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Full Length Article

Cloning and Analysis of QTL Linked to Blast Disease Resistance in Malaysian Rice Variety Pongsu Seribu 2

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Abstract

Blast, caused by *Magnoporthe oryzae,* is considered to be a global rice disease around the world including Malaysia. Limited information is available on the identification of quantitative trait loci (QTL) and linked markers associated with blast resistance within local rice varieties e.g. Pongsu Seribu 2. Partial resistance against blast disease is widely observed in this variety with mapping of QTLs linked to resistance genes. However, knowledge about the blast resistance genes on chromosome 3 is very limited and no specific blast resistance gene have been identified on chromosome 3 in rice genome of Pongsu Seribu 2. In order to find the similarity of sequence, QTL qRBr-3.1 located on chromosome 3 in Pongsu Seribu 2 was cloned and compared with identified blast resistant genes from other varieties. Sequence analysis of cloned fragment revealed a tandem of (CA)23 repeats. The similarity of sequence was searched in Basic Local Alignment Search Tool (BLAST), which expressed similarity with different clones of rice located on chromosome 3. The cloned QTL fragment also expressed the similarity of 46% with *Pi-b,* 52% with *Pi-kh,* 23% with *Pi-9* and 38% with *Pi-zt,* blast resistance genes located at different chromosomes in rice. The QTL fragment produced none of distribution of leucine rich repeats (LRRs) and nucleotide binding site (NBS-LRR). However, the domain contains maximum distribution of leucine amino acid which is responsible for the pathogen recognition in host-plant interaction and play important role in resistance mechanism against diseases. This result concluded that Pongsu Seribu 2 has homology to other resistant genes which are allelic to *Pi-b, Pi9, Pi-zt and Pi-kh* at different chromosomes. © 2014 Friends Science Publishers

Keywords: Partial resistance; QTL; Microsatellite marker; Six frame translation; Leucine rich repeat

Introduction

Rapid growth in the world population has increased the demand of food crops especially rice (Khush, 2005). Further, increased human population has also reduced inputs for the agriculture especially cultivated land and water. Moreover, according to FAO survey weeds, disease, insects are responsible for yield loss up to 25%.

Magnoporthe oryzae, the main causing fungus of blast disease in rice, is considered as severe pathogen damaging the rice leaves, stems and flowers. It is a plant-pathogenic fungus and is member of the *M. grisea* complex which contains at least two biological species that have clear genetic differences and do not interbreed. *M. oryzae* and *M. grisea* are complex members (Couch and Kohn, 2002). The strains isolated from *Digitaria* have been defined as *M. grisea*, and the isolates from rice were named as *M. oryzae*. Members of the *M. grisea* complex can also infect other agriculturally important cereals including wheat, rye, barley, and pearl millet causing diseases called blast disease or blight disease. The usage of the names *M. grisea* and *M. oryzae* has generally reflected the host from which the fungus was isolated rather than any morphological differences, with the name *M. oryzae* applied to and from rice and *M. grisea* to isolates from cereals and other grasses (Sprague, 1950). Rossman *et al*. (1990) confirmed the morphological similarity after examination of the type specimens of *M. grisea* and *M. oryzae*. Fully fertile matings between isolates from rice and isolates from other grasses were interpreted as evidence for the existence of a single biological species (Yaegashi and Udagawa, 1978).

In Malaysia, blast caused more than 50% losses in rice production (Talbot, 2003; Miah *et al*., 2013). The recent outbreak of the disease occurred in 2006 that destroyed 60%

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of area under rice cultivation in Malaysia (Rahim, 2010). The *M. oryzae* can infect the rice crop at any stage of its growth from young shoots to flowering stage (Sharma *et al*., 2008). The *M. oryzae* cannot bring up under permanent control due to its great diversity. However, the development of durable resistant rice varieties is the vital solution to control the infections of this pathogen.

Pongsu Seribu 2 is a local rice variety grown in Malaysia for decades, which posses the partial resistance against the leaf blast in Malaysia (Rahim, 2010). The partial resistance is polygenic and durable conferred by the QTLs and also stable against different races of *M. oryzae* (Zenbayashi *et al*., 2002).

Molecular markers such as SSRs, RFLP, RAPD and AFLP are important tools to determine the genetic diversity in rice (Rafii *et al.,* 2012; Latif *et al*., 2011; Miah *et al*., 2013). Further, identification of QTL linked with blast resistance genes made it effortless to clone and characterize the resistant genes in rice. The functional characterizations and cloning of blast resistance genes indicate that most blast resistant genes belongs to nucleotide binding site-leucine rich repeat (NBS-LRR) family. A number of resistant genes have been mapped and isolated by cloning strategy in the last decade in rice and other cereal crops (Eitas and Dangl, 2010).

The cloning of single QTL of the interest is now possible by merging marker technology with genomic resources such as bacterial artificial chromosome (BAC), yeast artificial chromosome (YAC), linkage map construction and by exploiting developed plant material (Paran and Zamir, 2003; Salvi and Tuberosa, 2005).

QTL and its linked markers associated with blast disease in rice have been mapped by various researchers (Ashkani *et al.,* 2013a; Sharma *et al.,* 2005). In Pongsu Seribu 2 variety, partial resistance is conferred by QTL mapping against *M. oryzae* pathoype P7.0 and 5.0 (Ashkani *et al*., 2013a, b). The genetic dissection of blast resistance by QTLs mapping, gene tagging, marker assisted selection, gene cloning and functional characterization of blast resistance genes explore knowledge about the resistance pattern and host pathogen interaction.

The QTL mapping and identification of molecular markers linked with blast resistance genes was already mapped in Pongsu Seribu 2 by Ashkani *et al*. (2013a, b). The links suggested recognized location of QTLs on various chromosomes. In this study we cloned QTL qRBr-3.1 Sirithunya *et al*. (2002) and Ashkani *et al*. (2011) linked to SSR marker RM168 on chromosome 3 to identify the nucleotide sequence responsible for the blast resistance in rice. Studies were also done to find the similarity with other identified genes sequences.

Material and Methods

Plant Material

Seed of Pongsu Seribu 2, a resistant rice variety to pathotype P7.2 was collected from the Malaysian Agricutural Research and Development Institute (MARDI). The seeds pre-soaked in water for 24 h were grown on moisten filter paper in Petri dishes. After 2 days, the germinated seeds were transferred to greenhouse under controlled conditions to avoid the adverse effects of abiotic and biotic factors. After 30 days, the fresh young leaves were harvested and stored at -80°C.

Magnaporthe Oryzae Pathotype

The most virulent *M. oryzae* pathotype P7.2 (Filippi and Prahbu, 2001) was obtained from the Malaysian Agricultural Research and Development Institute (MARDI), Seberang Perai. The pathotype used in experiment was selected based on their virulence towards Pongsu Seribu 2 (PS2), where PS2 was resistant to the pathotype P7.2.

Media Preparation and Pathogenecity Test of *Magnaporthe Oryzae*

Potato dextrose agar (PDA) was used as a media for growing selected pathotype of *M. oryzae*. PDA was prepared by mixing 100 g of potato in 1 L of water and boiled at 70°C for 1 h. The potatoes were then filtered out and the solution was added up to 1.0 L with water. After that, agar (13 g/L) was added into the solution and was autoclaved. The solution was then poured in 9 cm diameter Petri dish in the laminar flow cabinet and sealed with tape to avoid contamination (Filippi and Prahbu, 2001). Single spore isolation was carried out for the selected pathotype. The isolation was carried out to make sure that the isolate was pure. *Isolate was* cultured at room temperature in the petridish. The colonies were then exposed under the fluorescent light at room temperature and covered with wet cotton muslin cloth for 5 days to induce sporulation. Relatively high humidity and suitable aeration are two important factors for optimum production of conidia. Spores for inoculation were prepared as described by Chen (2001) and the concentration was adjusted to 1 x 10^5 spores/conidia per ml. The aerial mycelia were slightly washed off by gentle rubbing with a water soaked paintbrush. Later, the brush was soaked in sterile distilled water. The spore suspensions were filtered through nylon gauze mesh and adjusted to a concentration of 1 x 10^5 spores/mL using a haemocytometer. Before inoculation, 0.05 % Tween 20 was added to the suspension to increase the adhesion of the spores to the plant.

Twenty-two day old plants (10 plants per line), with three or four fully expanded leaves, were inoculated by spraying with 25 mL aqueous spore suspension (1 x 10^5 spores/mL) onto the leaves until run-off using an atomiser connected to an air compressor. Inoculated plants were incubated in moisture/dew chamber and the relative humidity was maintain 100% for 24 h at 25 to 28ºC after, which they were placed in the greenhouse (controlled environment) at temperatures ranging from 25 to 30ºC

(Filippi and Prahbu, 2001). Ten days after inoculation, blast disease having characteristic eye shaped symptom was scored.

DNA Extraction

Fresh young leaves of Pongsu Seribu 2 were used for genomic DNA isolation by CTAB method (McCouch *et al*., 1988) with minor modifications. 1 g Leaf was first grounded in the liquid nitrogen by using a pestle and then immediately transferred to microtubes. The 2% CTAB buffer of 1000 µL cetyltrimethyl ammonium bromide and 3 µL of β-mercaptoethanol were added in microtube. Samples were incubated in micro centrifuge at 65°C for 1 h by gently shaking the tube at 5 min interval. After incubation isoamyl alcohol (24:1, v/v) was added and tubes were gently inverted to homogenize the mixture. The sample was then centrifuge at 1300 rpm for 10 min to collect the supernatant layer. The supernatant layer was transferred to new 50 mL falcon tube and 600 µL of cold (-20°C) isopropanol was added to the mixture and kept in refrigerator for 30 min at -20°C. The sample was then centrifuged at 13000 rpm for 10 min until the DNA pooled out. The supernatant was removed with care to avoid any damage to pooled DNA pellet. The DNA pellet was washed with 70% alcohol for two/three times until it became very clear and kept for drying. Further, 1 µL of RNAs was added in DNA pellet to remove RNA and 50 mL of Tris-EDTA (Ethylenediamine Tetraacetic Acid; buffered solution) to make it diluted and then kept 30 min at 37°C for incubation. DNA concentration was checked by using nano-drop spectrophotometry.

PCR Amplification and Gel Electrophoresis

The genomic DNA of Pongsu Seribu 2 was used as template and amplified by the primer pair forward (5′TGCTGCTTGCCTGCTTCCTTT-3') and reverse primers (5′ GAAACGAATCAATCCACGGC-3'). PCR reactions were performed according to McCouch *et al*. (2002a, b) with minor modifications, by adjusting total volume to 15 µL for each sample. The PCR reaction was settled down by adding template DNA 40ng and dNTP 100 µM with (0.2 Mm of each dGTP, dCTP, dTTP and dTTP), 1.5 mM MgCl2, individual primers (forward and reverse) 1.0 µM, 1X PCR buffer (50 mM KCl, pH 8.310 mM Tris-HCl) and 0.2 U *Taq* polymerase. The PCR reaction was performed in thermocycler machine (GeneAmp System 9700 - Applied Biosystems, Foster City, CA). The temperature for initial de-naturation was 94°C for 5 min followed by 35 cycles at 94°C for 30 sec, annealed at 55°C for 30 sec, elongated at 72°C for 30 sec and final extension was done for 5 min at 72°C prior to cool at 4°C. After amplification, the gel electrophoresis was carried out by resolving the amplified product in 3% metaphore agarose gel. The gel was stained with 0.2 µg of midori green DNA stain at 80 Volts for 1.5 h to detect the DNA fragments. Finally, the gel was visualised under UV light and analysed by using Bio imaging system (Fig. 1).

Competent Cells Preparation

Competent cells (DH5α) were multiplied for overnight at 37°C by incubating in the free antibiotic Lysogeny broth (LB) medium. Single colonies were picked from the grown competent cells and re-grown on antibiotic free LB-agar plats at 37°C. The 5 ml of overnight competent cell culture was then put in the conical flask containing 50 mL antibiotic free SOB medium supplemented with 10 mM $MgSO_4.7h_2O$ and 0.2% glucose. This culture was incubated at 37ºC on orbital shaker for 90-100 min until OD_{600} value reached at 0.6 (shaking at 220 rpm/min). After incubation, culture was centrifuge at 3000 rpm for 10 min to pellet out bacteria and gently re-suspended in 30 mL cold 50 mM CaCl2 by using a 100 mL falcon tube. The tubes were than thaw on ice for 30 min before re-pelleting by centrifugation at 3000 rpm for 10 min at 4ºC. 50% ice-cold glycerol (final concentration 15%) was added after the re-suspension in 2.1 mL ice cold Mm $CaCl_2$ on ice. Cells obtained were placed in 100 µL microcentrifuge tubes and flash-frozen in liquid nitrogen by storing at 80°C.

Ligation with Vector

The Qiagen Gel extraction kit was used to purify the PCR fragment following the manufacturer protocol. For ligation, PGEM-T easy vector system I kit (promega) was used. The total volume of reaction was 15 µL consisting of 7.5 µL 2x rapid ligation buffer (Tris-HCl, 60 mM pH7.8; mM $MgCl_2$ 20; mM DTT 20; ATP 2 mM; PEG 10%); 0.9 µL PGEM-T Easy plasmid; 1.2 µL T4 DNA Ligase; 5.4 µL DNA and kept for overnight incubation at 4°C.

Transformation in Competent Cells

The 15 µL ligated mixture was added to 100 µL competent cells (DH5α) in a 2.0 mL micro centrifuge tube and kept on ice for 30 min prior to heat shocking for 1 min at 45°C. The transformed cells were immediately incubated on ice for 10 min. Then, the transformed cells were added in antibiotic-free LB medium and again incubated at 37°C for 100 min on an orbital incubator shaker at 220 rpm/min. Later, the transformed cell culture was plated on LB medium supplemented (100 g/mL) ampicillin, X-gal 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (40 ng/mL) and IPTG isopropyl-β-Dthiogalactopy-ranoside (0.5 mM) and incubated over night at 37°C.

Selection of Recombinant Cell and BAC Plasmid DNA Isolation

Ten colonies picked were grown in 100 mL LB medium supplemented with ampicillin (40 µg/mL) and incubated overnight on incubator shaker at 37ºC (250 rpm/min).

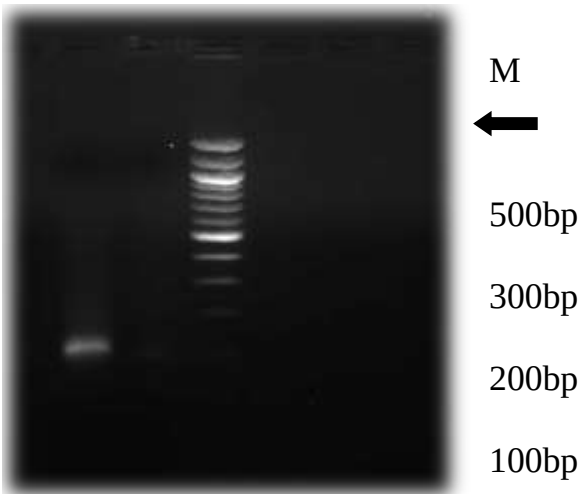

Fig. 1: Amplified genomic DNA of Pongsu Seribu2 variety by using primer RM168. The PCR product was separated by 3% metaphore agarose gel. M=100 bp ladder (Promega)

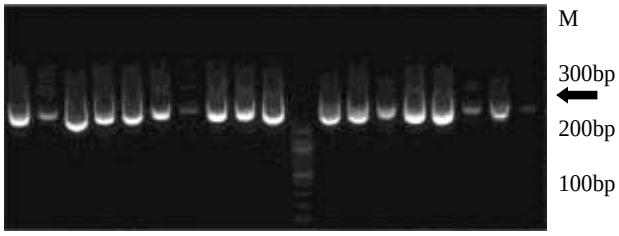

Fig. 2: Recombinant plasmid after insertion into the competent cells cells M=100 bp ladder (Promega)

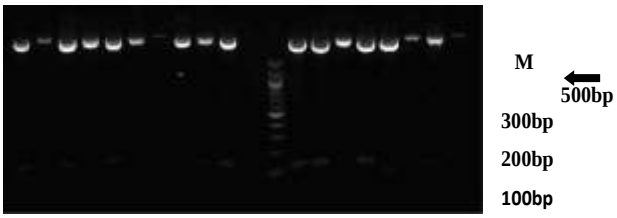

Fig. 3: Confirmation of presence of desired QTL amplified with RM168 primer after digestion with restriction enzyme *EcoR1*M=100 bp ladder (Promega)

Recombinant clones were identified by white colonies and further confirmed by Universal M13 Primers amplification according to manufacture protocol. The PCR products were visualised by resolving the product under 1.5 agrose gel containing 1 µL of ethidium bromide. The total volume of PCR products for confirmation was 15 µL by using the Qiagen Miniprep-Kit, according to the manufacturer's protocol. Finally, 100 ng/µL plasmids were digested by using the restriction enzyme EcoR1, 0.1U to confirm the size of the desired DNA fragments (Fig. 2 and 3).

DNA Sequencing Clones and Analysis of Sequence

Plasmid DNA (1 µL) was diluted in 9 µL dH_2O for verification of clones by using restriction analysis as described above and double standard plasmid DNA was sequenced commercially (NHK Company). The M13 F and M13 R primers were used according to the instruction of the ABI3700 capillary sequencing system. Sequence data were analysed by the CHROMAS 2.01 software. DNA sequence was aligned by using the CLC workbench 6.0 software.

Results

Sequence Analysis

The clones after restriction analysis were purified and sequenced. RM168 primer amplified 116 bp bands approximately and revealed a tandem of (CA)23 repeats (Fig. 4).

Pathotype Virulence

The resistance and susceptibility of Pongsu Seribu 2 was determined based on disease reaction. Pathotype P7.2 was collected and used to score the disease severity. Pongsu Seribu 2 appeared resistance for pathotype P7.2.

Identification of Similarity of a Sequence using BLAST and Alignment

The homology of cloned QTL fragment was investigated against other identified nucleotide sequence of blast resistance genes by using BLAST (www.ncbi.nlm.nih.gov) and CLC workbench software. From BLAST analysis, cloned QTL fragment showed great similarity with *Oryza sativa* Japonica Group chromosome 3 clones OSJNBb0024B16 map S2606, complete sequence with query cover 51% and E value ie-20, and accession no. AC093017.10.

We also aligned the sequences of cloned QTL fragment with other identified blast resistant genes. Cloned QTL fragment revealed 52, 46, 38 and 23% similarity with *Pi-kh Pi-b Pi-zt* and *Pi9* genes, respectively (Table 1).

Six Frame Protein Translation of Cloned Fragment

Sequenced fragment translated into amino acid by using six frame translations in CLC workbench 6.0 software was used to confirm the secondary structure and leucine rich repeats in the sequence. After translation, frame +3 showed maximum distribution of leucine amino acid (Fig. 5).

Discussion

The PCR products of RM168 indicated that Pongsu Seribu

Table 1: Identity between the QTL cloned fragment and other identified blast resistance genes. Alignment was done using CLC workbench 6.0 software. The identity is represented in percentage (%)

	QTL qRBr-3.1 Cloned fragment)	*Pib*	*Pi-kh*	*Pi9*	*Pi-zt*
QTL qRBr-3.1 Cloned fragment	100	46	52	23	38
Pib		100	39	27	44
Pi-kh			100	32	43
Pi9				100	33
Pi-zt					100

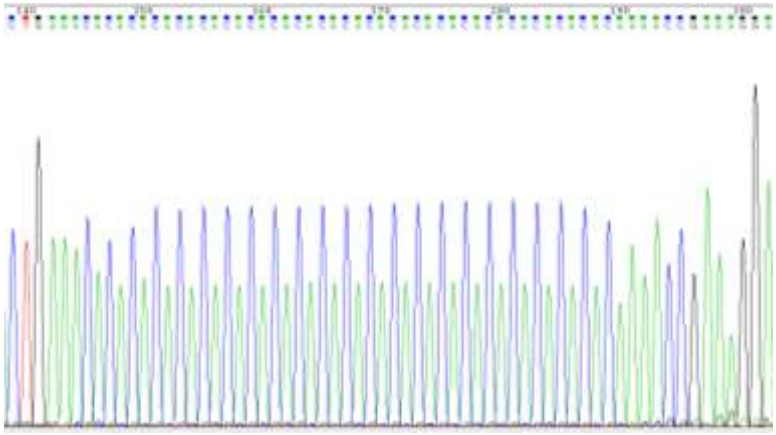

Fig. 4: Pattern of SSR repeats after cloning the QTL qRBr 3.1 and sequence was analysed in chromas software 2.01

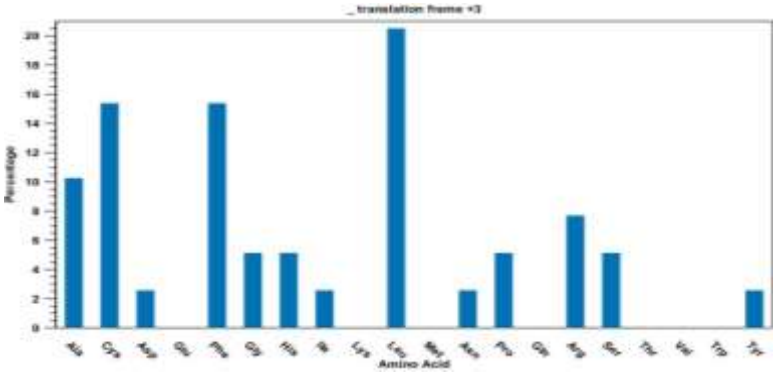

Fig. 5: Distribution of amino acids in cloned DNA fragment of Pongsu Seribu 2. Leucine showing maximum distribution

2 variety contained resistant genes linked with blast disease as reported by Ashkani *et al.* (2011). Therefore, the application of SSR markers is useful to identify the resistant genes in Pongsu Seribu 2 variety. Although the blast resistance specific gene in Pongsu Seribu 2 is yet not identified but screening against blast pathogen oryzae pathotype P7.2 has confirmed the resistance against the blast disease. From sequence analysis, it was identified that SSR markers, the molecular mapping, map-based cloning and other molecular techniques may be applied to isolate the blast resistance genes from Pongsu Seribu 2. The result indicates that combination of major blast resistance genes and QTLs are required to obtain maximum level of resistance in Pongsu Seribu 2 variety. Plant disease resistance contain leucine rich repeats with nucleotide binding sites, which is one of the largest resistance family (Liu *et al.*, 2010; Chen *et al.*, 2011; Okuyama *et al.*, 2011; Yuan *et al.*, 2011; Zhai *et al.*, 2011). It showed that translated QTL fragment did not contain conserved domain instead contained leucine rich repeats, confirming Pongsu Seribu 2 resistivity against the pathogen during host-plant interaction. Further, the alignment with resistant genes revealed that Pongsu Seribu 2 contained different blast resistance genes on chromosome 3. The new cloned QTL fragment showed 46% similarity with *Pi-b* gene, previously cloned on chromosome 2 (Sallaud *et al.*, 2003; Fjellstrom *et al.*, 2004) and 52% similarity with *Pi-kh* gene, previously mapped in Tetep rice variety on chromosome 12 (Fjellstrom *et al.*, 2004; Sharma *et al.*, 2005). Moreover, cloned QTL fragment showed 23% similarity with *Pi9* gene cloned by Ou *et al.* (2006) located on chromosome 6 and 38% similarity with *Pi-zt* gene, cloned by Zhou *et al.* (2006) located on chromosome 6. The isolation of mRNA from Pongsu Seribu 2 variety to produce complementary cDNA library for gene expression would further confirm the gene structure, function and regulation of the blast

resistance genes in Pongsu Seribu 2. Further analysis of protein in Pongsu Seribu 2 will help to identify the motif and terminals to find its complete protein structure. PCR product derived from genomic DNA of Pongsu Seribu 2 variety and amplified by microsatellite primers RM168 revealed a tandem repeat of (CA)23 linked to blast resistance genes. It was also found that protein sequence of LRR regions related to biotic stresses showed homology to other blast genes (*Pib, Pi-kh, Pi-zt and Pi-9)* located at different chromosomes. Therefore, this work is important in isolating the blast resistance genes for Pongsu Seribu 2 variety that can be utilised in breeding of blast resistant local Malaysian rice varieties.

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