the stereomicroscope on ascus is easier than ascospore. The ascospore culture method was used to cross *M. grisea* isolates collected from wheat and foxtail mellet. Eighty F_1 progenies were derived from 10 mature asci in which individual ascus contained 8 ascospores (Murakami *et al*., 2003). The same method was used to identify a gene-for gene relationship underlaying species-specific parasitism of Avena/ Triticum isolates of *Magnaoporthe grisea* on wheat cultivar (Takabayashi *et al*., 2002). On the other hand, the ascus culturing method was used to establish a new cross of the rice blast fungus derived from Japanese differential strain Ina168 and hermaphroditic rice pathogen Guy11 (Fukiya *et al*., 2001). To produce a large population size, we recommend the use of ascus culturing method.

II. Determination of new virulence phenotype arising from genetic recombination

Hypervariation in blast fungus is well known as the pathogen is able to adapt to tackle new released blast resistant cultivars in a short time. An increase in mutation or new genetic recombination could affect the applicability of resistance gene in breeding programme. The knowledge of population structure and pathogen diversity is necessary to develop resistant cultivars. Classification of blast pathogen based on resistance reactions on near isogenic lines has been published previously, but time and

labour are the major pitfalls of this method. Development in molecular biology techniques hastens characterization of the pathogen over decades due to extensive use and precise interpretation (Zeigler, 1998). So far, different kinds of molecular markers have been exploited to resolve the pathogen complexity such as MGR586 , Pot-2 based rep-polymerase chain reaction, amplified fragment length polymorphism, microsatellite repeat marker and avirulence markers (Babujee and Gnanamanickam, 2000; Couch *et al*., 2005; Kaye *et al*., 2003).

The importance of blast disease has been recognized over decades especially in Thailand. Although pathogenicity in response to breeding programmes in Thailand has been investigated with success (Mekwatanakarn *et al*., 2000; Sirithunya *et al*., 2004), fundamental knowledge of fungal diversity is still not understood. The representatives of blast fungus were screened for either disease reactions or with molecular markers. High genetic diversity in Thailand was found especially in the north. Discovery of different mating types in sampling isolates collected from the rice fields allows the presumption of sexual reproduction occurring in the natural habitat in Thailand especially in rice research stations (Sirithunya *et al*., 2007).

Calculated indices of the blast fungus in the north and northeast of Thailand indicates the opportunity of sexual fertilization made by vast and diversification of pathogens mating type. Sexual recombination by crossing two isolates in laboratory confirms the ability of sexual reproduction in northern and north-eastern areas which resemble like those in China where the sexual reproduction has been found in nature (Hayashi *et al*., 1997). In addition to sexual reproduction, asexual reproduction also surges the genetic diversity of pathogens especially with *M. grisea* that could have heterokaryosis.

A sexual recombination between different host infected isolates often produces some abnormal recombinants such as white mycelium genotype. This genotype is wildly known as *buf* mutant genotype. The *buf* locus corresponds to a lack of melanin synthesis resulting in a white colony. The *buf* mutant genotype normally fails to penetrate host plant due to the weakening of appressorium penetration into the host

cell wall (Farman, 2002). In our study, up to 17% of haploid progenies derived from the cross THL1000 and THL16 were buff-colored colonies.

Study of the genetic structure of Thai blast fungus populations in Thailand, revealed higher genetic relationship between rice-infected isolates with barley-infected isolates compered to wildrice-or weed-infected isolates (Sirithunya *et al*., 2007). Although crossing between two isolates from different hosts may be a rare event, but a possibility in nature. A transgressive segregation of avirulence can arise from the genetic recombination between two isolates. In our studies, the population derived from wildrice-infected isolate THL1000 and rice-infected isolate THL16 showed less virulence toward rice hosts. The population derived from rice- infected isolates, B1-2 and barley-infected isolates, TH16 showed higher virulence toward rice hosts. It might be noted that virulence arose from complementary *Avr* gene action.

III. Linkage map construction of blast pathogen population using molecular markers

We used microsatellite repeat distributed throughtout each chromosome of blast pathogen to construct the linkage map of two haploid populations. It showed 54.7 and 57.3 % of markers showed polymorphism between THL1000 and THL16 and B1-2 and TH16 respectively. These indicated that blast pathogen used in this study showed about 50 percentages of genetice background differences. We used microsatellite markers to screen for polymorphic markers between parental isolates of each cross. It showed 43, 59 and 22% of polymorphic markers in cross Guy11x2539, CH63xTH16 and 95-23-4ax94-64-1b respectively. The close relates genetic background isolates had lower polymorphism markers than distant genetic background (Kaye *et al*., 2003).

Two linkage maps were constructed from two crosses of *M. grisea* in the present study. Based on location of markers on reference population, Guy11x2539, 7-linkage maps were constructed for each cross. Compaired to the reference map, our map showed lacking of molecular marker on 14 telomeric regions of 7 chromosomes. This may be due to avirulence gene identification in these mapping populations. It is

worth noting that many *Avr* genes in *M. grisea* having key roles in pathogen–host interactions were located in the telomeric regions (Orbach *et al*., 2000; Valent and Chumley, 1991). However, to generate the complete genetic map, we can find more gentic information in avirulence gene identification than using trail analysis.

A high-density genetic map of the rice blast fungus *M. grisea* from Guy11x2539 cross was constructed by adding 87 cosmid-derived RFLP markers to (Farman and Leong, 1995) and the integration of this map with that of Sweigard *et al*. (1995). The new map consists of 203 markers representing 132 independently segregating loci and spans approximately 900 cM with an average resolution of 4.5 cM. Mapping of 33 cosmid probes from the genetic map generated by Sweigard *et al*., (1995) has allowed the integration of two *M. grisea* maps. The integrated map showed that the linear order of markers along all seven chromosomes in both maps is in good agreement. The molecular map serves to significantly increase the number of phenotypic and molecular markers that are available to fungal researchers (Nitta *et al*., 1997). Since, we have 2 mapping population and some of markers in each are the same, the 2 linkage maps can be intregrate together. It showed total genetic lengh of 832 cM of with an average resolution of 10.5 cM per marker. The integrated map to the existing genetic map of *M. grisea* resulted in a clearer picture of genome organization and evolution in *M. grisea*. In particular, mapping of markers enabled the documentation of several interesting genomic rearrangements that would otherwise have additionol genetic information (Nitta *et al*., 1997).

IV. Mapping of avirulence genes in two developed blast pathogen populations

Discovery of the perfect stage of blast fungus in *in vitro* enables possibility of genetic analysis. By using these spacific isolates, more than 40 *Avr* genes have been identified in many laboratories. Use of numerous molecular markers developed for *M. grisea* has greatly facilitated the mapping of the avirulence gene to a specific chromosome region. The three genetically independent *Avr* genes, *AvrPik*, *AvrPiz*, and *Avr-Pizt*, were identified using random amplified polymorphic DNA (RAPD) analysis, and a partial linkage map was constructed with this type of markers closely linked to

the *Avr* genes (Luo *et al*., 2004). The combining of two molecular markers RAPD and restriction fragment length polymorphism (RFLP) markers were used to identify and construct a partial genetic map of *Avr-Hattan 3*. This avirulence gene corresponding to *Pi-k* locus in Hattan 3 rice variety (Yasuda *et al*., 2005). Since the genome sequence of *M. grisea* is available the microsatellite markers have been developed and widely used in genome mapping. The Microsatellite markers were required to determine the chromosome position of the avirulence gene. Use of the microsatellite markers analysis can facilitate the identification of markers linked more closely to the trait of interest. Most importantly, it provides information on chromosome location without the expense of creating a full genetic map (Chen *et al*., 2006; Dutech *et al*., 2007; Hutamekalin *et al*., 2001; Kaye *et al*., 2003; Santoso *et al*., 2007).

Avirulence genes of blast fungus *Magnaporthe grisea* were studied using Quantitative trait loci (QTL) approach. Two haploid mapping populations derived from THL1000xTHL16 and B1-2xTH16 crosses were developed by artificial crossing in the laboratory. THL1000 was isolated from a wild rice *Oryza rufipogon*. B1-2 was isolated from cultivated rice, *Oryza sativa*, and TH16 was isolated from barley, *Hordium vulgare*. Eighty eight and two hundred eighty eight haploid progenies were successfully developed from THL1000xTHL16 and B1-2xTH16 crosses respectively. These populations were used to study the arising of new virulence pathotype from the genetic recombination and to identify the genomic location of avirulence (*Avr*) gene specific to the resistance gene in 10 rice cultivars such as Sariceltik, Jao Hom Nin (JHN), C101TTP, C101PKT, C104PKT, C105TTP, Azucena, CNT1, IR64 and IR57514. The location of avirulence loci on intregated map showed the cluster of avirulence loci located on chromosome 2 and 4 (Figure 18). Mating type characteristic had been identified as single gene control located on chromosome 7 of *M. grisea* and it was mapped in the same loci (Kaye *et al*., 2003).

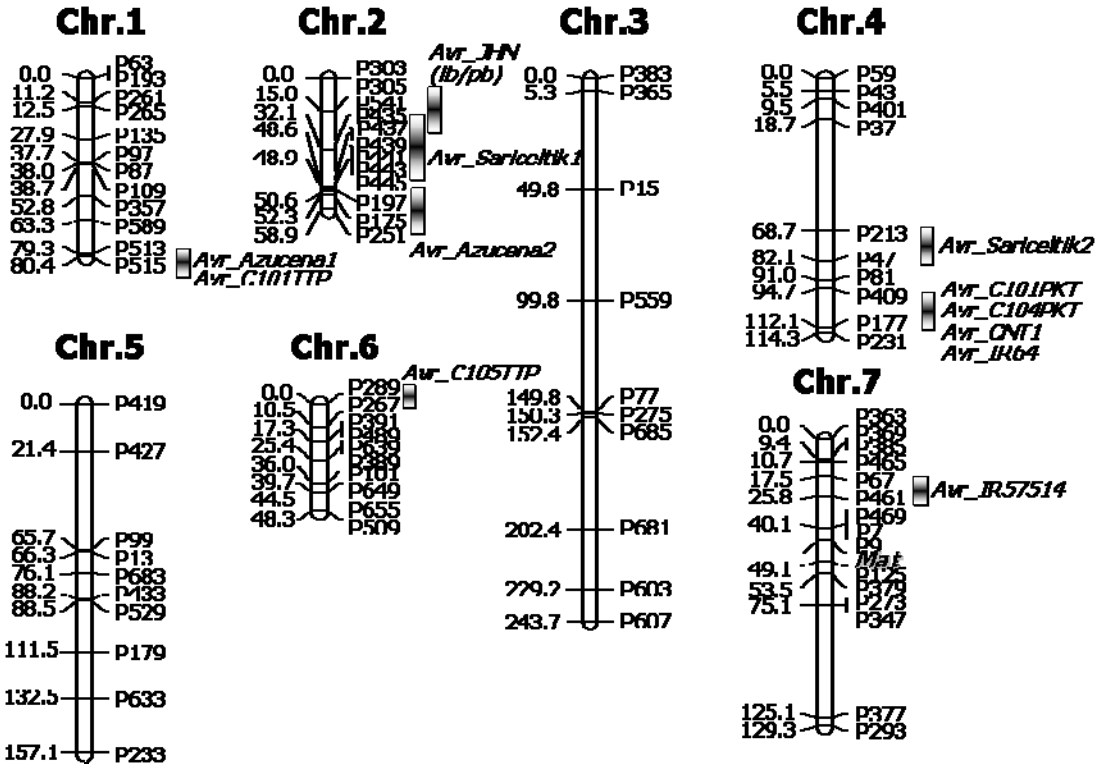

Figure 18 Integrated map of THL1000xTHL16 and B1-2xTH16 crosses. The location of genetic material controlling avirulence characteristic toward JHN rice variety for leaf blast, *Avr_JHN(lb)*, avirulence characteristic on JHN rice variety for panicle blast, *Avr_JHN(pb)*, *Avr_Sariceltik*, *Avr_101TTP*, *Avr_C101PKT*, *Avr_C104PKT*, *Avr_C105TTP*, *Avr_Azucena*, *Avr_CNT*, *Avr_IR64* and mating type (*Mat*) characteristic are shown beside this linkage map.

Recent progress in genetic mapping and molecular characterization of *Avr* genes indicate that the chromosomal position or organization is relative to their variability. Interestingly, five known *Avr* genes of *M. grisea*, *Avr-Pita*, *Avr1-TSUY*, *Avr1-Ku86*, *Avr1-MedNoi*, and *PWL1* were also mapped at the respective telomeres (Kang *et al*., 1995; Sweigard *et al*., 1995; Valent and Chumley, 1991). The presence of these genes in highly dynamic chromosome ends may provide a selective advantage to *M. grisea* by allowing them to rapidly adapt to new *R* genes in host plants (Orbach *et al*., 2000; Valent and Chumley, 1991). In this study, some of *Avr* loci could be hidden on the telomerice region because our genetic maps lacked of telomeric

markers. It should be added to these markers on the maps to get more information of avirulence loci.

Transgressive segregation for virulence and avirulence characteristics were observed in rice cultivars Azucena, C101TTP, IR64 and IR57514. Two *Avr* loci that are specific to the resistance genes in Sariceltik were mapped on *M. grisea* chromosomes 2 and 4 in the THL1000xTHL16 haploid population. THL1000 contributed both alleles for the avirulence characteristic. Ten Avr loci that are specific to the resistance genes in rice cultivars JHN, C101TTP, C101PKT, C104PKT, C105TTP, Azucena, CNT1, IR64 and IR57514 were mapped on chromosomes 1, 2, 4, 6 and 7 in the B1-2xTH16 haploid population. THL16 contributed *Avr* alleles specific to the resistance genes carried by JHN, C101PKT, C104PKT, C105TTP, CNT1, IR64. B1-2 contributed *Avr* alleles specific to the resistance genes carried by Azucena, C101TTP and IR57514. Transgressive segregation of virulence or avirulence isolates can be formed from an interaction between some genetic loci on a new genetic back ground of a new genetic recombination (Dean *et al*., 2005; Farman, 2002; Valent and Chumley, 1991; Valent *et al*., 1991).

The Jao Hom Nin rice variety has show broad spectrum resistance to Thai blast pathogen population. The position of resistance gene has been identify (Noenplab *et al*., 2006). These resistant genes are located on chromosome 1 and 11. The resistant gene on chromosome 11 showed major effect while gene on chromosome 1 showed minor effect. The resistant characteristic in two stage of rice at seedling and flowering stage could be related to the same region on these chromosomes. It could be the same gene that governs both leaf and panicle blast resistance (Noenplab *et al*., 2006; Sirithunya *et al*., 2004). The inoculation of 346 Thai blast isolates choosen from all rice growing areas in Thailand, we found only 2 isolates fully pathogenic on this rice variety. Moreover, the 124 blast isolates from worldwide were used to inoculate on JHN, and it showed high susceptibility to 7 blast isolates which composed of 2 isolates from Colombia and 5 isolates from China. This result indicated that JHN rice tends to be resistant to Thai blast population, but it tends to be susceptible to blast pathogen from China. Since JHN rice is a mutation line of Chinese rice variety and it may have

genetice background and may be result of coevolution between with pathogen in original rice-pathogen ecosystem.

V. Developing new microsatellite markes and narrow down avirulence locus

Map-based cloning is an efective method to isolate the target genes, especially when no expression products and functional information of target genes are available (Peter *et al*., 2003). As for fungal pathogens, the diversity and lack of sequence similarity or conservation in the fungal *Avr* gene products were demonstrated by comparison of *Avr* genes identifed in *M. grisea*, *Cladosporium fulvum* and *Rhynchosporium* (Dean *et al*., 2005). However, this was tediously hampered by the quick identifcation of anchor markers for chromosome walking to the target locus. Fortunately, the emergence of genomic sequences of the fungus pathogen offers powerful tool to increase the density of markers in the genetic map and to assemble the contiguous clones (contig) in the physical map, where a target gene is located (Chen *et al*., 2006; Dean *et al*., 2005; Liu *et al*., 2002; McCouch *et al*., 1994). This has made map-based cloning perform in *M. grisea* much more efficiently. Doubtlessly, construction of sequence-based map of the target gene is a crucial step for map-based cloning.

A total of 242 progeny isolates from CHL346xCHL42 cross were used for establishing a mapping population. Linkage analysis to identify the *AvrPi15* gene on chromosome 6 was carried out by using microsatellite markers to narrow down the chromosomal location of the *AvrPi15* locus, two additional markers, MS6-17 and STS6-6, which were developed based on the sequences of telomeric region 11 (TEL11). The results showed that MS6-17 and STS6-6 were associated with the locus by 3.3 and 0.8 cM, respectively. This data is useful to identify the *AvrPi15* gene using chromosome walking strategy (Ma *et al*., 2006). The *Avr-JHN* locus flanking by Pyms305 and Pyms437 markers that spanned 1,177.5 kp on physical map was narrowed down by adding markers and increasing the population size up to 286 progenies. The new patial map was constructed on this genetic region. The new linked

microsatellite markers, MR14 and MR18, were located within the QTL region of *Avr-JHN*. These flaking markers spanned 185.6 kb.

CONCLUSION

Based the experimental results and discussion of the present study, the conclusion can be drawn as follow:

1. Sexual genetic recombination can be developed in laboratory by growing opposite mating type isolates together under specific controlled conditions.

2. Ascus culture is easiler and more practical method than ascospore culture method in developing large mapping population.

3. New virulence and avirulence genotypes arose from sexual recombination. The examples are the results of two haploid populations developed by crossing two isolates derived from different hosts.

4. Sexual recombination produced by crossing two isolates with diverse genetic relationship such as THL1000 and THL16 generated a high percentage of mutant progenies with lacking of melanin systhesis.

5. We did not find any mutant progeny was found when cross between two pathogenic isolates B1-2 and TH16 were made.

6. New virulence progenies were found from the B1-2xTH16 population. These progenies infected the rice varieties which could not be infected by B1-2 and TH16.

7. Jao Hom Nin (JHN) is a broad spectrum blast resistance variety. In the inoculation experiment with 346 Thai blast isolates, only 2 isolates showed full pathogenicity on this variety. Inoculation experiment with 124 worldwide blast isolates showed that JHN is susceptible to only 7 blast isolates, 2 isolates from Colombia and 5 isolates from China. These results support that JHN possesses the resistance genes against a broad spectrum isolates.

8. Order and location of microsatellite markers in our linakge maps corresponded well to those of the refernce map derived from Guy11x2539.

9. Virulence characteristic seggration ratio of 1:1 in both leaf and panicle blast indicated that avirulence gene conresponding to resistance gene in JHN is controlled by single gene.

10. Avirulence genes corresponding to resistance to leaf and panicle blast is located between Pyms305 and Pyms435 markers on chromosome 2.

11. Two recombinants were discovered in the blast progenies which indicated a tighty linked of *Avr-JHN(lb)* and *Avr-JHN(pb)*.

12. The genomic locations of *Avr* genes were on chromosome 1 for *Avr_Azucena1* and *Avr_C101TTP*; chromosome 2 for *Avr_JHN*, *Avr_Sariceltik* and *Avr_Azucena2*; chromosome 4 for *Avr_Sariceltik2*, *Avr_C101PKT*, *Avr_C104PKT*, *Avr_CNT1* and *Avr_IT64*; chromosome 7 for only *Avr_IR57514*.

13. The *Avr-JHN* locus was narrowed down to 185.6 kb by adding 23 microsatellite markers and increasing the population size to 286 individuals. The 185.6 kb contained 90 predicted genes.

LITERATURE CITED

Agrios, G.N. 1997. **Plant Pathology: Third edition** Academic Press, San Diego, USA.

Alexopolos, C.J., C.W. Mims and M. Blackwell. 1996. **Introductory mycology 4th.** John Wiley and Sons, Inc, New York. 432 p.

Babu, R., S.K. Nair, B.M. Prasanna and H.S. Gupta. 2004. Integrating marker-assisted selection in crop breeding – prospects and challenges. **Current Science** 87: 607-619.

Babujee, L. and S.S. Gnanamanickam. 2000. Molecular tools for characterization of rice blast pathogen (*Magnaporthe grisea*) population and molecular marker-assisted breeding for disease resistance. **Current Science** 78: 248-257.

Baker, B., P. Zambryski, B. Staskawicz and S.P. Dinesh-Kumar. 1997. Signal in plant-microbe interaction. **Scinece** 276: 726-733.

Bechinger, C., K.F. Giebel, M. Schnell, P. Leiderer, H.B. Deising and M. Bastmeyer. 1999. Optical measurements of invasive forces exerted by appressoria of a plant pathogenic fungus. **Science** 285: 1896-1899.

Berger, S. 2002. Jasmonate-related mutants of Arabidopsis as tools for studying stress signaling. **Planta** 214: 497-504.

Bleeker, A.B. and H. Kende. 2000. Ethylene: A gaseous signal molecule in plants. **Annu. Rev. Cell Dev. Biol.** 16: 1-18.

Bohnert, H.U., I. Fudal, W. Dioh, D. Tharreau, J.-L. Notteghem and M.-H. Lebrun. 2004. A putative polyketide synthase/peptide synthetase from *Magnaporthe grisea* signals pathogen attack to resistant rice. **Plant Cell** 16: 2499-2513.

Broman, K.W. 2001. Review of statistical methods for QTL mapping in experimental crosses. **Lab Animal** 30: 44-52.

Brondani, C., R.P.V. Brondani, L.d.R. Garrido and M.E. Ferreira. 2000. Development of microsatellite markers for genetic analysis of *Magnaporthe grisea*. **Genetics and Molecular Biology** 23: 753-762.

Bryan, G.T., K.-S. Wu, L. Farrall, Y. Jia, H.P. Hershey, S.A. McAdams, K.N. Faulk, G.K. Donaldson, R. Tarchini and B. Valent. 2000. A single amino acid difference distinguishes resistant and susceptible alleles of the rice blast resistance gene Pi-ta. **Plant Cell** 12: 2033-2046.

Chao, C.C.T. and A.H. Ellingboe. 1991. Selection for mating competence in *Magnaporthe grisea* pathogenic to rice. **Can. J. Bot.** 69: 2130-2134.

Chen, Q.H., Y.C. Wang and X.B. Zheng. 2006. Genetic analysis and molecular mapping of the avirulence gene *PRE1*, a gene for host-species specificity in the blast fungus *Magnaporthe risea*. **Genome** 49: 873-881.

Couch, B.C., I. Fudal, M.-H. Lebrun, D. Tharreau, B. Valent, P.v. Kim, J.-L. Notteghem and L.M. Kohn. 2005. Origins of host-specific populations of the blast pathogen *Magnaporthe oryzae* in crop domestication with subsequent expansion of pandemic clones on rice and weeds of rice. **Genetics** 170: 613-630.

Crawford, M.S., F.G. Chumiey, C.G. Weaver and B. Valent. 1986. Characterization of the heterokaryotic and vegetative diploid phase of *Magnaporthe grisea*. **Genetics** 114: 1111-1129.

Dangl, J.L. and J.M. McDowell. 2006. Two modes of pathogen recognition by plants. **PNAS**. 103: 8575-8576.

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

Deslandes, L., J. Olivier, N. Peeters, D.X. Feng, M. Khounlotham, C. Boucher, I. Somssich, S. Genin and Y. Marco. 2003. Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. **Proc. Natl. Acad. Sci. USA.** 100: 8024-8029.

Dutech, C., J. Enjalbert, E. Fournier, F. Delmotte, B. Barres, J. Carlier, D. Tharreau and T. Giraud. 2007. Challenges of microsatellite isolation in fungi. **Fungal Genetics and Biology** 44: 933-949.

Ebbole, D.J. 2007. *Magnaporthe* as a model for understanding host-pathogen interactions. **Annu. Rev. Phytopathol.** 45: 437-456.

Farman, M.L. 2002. Meiotic deletion at the *BUF1* locus of the fungus *Magnaporthe grisea* is controlled by interaction with the homologous chromosome. **Genetics** 160: 137-148.

Farman, M.L. and S.A. Leong. 1995. Genetic and physical mapping of telomeres in the rice blast fungus, *Magnaporthe grisea*. **Genetics** 10: 479 - 492.

Farman, M.L., Y. Eto, T. Nakao, Y. Tosa, H. Nakayashiki, S. Mayama and S.A. Leong. 2002. Analysis of the Structure of the *Avr1-CO39* Avirulence Locus in Virulent Rice-Infecting Isolates of *Magnaporthe grisea*. **MPMI**. 15: 6-16.

Flor, H.H. 1946. Genetics of pathogencity in *Melampsora lini*. **J. Agric. Res.** 73: 335-357.

Flor, H.H. 1971. Current status of the gene-for-gene concept. **Annu. Rev. Phytopathol.** 9: 275-296.

Fukiya, S., M. Kodama, H. Kito, T. Sone and F. Tomita. 2001. Establishment of a new cross of the rice blast fungus derived from Japanese differential strain Ina168 and hermaphroditic rice pathogen Guy11. **Biosci. Biotechnol. Biochem.** 65: 1464-1473.

Genovesi, A.D. and D.W. Magill. 1976. Heterokaryosis and parasexuality in *Pyricularia oryzae* Cuvara. **Can. J. Microbiol** 22: 531-536.

Goff, S.A., D. Ricke, T.H. Lan, G. Presting, R. Wang, M. Dunn, J. Glazebrook, A. Sessions, P. Oeller, H. Varma, D. Hadley, D. Hutchison, C. Martin, F. Katagiri, B.M. Lange, T. Moughamer, Y. Xia, P. Budworth, J. Zhong, T. Miguel, U. Paszkowski, S. Zhang, M. Colbert, W.L. Sun, L. Chen, B. Cooper, S. Park, T.C. Wood, L. Mao, P. Quail, R. Wing, R. Dean, Y. Yu, A. Zharkikh, R. Shen, S. Sahasrabudhe, A. Thomas, R. Cannings, A. Gutin, D. Pruss, J. Reid, S. Tavtigian, J. Mitchell, G. Eldredge, T. Scholl, R.M. Miller, S. Bhatnagar, N. Adey, T. Rubano, N. Tusneem, R. Robinson, J. Feldhaus, T. Macalma, A. Oliphant and S. Briggs. 2002. A draft sequence of the rice genome (*Oryza sativa* L. ssp. japonica). **Science** 5: 92-100.

Hammond-Kosack, K. and J.D.G. Jones. 2000. Chapter21: Responses to plant pathogens, p. 1102-1203, *In* B. B. Buchanan, W. Gruissem and R. L. Jones, eds. **Boichemistry & Molecular Biology of Plant**. ASPP, Rockville, Maryland, USA.

Hanlin, R.T. 1998. **Illustraed gernea of ascomycetes: Volume II** APS Press, St. Paul, Minnesota, USA.

Hayashi, N., C.Y. Li, J.L. Li and H. Naito. 1997. In vitro production on rice plants of perithecia of *Magnaporthe grisea* from Yunnan, China. **Mycol. Res.** 10: 1308-1310.

Heath, M.C. 1981. A generalized concept of host-parasite specificity. **Phytopathology** 71: 1121-1123.

Heath, M.C. 1991. The role of gene-for-gene interactions in the determination of host species specificity. **Phytopathology** 81: 127-130.

Hebert, T.T. 1971. The perfect stage of *Pyricularia grisea*. **Phytopathology** 61: 83-87.

Howard, R.J. and B. Valent. 1996. Breaking and entering: host penetration by the fungal rice blast pathogen *Magnaporthe grisea*. **Annual Review of Microbiology** 50: 491-512.

Hutamekalin, P., T. Veerapraditsin, S. Pimpisitthavorn, T. Sriwongchai and P. Sirithunya. 2001. AFLP analysis of blast pathogen diversity of Thailand, p. 42-48, *In* S. Jamratlertluk and V. Jindamanee, eds. **Functional Genomics of rice and seed biotechnology**, Queen Sirikit National Convention Center. Bangkok, Thailand.

IRRI. 2002. **Standard evaluation system for rice (SES).** International Rice Research Institute, Philippine. 56 p.

Jia, Y., M.A. Sean, B.T. Gregory, H.P. Howard and B. Valent. 2000. Direct interaction of resistance gene and avirulence gene products confers rice blast ressitance. **The EMBO Journal** 19: 4004-4014.

Kang, S., J.A. Sweigard and B. Valent. 1995. The PWL host specificity gene family in the blast fungus *Magnaporthe grisea*. **Mol. Plant-Microbe Interact.** 8: 939-948.

Kang, S., M.H. Lebrun, L. Farrall and B. Valent. 2001. Gain of Virulence Caused by Insertion of a *Pot3* Transposon in a *Magnaporthe grisea* Avirulence Gene. **MPMI**. 14: 671-674.

Kato, H. 2001. Rice blast disease. **The Royal Society of Chemistry** 10: 23 - 25.

Kato, H., M. Yamamoto, T. Yamaguchi-Ozaki, H. Kadouchi, Y. Iwamoto, H. Nakayashiki, Y. Tosa, S. Mayama and N. Mori. 2000. Pathogenicity, mating ability and DNA restriction fragment length polymorphisms of Pyricularia populations isolated from Gramineae, Bambusideae and Zingiberaceae plants. . **J. Gen. Plant Pathol.** 66: 30-47.

Kawchuk, L.M., J. Hachey, D.R. Lunch, F. Kulcsar, G. Van Rooyen, D.R. Waterer, A. Robertson, E. Kokko, R. Byers, R.J. Howard, R. Fischer and D. Prufer. 2001. Tomato Ve disease resistance genes encode cell surface-like receptors. **Proc. Natl. Acad. Sci. USA**. 98: 6511-6515.

Kaye, C., J. Milazzo, S. Rozenfeld, M.-H. Lebrun and D. Tharreau. 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.

Kiyosawa, S. 1966. Studies on inheritance of resistance of rice varieties to blast, 3. Inheritance of resistance of a rice variety Pi No. I to the blast fungus. **Jpn. J. Breed** 16: 243-250.

Lander, E.S., P. Green, J. Abrahamso, A. Barlow, M.J. Daly, S.E. Lincoln and N. L. 1987. Mapmaker: an interactive computer package for construcing primary genetics linkage map of experimental and natural population. **Genomics** 1: 174-181.

Lauge, R. and P.J.G.M. Dewit. 1998. Fungal avirulence gene: structure and posible functions. **Fungal Genetics and Biology** 24: 285-297.

Leach, J.E. and F.F. White. 1996. Bacterial avirulences. **Annu. Rev. of Phytopathol** 34: 153-179.

Leung, H., R.J. Nelson and J.E. Leach. 1993. Population structure of plant pathogenic fungi. **Advance in Plant Pathology** 10: 157-205.

Leung, H., S. Borromeo, M.A. Bernardo and J.L. Notteghem. 1988. Genetic analysis of virulence in the rice blast fungus *Magnaporthe grisea*. **Phytopathology** 78: 1227-1233.

Lin, F., S. Chen, Z. Que, L. Wang, X. Liu and Q. Pan. 2007. The Blast Resistance Gene Pi37 Encodes a Nucleotide Binding Site–Leucine-Rich Repeat Protein and Is a Member of a Resistance Gene Cluster on Rice Chromosome 1. **Genetics** 177: 1871-1880.

Lisker, N., J. Katan and Y. Henis. 1975. Sequential production of polygalacturonase, cellulase and pectine lyase by *Rhizoctonia solani*. **Can. J. Microbiol.** 21: 1298-1306.

Liu, G., G. Lu, L. Zeng and G.L. Wang. 2002. Two broad-spectrum blast resistance genes, *Pi9(t)* and *Pi2(t)*, are physically linked on rice chromosome 6. **Mol Genet Genomics** 267: 472-480.

Liu, X., F. Lin, L. Wang and Q. Pan. 2007. The in silico map-based cloning of *Pi36*, a rice coiled-coil–nucleotide-binding site–leucine-rich repeat gene that confers race-specific resistance to the blast fungus. **Genetics** 176: 2541 - 2549.

Ludere, R. and M.A.J. Jooste. 2001. Avirulence proteins of plant pathogens: determinants of victory and defeat. **Molecular Plant Pathology** 2: 355-364.

Luderer, R., F.L.W. Takken, P.J.G.M. De Wit and M.H.A.J. Joosten. 2002. Cladosporium fulvum overcomes *Cf-2*-mediated resistance by producing truncated *AVR2* elicitor proteins. **Molecular Microbiology** 45: 875 - 884.

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

Ma, J.H., L. Wang, S.J. Feng, F. Lin, Y. Xiao and Q.H. Pan. 2006. Identification and fine mapping of AvrPi15, a novel avirulence gene of *Magnaporthe grisea*. **Theor. Appl. Genet.** 113: 875-883.

Manly, K.F. and J.M. Olson. 1999. Overview of QTL mapping software and introduction to map manager QT. **Mammalian Genome** 10: 327-334.

McCouch, S.R., R.J. Nelson, J. Tohme and R.S. Zeigler. 1994. Mapping of blast resistance genes in rice, p. 167 - 186, *In* R. S. Zeigler, S. A. Leong and P. S. Teng, eds. **Rice Blast disease**. CAB International, Cambridge, UK.

McDowell, J.M. and B.J. Woffenden. 2003. Plant disease resistance genes: recent insights and potential applications. **Trends in Biotechnology** 21: 178-183.

Mekwatanakarn, P., W. Kositratana, M. Levy and R.S. Zeigler. 2000. Pathotype and avirulence gene diversity of *Pyricularia grisea* in Thailand as determined by rice lines near-isogenic for major resistance genes. **Plant Disease** 80: 60-70.

Merhrotra, R.S. and A. Aggarwal. 2003. **Plant pathology** Tata McGraw-Hill, New Delhi, India. 846 p.

Meyer, G. and P. Prasertsri. 2007. **Thailand grain and feed rice crop survey 2007** GAIN Report. Ministry of Agriculture. 45 p.

Murakami, J., R. Tomita, T. Kataoka, H. Nakayashiki, Y. Tosa and S. Mayama. 2003. Analysis of host specificity of *Magnaporthe grisea* toward foxtail mellet using a genetic cross between isolates from wheat and foxtail mellet. **Phytopathology** 93: 42-45.

Nitta, N., M.L. Farman and S.A. Leong. 1997. Genome organization of *Magnaporthe grisea*: integration of genetic maps, clustering of transposable elements and identification of genome duplications and rearrangements. **Theor. Appl. Genet.** 10: 875-883.

Noenplab, A., A. Vanavichit, T.Toojinda, P. Sirithunya, S. Tragoonrung, S. Sriprakhon and C. Vongsaprom. 2006. QTL mapping for leaf and neck blast resistance in Khao Dawk Mali105 and Jao Hom Nin recombinant inbred lines. **ScienceAsia** 32: 133-142.

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

Osbourne, A.E. 1996. Preformed antimicrobial compounds and plant defence against fungal attack. **Plant Cell Physiol.** 8: 1821-1831.

Ou, S.H. 1980. Pathogen variability and host resistance in rice blast disease. **Annu. Rev. Phytopathology** 18: 167-187.

Peter, J.L., F. Cnudde and T. Gerats. 2003. Forward genetics and mapbased cloning approaches. **Trend Plant Sci.** 10: 484-491.

Pieterse, C.M.J., J.A. Van-Pelt, S.C.M. Van-Wees, J. Ton, K.M. Leon-Kloosterziel, J.J.B. Keurentjes, B.W.M. Verhagen, M. Knoester, I. Van der Sluis, P.A.H.M. Bakker and L.C. Van Loon. 2001. Rhizobacteria-mediated induced systemic resistance: triggering, signalling and expression. **European J. of Plant Pathology** 107: 110-121.

Pongam, P., T.C. Osborn and P.H. Williams. 1998. Genetic analysis and identification of amplified fragment length polymorphism markers linked to the *alm1* avirulence gene of *Leptosphaeria maculans*. **Phytopathology** 88: 1068-1072.

Qu, S., G. Liu, B. Zhou, M. Bellizzi, L. Zeng, L. Dai, B. Han and G.-L. Wang. 2006. The Broad-Spectrum Blast Resistance Gene *Pi9* Encodes a Nucleotide-Binding Site–Leucine-Rich Repeat Protein and Is a Member of a Multigene Family in Rice. **Genetics** 172: 1901-1914.

Ramalingam, J., C.M.V. Cruz, K. Kukreja, J.M. Chittuor, J.L. Wu, S.W. Lee, M. Baraoidan, M.L. George, M.B. Cohen, S.H. Hulbert, J.E. Leach and H. Leung. 2003. Candidate defence gene from rice, barley and maize and their assosiation with quailitative and quantitative resistance in rice. **Mol. Plant-Microbe Interaction** 16: 14-24.

Rivas, S., A. Rougon-Cardoso, M. Smoker, L. Schauser, H. Yoshioka and J.D. Jones. 2004. CITRX thioredoxin interacts with the tomato *Cf-9* resistance protein and negatively regulates defence. **EMBO J.** 23: 2156-2165.

Roumen, E., M. Levy and J.L. Notteghem. 1997. Characterisation of the European pathogen population of *Magnaporthe grisea* by DNA finger printing and pathotype analysis. **European J. of Plant Pathology** 103: 363-371.

Sallaud, C., M. Lorieux, E. Roumen, D. Tharreau, R. Berruyer, P. Svestasrani, O. Garsmeur, A. Ghesquiere and J.L. Notteghem. 2003. Identification of five new blast resistance genes in the highly blast-resistant rice variety IR64 using a QTL mapping strategy. **Theor. Appl. Genet.** 106: 794-803.

Santoso, H.A., D. Aadriantsinialona, J.L.N. D . W. UTAMI, M.H. Lebrun and D. Tharreau. 2007. Microsatellite markers for population studies of the rice blast fungus, *Magnaporthe grisea*. **Molecular Ecology Notes** doi: 10.1111/j.1471-8286.2006.01672.x.

Sax, K. 1923. The association of size differences with seed-coat pattern and pigmentation in *Phaseolus vulgaris*. **Genetics** 8: 552-560.

Sesma, A. and A.E. Osbourn. 2004. The rice leaf blast pathogen undergoes developmental processes typical of root-infecting fungi. **Nature Genet**. 431: 582-586.

Sexton, A.C., M. Paulesen, J. Woestemeyer and B.J. Howlett. 2000. Cloning, characterization and chromosomal location of three genes encoding host-cell-wall-degrading enzymes in Leptosphaeria maculans, a fungal pathogen of *Brassica spp*. **Gene** 248: 89-97.

Sharma, T.R., M.S. Madhav, B.K. Singh, P. Shanker, T.K. Jana, V. Dalal, A. Pandit, A. Singh, K. Gaikwad, H.C. Upreti and N.K. Singh. 2005. High-resolution mapping, cloning and molecular characterization of the *Pi-kh* gene of rice, which confers resistance to *Magnaporthe grisea*. **Mol. Gen. Genomics** 274: 569-578.

Shen, W.-C., P. Bobrowicz and D.J. Ebbole. 1999. Isolation of Pheromone Precursor Genes of *Magnaporthe grisea*. **Fungal Genetics and Biology** 27: 253-263.

Shi, J., Y. Wei and P.H. Goodwin. 2000. A comparison of the pectate lyase genes *pel-1* and *pel-2* of *Colletotrichum gloeosporioides* sp. malvae and the relationship between their expression in culture and during necrotrophic infection. **Gene** 243: 139-150.

Sirithunya, P., S. Tragoonrung, A. Vanavichit, N. Pa-In, C. Vongsaprom and T. Toojinda. 2002. Quantitative trait loci associated with leaf and neck blast resistance in recombinant inbred line population of rice (*Oryza sativa*). **DNA Res.** 9: 79-88.

Sirithunya, P., S. Sriprakhon, C. Vongsaprom, T. Sreewongchai, A. Vanavichit and T. Toojinda. 2004. Discovery of broad spectrum blast resistance in rice, p. 160, *In* A. Vanavichin, ed. **The 1st International Conference on Rice for the Future.** 31 August – 3 September 2004, Kasetsart University, Bangkok, Thailand.

Sirithunya, P., T. Sreewongchai, S. Sriprakhon, T. Toojinda, S. Pimpisithavorn, C. Kosawang and P. Smitamana. 2007. Assessment of Genetic Diversity in Thai Isolates of Pyricularia grisea by Random Amplification of Polymorphic DNA. **J. Phytopathology** doi: 10.1111/j.1439-0434.2007.01341.x.

Song, F. and R.M. Goodman. 2001. Molecular biology of disease resistance in rice. **Physiological and Molecular Plant Pathology.** 59: 1-11.

Soubabere, O., V. Jorge, J.L. Notteghem, M.H. Lebrun and D. Tharreau. 2001. Sequence characterized amplified region markers for the rice blast fungus, *Magnaporthe grisea*. **Molecular Ecology Notes** 1: 19-21.

Sriboonjit, S. and A. Viboonpong. 2000. Evaluation of neck blast disease affected to KDML rice production by stochastic frontier method. **CMU Econonic Journal** 3: 39-52.

Sweigard, J.A., A.M. Carroll, S. Kang, L. Farrall, F.G. Chumley and B. Valent. 1995. Identification, cloning, and characterization of *PWL2*, a gene for host species specificity in the rice blast fungus. **Plant Cell** 7: 1221-1233.

Takabayashi, N., Y. Tosa, H.S. Oh and S. Mayama. 2002. A gene-for gene relationship underlaying species-specific parasitism of Avena/ Triticum isolates of *Magnaoporthe grisea* on wheat cultivar. **Phytopathology** 92: 1182-1188.

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

Tang, X., R.D. Frederick, J. Zhou, D.A. Halterman, Y. Jia and G.B. Martin. 1996. Initiation of plant disease resistance by physical interaction of *AvrPto* and *Pto* kinase. **Science** 274: 2060-2063.

Tanksley, S.D. 1993. Mapping polygenes. **Annu. Rev. Genet**. 27: 205-233.

Tenjo, F.A. and J.E. Hamer. 2002. Pathogenic development in *Magnaporthe grisea*, p. 1-15, *In* H. D. Osiewacz, ed. **Molecular Biology of Fungal Developmemt**. Johann Wolfgang Goethe-Universität, Frankfurt, Germany.

Uddin, W., G. Viji and P. Vincelli. 2003. Gray Leaf Spot of Perennial Ryegrass Turf: An Emerging Problem for the Turfgrass Industry. **USGA Turfgrass and Environmental Research Online** 2: 1-18.

Valent, B. and F.G. Chumley. 1991. Molecular genetic analysis of the rice blast fungus, *Magnaporthe Grisea*. **Annual Review of Phytopathology** 29: 443-467.

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

Van'T Slot, K.A.E. and W. Knogge. 2002. A dual role for microbial pathogen-derived effector proteins in plant disease and resistance. **Critical Reviews in Plant Science** 21: 229-271.

Van der Biezen, E.A. and J.D.G. Jones. 1998. The NB-ARC domain: a novel signaling motif shared by plant resistance gene products and regulators of cell death in animals. **Curr. Biol.** 8: R226-R227.

Van Wees, S.C., M. Luijendijk, I. Smoorenburg, L.C. Van Loon and C.M. Pieterse. 1999. Rhizobacteria-mediated induced systemic resistance (ISR) in Arabidopsis is not associated with a direct effect on expression of known defence-related genes but stimulates the expression of the jasmonate-inducible gene Atvsp upon challenge. **Plant Mol. Biol.** 41: 537-549.

Vanavichit, A., S. Tragoonrung and T. Toojinda. 2005. Genomic Mapping and Positional Cloning, with Emphasis on Plant Science, p. 103-128, *In* C. W. Sensen, ed. **Handbook of Genome Research**. WILEY-VCH Verlag GmbH & Co. KGaA., Weinheim.

Wang, Z.-X., M. Yano, U. Yamanouchi, M. Iwamoto, L. Monna, H. Hayasaka, Y. Katayose and T. Sasaki. 1999. The Pib gene for rice blast resistance belongs to the nucleotide binding and leucine-rich repeat class of plant disease resistance genes. **The Plant Journal** 19: 55-64.

Xu, J.-R., Y.-L. Peng, M.B. Dickman and A. Sharon. 2006. The dawn of fungal pathogen genomics. **Annual Review of Phytopathology** 44: 337-366.

Yaegashi, H. 1978. Inheritance of pathogenicity in crosses of *Pyricularia* isolates from weeping lovegrass and finger millet. **Annu. Phytopathol. Soc. Jpn.** 44: 626-632.

Yamasaki, Y. and S. Kiyosawa. 1966. Studies on inheritance of resistance of rice varieties to blast, 1. Inheritance of resistance of Japanese varieties to several strains of the fungus. **Bull. Natl. Inst. Agr. Sci.** 14: 39-69.

Yasuda, N., M.T. Noguchi and Y. Fujita. 2005. Identification of an avirulence gene in the fungus *Magnaporthe grisea* corresponding to a resistance gene at the *Pik* locus. **Phytopathology** 88: 768-772.

Young, N.D. 1996. QTL mapping and quantitative disease resistance in plants. **Annual Review of Phytopathology** 34: 479-501.

Zeigler, R.S. 1998. Recombination in *Magnaporthe grisea*. **Annual Review of Phytopathology** 36: 249-275.

Zeigler, R.S., R.P. Scott, H. Leung and R.J. Nelson. 1997. Evidence of parasexual exchange of DNA in rice blast fungus challenges its exclusive clonality. **Phytopathology** 87: 284-294.

Zeigler, R.S., J. Thome, R.J. Nelson, M. Levy and F.J. Correa-Victoria. 1994. Lineage exclusion: a proposal for linking blast population analysis to resistance breeding, p. 267-296, *In* R. S. Zeiler, L. S.A. and P. S. Teng, eds. **Rice blast disease**. CAB International, Cambridge, UK.

Zhou, B., S. Qu, G. Liu, M. Dolan, H. Sakai, G. Lu, M. Bellizzi and G.-L. Wang. 2006. The Eight Amino-Acid Differences Within Three Leucine-Rich Repeats Between *Pi2* and *Piz-t* Resistance Proteins Determine the Resistance Specificity to *Magnaporthe grisea*. **MPMI**. 19: 1216-1228.

APPENDIX

Appendix Table 1 Pathogenicity on leaf blast of progenies dereived from B1-2xTH16 cross on 10 rice varieties and JHN rice on leaf (lb) and panicle blast (pb) evaluation.

Entry	C101TTP	Azucena	CNT1	IR68144	IR62266	IR57514	IR64	SPR1	SPR60	KDML105	JHN (lb)	JHN (pb)
B47	6.0	4.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.5	100.0
B48	6.0	5.0	2.0	3.0	4.0	1.0	2.0	2.5	2.5	3.5	2.0	4.3
B49	6.0	5.0	0.0	0.0	6.0	0.0	0.0	4.0	1.0	2.0	4.5	90.0
B53	6.0	6.0	0.0	1.0	1.0	0.0	2.0	4.0	4.0	2.0	1.5	10.0
B55	6.0	6.0	6.0	0.0	2.0	0.0	4.0	5.0	2.5	2.0	1.5	10.0
B56	6.0	6.0	5.0	0.0	6.0	0.0	4.5	0.0	0.0	2.0	5.0	95.0
B57	4.0	4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0
B58	6.0	6.0	3.0	6.0	6.0	4.0	0.0	5.5	5.0	4.5	4.5	87.5
B59	6.0	4.0	5.0	1.0	5.0	0.0	5.0	4.0	0.0	0.0	0.5	4.8
B60	2.0	6.0	2.0	2.0	2.0	2.0	0.0	0.0	2.0	4.5	0.5	19.5
B62	6.0	5.0	5.0	5.5	2.0	1.0	4.5	6.0	1.0	2.0	2.0	27.5
B63	5.5	5.0	4.5	0.0	2.0	1.0	1.0	0.0	4.0	2.0	0.5	4.8
B64	2.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	100.0
B69	5.0	6.0	6.0	0.0	4.0	5.0	5.0	4.0	0.0	0.0	2.5	3.3
B70	6.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	-
B71	2.0	6.0	6.0	5.0	6.0	1.0	1.0	6.0	6.0	6.0	2.0	16.0
B72	5.0	0.0	0.0	2.0	0.0	4.0	0.0	2.0	0.0	0.0	5.0	85.0
B73	6.0	6.0	3.0	1.0	2.0	2.0	0.0	2.0	2.0	2.0	1.0	20.2
B74	1.0	4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0	45.0
B75	6.0	6.0	6.0	3.0	6.0	0.0	5.0	2.0	5.0	0.0	2.0	23.3
B76	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.5	5.5	100.0
B78	6.0	4.0	2.0	2.0	4.5	0.0	0.0	1.0	1.0	1.0	1.5	95.0
B79	2.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.0
B80	2.0	2.0	2.0	1.0	1.0	0.0	0.0	0.0	4.0	4.5	1.0	10.0
B81	5.0	5.0	0.0	0.0	2.0	4.0	0.0	2.0	4.5	0.0	0.0	12.5
B82	5.5	3.5	4.0	0.0	0.0	1.0	0.0	1.0	2.0	0.0	4.5	100.0
B83	5.0	6.0	4.5	2.0	6.0	5.0	6.0	6.0	4.0	5.0	4.5	100.0
B84	6.0	5.0	2.0	0.0	0.0	0.0	0.0	1.0	2.0	2.0	1.5	7.5
B85	5.0	6.0	5.0	6.0	6.0	2.0	6.0	6.0	6.0	6.0	6.0	65.0
B86	2.0	6.0	3.0	4.0	6.0	0.0	0.0	5.0	6.0	4.0	5.5	100.0
B87	2.0	6.0	2.0	3.0	2.0	0.0	0.0	2.0	2.0	0.0	1.0	5.0
B88	6.0	6.0	6.0	4.5	0.0	0.0	5.0	5.5	6.0	5.0	0.0	30.0

Appendix Table 1 (Continued)

Entry	C101TTP	Azucena	CNT1	IR68144	IR62266	IR57514	IR64	SPR1	SPR60	KDML105	JHN (lb)	JHN (pb)
B90	2.0	4.0	0.0	0.0	0.0	0.0	0.0	0.0	4.0	0.0	4.5	85.0
B91	4.0	2.0	5.0	5.0	2.0	0.0	4.0	5.0	4.0	3.5	5.0	100.0
B105	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	80.0
B106	5.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	18.6
B107	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	100.0
B109	4.0	0.0	0.0	0.0	2.0	0.0	0.0	0.0	0.0	1.0	1.0	12.5
B110	4.0	4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	19.4
B111	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.5	100.0
B112	5.0	5.0	5.5	4.0	4.5	0.0	0.0	5.0	4.0	2.0	0.0	20.0
B113	2.0	4.0	0.0	1.0	2.0	1.0	0.0	0.0	0.0	0.0	1.0	20.0
B114	4.0	5.5	0.0	0.0	0.0	0.0	0.0	3.4	2.0	2.0	5.0	96.3
B115	5.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	15.0
B116	2.0	6.0	0.0	0.0	5.0	0.0	0.0	2.5	0.0	0.0	2.5	16.7
B117	6.0	6.0	5.0	2.0	6.0	2.0	5.0	0.0	2.0	2.0	2.5	14.0
B118	5.5	4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	80.0
B120	5.5	4.5	4.5	0.0	5.0	0.0	0.0	0.0	0.0	0.0	1.0	17.5
B121	6.0	6.0	0.0	0.0	3.5	0.0	0.0	0.0	0.0	1.0	5.0	54.0
B122	4.5	0.0	3.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	90.0
B124	6.0	5.0	2.0	0.0	4.0	0.0	0.0	2.0	0.0	0.0	0.0	10.0
B125	6.0	5.0	3.0	0.0	5.0	0.0	1.0	0.0	1.0	2.0	0.5	3.1
B126	-	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.0	10.0
B129	4.5	6.0	1.0	5.0	3.0	4.0	0.0	5.5	4.0	3.5	2.0	6.5
B131	4.5	5.0	0.0	0.0	4.0	0.0	0.0	4.0	0.0	0.0	1.0	100.0
B132	2.0	0.0	4.0	4.0	0.0	0.0	3.0	0.0	4.5	0.0	5.0	100.0
B134	0.0	3.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	26.7
B135	3.0	2.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.5	95.0
B138	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.5	-
B139	3.0	5.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	1.0	11.5
B140	6.0	6.0	0.0	0.0	2.0	0.0	0.0	0.0	0.0	2.0	2.0	13.8
B141	4.5	4.0	4.5	1.0	5.0	3.0	5.5	4.5	2.0	1.0	1.5	10.0
B143	6.0	6.0	0.0	6.0	2.0	0.0	5.0	4.0	6.0	4.5	5.0	100.0
B144	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	21.0
B145	4.0	5.5	4.0	4.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	40.0
B147	6.0	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.5	100.0
B150	4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	15.0

Appendix Table 1 (Continued)

Entry	C101TTP	Azucena	CNT1	IR68144	IR62266	IR57514	IR64	SPR1	SPR60	KDML105	JHN (lb)	JHN (pb)
B151	6.0	6.0	4.5	5.0	6.0	5.0	1.0	5.0	5.0	6.0	0.5	12.0
B152	6.0	5.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	18.8
B154	4.5	5.0	0.0	0.0	1.0	1.0	0.0	0.0	0.0	1.0	0.5	70.0
B155	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.5	100.0
B156	5.0	4.0	3.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	12.5
B157	2.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.5
B158	4.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.5	0.0
B160	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	47.5
B161	6.0	6.0	6.0	6.0	0.0	0.0	6.0	5.0	5.0	6.0	5.5	60.0
B163	5.0	5.0	4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	65.0
B164	2.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	20.0
B166	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.0	100.0
B167	6.0	4.5	6.0	0.0	4.0	0.0	0.0	5.0	4.0	0.0	5.0	100.0
B168	5.0	6.0	2.0	5.0	5.0	4.0	0.0	6.0	6.0	6.0	2.0	10.0
B169	5.0	5.0	0.0	0.0	4.0	0.0	0.0	0.0	0.0	0.0	5.0	100.0
B170	6.0	5.0	2.0	1.0	5.0	0.0	0.0	2.0	2.0	2.0	3.0	25.0
B171	0.0	5.0	5.0	4.0	4.0	0.0	4.0	4.0	4.0	5.0	4.5	95.0
B172	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	100.0
B173	5.5	5.5	4.0	0.0	1.0	0.0	1.0	0.0	0.0	2.0	5.0	100.0
B174	4.0	4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.5	35.0
B175	6.0	6.0	6.0	6.0	6.0	5.0	5.0	5.0	5.0	4.0	1.0	40.0
B176	6.0	4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	60.0
B177	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.5	100.0
B178	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	100.0
B179	4.0	1.0	4.0	2.0	2.0	0.0	0.0	5.0	5.0	2.0	0.5	90.0
B180	6.0	6.0	5.0	1.0	5.0	0.0	4.0	2.0	0.0	0.0	4.5	100.0
B181	2.5	4.0	4.0	0.0	2.0	2.0	1.0	0.0	1.0	1.0	0.0	7.5
B182	5.0	5.0	0.0	5.0	0.0	3.0	0.0	6.0	5.0	0.0	1.0	2.0
B183	5.5	6.0	4.5	0.0	4.0	0.0	0.0	0.0	3.5	1.0	5.0	100.0
B184	5.0	5.0	4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	90.0
B185	3.0	2.0	0.0	0.0	2.0	0.0	2.0	0.0	0.0	0.0	1.5	18.5
B186	2.0	5.0	3.0	0.0	2.0	3.0	3.0	0.0	0.0	0.0	5.5	70.0
B187	2.0	4.5	4.0	0.0	0.0	0.0	0.0	0.0	0.0	4.0	3.0	85.0
B188	5.0	4.0	4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	6.7
B189	4.0	5.0	1.0	4.0	0.0	4.0	2.0	4.0	4.0	6.0	5.0	100.0

Appendix Table 1 (Continued)

Entry	C101TTP	Azucena	CNT1	IR68144	IR62266	IR57514	IR64	SPR1	SPR60	KDML105	JHN (lb)	JHN (pb)
B190	4.0	0.0	4.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	95.0
B191	2.0	5.0	6.0	5.0	6.0	0.0	5.0	5.5	6.0	5.0	5.5	100.0
B192	2.0	3.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.5	95.0
B195	0.0	4.0	4.0	3.0	4.0	4.0	0.0	4.5	4.0	4.5	4.5	100.0
B199	6.0	6.0	2.0	0.0	1.0	0.0	0.0	0.0	0.0	2.0	5.0	95.0
B200	5.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	15.0
B201	2.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0	100.0
B202	5.0	4.0	0.0	1.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	100.0
B203	6.0	6.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	2.0	0.0	10.0
B204	6.0	5.0	0.0	0.0	4.0	0.0	0.0	0.0	0.0	0.0	2.0	55.0
B205	5.0	3.0	5.5	0.0	1.0	1.0	0.0	0.0	0.0	0.0	0.0	90.0
B207	6.0	0.0	4.0	0.0	0.0	2.0	0.0	0.0	0.0	0.0	1.0	9.2
B208	3.0	3.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	15.0
B209	6.0	3.0	0.0	1.0	4.5	4.5	0.0	0.0	0.0	0.0	1.5	45.0
B210	6.0	5.0	0.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0	4.5	95.0
B1-2	5.0	6.0	5.0	5.0	5.0	0.0	0.0	0.0	0.0	0.0	5.5	95.0
TH16	5.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.8	20.0

CURRICULUM VITAE

NAME : Mr. Tanee Sreewongchai

BIRTH DATE : October 18, 1972

BIRTH PLACE : Chaiyaphum, Thailand

EDUCATION :

YEAR	INSTITUTE	DEGREE
1997	Kasetsart Univ.	B.Sc.(Agriculture)
2000	Kasetsart Univ.	M.S.(Agriculture)

SCHORASHIP/AWARDS : Grants for thesis from

- The Rockefellor Foundation under the project of Human Resources Development in Genome Research
- The National Center for Genetic Engineering and Biotechnology (BIOTEC) under the project of Mapping and Identification of Avirulence Genes in Thai Population of Blast Fungus (*Magnaporthe grisea*) Corresponding Rice Resistance Genes