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THESIS

IDENTIFICATION OF BLAST RESISTANCE QTLs IN TWO RICE RIL POPULATIONS AND MARKER ASSISTED SELECTION FOR PYRAMIDING OF FOUR QTLs IN RD6 RICE VARIETY

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Blast disease, caused by the fungus, *Pyricularia grisea* Sacc, is the most destructive diseases in rice worldwide. To improve durable and broad spectrum resistance in rice, understanding the host-pathogen interaction is required. In this study, the blast strains B71, B88, B124, B151, B161, B209, B248 and B259 derived from sexual recombinations of B1-2 and TH16 were used to demonstrate the arising of new virulence strains that cause a change of the virulence spectrum in rice varieties. Recombinant strains B71, B124, B151, B161 and B248 had shown more virulence than the parental strains on the tested rice varieties. The strain B124 is the most aggressive strain with broader virulence spectrum. The blast strain B1-2, reported to overcome the broad spectrum resistant QTL of Jao Hom Nin (JHN) rice variety, was used to identify its corresponding resistance QTL using two rice mapping populations derived from crosses of KDML105 x JHN and IR57514 x KDML105. Four QTLs, $qBL1_{IR}$, $qBL2_{KD}$, $qBL6_{IR}$ and $qBL8_{KD}$ were identified on chromosomes 1, 2, 6 and 8 respectively. The $qBL1_{IR}$ and $qBL6_{IR}$ mapped to RM495-RM84 and GT11-RM564 intervals respectively are associated with the infection efficiency. The $qBL2_{KD}$ and $qBL8_{KD}$ mapped to RM213-RM208 and RM310-RM72 intervals respectively are associated with the extension of lesion size. Four QTLs, $qBL1_{JHN}$, $qBL2_{IR64}$, $qBL11_{JHN}$ and $qBL12_{Azu}$, for blast resistance were pyramided into Thai glutinous rice variety RD6 using marker assisted selection (MAS). The introgression lines carrying combinations of QTLs showed lower level of infection and broader resistance spectrum than the original RD6, especially the introgression lines carrying the $qBL11_{JHN}$. The information of genetic variation of blast fungus, mapping locations of resistance genes and their closely linked markers will be useful for blast resistance rice breeding program in the future.

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LIST OF ABBREVIATIONS

AFLP	=	amplified fragment length polymorphism
Avr	=	avirulence
BSR	=	broad spectrum resistance
CAPS	=	cleaved amplified polymorphic sequence
CC	=	coiled-coil
cDNA	=	complementary DNA
Chr	=	chromosome
cm	=	centimeter
cM	=	centimorgan
CN1	=	Chainat 1
^{o}C	=	degree celcius
dH_2O	=	distilled water
dNTP	=	deoxynucleotide triphosphate
DR	=	durable resistance
EST	=	expressed sequence tag
g	=	gram
HR	=	hypersensitive response
IL-1R	=	interleukin-1 receptor
ISSR	=	inter-simple sequence repeat
IRRI	=	International Rice Research Institute
JHN	=	Jao Hawm Nin
KDML105	=	Khao Dawk Mali 105
LRR	=	leucine rich repeat
LZ	=	leucine zipper
MAB	=	marker assisted backcrossing
MAP	=	marker assisted pyramiding
MAS	=	marker assisted selection
Mb	=	mega base pairs
ml	=	milliliter
mM	=	millimolar

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LIST OF ABBREVIATIONS (Continued)

NBS	=	nucleotide binding site
ng	=	nanogram
NIL	=	near isogenic line
PCR	=	polymerase chain reaction
PVE	=	phenotypic variance explained
QTL	=	quantitative trait loci
R	=	resistance
RAPD	=	random amplified polymorphic DNA
RFLP	=	restriction fragment length polymorphism
RIL	=	recombinant inbred line
rpm	=	round per minute
RSR	=	race-specific resistance
SCAR	=	sequence characterized amplified region
sCIM	=	simplified composite interval mapping
sec	=	second
SIM	=	simple interval mapping
SNP	=	single nucleotide polymorphism
SSR	=	simple sequence repeat
SSLP	=	simple sequence length polymorphism
STR	=	short tandem repeat
STS	=	sequence tagged site
TIR	=	toll/interleukin-1/resistance
TM	=	transmembrane domain
VNTR	=	variable number of tandem repeat
µl	=	microliter
µm	=	micrometer
µM	=	micromolar
χ^2	=	chi-square

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IDENTIFICATION OF BLAST RESISTANCE QTLs IN TWO RICE RIL POPULATIONS AND MARKER ASSISTED SELECTION FOR PYRAMIDING OF FOUR QTLs IN RD6 RICE VARIETY

INTRODUCTION

Rice (*Oryza sativa* L.) is the economically valuable crop that provides the main source of food for more than half of the world's population (Delseny*, et al.*, 2001). Rice has been grown and consumed in most of developing countries around the world but an increasing of rice production has not been enough to meet the need of population growth. Rice production confronts many problems such as unsuitable environment, biotic and abiotic stresses, that caused of yield losses. Among them, diseases are the most limiting factor causing a yield loss (Madden and Wheelis, 2003).

Blast disease is one of the most important biotic constraints in rice worldwide (Ou, 1985). The fungus *Pyricularia grisea* Sacc is causal organism of the disease. The fungus can infect rice plants at any stage of growth and the symptom can be seen on the leaf (blade, sheath and ligule), stem nodes and panicle (rachilla and neck). Blast disease can cause a severe loss of yield up to 50-85% (Teng and Revilla, 1996; Gnanamanickam, 2009). In Thailand, blast epidemic was reported on November 2002 and damaged 75,840 rai of rice growing areas (http://www.doae.go.th). Tremendous genetic diversity of the blast fungus resulted from mutation (Xiong*, et al.*, 2007; Zhou*, et al.*, 2007), sexual hybridization (Schardl and Craven, 2003), parasexualism (Genovesi and Magill, 1976; Zeigler*, et al.*, 1997) and heterocaryosis (Genovesi and Magill, 1976; Chen and Wu, 1977; Crawford*, et al.*, 1986), that believed to cause a development of novel pathotype (Ou, 1980) has been documented. Increment of the prevalence of a previously rare pathotype and novel pathotype has been reported to cause a breakdown of resistance of rice varieties soon after released (Kiyosawa, 1982). Epidemic of blast disease in the rice production areas is not only determined

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by hyper-variation of the blast fungus itself but also induced by climate and rice variety. High genetic diversity of the pathogen is reported due to a long period of co-existence, development and evolution, with host. Disease reactions on differential varieties and molecular marker analysis had shown an extremely diverse of genetic found in Thailand especially in the Northern part (Mekwatanakarn*, et al.*, 2000; Sirithunya*, et al.*, 2008).

The frequently occurring of epidemics has concerned rice breeders to search for broad spectrum resistance (BSR) (Mackill and Bonman, 1992; Chen*, et al.*, 1996; Liu*, et al.*, 2002; Zhu*, et al.*, 2004; Liu*, et al.*, 2007b; Yang*, et al.*, 2008) and durable resistance (DR) (Bonman, 1992; Luu and Bui, 1999; Pham and Le, 2007) rather than race-specific resistance (RSR) (Chantret*, et al.*, 1999; Yang*, et al.*, 2008; Zhao*, et al.*, 2009). Identification of BSR and DR is of great interests to the rice breeders. Nowadays, more than 73 resistance genes and 347 QTLs were reported from different rice cultivars and more than 50 blast resistance genes have been identified in rice. Most of the known blast resistance genes are located on chromosomes 6, 11 and 12. The resistant genes; *Pi-1*(t), *Pi2*, *Pi9*, *Pi20*(t) *Pi27*(t), *Pi39*(t) and Pik^h have been recognized to confer broad spectrum resistance to many isolates collected from various regions (Mackill and Bonman, 1992; Chen*, et al.*, 1996; Luu and Bui, 1999; Liu*, et al.*, 2002; Zhu*, et al.*, 2004; Liu*, et al.*, 2007b; Li*, et al.*, 2008; Yang*, et al.*, 2008). Some major resistance genes; *Pia*, *Pib*, *Pii*, *Pit*, *Pi3*(t), *Pi5*(t), *Pi12*(t) and *Pi19*(t) showed race specific resistance to blast isolates collected from China (Yang*, et al.*, 2008). Of all, nine cloned genes including *Pi2*, *Pi9*, *Pi36*, *Pi37*, *Pi-b*, *Pi-d2*, $Pi\text{-}k^h$, *Pi-ta* and *Piz-t* (Wang*, et al.*, 1999; Bryan*, et al.*, 2000; Sharma*, et al.*, 2005; Chen*, et al.*, 2006; Qu*, et al.*, 2006; Zhou*, et al.*, 2006; Lin*, et al.*, 2007; Liu*, et al.*, 2007a) their sequence information indicated that the largest class of the cloned blast resistance genes encode proteins with a nucleotide binding site (NBS) and C-terminal leucine rich repeats (LRR).

In Thailand, broad spectrum resistance to blast disease was studied and some of them such as JHN, IR64 and Azucena had shown high resistance to a broad range of spectrum of Thai blast isolates. They were used as plant materials to identified and mapped the resistance genes. In rice variety JHN, two resistance QTLs were identified

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and mapped on chromosome 1 and chromosome 11 using recombinant inbred lines (RIL) derived from a cross between KDML105 and JHN (Sirithunya*, et al.*, 2004; Noenplab*, et al.*, 2006). The QTL was also studied in the doubled haploid population derived from a cross between IR64 and Azucena. Two major QTLs on chromosomes 2 and 12 were identified by Rice Gene Discovery Unit (RGDU).

Improvement disease resistance cultivars are the most effective and environmentally friendly way to control blast disease (Hulbert*, et al.*, 2001). DNA marker technology has enormous potential to determine the chromosome position of resistance gene and to increase the efficiency and precision of selection in the breeding program. Marker assisted selection (MAS) using markers tightly linked to resistance genes or quantitative trait loci (QTL) has been successfully applied for backcross and pyramid breeding program. Four QTL for blast resistance were introgressed into Thai glutinous rice variety RD6 using MAS. Introgression lines of RD6 developed by RGDU that contained resistance QTLs on chromosomes 1 and 11 from JHN and introgression lines of RD6 that carried two QTLs on chromosomes 2 and 12 from IR64 and Azucena, respectively were developed by Rice Department and Plant Breeding Research Center for Sustainable Agriculture. These introgression lines had shown broad spectrum resistance to many Thai blast isolates. Consistent with the previous reports, the introgression lines contained resistance gene showed higher level of resistance and/or wider spectrum of resistance to the pathogen (Huang*, et al.*, 1997).

Improving durable and broad resistance required an understanding of the host-pathogen interaction. In this study, we used the blast strains B71, B88, B124, B151, B161, B209, B248 and B259 derived from a sexual recombination of B1-2 and TH16 to demonstrate the arising of new avirulence gene of *P. grisea* Sacc causing a change of the virulence spectrum in differential rice varieties. The blast strain B1-2 that can breakdown the resistance variety Jao Hawm Nin (JHN) was used to identify the locations and effects of resistance QTLs in two rice mapping populations, KDML105 x JHN and IR57514 x KDML105. The blast strain B161 was also used to identify the genes in the IR57514 x KDML105 mapping population. The mapping locations of

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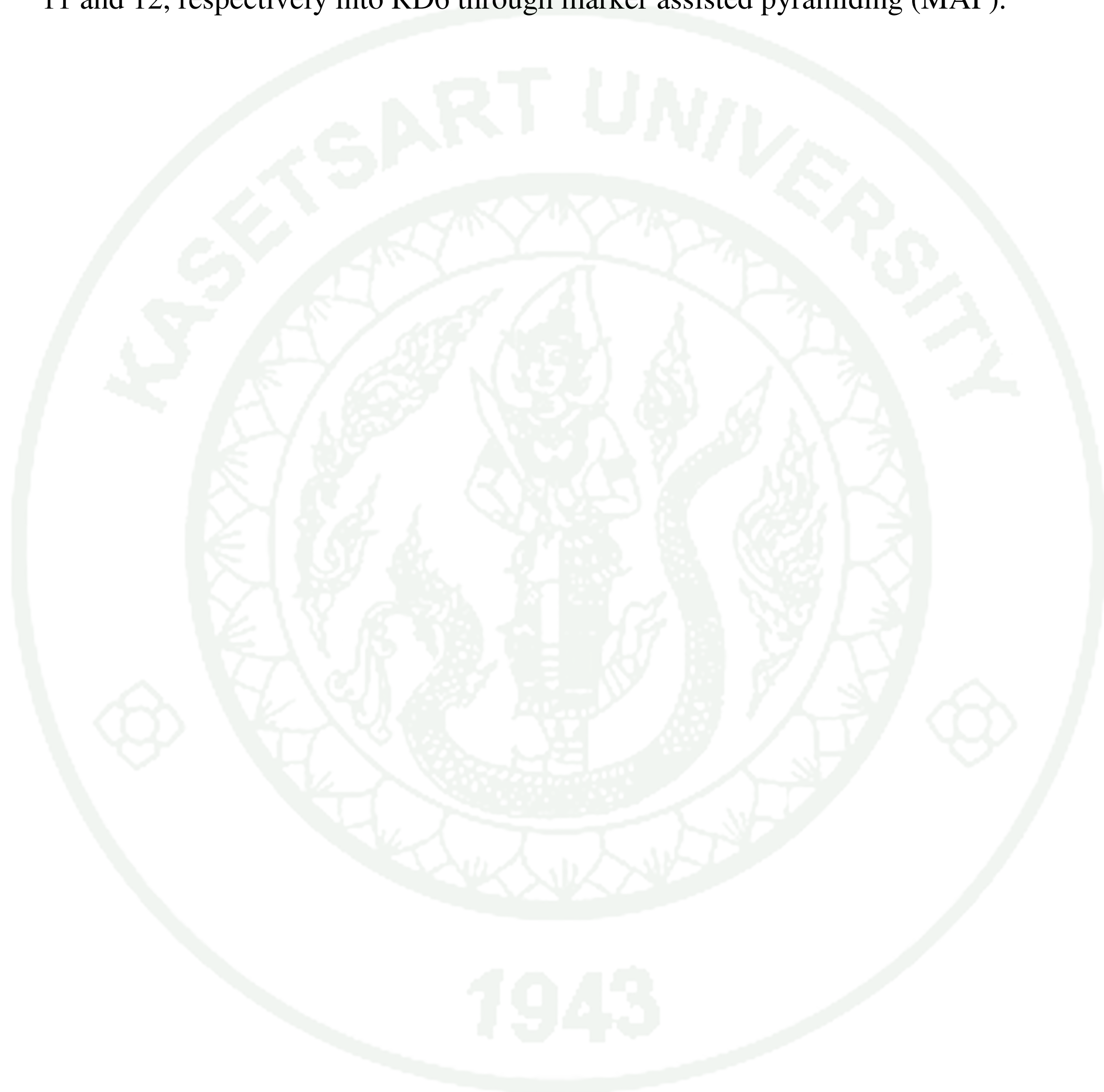
resistance QTL and their closely linked markers which reported in this study will be useful for breeders to improve blast resistance through MAS. Moreover, to improve broad spectrum blast resistance in Thai rice, we had combined four blast resistance QTLs as $qBL1_{JHN}$, $qBL2_{IR64}$, $qBL11_{JHN}$ and $qBL12_{Azu}$ located on chromosomes 1, 2, 11 and 12, respectively into RD6 through marker assisted pyramiding (MAP).



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OBJECTIVES

1. To study a virulence spectrum of recombinant strains derived from a sexual recombination between B1-2 and TH16 in rice differential lines.

2. To map the resistance QTLs against B1-2 strain using two recombinant inbred line (RIL) populations derived from crosses between IR57514 and KDML105 and KDML105 and JHN.

3. To identify resistance QTLs against recombinant strain B161 using the RILs of IR57514 x KDML105.

4. To pyramid four QTLs for blast resistance using marker assisted selection.

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LITERATURE REVIEW

1. Rice

Rice (*Oryza sativa* L.) is one of the staple foods for more than half of the world population and is considered as the lifeline of Asia. Indica and japonica are the two main groups of *O. sativa* that grow in different ecosystems. Genome of rice consists of 12 chromosomes. The estimation nuclear DNA content of rice genome is 430 Mb per haploid cell (Arumuganathan and Earle, 1991). The small genome of rice is a valuable point of comparison for comparative studies involving monocots. To date rice genome sequences had completely done by the International Rice Genome Sequencing Project (2005), collaboration of Japan and International Community. The rice genome information is publicly available in well established databases on the internet. The complete genome sequence has provided a comprehensive and highly reliable analysis of predicted genes. The primarily maps of rice fulllength cDNA sequences in Nipponbare contain the manually curated annotation of gene models. So far, about 30,000 rice genes are unambiguously attributed to the genome sequence. Rice has become a very valuable crop not only in agriculture but also in basic, functional and applied genomics research. Since the first rice molecular genetic map was published (McCouch*, et al.*, 1988), molecular analysis of the rice genome has made significant progress.

2. Blast disease and symptom

The causal agent of rice blast disease is *Pyricularia grisea* Sacc. The perfect stage of *P. grisea* is *Magnaporthe grisea* (Hebert) Barr. It should be synonymized as *P. oryzae*. Rice blast disease is also a serious problem for all rice growing regions worldwide (Madden and Wheelis, 2003). It can cause a significant yield loss up to 50% when it occurs (Gnanamanickam, 2009). In severe case, it causes up to 85% yield reduction (Teng and Revilla, 1996). There was estimated that the yield loss due to blast disease in each year can feed 60 million peoples. In Mahasarakham, Thailand,

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76,000 rai of rice field were severely disseminated by blast disease. This represents a worth of loss more than 500 millions Baths (ประชาชาติธุรกิจ, 2553).

Figure 1 Blast disease symptoms in rice

Source: http://www.knowledgebank.irri.org, http://www.globalplantclinic.org, http://www.jircas.affrc.go.jp, http://www.ipmcenters.org and http://extension.missouri.edu

The fungus can infect all above ground parts of the rice plant such as leaf, node, internodes, leaf sheath, collar, neck and panicles (Figure 1). In general, the infected leaf and panicle neck are the most common symptom found in the rice fields. On the leaves, lesions are typical spindle shaped, wide in the center and pointed toward either end. The center of the spots appears pale green or dull grayish green changing to grey and the periphery has a dark brown band with a yellow halo around the lesion. The lesions run parallel to the long axis of the leaf. Under favorable conditions, lesions on the susceptible leave expand rapidly and tend to coalesce. Size

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of the lesion is 1-1.5 cm long and 0.3-0.5 cm broad. Lesion may kill the entire leaf in susceptible varieties but they remain pin-head sized brown points in the resistant plants. An early seedling infection may give a burnt appearance to the growing areas. More or less similar spots also develop on the leaf sheath. At flowering stage, the pathogen can attacks the peduncles, which are engirdled and the lesion becomes brownish-black. This stage, commonly referred to rotten neck or neck rot or neck blast or panicle blast, is very serious for grain development. When young neck is infected, the panicles become white in color (also called white head) that liked insect damage and number of mature panicle and no grain filling. In late infection causes incomplete grain filling, poor grain quality and rachis may breakdown. Nodal infection is usually observed after heading. Lesions may also develop on internodes in severe infection. Collar blast also occurs when the pathogen infects the collar that can kill the entire leaf blade. In addition, the invasion of fungus at root also can lead to the development of disease symptoms on the above ground parts of the plant (Sesma and Osbourn, 2004).

3. The pathogen of rice blast disease

P. grisea is usually generated spores, called conidia, which can be easily dispersed by the wind and splashing rain. A single conidium develops at the tip of each structure called conidiophore which can construct 1-20 conidia per conidiophore. The conidiophores are pale brown, smooth and straight or bending. The conidia of fungus are usually three-celled or two septate, pyriform (pear shaped) to obclavate, hyaline or colorless to pale olive and measure 8-10 x 19-27 µm, with a distinctly protruding basal hilum (http://www.knowledgebank.irri.org). The production of spores relate with the increasing of humidity. A typical lesion on leaf can produces 4,000 to 6,000 conidia every night for two weeks or more (Sharma, 2006). The pathogen can continue to live in plants from one crop season to another in the following year (Ou, 1985; Guerber and TeBeest, 2006). In temperate zones, sources of inoculums are rice straw residues, seed, grassweeds host, and perhaps also teleomorph (perfect state), rice grown at distant locations and sclerotia and chlamudospores. The pathogen overwinters as mycelium and conidia of disease on

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straw and seed. In tropical regions, airborne conidia, available throughout the year are the source of primary infection. The feature of blast fungus was showed in Figure 2.

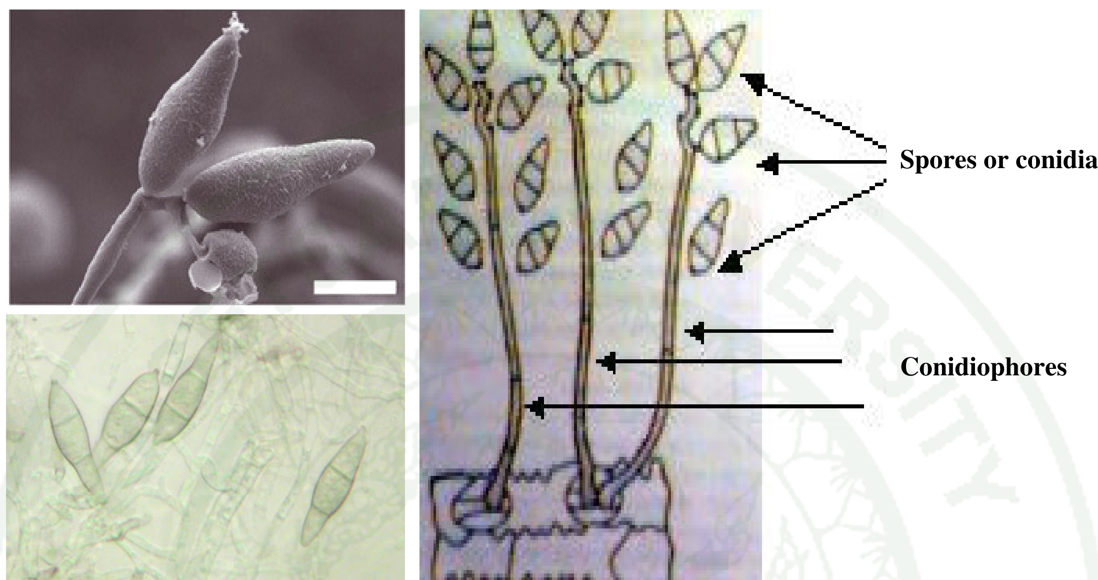

Figure 2 *Pyricularia grisea*, conidia and conidiophores
Source: Wilson and Talbot (2009), www.uark.edu and www.knowledgebank.irri.org

4. The infection of rice blast fungus

The mature conidia are wind disseminated. Infection is most likely after long periods of rain or high humidity with little or no wind movement and relatively warm nights. Germination occurs between 10 ^{0}C and 33 ^{0}C, optimum being 25 ^{0}C to 28 ^{0}C (Sharma, 2006). Infection by rice blast fungus is initiated when a conidium lands on a host leaf surface (Figure 3). The hydrated conidium attaches firmly to the leaf cuticle by releasing mucilage from the spore tip for preventing dislodgement by wind and rain (Hamer, *et al.*, 1988). After a few hours, the germ tube tip will form an appressorium and accumulate tugor pressure inside the appressorium to swell for hooking. To induce appressorium formation, a hydrophobic surface is required (Hamer, *et al.*, 1988; Howard, *et al.*, 1991; Lee and Dean, 1994) and chemical on the leaf surface were also found to induce the formation (Gilbert, *et al.*, 1996). During maturation, the appressorium becomes melanized, except at a well defined pore between the appressorium and the leaf substratum (Howard and Valent., 1996).

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Afterward, a penetration peg is driven through the leaf surface and a hypha emerges through the pore and penetrates directly into the rice leaf. This stage, the symptom can be to sight brown pinpoint lesions and then the lesions will be extended. Maturate fungus produce a large amount of conidia, carried by air to near by plant, and spread the disease.

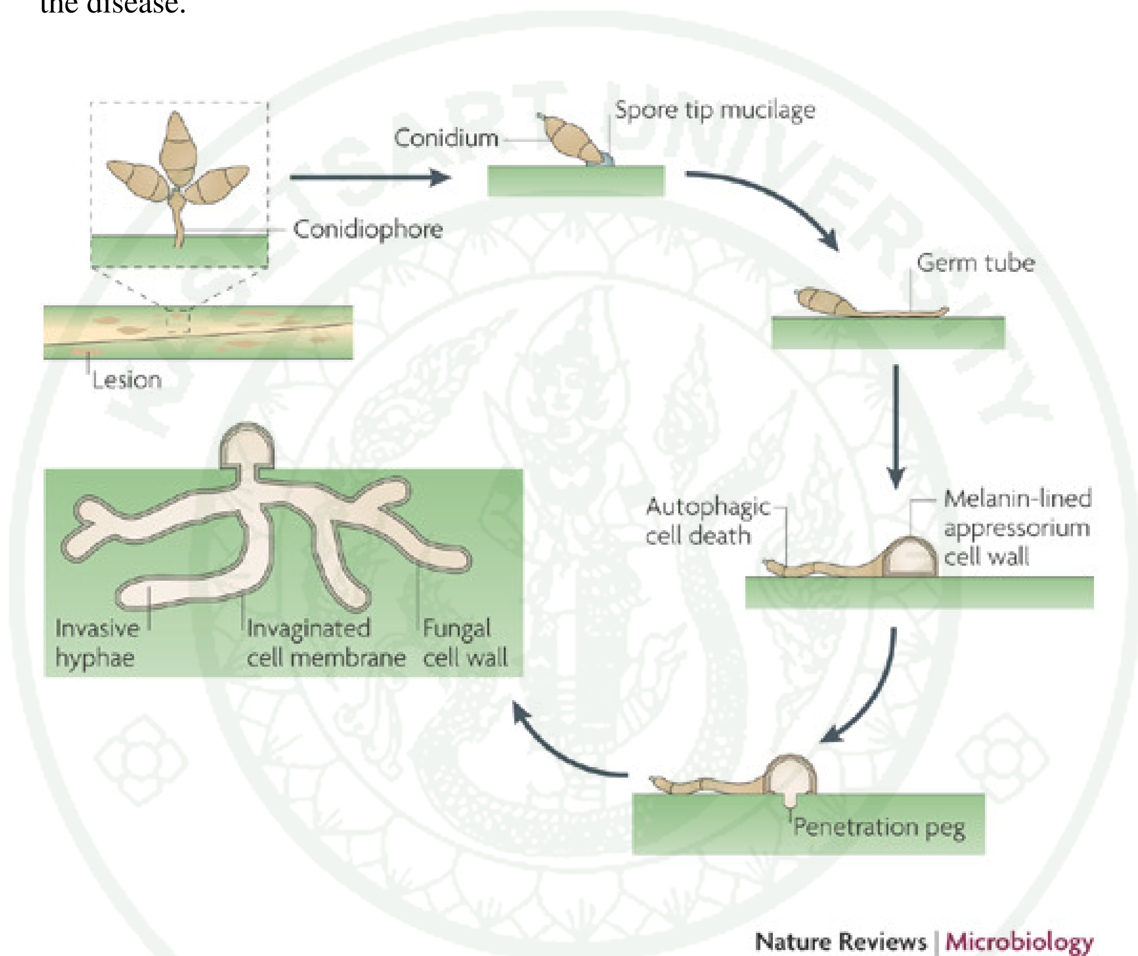

Figure 3 Blast infection cycle

Source: Wilson and Talbot (2009)

5. Plant-pathogen interactions

Developments of diseases are occurring when aggressive pathogens infects susceptible host in appropriated time. Pathogens have one of three main strategies to attack plants: necrotrophy, biotrophy, or hemibiotrophy. Necrotrophs first kill host cells and then metabolize their contents which the cell death is often induced by

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toxins and/or enzymes targeted to specific substrates (Walton, 1996). Biotrophic and hemibiotrophic pathogens invade living cells and subvert metabolism to favor their growth and reproduction.

Plants need to defend themselves against the attack from pathogens such as bacteria, fungi, virus, viroid, invertebates and other plants. So, plants have evolved defense systems to battle against these potential pathogens. 1) An exterior barrier of passive defense includes the waxy cuticle and the plant cell wall. This defense system, some pathogens are entering either through wounds or stomata and many pathogens are able to destroy this barrier by producing enzymes to digest cuticle or cell wall. 2) This barriers of defense are containing many antimicrobial compounds affected directly inhibiting pathogen growth. 3) Plant defense mechanisms are stimulated through the plant signaling molecules, salicylic acid, jasmonic acid and ethylene. One of the most effective inducible defense mechanisms can be explained by gene for gene concept (Flor, 1971). The specificity of a plant-pathogen interaction is determined by the interaction of a product encoded by dominant avirulence (*Avr*) gene in the pathogen and a product of the dominant resistance (*R*) gene in the plant. Therefore, the basis of the plant resistance reaction is a specific recognition between the two components. Resistance occurs only when both, a dominant *R* gene from the plant as well as the dominant *Avr* gene from the pathogen are present. This recognition triggers further physiological defense reactions regulating in a rapid cell death (hypersensitive response, HR) and the accumulation of molecules toxic to the pathogen.

6. Blast resistance in rice

Blast resistance in rice has been generally classified into two types as complete (qualitative) and partial (quantitative) resistances (Ezuka, 1972). Complete resistance, used in term of incompatible interaction, is a result of the interaction between a dominant *R* gene in plant and a corresponding *Avr* in pathogen. Most of complete resistance is usually controlled by a major gene. Partial resistance, caused by compatible interaction, is non race specific and likely polygenic (Kiyosawa, 1981). The relationship between complete resistance in rice and blast pathogen is according

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to a gene for gene model. However, some major genes have been identified to control partial resistance to blast such as *Pif* (Toriyama*, et al.*, 1968), *Pb1* (Fujii*, et al.*, 2000), *pi21* (Fukuoka and Okuno, 2001), *Pi34* (Zenbayashi*, et al.*, 2005) and *Pi35*(t) (Nguyen*, et al.*, 2006).

According to interactions between R genes and pathogen strains, blast resistance can be classified into two groups as race specific and broad spectrum resistance. The race specific resistance is effective to only particular blast isolate. For instance, some major resistance genes, *Pia*, *Pib*, *Pii*, *Pit*, *Pi3*(t), *Pi5*(t), *Pi12*(t) and *Pi19*(t) showed race specific resistance to blast isolates collected from China (Yang, *et al.*, 2008). In term of broad spectrum resistance, the definition is commonly used in two meanings (Qu*, et al.*, 2006). The first one is defined as the resistance to a large number of, but not all, strains of one pathogen. The second mean is effective against two or more unrelated pathogens. The broad spectrum for rice blast resistance is normally following the first definition. The resistant gene *Pi-1*(t), *Pi2*, *Pi9*, *Pi20*(t) *Pi27*(t), *Pi39*(t) and *Pikh* have been recognized to confer broad spectrum resistance to many isolates collected from various region (Mackill and Bonman, 1992; Chen*, et al.*, 1996; Luu and Bui, 1999; Liu*, et al.*, 2002; Zhu*, et al.*, 2004; Liu*, et al.*, 2007b; Yang*, et al.*, 2008). Other type of resistance called durable resistance which remains effective during its prolonged and widespread use in environments favorable to the disease spread (Johnson, 1981). The previous studies indicated that multiple major genes conferring broad spectrum qualitative resistance and minor genes conferring quantitative resistance are the genetic bases of durable resistance to different pathogenic races (Wang*, et al.*, 1994; Sallaud*, et al.*, 2003). Whether a broad-spectrum resistance gene is durable or not in multiple locations during a relatively long time is still debatable.

7. Diversity of blast pathogen

The resistance of rice cultivars to the blast disease often breaks down in a few years after released. One of the causes of resistance breakdown is an increment of the prevalence of previously rare pathotypes or the development of novel pathotypes (Ou, 1980). Mutation (Xiong*, et al.*, 2007; Zhou*, et al.*, 2007), sexual hybridization

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(Schardl and Craven, 2003; Sreewongchai, 2009), parasexualism (Genovesi and Magill, 1976; Zeigler*, et al.*, 1997)and heterocaryosis , (Genovesi and Magill, 1976; Chen and Wu, 1977; Crawford*, et al.*, 1986) have been reported as causes of novel pathotype development (Ou, 1980).

Diversity of blast pathogen was found higher in northern, north-eastern and central than eastern and southern parts of Thailand (Sirithunya, et al., 2008). Amplified fragments length polymorphism (AFLP) fragments generated by using 6 primer combinations were classified 779 isolates into 16 main lineages (Hutamekalin, et al., 2001). Significant differences in pathotype diversity were detected using 527 blast isolates collected across sites, seasons from five sites in Thailand which inoculated onto 15 near isogenic lines (NILs) (Mekwatanakarn, et al., 2000).

8. Molecular marker

Molecular marker, a fragment of DNA sequence, can be classified into different groups based on: mode of transmission (biparental nuclear inheritance, maternal nuclear inheritance, maternal organelle inheritance, or paternal organelle inheritance), Mode of gene action (dominant or codominant markers) and method of analysis (hybridization-based or polymerase chain reaction (PCR)-based markers). The hybridization-based marker is represented by Restriction Fragment Length Polymorphism (RFLP). This technique is based on restriction enzymes which only cut the DNA molecule where there is specific DNA, called restriction sites, which reveal a pattern difference between DNA fragment sizes in individual organisms.

PCR (Polymerase Chain Reaction) is an efficiency technique to double the amount of target DNA. The advantages of PCR technique compared to hybridization-base methods include: require small amount of DNA, high polymorphism and no need to know about prior information. PCR-based marker has two types depending on the primers used for amplification (Semagn*, et al.*, 2006). Type I is arbitrary or semi-arbitrary primed PCR techniques that developed without prior sequence information. Type II is site-targeted PCR techniques that developed from known DNA sequences.

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Random amplified polymorphic DNA (RAPD) is the marker that used a single arbitrary short primer (8-12 nucleotides) to randomly amplify DNA template without prior knowledge of the target sequence. PCR product is produced when, have an appropriate annealing temperature, the single primer binds to sites on opposite strands of the genomic DNA that are within an amplifiable distance, generally less than 3,000 base pairs.

Amplified fragment length polymorphism (AFLP) method uses the combination of restriction digestion and PCR technology by ligating primer recognition sequences (adaptors) to the restricted DNA. AFLP consists of two steps. The first step, two restriction enzymes, combination of rare cutter (EcoRI or PstI) and frequent cutter (Msel or Taql), are used to digest genomic DNA. Then, adaptors are ligated to both ends of the fragments to provide known sequences for PCR amplification. For primer, it is able to anneal to fragments which have the adaptor sequence plus the complementary base pairs to the additional nucleotides called selective nucleotides (increased 1-3 nucleotides).

Microsatellites are known as simple sequence repeats (SSRs), short tandem repeats (STRs) or simple sequence length polymorphisms (SSLPs) or variable number of tandem repeats (VNTRs). In higher organisms, multiple copies of simple repetitive DNA sequences are minisatellites and microsatellites which arranged in arrays of vastly differing size. The repeated sequence is often simple, consisting of two, three or four nucleotides (di-, tri-, and tetranucleotide repeats, respectively).

Over the three types of PCR based marker, six molecular markers are broadly used in plant genome studies including: inter-simple sequence repeat (ISSR), a use of microsatellites as single primer (15-30 nucleotides) to amplify an inter simple sequences, expressed sequence tag (EST), a short subsequence of a transcribed cDNA sequence, cleaved amplified polymorphic sequence (CAPS), originally named PCR-RFLP, a combination of the PCR and RFLP, sequence characterized amplified region (SCAR) a sequences of the two ends of the RAPD markers that appeared for specific purposes, sequence tagged site (STS), a short unique sequence which found nowhere else in the genome and characterized by

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sequencing either an RFLP probe and AFLP fragment and single nucleotide polymorphism (SNP), a single base change in a DNA sequence when comparative two DNA sequence.

Nowadays, SSR marker is used to identify the presence of a specific gene or combination of genes that carry desirable traits because of a highly polymorphic even between closely related lines, randomly distributed throughout the genome, required low amount of DNA, codominant action, low cost, highly informative, exchangable between laboratories, and highly transferable between populations. In the rice genome, a total of 18,828 SSRs sequences have been detected by the Rice Genome Mapping project, 2005. Many blast resistance genes were identified by using these SSR markers. For example, in Chinese native cultivar "Q14", *Pi27*(t) is identified determined based on a linkage analysis with SSR markers in the F_2 population developed from Q14 x Q16 cross (Zhu, *et al.*, 2004). *Pi34* is identified in japonica rice, Chubu 32, through RFLP and SSR markers in F_3 population from Koshinikari/Kasalath and Chubu 32 (Zenbayashi, *et al.*, 2005). Nguyen, *et al.* (2006) identified *Pi35*(t) in Hokkai 188 through a linkage analysis with SSR markers in the $F_{2:3}$ progenies from a cross between Hokkai 188 and Danghang-Shali. *Pi39*(t) is identified in Chinese native cultivar, Q15, by using a linkage analysis with SSR markers in F_2 population from a cross of Q15 x Tsuyuake (Liu, *et al.*, 2007b).

9. Identification of blast resistance gene

Genetic studies of blast resistance provide a large amount of information that can be used to improve the resistance in rice cultivars in the breeding program. Since the first publication of resistance to rice blast (Sasaki, 1923), many blast R genes have been identified in both indica and japonica rice. The first Pi gene in rice was named by Kiyosawa (1966). Currently, more than 73 blast resistance genes and approximately 347 QTLs have been identified and documented.

Most of the known blast resistance genes are located on chromosomes 6, 11 and 12. On chromosome 6, at least 13 genes, *Pi2* = *Piz*^{5}, *Piz*, *Piz-t*, *Pi8*(t), *Pi9*, *Pi13*(t), *Pi22*(t), *Pi25*(t), *Pi26*(t), *Pi27*(t), *Pi-d(t)2*, *Pi-gm*(t) and *Pi40*(t) have been

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mapped near the region of centromere (Pan, *et al.*, 1996; Pan, *et al.*, 1998; Ahn, *et al.*, 2000; Zhuang, *et al.*, 2002; Sallaud, *et al.*, 2003; Chen, *et al.*, 2004; Hayashi, *et al.*, 2004; Wu, *et al.*, 2005; Deng, *et al.*, 2006; Qu, *et al.*, 2006; Zhou, *et al.*, 2006; Jeung, *et al.*, 2007). On the long arm of chromosome 11, at least 8 genes as *Pi1*, *Pi7*, *Pi18*, *Pi34*, *Pi38*, *Pi44*, and *Pilm2* and six alleles of the *Pik* locus (*Pik*, Pik^p, Pik^m and Pik^h) have been mapped (Wang, *et al.*, 1994; Chen, *et al.*, 1999; Ahn, *et al.*, 2000; Tabien, *et al.*, 2000; Sharma, *et al.*, 2005; Zenbayashi, *et al.*, 2005; Gowda, *et al.*, 2006; Hayashi, *et al.*, 2006; Li, *et al.*, 2007; Fuentes, *et al.*, 2008; Wang, *et al.*, 2009). On chromosome 12, at least 10 resistance genes and their allelic *Pita*, $Pita^2$, *Pitq6*, *Pi12*(t), *Pi19*(t), *Pi20*(t), *Pi24*(t), *Pi31*(t), *Pi32*(t) and *Pi39*(t) have been mapped in the region near the centromere (Inukai, *et al.*, 1996; Hayashi, *et al.*, 1998; Bryan, *et al.*, 2000; Tabien, *et al.*, 2000; Zhuang, *et al.*, 2002; Sallaud, *et al.*, 2003; Hayashi, *et al.*, 2006; Liu, *et al.*, 2007b; Li, *et al.*, 2008). Of these, only nine blast resistance genes, *Pi2*, *Pi9*, *Pi36*, *Pi37*, *Pi-b*, *Pi-d2*, Pik^h, *Pi-ta* and *Piz-t*, have been cloned (Wang, *et al.*, 1999; Bryan, *et al.*, 2000; Sharma, *et al.*, 2005; Chen, *et al.*, 2006; Qu, *et al.*, 2006; Zhou, *et al.*, 2006; Lin, *et al.*, 2007; Liu, *et al.*, 2007a). Eight of the cloned genes (*Pi2*, *Pi9*, *Pi36*, *Pi37*, *Pib*, Pik^h, *Pi-ta* and *Piz-t*) have a sequence containing both of nucleotide binding site (NBS) and leucine rich repeat (LRR), while *Pi-d2* encodes a receptor-like kinase protein with a predicted extracellular domain of a bulb-type mannose specific binding lectin (B-lectin).

10. Marker assisted selection (MAS) in rice

MAS is the process of using molecular markers to assist the selection of plant material in breeding program. It has many advantages when compared with conventional phenotypic selection (Collard, *et al.*, 2008; Xu and Crouch, 2008) as (1) it could save time, effort, resources and money for some trait that difficult to management, measuring, low heritability and selection depends on specific environment effect, (2) selection can be done at any growth stage and be quickly eliminated undesirable genotype since seedling stage, (3) the desirable plants can be selected at early generation in breeding schemes and can separate homozygous plant and heterozygous plant.

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The MAS has been successfully applied in rice breeding program especially for backcrossing (MAB) and pyramiding (MAP). MAB is the process of using markers to select the target loci (foreground), narrow down the donor segment (recombinant) and recovery of the recurrent parent genome (background) during backcrossing (Hospital, 2001; Collard and Mackill, 2008). The basis of MAB approach is to develop near isogenic line (NIL) carrying the target genes with surly precision than conventional backcrossing. Many rice cultivars were improved by MAB. Pyramiding is the process of combining genes or QTLs from different parents into a single genotype (Collard, *et al.*, 2008). MAB and MAP have been performed to improve two importance diseases, blast and bacterial blight, in rice (Hittalmani, *et al.*, 2000; Davierwala, *et al.*, 2001; Liu, *et al.*, 2003; Toojinda, *et al.*, 2005; Sundaram, *et al.*, 2008; Sreewongchai, *et al.*, 2010). Moreover, these two approaches also used for improvement of other abiotic stress tolerance (Toojinda, *et al.*, 2005; Siangliw, *et al.*, 2007), resistance to brown planthopper (BPH) (Jairin, *et al.*, 2009), quality (Zhou, *et al.*, 2003) and agronomic traits (Nas, *et al.*, 2005).

Liu, *et al.*, (2003) reported on the use of MAB to incorporate a broad spectrum blast resistant gene *Pi1* into an elite hybrid maintainer line, Zhenshan 97. Introgression lines of Zhenshan 97 had significantly improved their resistance to Chinese races. According to RGDU reports, the introgression lines of RD6 contained resistance QTL on chromosomes 1 and 11 from JHN and the introgression lines of RD6 carried two resistance QTLs on chromosomes 2 and 12 from IR64 and Azucena, respectively, showed significant improvement of blast resistance to Thai isolates. Toojinda *et al.* (2005) also reported the use of MAB in transferring favorable alleles of QTL for blast resistance into genetic background of KDML105. Introgression lines of KDML105 showed higher and broader resistance than original KDML105. MAP was used to combined QTL for resistance possessed by IR64 and JHN (Sreewongchai, *et al.*, 2010). The pyramiding lines showed high resistance to broad range of spectrum. Hittalmani, *et al.*, (2000) reported that introgression lines containing the three major blast resistance genes, *Pi1*, Piz^5 and *Pita*, showed higher resistance level to the compatible isolates from Philippine than present alone.

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MATERIALS AND METHODS

1. A virulence spectrum of recombinant strains derived from a sexual recombination between B1-2 and TH16

Materials: A virulence spectrum of recombinant strains derived from a sexual recombination between B1-2 and TH16 (Sreewongchai, *et al.*, 2009) was assessed by inoculating ten rice varieties with eight blast isolates. Rice varieties included KDML105, RD6, JHN, Chainat 1 (CN1), IR57514, IR64, Azucena, FL496, FL530 and Sariceltic. The eight blast isolates included B71, B88, B124, B151, B161, B209, B248 and B259. These isolates were selected from a haploid population derived from a sexual recombination of B1-2 and TH16 isolates.

Blast evaluation: The ten rice varieties were evaluated for their blast resistance at seedling stage using an artificial inoculation. Lesion scores were taken following protocol as described by Roumen, *et al*. (1997).

2. Identification of race specific resistance to the blast isolates B1-2

Plant materials: Genes for race specific resistance to the blast isolates B1-2 were identified using two RIL populations. The first population was derived from a cross between KDML105 and Jao Hom Nin (JHN) (Toojinda, *et al.*, 2003) and consisted of 240 individuals. The second population consisted of 76 individuals derived from a cross between IR57514 and KDML105, developed by Rice Gene Discovery Unit (RGDU). JHN is a non-glutinous rice variety with broad spectrum resistance to blast isolates found in Thailand. It also has an excellent nutritional and cooking quality such as high iron, protein and antioxidant. IR57514 developed by IRRI is a drought and submergence tolerant variety that is well adapted to rainfed lowland areas. This variety confers high level of resistance to many Thai blast isolates. KDML105 is a popular commercial variety. It is non-glutinous aromatic rice with low amylose content. It widely grows in the North and Northeast of Thailand and very susceptible to blast disease.

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Blast isolates: Blast strain B1-2 was collected by Ubon Ratchatani Rice Research Center. It is a rare pathotype found in rainfed lowland areas. KDML105 and RD6 had shown a resistant reaction to B1-2 while JHN had shown moderate susceptible. Three parental cultivars, KDML105, JHN and IR57514 had shown differential responses to B1-2.

Blast evaluation: B1-2 was used to inoculate progenies derived from the KDML105 x JHN and KDML105 x IR57514 crosses at seedling stage using an artificial inoculation. B161 was used to inoculate to progenies derived from the KDML105 x IR57514 cross. Seven days after inoculation, lesion scores were taken following a protocol as described by (Roumen, *et al.*, 1997).

Locating resistance QTLs: In KDML105 x JHN population, modified bulked segregant analysis (Korinsak, *et al.*, 2009) was applied to identify the location of the resistance QTLs. Ten most resistant and 10 most susceptible RILs were selected based on their lesion score. Genomic DNA of the selected RILs and their parents were genotyped with 164 microsatellite markers obtained from the public database (http://www.gramene.org/). Ninety nine microsatellite markers exhibiting differential patterns between resistant and susceptible were genotyped individual RILs. Marker genotypes and disease score were analyzed for their association using STATGRAPHIC 3.0 program and nQTL (Sreewongchai, 2009). The analysis was followed the protocol described by (Siangliw and Sreewongchai, 2006).

In IR57514 x KDML105 population, eighty four microsatellite markers covering 12 chromosomes (Juntaboon *et al*. personal contact) were genotyped the 76 RIL. Linkage map was constructed following the protocol described by (Siangliw and Sreewongchai, 2006). QTL analysis was used to determine the association between genotypic data and disease score. The simple interval mapping (SIM) and the simplified composite interval mapping (sCIM) procedures of the software were applied for data analysis. Each data set was analyzed with 5 cM walk speed, 1,000 permutations and 5% of Type I error.

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3. Pyramiding QTLs for blast resistance

Development of plant material: Two rice varieties, JHN, IR64 and Azucena were used as original source of broad spectrum resistant QTL for blast disease. Four QTLs were introgressed into the glutinous rice variety RD6 by marker assisted backcrossing (MAB) as shown in Figure 1. Introgression line, RGDU334-3-11-1-1-179 developed by RGDU which carried $qBL1_{JHN}$ and $qBL11_{JHN}$ from JHN was used as female parent while two introgression lines, BC_4F_2-160-19-3-8-22 and BC_4F_2-160-19-3-15-3 developed by Plant Breeding Research Center for Sustainable Agriculture which carried $qBL2_{IR64}$ and $qBL12_{Azu}$ from IR64 and Azucena, respectively, were used as male parent. The crosses were made in wet season 2007.

Four F_1 plants were backcrossed to RGDU334-3-11-1-1-179 to produce 100 BC_1F_1 plants. Hereafter, nine molecular markers, RM212, RM319, RM207, RM48, RM144, RM224, RM277, RM313 and BADH (Table 1) were used for genotypic identification of individual BC_1F_1 plant. Two BC_1F_1 plants identified carrying heterozygous alleles of 9 marker loci and one BC_1F_1 plants identified carrying homozygous allele of the flanking markers on chromosome 11 and heterozygous allele of the rest of markers loci were used to produce 281 BC_1F_2 by selfing. Sixty four BC_1F_2 plants identified by MAS to carry some of homozygous allele of the selected marker loci were self pollinated to generate 960 BC_1F_3 plants (15 BC_1F_3 plants per each BC_1F_2 lines). The same set of markers was used to genotype individuals. All combinations of QTLs were identified. One hundred and eighty eight BC_1F_3 plants were selected. The QTL combinations were showed in Table 2. Thirty two introgression lines selected from each combination types (2 lines per combination type) and their original parents were used for evaluation of blast resistance (Table 2). The process of pyramiding was illustrated in Figure 4.

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Table 1 List of molecular markers for marker assisted selection used for development of RD6 pyramiding blast resistance QTL population.

QTLs	Markers	Chr.	Sequence (5'-->3')
$qBL1_{JHN}$	RM212	1	F: CCACTTTCAGCTACTACCAG R: CACCCATTTGTCTCTCATTATG
	RM319	1	F: ATCAAGGTACCTAGACCACCAC R: TCCTGGTGCAGCTATGTCTG
$qBL2_{IR64}$	RM207	2	F: CCATTCGTGAGAAGATCTGA R: CACCTCATCCTCGTAACGCC
	RM48	2	F: TGTCCCACTGCTTTCAAGC R: CGAGAATGAGGGACAAATAACC
$qBL11_{JHN}$	RM144	11	F: TGCCCTGGCGCAAATTTGATCC R: GCTAGAGGAGATCAGATGGTAGTGCATG
	RM224	11	F: ATCGATCGATCTTCACGAGG R: TGCTATAAAAGGCATTCGGG
$qBL12_{Azu}$	RM277	12	F: CGGTCAAATCATCACCTGAC R: CAAGGCTTGCAAGGGAAG
	RM313	12	F: TGCTACAAGTGTTCTTCAGGAC R: GCTCACCTTTTGTGTTCCAC
Aroma	BADH	8	F: TGCTCCTTTGTCATCACACC R: TTTCCACCAAGTTCCAGTGA

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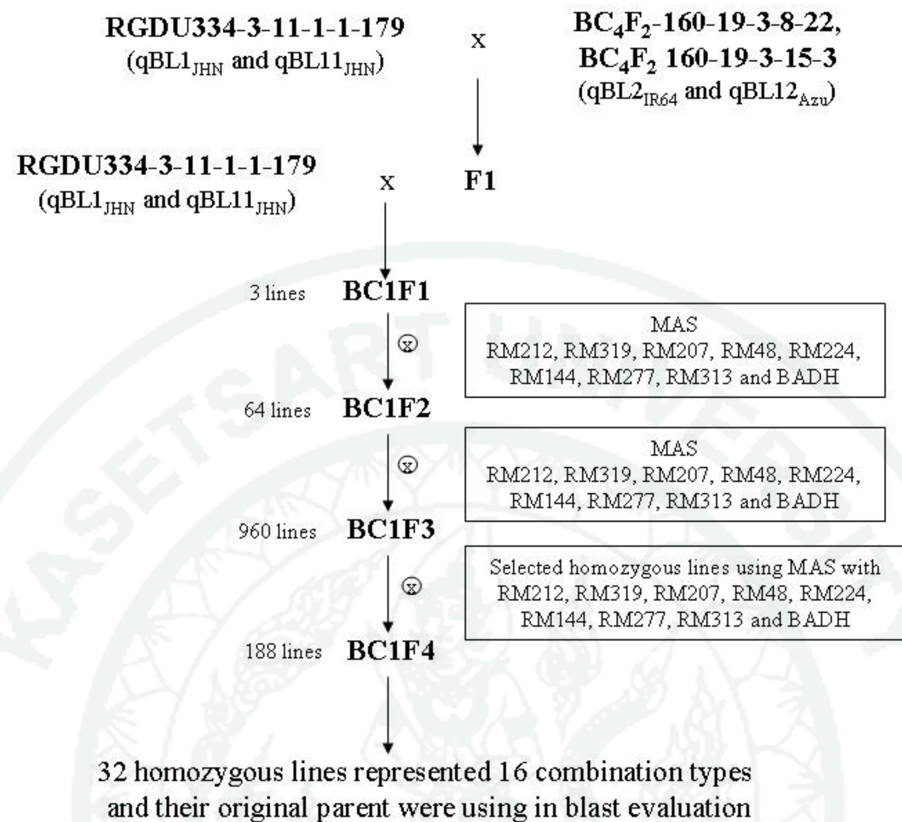

Figure 4 Breeding scheme for the pyramiding blast resistance QTL on chromosome 1, 2, 11 and 12 in RD6 rice variety.

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Table 2 List of the BC_1F_3 introgression lines, original parents and their QTL combinations.

Entry	QTL combination	Varieties/ Introgression lines
1	-	RD6
2	$qBL1_{JHN} + qBL11_{JHN}$	JHN
3	$qBL2_{IR64}$	IR64
4	$qBL12_{Azu}$	Azucena
5	-	RGDU07079-6-122-1
6	-	RGDU07079-6-176-9
7	$qBL1_{JHN}$	RGDU07079-6-122-5
8	$qBL1_{JHN}$	RGDU07079-6-176-13
9	$qBL2_{IR64}$	RGDU07079-6-122-12
10	$qBL2_{IR64}$	RGDU07079-7-228-8
11	$qBL11_{JHN}$	RGDU07123-12-247-2
12	$qBL11_{JHN}$	RGDU07079-7-190-6
13	$qBL12_{Azu}$	RGDU07079-7-227-11
14	$qBL12_{Azu}$	RGDU07079-7-236-7
15	$qBL1_{JHN} + qBL2_{IR64}$	RGDU07079-6-131-6
16	$qBL1_{JHN} + qBL2_{IR64}$	RGDU07079-6-131-8
17	$qBL1_{JHN} + qBL11_{JHN}$	RGDU07123-12-203-5
18	$qBL1_{JHN} + qBL11_{JHN}$	RGDU07123-12-231-8
19	$qBL1_{JHN} + qBL12_{Azu}$	RGDU07079-7-195-7
20	$qBL1_{JHN} + qBL12_{Azu}$	RGDU07079-7-218-11
21	$qBL2_{IR64} + qBL11_{JHN}$	RGDU07123-12-164-15
22	$qBL2_{IR64} + qBL11_{JHN}$	RGDU07123-12-200-2
23	$qBL2_{IR64} + qBL12_{Azu}$	RGDU07079-7-219-13
24	$qBL2_{IR64} + qBL12_{Azu}$	RGDU07079-7-228-13
25	$qBL11_{JHN} + qBL12_{Azu}$	RGDU07079-6-140-4
26	$qBL11_{JHN} + qBL12_{Azu}$	RGDU07079-7-186-8
27	$qBL1_{JHN} + qBL2_{IR64} + qBL11_{JHN}$	RGDU07123-12-200-15
28	$qBL1_{JHN} + qBL2_{IR64} + qBL11_{JHN}$	RGDU07079-6-113-1
29	$qBL1_{JHN} + qBL2_{IR64} + qBL12_{Azu}$	RGDU07079-7-180-14
30	$qBL1_{JHN} + qBL2_{IR64} + qBL12_{Azu}$	RGDU07079-7-235-15
31	$qBL1_{JHN} + qBL11_{JHN} + qBL12_{Azu}$	RGDU07123-12-245-6
32	$qBL1_{JHN} + qBL11_{JHN} + qBL12_{Azu}$	RGDU07079-7-190-11
33	$qBL2_{IR64} + qBL11_{JHN} + qBL12_{Azu}$	RGDU07123-12-147-15
34	$qBL2_{IR64} + qBL11_{JHN} + qBL12_{Azu}$	RGDU07123-12-189-2
35	$qBL1_{JHN} + qBL2_{IR64} + qBL11_{JHN} + qBL12_{Azu}$	RGDU07123-12-165-7
36	$qBL1_{JHN} + qBL2_{IR64} + qBL11_{JHN} + qBL12_{Azu}$	RGDU07079-7-235-5

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Blast isolates: Ninety six blast isolates were used for disease evaluation of the introgression lines. These isolates represent the genetic diversity found in the rainfed lowland in Thailand (Hutamekalin, *et al.*, 2001). The list of blast isolates was showed in a Table 8.

Blast evaluation: The blast evaluation was performed by using an artificial inoculation at seedling stage. The original parents (RD6, JHN, IR64 and Azucena), eight single QTL introgression lines, two introgression lines without the QTL and susceptible check, Saricletik, were tested for blast resistance using ninety six blast isolates. Eleven pathogenic isolates to RD6 but fail to infect JHN, IR64 and Azucena were selected for validation of the 22 introgression lines that combined two, three or four resistance QTL. The disease assessment was followed a protocol as described by (Roumen, *et al.*, 1997).

4. DNA marker genotyping

4.1 Extraction of genomic DNA

Genomic DNA was isolated from leaves using DNA Trap Kit (DNA Technology Laboratory, Thailand). Fresh leaves were placed in a 1.5 ml tube and ground with liquid nitrogen using a sterile plastic rod. Ground tissue was added 1,000 µl of Extraction buffer and incubated at 65 ^{o}C for one hour. After, the tube was placed on ice for 5 min. followed by adding 100 µl of Neutralizing buffer and mixed it together and put its on ice again for 5 min. Centrifuge the tube at 14,000 rpm for 10 min. and then transferred the supernatant into a new 1.5 ml tube. Added 500 µl of Trapping buffer and mixed thoroughly. After briefly centrifuge and discarde the supernatant, pellets were washed by 500 µl of wash buffer I, propanol. Collected the pellets by centrifuge the tube at 2,000 rpm for 1 min. and removed the supernatant. Wash pellets again with 500 µl of washing buffer II, 95% ethanol, and air dried. DNA was eluted from the pellets by adding 100 µl of distilled water (dH_2O) and incubating at 65 ^{o}C for 30 min. The DNA concentration was compared with the intensity of standard DNA concentration band.

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4.2 Polymerase Chain Reaction (PCR) amplification condition

PCR was performed in a 10 µl reaction mixture containing 20-25 ng of template DNA, 1X PCR buffer, 2.5 mM $MgCl_2$, 0.25 µM of each primer, 0.2 mM of each dNTP, and 0.5 unit Taq polymerase. Amplification began with a denaturation step of 94 ^{o}C for 3 min followed by 35 cycles (30 sec at 94 ^{o}C, 30 sec at 55 ^{o}C and 2 min at 72 ^{o}C) and completed by 7 min incubation at 72 ^{o}C. The PCR products were separated by electrophoresis in 4.5% denaturing polyacrylamide gel and the banding patterns were visualized using silver staining.

5. Assessment of blast resistance

Blast isolates were cultured on the agar (Rice bran 20 g, Agar 20 g, Yeast extract 2 g and dH_2O 1 liter) in Petri dish, incubated at 27 ^{o}C for 7 days. The mycelia were scarped with glass rod then plates were transferred to a cabinet with black light for 2 days to induce sporulation. On the day of inoculation, dH_2O was added into the Petri dish; the mycelial mat was scraped with glass rod. The suspension was transferred to test tube and adjusted a concentration to 5 x 10^5 conidia per ml in 0.5% gelatin.

Inoculum was sprayed onto 14 day-old rice seedling that planted in plastic tray with soil at the greenhouse by using air brush spray. Inoculated plants were incubated at 25 ^{o}C, 100% humidity for over night (at least 16 hours). On the next day, the inoculated plants were returned to the greenhouse. Lesion scores were recorded at 7 days after inoculation. Lesion score was scored based on infection type or disease severity index using a scale of 0-6. The classification of resistance and susceptible was followed as described by (Roumen*, et al.*, 1997). Lesion scores are showed in Figure 5.

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Figure 5 Lesion scores based on 7 lesions type categories as

0 = No evidence of infection

1 = Brown pinpoint smaller than 0.5 mm., without sporulation

2 = Brown pinpoint smaller than 0.5-1 mm., without sporulation

3 = mall eyespot about 1-3 mm., lesion capable of sporulation gray center

4 = Small eyespot about 3 mm. or more longs, lesion capable of sporulation gray center and dark margin

5 = Susceptible sporulation type, coalescence lesion without dark margin

6 = Susceptible sporulation type, lesion without dark margin

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RESULTS

1. A virulence spectrum of recombinant strains derived from a sexual recombination between B1-2 and TH16

Virulence spectrum of recombinant isolates, B71, B88, B124, B151, B161, B209 and B248 was identified through ten rice varieties as presented in Table 3 Virulence or avirulence of pathogenic isolates to rice has been identified toward particular rice varieties. Parental blast strain B1-2 isolated from rice showed virulent reactions to rice varieties Saliceltik and JHN while TH16 isolated from barley showed a virulent reaction only to Saliceltik. Pathogenicity is a complex phenotype, involving such distinct components as infection efficiency (determines lesion number), rate of lesion development, extent of colonization (determines lesion size), and efficiency of sporulation. In our study, we used lesion size as determined by the disease score to compare the pathogenicity. Through 10 rice varieties, B1-2 was more virulent than TH16. Recombinant strains B71, B124, B151, B161 and B248 had shown higher pathogenicity on rice varieties than the parental strains. The strain B124 is the most virulence in our study. It can infect most rice varieties except JHN, IR64 and CN1. According to our result, the sexual recombination not only yielded virulence strains but also avirulence strains. For examples, B71, B151, B161, B248 and B259 strains, fail to infect CN1 while the B1-2 and TH16 can.

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Table 3 Pathogenicity of the eight recombinant strains and their parents based a differential reaction on the ten rice varieties. Disease scores were rated at seedling stage following the 0-6 scales described by International Rice Research Institute (IRRI).

Varieties	Blast isolates									
	B71	B88	B124	B151	B161	B209	B248	B259	B1-2	TH16
IR57514	1	1	3	2	1	1	2	0	1	1
KDML105	2	2	3	1	2	1	3	1	2	1
JHN	1	2	2	1	1	1	1	2	4	1
RD6	2	2	3	1	3	1	2	1	2	1
IR64	2	1	1	1	2	1	1	1	2	1
Azucena	2	2	5	4	3	2	2	2	2	2
FL496	3	2	3	2	3	1	3	1	2	1
FL530	3	2	3	1	2	1	2	1	2	1
CN1	0	1	2	0	0	1	0	0	1	1
Sariceltick	6	6	6	6	6	6	6	6	6	6

2. Identification of race specific resistance in two RIL populations

2.1 KDML105 x JHN population

2.1.1 Phenotypic distribution

Frequency distribution of lesion score obtained on 240 RILs is illustrated in Figure 6. Averages of lesion score were were ranged from 0.4 - 1.4 and 3.3 – 4.7 for KDML105 and JHN respectively. When lesion scores of the RILs were classified into 2 groups, resistant (0, 1 and 2) and susceptible (3, 4, 5 and 6), the chi-square (χ^2) test showed a goodness-of-fit with a 1R:3S ratio (χ^2 = 5.0) at probability value of 0.01. It is indicated that the lesion scores of the RIL are probably determined by two independent genes with an additive action.

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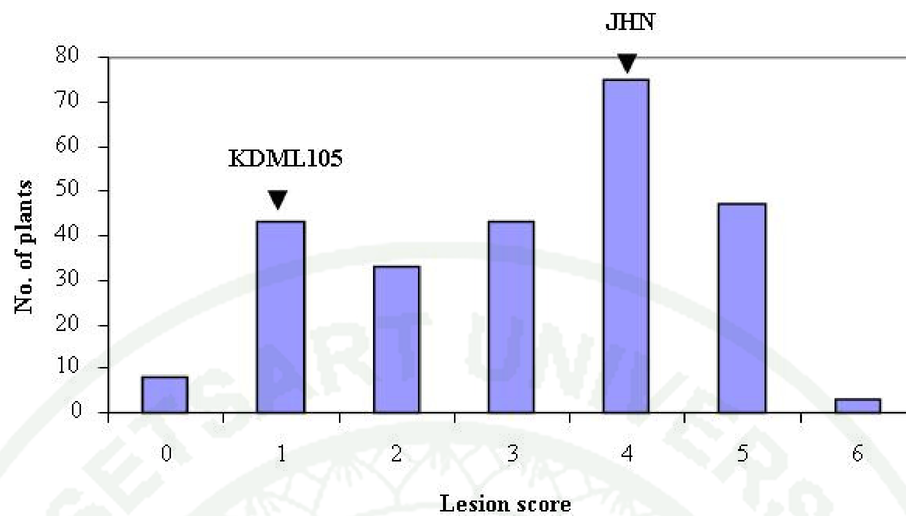

Figure 6 Distribution frequency of the lesion scores obtained from 240 RIL of a cross between KDML105 and JHN after inoculated with blast fungus isolate B1-2. The average lesion score of KDML105 and JHN were ranged from 0.4 - 1.4 and 3.3 – 4.7 respectively.

2.1.2 Identification of closely linked markers

Ninety nine markers showed very clear polymorphisms between KDML105 and JHN were genotyped 20 RIL individuals (10 resistant and 10 susceptible) and their parents. Only 4 microsatellite markers, RM208, RM213, RM72 and RM310 had shown discriminated bands between the resistance and susceptible RILs but the markers located outside this four markers as RM6 and RM207 on chromosome 2 and RM152 and RM44 on chromosome 8 had not identified clear band pattern between two groups (Table 4 and 5 and Figure 7 and 8). These markers were used to genotype the whole RIL population and constructed a partial linkage map for QTL analysis.

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Table 4 Marker genotypes of 10 most resistant, 10 most susceptible RILs on chromosome 2. KD = homozygous KDML105, JHN = homozygous JHN and M = missing data.

Entry	**Resistance reaction**	**Genetic position (Mb)**	**29.5**	**34.6**	**35.1**	**35.3**
		RIL lines	**RM6**	**RM213**	**RM208**	**RM207**
1	Resistance	46	JHN	JHN	JHN	JHN
2	Resistance	62	JHN	KD	JHN	JHN
3	Resistance	89	KD	KD	KD	KD
4	Resistance	91	KD	KD	KD	KD
5	Resistance	144	JHN	JHN	JHN	JHN
6	Resistance	162	JHN	KD	KD	KD
7	Resistance	172	JHN	KD	KD	KD
8	Resistance	197	KD	KD	KD	KD
9	Resistance	318	JHN	KD	KD	KD
10	Resistance	332	KD	JHN	KD	KD
11	Susceptible	8	JHN	JHN	JHN	JHN
12	Susceptible	23	JHN	JHN	JHN	JHN
13	Susceptible	55	KD	JHN	JHN	JHN
14	Susceptible	136	JHN	JHN	JHN	KD
15	Susceptible	139	KD	M	KD	KD
16	Susceptible	159	JHN	JHN	JHN	JHN
17	Susceptible	266	JHN	JHN	JHN	JHN
18	Susceptible	276	JHN	JHN	JHN	JHN
19	Susceptible	341	KD	JHN	JHN	KD
20	Susceptible	350	JHN	JHN	JHN	JHN

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Table 5 Marker genotypes of 10 most resistant, 10 most susceptible RILs on chromosome 8. KD = homozygous KDML105, JHN = homozygous JHN and M = missing data.

Entry	Resistance reaction	Genetic position (Mb)	0.6	5.1	6.7	11.7
		RIL lines no.	RM152	RM310	RM72	RM44
1	Resistance	46	JHN	KD	KD	KD
2	Resistance	62	KD	KD	KD	JHN
3	Resistance	89	JHN	KD	KD	JHN
4	Resistance	91	JHN	KD	KD	KD
5	Resistance	144	KD	KD	KD	KD
6	Resistance	162	KD	KD	KD	KD
7	Resistance	172	KD	KD	KD	KD
8	Resistance	197	KD	KD	KD	JHN
9	Resistance	318	JHN	KD	KD	KD
10	Resistance	332	KD	KD	KD	KD
11	Susceptible	8	KD	JHN	JHN	JHN
12	Susceptible	23	JHN	JHN	JHN	KD
13	Susceptible	55	KD	KD	JHN	JHN
14	Susceptible	136	JHN	JHN	JHN	KD
15	Susceptible	139	JHN	JHN	JHN	KD
16	Susceptible	159	JHN	KD	KD	KD
17	Susceptible	266	KD	JHN	JHN	KD
18	Susceptible	276	KD	JHN	JHN	JHN
19	Susceptible	341	KD	JHN	JHN	JHN
20	Susceptible	350	JHN	JHN	KD	KD

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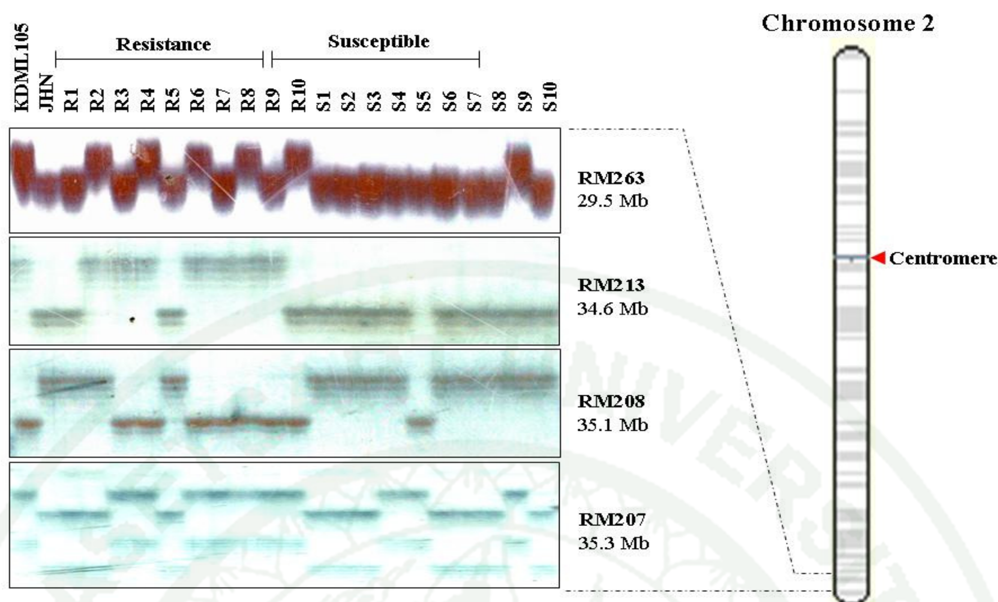

Figure 7 The position of SSR markers located on chromosome 2. Two markers, RM213 and RM208, had shown the discriminated banding pattern between the resistance and susceptible RILs while two orther markers RM263 and RM207 hadn't.

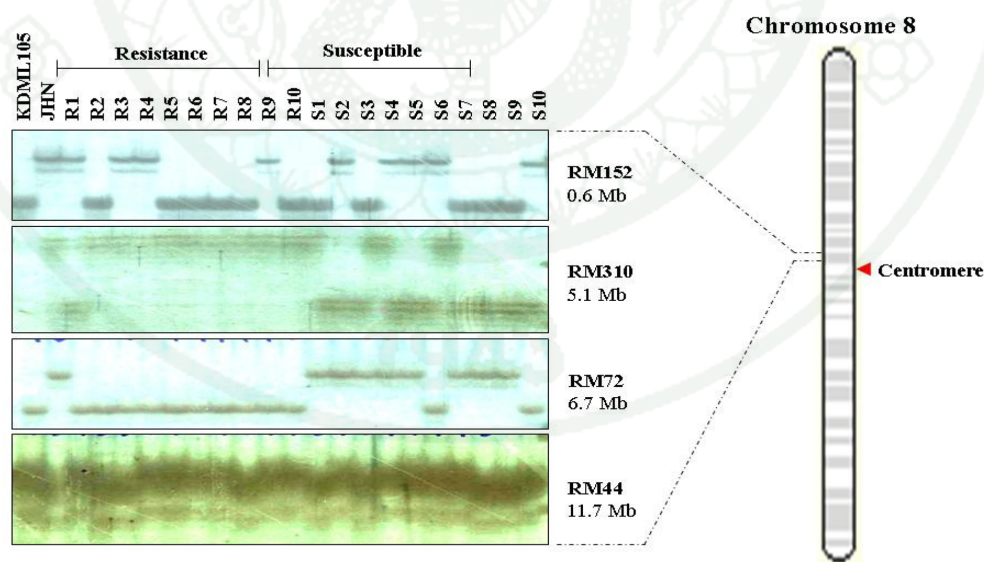

Figure 8 The position of SSR markers located on chromosome 8. Two markers, RM310 and RM72, had shown the discriminated banding pattern between the resistance and susceptible RILs while two orther markers RM152 and RM44 hadn't.

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2.1.3 Association between phenotype and genotype and QTL mapping

Four microsatellite markers, RM72, RM208, RM213 and RM310, were used to construct the two partial linkage group using MAPMEKER 3.0 software. These linkage groups were located on long arm of chromosome 2 (RM208 and RM213) and on short arm of chromosome 8 (RM72 and RM310). The distance between RM208 and RM213 was 7.6 cM while RM72 located away from RM310 at 6.1 cM.

QTL analysis identified two QTL, $qBL2_{KD}$ and $qBL8_{KD}$ located on chromosome 2 and 8 respectively determining the blast resistance against B1-2 as showed in Table 6. This result confirms the association between lesion score and microsatellite markers identified by modified bulk segregrant analysis. Small effect QTL, $qBL2_{KD}$ was detected between RM213 and RM208 with LOD score of 1.83 while large effect QTL, $qBL8_{KD}$ was detected between RM310 and RM72 with LOD score of 53.61. The phenotypic variance explained (PVE) was 3.33 % and 65.12 % for $qBL2_{KD}$ and $qBL8_{KD}$ respectively. KDML105 contributed the favorable allele at both loci and the total PVE was 68.49 %. Figure 9 illustrated the association between lesion scores and allelic pattern of RM72. Allelic combination of RM310 and RM72 showed strong relationship with lesion scores (Figure 10). Most of susceptible RILs were skewed toward homozygous JHN allele.

Table 6 Blast resistance loci against isolate B1-2 identified in KDML105 x JHN population.

Isolate	QTLs	Chr.	Interval marker	LOD score	R2	Total R2	Contributor
B1-2	$qBL2_{KD}$	2	RM213-RM208	1.83	3.33**	68.49**	KDML105
	$qBL8_{KD}$	8	RM310-RM72	53.61	65.12**		

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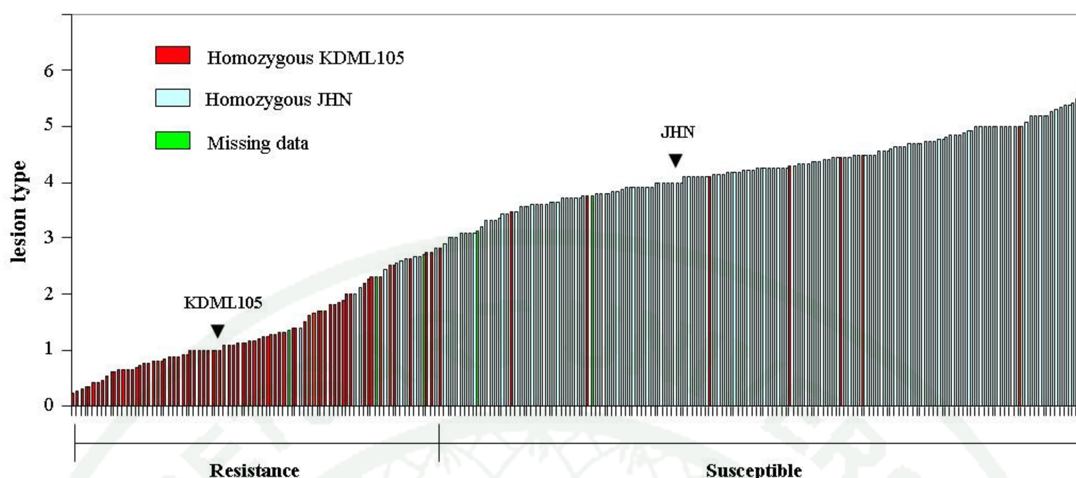

Figure 9 Distribution of lesion scores of 240 RIL and their association with the alleles of RM72. The red, light blue and green bars represented the homozygous KMDL105 allele, homozygous JHN allele and missing data, respectively.

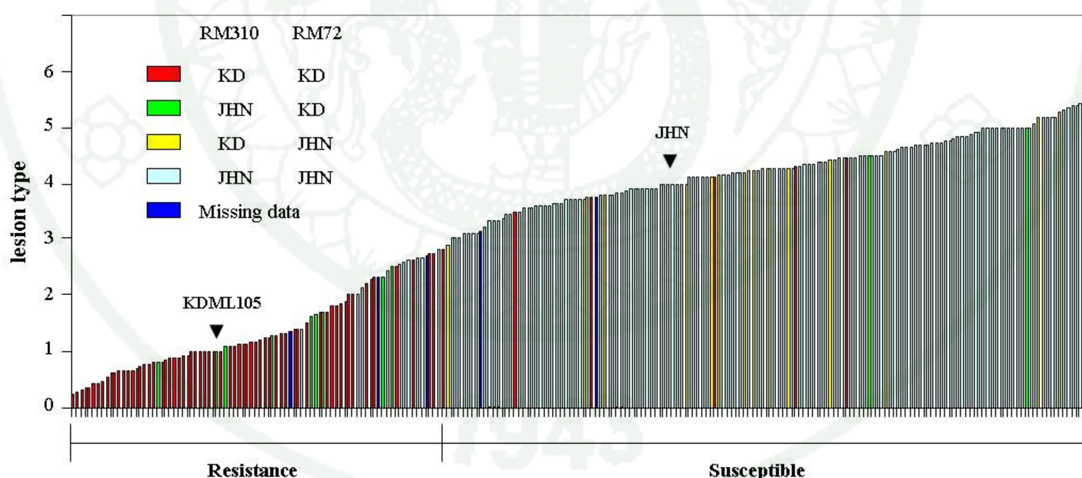

Figure 10 Distribution of lesion scores of 240 RIL and their association with the alleles of RM310 and RM72. Red bar = homozygous KMDL105 allele at RM310 and RM72, Green bar = homozygous JHN allele at RM310 and homozygous KMDL105 allele at RM72, Yellow bar = homozygous KMDL105 allele at RM310 and homozygous JHN allele at RM72, Light blue = homozygous JHN allele at RM310 and RM72 and Blue = missing data.

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2.2 IR57514 x KDML105 population

2.2.1 Phenotypic distribution

Blast isolates B1-2 and B161 had failed to infect IR57514 (lesion score 0) while it infects KDML105 (lesion score 1-2). The phenotypic distribution of lesion scores was showed in Figure 11. Lesion scores of IR57514 and KDML105 were 0.0 - 0.4 and 0.1 - 1.5 respectively for B1-2 and 0.0 - 0.6 and 0.9 – 1.9 respectively for B161. When infection efficiency was considered, lesion scores of the RIL were classified into 2 groups, low infection efficiency (lesion score of 0) and high infection efficiency (lesion scores of 1 and 2). The segregation ratio fitted with 1:1 at probability value of 0.01 for both of B1-2 and B161 at χ^2 = 0.84 and 5.26, respectively.

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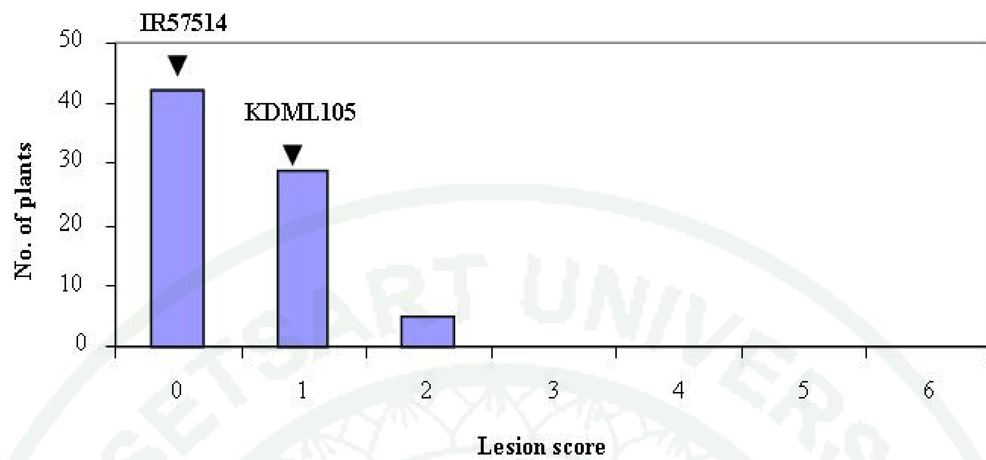
a) B1-2



b) B161

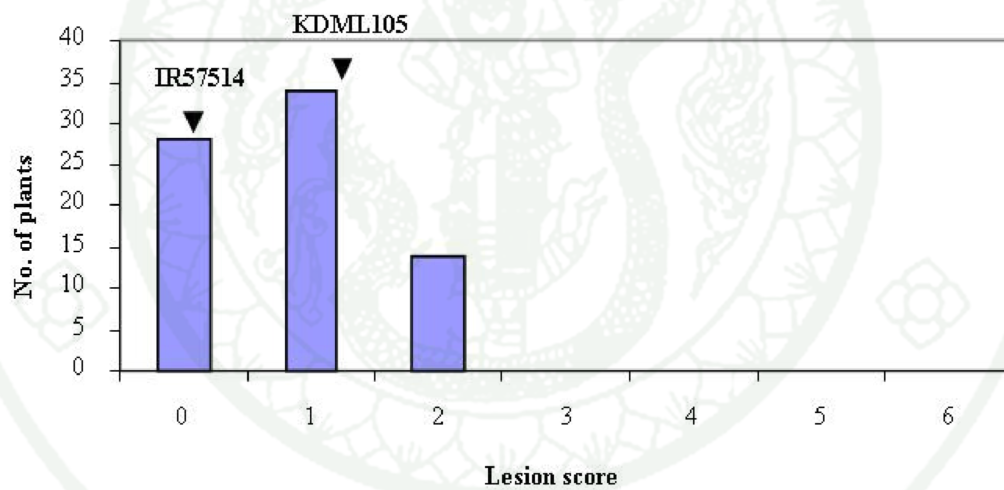


Figure 11 Frequency distributions of the lesion score obtained from 76 RIL
a) inoculated with B1-2, the average lesion scores were ranged from 0.0 - 0.4 and 0.1 - 1.5 for IR57514 and KDML105 respectively.
b) inoculated with B161, the average lesion scores were ranged from 0.0 - 0.6 and 0.9 – 1.9 for IR57514 and KDML105 respectively.

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2.2.2 Linkage map construct and QTL analysis

Linkage map of the IR57514 x KDML105 population was constructed based on eighty five microsatellite markers using MAPMEKER 3.0 software. Twelve linkage groups were determined in which each of linkage group spanned 126.3, 190.9, 56.3, 146.1, 116.1, 90.3, 136.3, 120.3, 4.3, 63.0, 55.9 and 105.7 cM on chromosomes 1-12 respectively. The 12 linkage groups spanned 1211.5 cM with an average marker density of 14.42 cM per marker. All linkage groups were showed in Appendix Figure 1.

Two QTLs were identified and located on chromosomes 1 (RM495-RM84) and 6 (RM314-RM564) against each isolate. For isolate B1-2, $qBL1.1_{IR}$ was detected with LOD score of 4.77 while $qBL6.1_{IR}$ was detected with LOD score of 5.79. The PVE of each QTL were 25.48 % and 28.73 % for $qBL1.1_{IR}$ and $qBL6.1_{IR}$ respectively. These two QTL explained 41.51 % of the variance of lesion scores. For the isolate B161, $qBL1.2_{IR}$ was detected with LOD score of 4.20 and $qBL6.2_{IR}$ was detected with LOD score of 7.50. The PVE of each QTL were 19.83 % and 35.52 % for $qBL1.2_{IR}$ and $qBL6.2_{IR}$ respectively. These two QTL explained 39.10 % of the variance of lesion scores. For all detected QTL, IR57514 contributed favorable alleles as showed in Table 7 and Figure 12.

Table 7 Blast resistant QTL detected for blast isolate B1-2 and B161 in IR57514 x KDML105 population**.**

Isolates	**QTLs**	**Chr.**	**Interval of QTL peak**	**LOD score**	**R^2**	**Total R^2**	**Contributor**
B1-2	$qBL1.1_{IR}$	1	RM495-RM84	4.77	25.48**	41.51**	IR57514
	$qBL6.1_{IR}$	6	GT11-RM564	5.79	28.73**		
B161	$qBL1.2_{IR}$	1	RM495	4.20	19.83**	39.10**	IR57514
	$qBL6.2_{IR}$	6	GT11-RM564	7.50	35.52**		

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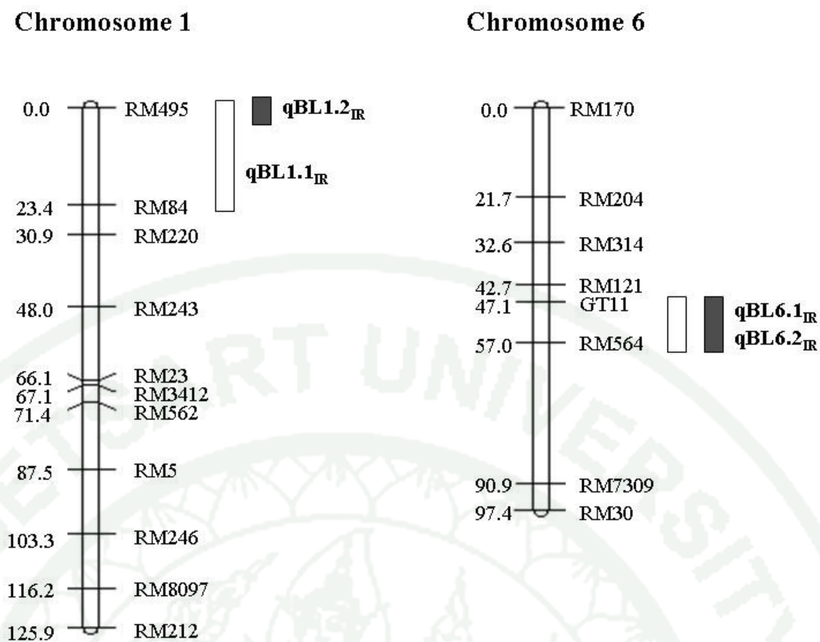

Figure 12 Locations of resistance QTL identified on chromosomes 1 and 6 in the IR57514 x KDML105 population. White bars were the resistance QTL against B1-2 isolate and gray bars were the resistance QTL against B161 isolate.

3. Pyramiding QTLs for blast resistance

3.1 Evaluation of blast resistance in parental and single QTL introgression lines.

The original parents (RD6, JHN, IR64 and Azucena), eight single QTL introgression lines, two introgression lines without the QTL and susceptible check Sariceltik were evaluated for blast resistance with 96 blast isolates. The lesion scores were presented in Table 8. We classified resistance reaction based on lesion scores into three types as resistance (R: lesion score 0, 1 and 2), moderate resistance (MR: lesion score 3 and 4) and susceptible (S: lesion score 5 and 6). RD6 showed high resistance to 60 isolates, moderate resistance to 16 isolates and susceptible to 20 isolates. Most of virulence isolates infecting the RD6 belongs to the AFLP group 3. Resistance variety, JHN showed high resistance to all isolates except B1-2 while IR64

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showed very high resistance to 92 isolates and moderate resistance to 4 isolate, THL47, THL84, THL557 and B1-2. Azucena showed high resistance to 72 isolates, moderate resistance to 14 isolates and susceptible to 10 isolates. The susceptible check, Sariceltik, showed susceptibility to all blast isolates.

The single QTL introgression lines carried $qBL1_{JHN}$ and $qBL11_{JHN}$ derived form JHN showed a broad spectrum resistance to isolates found in the rainfed lowland areas while single QTL introgression lines carried $qBL2_{IR64}$ and $qBL12_{Azu}$ derived from IR64 and Azucena, respectively, showed race specific resistance. The introgression lines without the QTL showed the same resistance patterns as the recipient RD6 for most of isolates except for the THL984 in which they showed more susceptible.

In case of high virulence to RD6 (RD6 showing susceptible), the single QTL introgression lines with $qBL1_{JHN}$ showed resistance and moderate resistance to most of tested isolates except for THL658 and THL983. The single QTL introgression lines with $qBL2_{IR64}$ or without any QTL showed susceptible to all of isolates while single QTL introgression lines with $qBL11_{JHN}$ showed high resistance to all isolates. The single QTL introgression lines with $qBL12_{Azu}$ showed moderate resistance to most of the isolates.

In case of virulence to RD6 (RD6 showing moderate resistance), the single QTL introgression lines with $qBL1_{JHN}$ or $qBL12_{Azu}$ showed resistance and moderate resistance to these isolates. The single QTL introgression lines with $qBL2_{IR64}$ showed moderate resistance and susceptible to most of these isolates except for THL156. The single QTL introgression lines with $qBL11_{JHN}$ were resistance to most of these isolates except for THL119, THL307 and B1-2 that showed reaction pattern like RD6. The introgression lines without any QTL were resistance and moderate resistance to most of these isolates except for THL930, THL934 and THL984.

In case of non virulence (RD6 showing resistance), the single QTL introgression lines with $qBL1_{JHN}$ or $qBL2_{IR64}$ or without any QTL showed resistance

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to all of these isolates. The single QTL introgression lines with $qBL11_{JHN}$ or $qBL12_{Azu}$ showed resistance to most of these isolates except for THL42 for $qBL11_{JHN}$ introgression lines and THL60 for $qBL11_{JHN}$ and $qBL12_{Azu}$ QTL introgression lines.

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Table 8 Resistance reactions of RD6, JHN, IR64, Azucena, Sariceltik, single and none QTL introgression lines and susceptible check, Sariceltik, inoculated with 96 blast isolates. Introgression line no. 1 = RGDU07079-6-122-5, 2 = RGDU07079-6-176-13, 3 = RGDU07079-6-122-12, 4 = RGDU07079-7-228-8, 5 = RGDU07123-12-247-2, 6 = RGDU07079-7-190-6, 7 = RGDU07079-7-227-11, 8 = RGDU07079-7-236-7, 9 = RGDU07079-6-122-1 and 10 = RGDU07079-6-176-9.

Entry	Isolates	RD6	JHN	IR64	Azucena	Sariceltik	Introgression lines no.										AFLP group	Blast collected province
							$qBL1_{JHN}$		$qBL2_{IR64}$		$qBL11_{JHN}$		$qBL12_{Azu}$		None QTL			
							1	2	3	4	5	6	7	8	9	10		
1	THL118	6	0	0	1	6	3	4	6	6	0	1	4	5	6	6	3	Chiang Rai
2	THL393	6	0	0	2	6	2	3	5	6	0	0	5	5	6	6	-	Nakorn ratchasima
3	THL658	6	0	1	1	6	5	-	6	6	1	1	5	5	5	6	3	Chiang Rai
4	THL983	6	0	0	1	6	4	5	6	5	0	1	5	4	5	6	3	Nongkhai
5	THL985	6	0	0	2	6	4	3	5	6	0	1	3	4	6	5	3	Nongkhai
6	THL1081	6	0	0	2	6	3	3	6	5	1	1	5	5	6	6	3	Nongkhai
7	THL456	6	0	0	2	6	4	4	6	6	1	1	5	5	6	6	3	Sakon Nakon
8	THL653	6	0	0	1	6	3	3	6	5	0	0	4	4	6	5	3	Chiang Mai
9	THL122	5	1	1	2	6	3	3	5	5	1	1	4	4	5	4	13	Chiang Rai
10	THL185	5	1	1	3	6	4	3	5	6	1	0	4	5	5	5	3	Chiang Rai
11	THL1067	5	0	0	1	6	2	2	5	6	0	0	4	4	6	6	-	Chiang Mai
12	THL266	5	0	1	2	6	4	4	5	6	1	1	4	5	5	5	3	Lampang
13	THL190	5	0	1	2	6	4	4	5	6	0	1	4	5	6	5	UN	Phitsanulok
14	THL810	5	0	0	2	6	2	2	6	5	1	1	5	4	5	6	3	Ubon Ratchathani
15	THL1119	5	0	1	2	6	4	3	6	5	1	1	4	4	6	6	3	Nongkhai
16	THL1135	5	0	0	3	6	3	4	6	6	1	1	5	5	6	6	6	Lamphun
17	THL289	5	1	1	2	5	4	3	5	5	1	1	4	4	5	5	-	Lampang

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Table 8 (continued)

Entry	Isolates	RD6	JHN	IR64	Azucena	Sariceltik	Introgression lines										AFLP group	Blast collected province
							$qBL1_{JHN}$		$qBL2_{IR64}$		$qBL11_{JHN}$		$qBL12_{Azu}$		None QTL			
							1	2	3	4	5	6	7	8	9	10		
18	THL291	5	0	0	1	6	2	3	5	4	0	0	4	3	4	5	3	Lampang
19	THL879	5	1	1	1	6	4	4	6	6	1	1	4	4	5	6	-	Chumpon
20	THL1066	5	0	0	1	6	2	1	5	5	0	0	4	5	6	6	UN	Chiang Mai
21	THL934	4	0	0	2	-	3	3	4	5	0	0	3	4	5	5	3	Nongkhai
22	THL930	4	0	0	0	5	1	2	4	5	0	0	2	3	5	4	3	Nongkhai
23	THL212	4	0	0	1	5	2	2	5	5	1	0	3	3	4	4	3	Chiang Rai
24	THL119	4	4	1	4	6	3	3	4	4	4	3	3	3	4	4	3	Chiang Rai
25	THL262	4	0	0	0	6	1	1	5	4	0	0	3	2	4	3	3	Lampang
26	THL690	4	0	1	1	6	3	3	4	4	1	1	4	3	3	4	6	Lamphun
27	THL868	4	0	0	2	6	2	2	4	4	0	0	3	3	4	4	UN	Sri saket
28	THL832	3	0	0	3	6	3	3	3	4	0	0	3	3	4	3	4	Mae Hong Son
29	THL984	3	0	0	2	6	3	3	6	5	1	0	4	5	6	6	3	Nongkhai
30	THL906	3	1	1	2	6	3	3	5	5	1	1	4	3	4	3	12	Yala
31	THL486	3	0	0	1	6	2	1	3	3	0	0	3	2	2	3	UN	Tak
32	THL759	3	0	0	2	6	1	2	2	3	0	1	2	2	2	2	4	Mae Hong Son
33	THL307	3	0	1	5	6	2	1	3	5	2	3	3	3	3	3	-	Chiang Mai
34	B1-2	3	5	4	3	6	3	3	3	3	4	3	3	3	4	4	-	Ubon Ratchathani
35	THL156	3	0	1	2	6	1	2	2	2	1	0	1	2	2	1	-	Chiang Mai
36	THL277	3	1	1	4	6	3	1	3	3	1	1	2	2	2	2	UN	Chai nat
37	THL252	2	0	1	2	6	2	3	3	4	0	0	2	3	3	3	-	Chai nat
38	THL897	2	0	1	1	6	2	2	2	2	1	1	2	3	3	2	UN	Suratthani

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Table 8 (continued)

Entry	Isolates	RD6	JHN	IR64	Azucena	Sariceltik	Introgression lines: qBL1$_{JHN}$		qBL2$_{IR64}$		qBL11$_{JHN}$		qBL12$_{Azu}$		None QTL		AFLP group	Blast collected province
							1	2	3	4	5	6	7	8	9	10		
39	THL210	2	0	0	1	6	1	1	1	-	-	0	2	1	2	2	1	Chiang Mai
40	THL142	2	2	1	4	6	1	1	1	1	1	1	1	1	1	2	-	Chiang Mai
41	THL496	2	2	1	4	6	2	1	2	2	2	1	2	2	1	1	UN	Chiang Mai
42	THL757	2	2	1	3	6	2	2	1	2	2	-	2	2	2	2	14	Mae Hong Son
43	PHI36	2	1	1	2	6	1	2	1	2	1	1	1	2	2	1	-	Chiang Mai
44	THL152	2	2	0	2	6	1	1	2	2	3	2	2	2	2	3	1	Chiang Mai
45	THL458	2	1	0	2	6	1	2	1	3	1	1	2	2	3	2	-	Surin
46	THL42	2	3	0	2	6	2	2	2	3	3	3	2	2	3	2	1	Chiang Mai
47	THL1077	2	0	1	1	5	1	1	1	1	0	0	1	2	2	2	UN	Lampang
48	TH12	2	3	1	5	6	1	2	1	2	2	2	1	2	1	1	UN	Khon Kaen
49	THL137	1	0	0	2	6	1	1	1	2	1	2	2	1	2	2	2	Chiang Mai
50	THL222	1	1	1	2	6	1	1	1	1	1	1	1	1	1	1	UN	Chiang Mai
51	THL498	1	1	1	2	6	1	1	1	1	1	1	1	1	1	1	UN	Chiang Mai
52	TH11	1	1	1	2	6	1	1	1	1	1	1	1	1	1	1	UN	Khon Kaen
53	THL968	1	0	0	1	6	1	1	2	1	1	1	2	1	1	1	-	Khon kaen
54	PHI40	1	0	1	2	5	2	1	2	2	1	1	2	2	2	2	-	Chiang Mai
55	THL60	1	3	1	5	6	0	0	0	3	4	2	3	2	2	2	1	Chiang Mai
56	THL473	1	0	0	0	4	0	0	0	1	1	0	0	0	1	0	UN	Phichit
57	THL981	1	0	0	2	6	1	1	1	1	0	0	1	1	1	1	3	Nongkhai
58	THL919	1	0	1	3	6	2	1	2	2	1	1	1	1	1	1	-	Song Khla
59	THL98	1	0	1	3	6	1	1	1	1	1	1	1	1	1	2	UN	Phitsanulok

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Table 8 (continued)

Entry	Isolates	RD6	JHN	IR64	Azucena	Sariceltik	Introgression lines										AFLP group	Blast collected province
							$qBL1_{JHN}$		$qBL2_{IR64}$		$qBL11_{JHN}$		$qBL12_{Azu}$		None QTL			
							1	2	3	4	5	6	7	8	9	10		
60	THL980	1	0	0	1	6	1	1	1	1	0	0	1	1	1	1	3	Nongkhai
61	TH31	1	1	1	2	6	1	1	1	1	1	1	2	1	1	1	UN	Khon Kaen
62	THL69	1	0	0	1	6	1	1	1	1	1	1	1	1	1	1	3	Phitsanulok
63	THL85	1	0	1	2	6	1	1	1	1	1	1	1	1	1	1	11	Phitsanulok
64	THL148	1	0	2	5	6	1	1	1	1	1	1	0	1	1	1	1	Chiang Mai
65	THL154	1	2	1	4	6	1	0	1	1	1	1	1	2	1	1	1	Chiang Mai
66	THL170	1	3	1	5	6	1	1	1	1	1	1	1	1	1	1	UN	Chiang Mai
67	THL186	1	0	1	2	5	1	1	1	1	0	0	2	1	1	1	UN	Phrae
68	THL443	1	1	1	2	6	1	1	1	1	1	1	1	1	1	1	UN	Lampang
69	THL831	1	0	0	1	6	2	1	2	2	0	1	2	2	2	2	-	Mae Hong Son
70	THL838	1	1	1	1	6	1	2	1	2	1	1	1	2	1	1	3	Sri saket
71	THL47	1	1	3	2	6	1	1	1	1	1	1	1	1	1	1	-	Chiang Mai
72	THL169	1	1	1	3	6	1	1	1	1	1	1	1	1	1	1	-	Chiang Mai
73	THL287	1	0	0	1	5	1	1	1	1	0	0	1	1	1	1	3	Lampang
74	THL316	1	0	0	1	6	1	1	1	1	0	0	0	1	1	1	-	Chiang Mai
75	THL717	1	0	1	3	6	1	1	1	1	1	1	1	1	1	1	11	Pathum thani
76	THL364	1	1	0	2	5	-	0	-	0	0	0	0	1	0	-	6	Nakorn ratchasima
77	THL972	1	1	1	0	4	1	1	1	1	1	1	1	1	1	1	-	Khon kaen
78	THL982	1	0	0	2	6	1	2	1	1	0	0	1	0	1	1	3	Nongkhai
79	THL84	1	0	4	2	6	0	1	0	1	0	0	1	0	0	1	7	Phitsanulok

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Table 8 (continued)

Entry	Isolates	RD6	JHN	IR64	Azucena	Sariceltik	Introgression lines										AFLP group	Blast collected province
							$qBL1_{JHN}$		$qBL2_{IR64}$		$qBL11_{JHN}$		$qBL12_{Azu}$		None QTL			
							1	2	3	4	5	6	7	8	9	10		
80	TH23	1	1	0	5	6	0	0	1	0	1	1	0	0	0	1	UN	Khon Kaen
81	THL183	0	0	0	0	5	0	1	0	1	0	0	0	1	1	1	UN	Chiang Rai
82	THL282	0	1	1	5	6	1	1	1	1	0	1	1	1	2	1	10	Phrae
83	THL882	0	0	0	5	6	0	0	2	2	0	0	1	1	0	1	5	Krabi
84	THL855	0	0	0	2	6	1	1	1	1	0	0	1	1	1	1	7	Prachin Buri
85	THL557	0	0	4	0	4	0	0	0	1	0	0	0	1	0	0	UN	Tak
86	THL153	0	1	0	2	5	0	0	1	1	0	0	0	0	0	0	1	Chiang Mai
87	TH26	0	1	1	5	6	1	1	1	1	1	1	1	1	1	1	UN	Khon Kaen
88	THL881	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	13	Chumpon
89	THL61	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	1	Chiang Mai
90	THL115	0	0	0	0	6	0	1	1	1	0	0	0	2	1	0	3	Chiang Rai
91	THL144	0	0	1	1	6	0	0	1	1	0	1	0	1	0	1	6	Chiang Mai
92	THL211	0	0	0	1	6	1	0	0	0	0	0	0	0	1	1	1	Chiang Mai
93	THL310	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	-	Chiang Mai
94	THL319	0	4	0	5	6	0	0	0	0	1	0	1	0	0	2	-	Chiang Mai
95	THL506	0	0	0	3	5	0	0	0	0	0	0	0	0	0	0	-	Phetchabun
96	THL954	0	0	0	0	5	1	0	1	0	0	0	0	1	0	0	3	Chantha Buri

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3.2 Validation of all combination of introgression lines

The introgression lines and the parents were inoculated with eleven blast isolates, THL122, THL190, THL266, THL456, THL658, THL690, THL810, THL868, THL984, THL1066 and THL1135 that showed a virulence reaction (Score 3-6) to the recipient variety RD6 and an avirulence reaction to JHN and IR64 (score 0-2). The resistance reactions of original parental lines and introgression lines were showed in Table 9.

Introgression lines with a single QTL $qBL1_{JHN}$ were found to be moderate resistant to the compatible isolates tested with an exception of THL658. Introgession lines with a single QTL $qBL2_{IR64}$ were susceptible to all isolates except THL690 and THL868. The introgression lines with $qBL11_{JHN}$ were highly resistant to all isolates tested. The introgression lines with $qBL12_{Azu}$ were moderately resistant to THL122, THL690 and THL868 and susceptible to the rest of isolates tested.

The introgression lines with more than two combinations, $qBL1_{JHN}$ + $qBL11_{JHN}$, $qBL2_{IR64}$ + $qBL11_{JHN}$, $qBL12_{Azu}$ + $qBL11_{JHN}$, $qBL1_{JHN}$ + $qBL2_{IR64}$ + $qBL11_{JHN}$, $qBL1_{JHN}$ + $qBL11_{JHN}$ + $qBL12_{Azu}$, $qBL2_{IR64}$ + $qBL11_{JHN}$ + $qBL12_{Azu}$ and $qBL1_{JHN}$ + $qBL2_{IR64}$ + $qBL11_{JHN}$ + $qBL12_{Azu}$ were highly resistant to all isolates. The introgression lines with $qBL1_{JHN}$ + $qBL2_{IR64}$, $qBL1_{JHN}$ + $qBL12_{Azu}$ and $qBL1_{JHN}$ + $qBL2_{IR64}$ + $qBL12_{Azu}$ were highly resistant to isolates THL868 and THL984 but moderately resistant to the rest of isolates tested. The introgression lines with $qBL2_{IR64}$ + $qBL12_{Azu}$ were highly resistant to isolate THL984 but moderately resistant to the rest of isolates tested.

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Table 9 Lesion score of all combination types in original parent and introgression lines after inoculated with eleven blast isolates.

Entry	QTL combination	Varieties/ Introgression lines	THL122	THL190	THL266	THL456	THL658	THL690	THL810	THL868	THL984	THL1066	THL1135	Reaction pattern
1	-	RD6	5	5	6	6	5	4	5	5	3	5	5	S
2	qBL1 + qBL11	JHN	0	0	0	0	0	0	1	0	0	0	0	R
3	qBL2	IR64	0	1	0	0	0	1	0	0	0	0	0	R
4	qBL12	Azucena	3	2	3	2	3	1	3	2	2	1	3	MR
5	-	Sariceltic	6	6	6	6	6	6	6	6	6	6	6	S
6	-	RGDU07079-6-122-1	5	6	5	6	5	3	5	5	6	6	6	S
7	-	RGDU07079-6-176-9	5	5	5	6	6	4	6	5	6	6	6	S
8	qBL1	RGDU07079-6-122-5	3	4	4	4	5	3	2	2	3	2	3	MR
9	qBL1	RGDU07079-6-176-13	3	4	4	4	-	3	2	2	3	1	4	MR
10	qBL2	RGDU07079-6-122-12	5	5	5	6	6	4	6	4	6	5	6	S
11	qBL2	RGDU07079-7-228-8	5	6	6	6	6	4	5	4	5	5	6	S
12	qBL11	RGDU07123-12-247-2	1	0	1	1	1	1	1	0	1	0	1	R
13	qBL11	RGDU07079-7-190-6	1	1	1	1	1	1	1	0	0	0	1	R
14	qBL12	RGDU07079-7-227-11	4	4	4	5	5	4	5	3	4	4	5	MR
15	qBL12	RGDU07079-7-236-7	4	5	5	5	5	3	4	3	5	5	5	S
16	qBL1 + qBL2	RGDU07079-6-131-6	3	3	3	3	4	3	2	0	1	2	2	MR
17	qBL1 + qBL2	RGDU07079-6-131-8	3	3	4	3	4	3	3	1	0	3	3	MR
18	qBL1 + qBL11	RGDU07123-12-203-5	0	0	0	0	0	1	0	0	0	0	0	R
19	qBL1 + qBL11	RGDU07123-12-231-8	0	0	0	0	0	0	0	0	0	0	0	R
20	qBL1 + qBL12	RGDU07079-7-195-7	3	3	3	3	4	4	2	1	1	2	3	MR
21	qBL1 + qBL12	RGDU07079-7-218-11	3	2	3	3	4	3	2	1	1	3	3	MR

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Table 9 (continued)

Entry	QTL combination	Varieties/ Introgression lines	THL122	THL190	THL266	THL456	THL658	THL690	THL810	THL868	THL984	THL1066	THL1135	Reaction pattern
1	-	RD6	5	5	6	6	5	4	5	5	3	5	5	S
2	qBL1 + qBL11	JHN	0	0	0	0	0	0	1	0	0	0	0	R
3	qBL2	IR64	0	1	0	0	0	1	0	0	0	0	0	R
4	qBL12	Azucena	3	2	3	2	3	1	3	2	2	1	3	MR
5	-	Sariceltic	6	6	6	6	6	6	6	6	6	6	6	S
22	qBL2 + qBL11	RGDU07123-12-164-15	0	0	0	1	0	1	0	0	0	0	0	R
23	qBL2 + qBL11	RGDU07123-12-200-2	0	0	1	0	0	1	0	0	0	0	0	R
24	qBL2 + qBL12	RGDU07079-7-219-13	4	4	4	5	3	4	3	4	2	4	3	MR
25	qBL2 + qBL12	RGDU07079-7-228-13	4	4	4	4	4	4	4	4	2	4	4	MR
26	qBL11 + qBL12	RGDU07079-6-140-4	0	0	0	1	1	0	0	0	0	0	0	R
27	qBL11 + qBL12	RGDU07079-7-186-8	0	0	0	0	1	1	0	0	0	0	0	R
28	qBL1 + qBL2 + qBL11	RGDU07123-12-200-15	0	0	0	0	1	1	0	0	0	0	0	R
29	qBL1 + qBL2 + qBL11	RGDU07079-6-113-1	0	0	0	0	1	1	0	0	0	0	0	R
30	qBL1 + qBL2 + qBL12	RGDU07079-7-180-14	3	3	3	3	3	4	2	1	0	3	3	MR
31	qBL1 + qBL2 + qBL12	RGDU07079-7-235-15	3	3	2	3	3	3	2	1	1	3	4	MR
32	qBL1 + qBL11 + qBL12	RGDU07123-12-245-6	0	0	0	0	1	1	0	0	0	0	0	R
33	qBL1 + qBL11 + qBL12	RGDU07079-7-190-11	0	0	0	0	1	1	0	0	0	0	0	R
34	qBL2 + qBL11 + qBL12	RGDU07123-12-147-15	0	0	0	0	0	1	0	0	0	0	0	R
35	qBL2 + qBL11 + qBL12	RGDU07123-12-189-2	0	0	0	0	1	0	0	0	0	0	0	R
36	qBL1 + qBL2 + qBL11 + qBL12	RGDU07123-12-165-7	0	0	0	0	1	0	0	0	0	0	0	R
37	qBL1 + qBL2 + qBL11 + qBL12	RGDU07079-7-235-5	0	0	0	0	1	1	0	0	0	0	0	R

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DISCUSSIONS

Increment of the prevalence of previously rare pathotypes and novel pathotype has been reported to cause a resistance breakdown of rice varieties soon after released. Genetic variations of blast fungus resulted from sexual and asexual reproductions have been reported (Crawford*, et al.*, 1986; Zeigler*, et al.*, 1997; Xiong*, et al.*, 2007; Zhou*, et al.*, 2007; Sreewongchai, 2009). Although in natural condition, the sexual reproduction of blast pathogens is rarely found (Srivastava*, et al.*, 2009). Sexual recombination is generated through meiosis that undergo an independent assortment of chromosomes to yield new recombinations which produced new genotypes (Milgroom, 1996). In this study, the blast strains B71, B124, B151, B161 and B248 derived from a sexual recombination of B1-2 and TH16 had shown the change of their virulence spectrums in differential rice varieties, especially in rice variety Azucena. Leung*, et al.* (1988), Grigg et al. (2001; 2003), Sreewongchai (2009) and (Silué and Notteghem, 1992), had reported that recombinant progenies are found to be more virulent than both parents. The same evidence was found in this study. For example, the recombinant isolates B71, B124, B151, B161 and B248 were more virulent than B1-2 and TH16 to rice variety Azucena. Thus, this is an important evidence indicating that sexual recombination can be one of powerful forces driving the natural evolution of virulence in the blast pathogen population. Moreover, the sexual recombination not only generated virulence strains but also avirulence strains such as B71, B151, B161, B248 and B259 which can not infect CN1 while the parental strains can.

QTLs conferring resistance to isolate B1-2 were mapped to different regions of the rice chromosomes in two mapping populations. In KDML105 x JHN population, two QTLs, $qBL2_{KD}$ and $qBL8_{KD}$ associated with the expansion of lesion size were mapped on chromosomes 2 and chromosome 8. Blast strain B1-2 infects both KDML105 and JHN. After infection, colonization was difference between KDML105 and JHN. The expansion of lesion size was found in JHN but it wasn't found in KDML105. In IR57514 x KDML105 population, two QTLs, $qBL1_{IR}$ and $qBL6_{IR}$ related with the infection efficiency (lesion number) were mapped on chromosomes 1 and chromosome 6. Blast strain B1-2 fails to infect IR57514 but it

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infects KDML105 without the expansion of lesion size. Our results indicated that the QTL associated with the expansion of lesion size after colonization and the infection efficiency are under difference genetic control. This evidence was supported by Wang, *et al.* (1994) and Wu, *et al.* (2004).

Resistant varieties carrying a single gene for race-specific resistance had proven short-lived, often lasting for only 2-4 years. This is particularly evident in large acreages of genetically uniform rice varieties, where adaptive selection pressurizes pressurize a *P. grisea* race-shift towards isolates that escape host recognition. Routine monitoring of the fungal population is needed to evaluate the durability of deployed R genes. The rapid identification of virulent strains can predict the spread of resistance breakdown and therefore help prevent blast epidemics. The blast strain B1-2 can overcome the broad spectrum resistance possessing by JHN. In this study, the location of the resistance QTLs corresponding to B1-2 was identified on chromosome 2 and chromosome 8 in the KDML105 x JHN population. KDML105 that is susceptible to most of blast strains contributes the resistant genes. This result indicated that the avirulence gene of blast isolate B1-2 might not be breaking down the resistance QTLs on chromosome 1 and chromosome 11 in JHN (Noenplab, *et al.*, 2006) but it can recognize with new susceptible loci (Sallaud, *et al.*, 2003).

The race specific resistance $qBL2_{KD}$ was mapped at the distal end of the long arm of chromosome 2 between RM213 and RM208 where five previously reported blast resistance genes, *Pib*, *Pi-g*(t), *Pi-tq5*, *Pi-y*(t) and *Pi25*(t) (Wang, *et al.*, 1999; Tabien, *et al.*, 2000; Sallaud, *et al.*, 2003; Zhou, *et al.*, 2004; Lei, *et al.*, 2005) were located. The $qBL8_{KD}$ was mapped on short arm of chromosome 8 between RM310 and RM72 where five blast resistance genes, *Pi11* (formerly *Pi-zh*), *Pi29*(t), *Pi33*, *Pi36* and *Pi-GD-1*(t) had reported (Zhu, *et al.*, 1993; Berruyer, *et al.*, 2003; Sallaud, *et al.*, 2003; Liu, *et al.*, 2004). By comparing the physical positions of the closely linked markers, the $qBL8_{KD}$ should not be an allelic of *Pi11*, *Pi29*(t), *Pi33* and *Pi-GD-1*(t) genes. However, more information has to be obtained to answer the question whether or not these genes are the same or difference. The answer to this question might be provided when these genes have been cloned. The resistance gene *Pi36* had been

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cloned in rice variety Kasalath by using in Silico Map-Based Cloning (Liu*, et al.*, 2007a), the *Pi36* locus can be a good candidate gene in our experiment.

The $qBL1_{IR}$ identified in IR57514 was located on short arm of chromosome 1 between RM495 and RM84 where *Pit* gene was reported (Kaji*, et al.*, 1997; Hayashi*, et al.*, 2006). The $qBL6_{IR}$ located on chromosome 6 was mapped between RM314 and RM564 where ten blast resistance genes, *Pi2* = *Piz*^{5}, *Pi9*, *Pi22*(t), *Pi25*(t), *Pi26*, *Pi27*, *Pi40*(t), *Pi-d(t)2*, *Pigm*(t) and *Piz-t* had been reported (Ahn*, et al.*, 2000; Zhuang*, et al.*, 2002; Sallaud*, et al.*, 2003; Chen*, et al.*, 2004; Wu*, et al.*, 2005; Deng*, et al.*, 2006; Qu*, et al.*, 2006; Zhou*, et al.*, 2006; Jeung*, et al.*, 2007). Therefore, we speculated the $qBL6_{IR}$ are either tightly linked or allelic of these reported genes.

Conventional phenotypic selection is unable or has a difficult to combine multiple resistance genes into a single genotype. The DNA markers can help breeders to overcome this limitation by identifying multiple resistance genes in pyramided plants. In our study, four blast resistance QTLs, $qBL1_{JHN}$, $qBL2_{IR64}$, $qBL11_{JHN}$ and $qBL12_{Azu}$ were introgressed and pyramided into RD6 through marker assisted selection (MAS). According to our results in Table 8, JHN and IR64 showed a broad spectrum resistance (92 of 96 isolates) to Thai isolates. It agrees with the report by Sirithunya *et al*. (2004). Moreover, introgression lines carrying the $qBL11_{JHN}$ exhibited a complete resistance to most of tested isolates while introgression lines carrying the $qBL1_{JHN}$ showed moderate resistance. Noenplab*, et al.*, (2006) reported that $qBL1_{JHN}$ and $qBL11_{JHN}$ had minor and major effects, respectively, against the KDML105 genetic background. We found that the $qBL2_{IR64}$ and $qBL12_{Azu}$ were not effective against the tested isolates in our experiment. The level of resistance controlled by qBL12Azu was higher than those by $qBL2_{IR64}$. It is in agreement with a previously study by RGDU.

Introgression lines with $qBL2_{IR64}$ and $qBL12_{Azu}$ had not shown an increment of resistance when compared with the original RD6. This result indicated that resistant alleles of the $qBL2_{IR64}$ and $qBL12_{Azu}$ loci didn't functioned against most of the virulent strains to the RD6 and there might be other resistant loci (Yang*, et al.*, 2008). The other reason is that the flanking markers employed for the selection were

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too narrow when compared with the sizes of the QTL studied by RGDU. Thus, indirect selection using inappropriate flanking marker could result in the lost of the target region in MAS.

The introgression lines carrying more than one QTL targets had lower level of infection and broader spectrum of resistance than original RD6, especially the lines that carrying the $qBL11_{JHN}$. Hittalmani et al. (2000) reported that the introgression lines containing the three major blast resistance genes, *Pi1*, Piz^5 and *Pita*, showed higher resistance level to the compatible isolates than those with one major gene. This evidence is due to the interaction between the resistance genes as additive, dominant and co-dominant (Ma, *et al.*, 2005; Utami, *et al.*, 2008). In case of THL984, the introgression lines contained the $qBL2_{IR64}$ + $qBL12_{Azu}$ and lines without QTL showed more susceptibility than the original RD6. It might be occurred by epistasis effect (Barendse, *et al.*, 2007). These results indicated that resistance genes do not function alone and the gene interactions affect the resistant phenotypes.

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CONCLUSION

1. Sexual recombination yields both virulent and avirulent strains and sexual recombination of B1-2 and TH16 demonstrate a change in virulence spectrum on the differential rice varieties.

2. The expansion of lesion size after colonization and the infection efficiency were controlled by difference QTLs.

3. Avirulence gene of the B1-2 strain does not break down the $qBL1_{JHN}$ and $qBL11_{JHN}$ but it can recognize the other susceptible loci that located on chromosome 2 (RM213-RM208) and chromosome 8 (RM310-RM72).

4. The resistance QTLs on chromosomes 1 and 11 ($qBL1_{JHN}$ and $qBL11_{JHN}$) conferred resistance to a broad range of spectrum of blast pathogen found in the rainfed lowland in Thailand while the resistance QTLs on chromosome 2 and chromosome 12 ($qBL2_{IR64}$ and $qBL12_{Azu}$), conferred race specific resistance.

5. The introgression lines carrying more than one QTL targets had a lower level of infection and broader spectrum of resistance than the original RD6.

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APPENDIX

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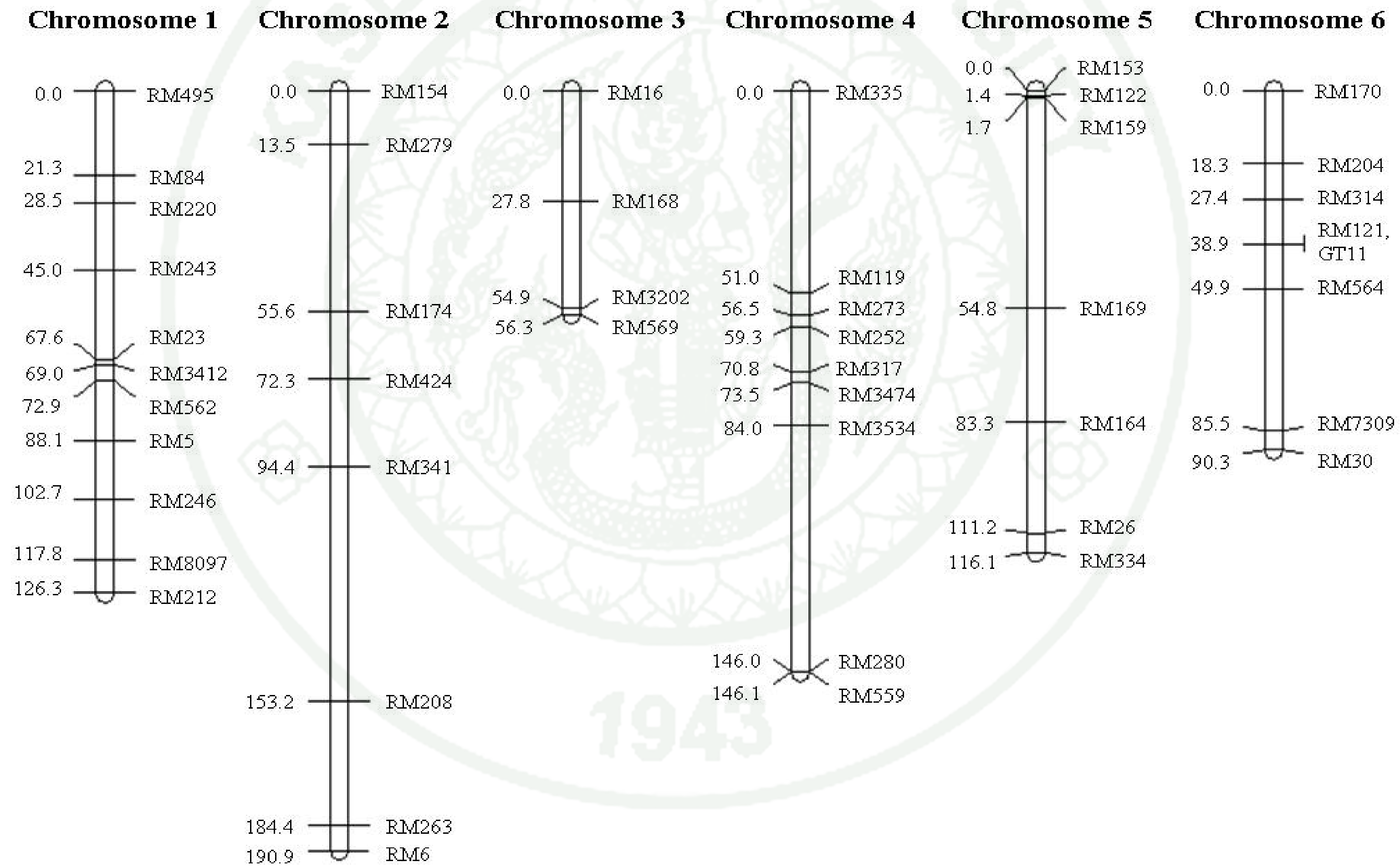
Appendix Figure 1 Twelve linkage groups constructed by using 84 SSR markers and 76 RIL of IR57514 x KDML105 population



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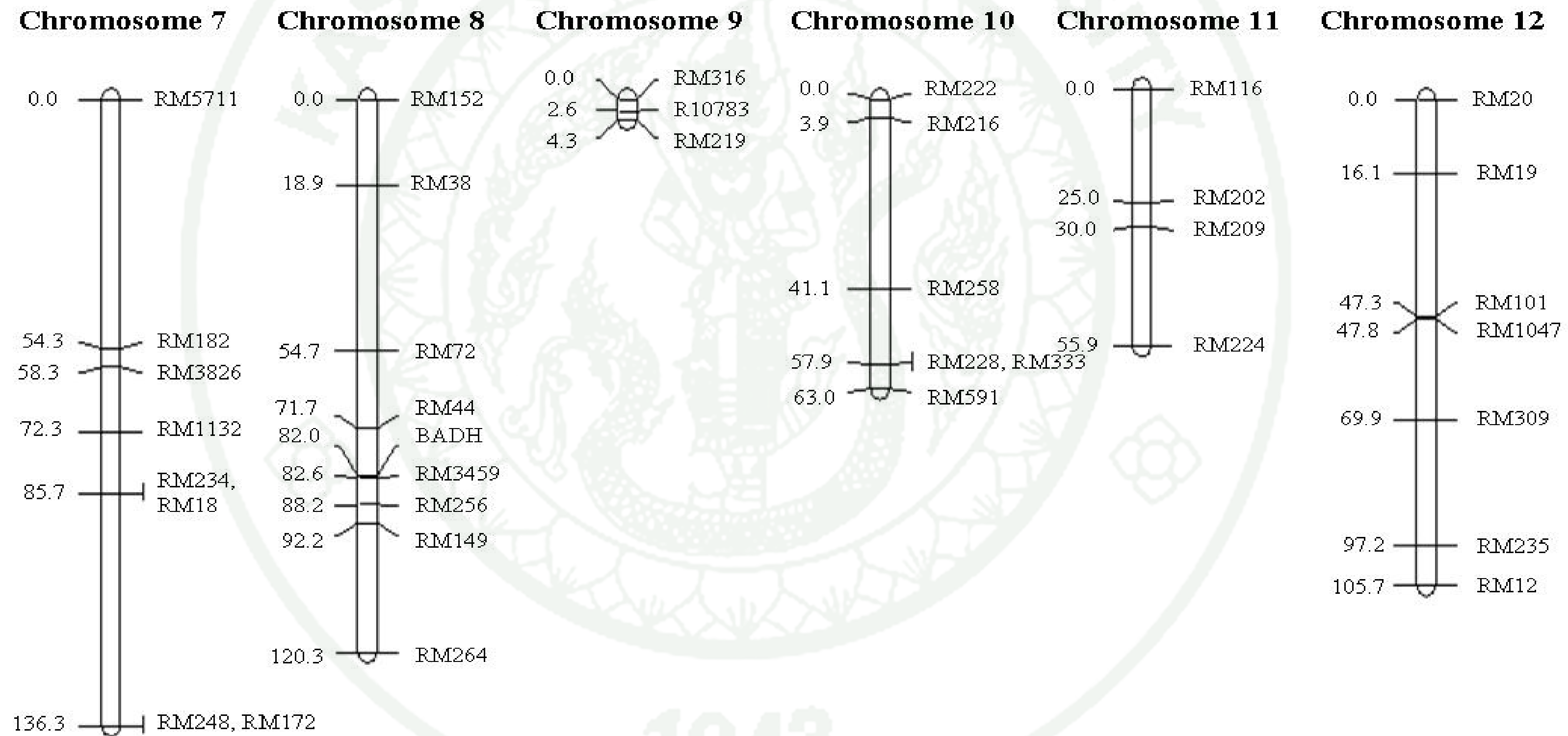
Appendix Figure 1 (continued)



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