DEVELOPMENT OF BLAST RESISTANT RICE VARIETY DERIVED FROM CROSSING BETWEEN MR219 AND PONGSU SERIBU 2 THROUGH MARKER-ASSISTED BACKCROSS BREEDING

TANWEER FATAH

FP 2015 46
DEVELOPMENT OF BLAST RESISTANT RICE VARIETY DERIVED FROM CROSSING BETWEEN MR219 AND PONGSU SERIBU 2 THROUGH MARKER-ASSISTED BACKCROSS BREEDING

By

TANWEER FATAH

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

November 2015
COPYRIGHT

All material contained within the thesis, including without limitation text, logos, icons, photographs and all other artwork, is copyright material of Universiti Putra Malaysia unless otherwise stated. Use may be made of any material contained within the thesis for non-commercial purposes from the copyright holder. Commercial use of material may only be made with the express, prior, written permission of Universiti Putra Malaysia.

Copyright © Universiti Putra Malaysia
DEDICATION

I dedicate this thesis to my beloved parents, wife, son, brother, his family and sister for their kind and loving support.
Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

DEVELOPMENT OF BLAST RESISTANT RICE VARIETY DERIVED FROM CROSSING BETWEEN MR219 AND PONGSU SERIBU 2 THROUGH MARKER-ASSISTED BACKCROSS BREEDING

By

TANWEER FATAH

November 2015

Chairman  :  Professor Mohd Rafii Yusop, PhD.
Faculty  :    Agriculture

Rice blast caused by fungus Magnaporthe oryzae is a major rice disease due to significant yield losses worldwide as well as in Malaysia. Cultivating blast-resistant rice varieties is the most effective, economical and practical approach to prevent blast disease. Marker-assisted backcross breeding contribute an effective and vital role in incorporating blast resistant genes into blast susceptible high yielding rice varieties.

In this study two blast resistant genes (putative Pi-b and Pi-kh) were identified from the Malaysian rice variety Pongsu Seribu 2 and revealed that it contain Nucleotide Binding Site-Leucine Rich Repeat (NBS-LRR) domain. The blast resistant genes (Pi-b and Pi-kh) were introgressed from Pongsu Seribu 2 variety using marker-assisted backcross breeding strategy into a high-yielding blast susceptible rice variety MR219. Therefore crosses were made between the MR219 used as recurrent parent and Pongsu Seribu 2 as donor parent to transfer blast resistant genes into MR219 without losing their actual quality and yield sustainability. Eleven SSR molecular markers linked to rice blast resistant genes were found polymorphic between the two parental varieties and used to find the potential relation with blast resistance in the present developed backcross population. The polymorphic markers were used in the further subsequent generation for confirmation of blast resistant genes. Out of the 11 markers, only 2 markers RM208 (located on chromosome 2 linked to Pi-b gene) and RM206 (located on chromosome 11 linked to Pi-kh gene) conferring blast resistance were confirmed and used in F₁, BC₁F₁, BC₂F₁ and BC₂F₂ generations providing resistance against most virulent Malaysian rice blast fungus M. oryzae pathotype P7.2.

300-SSR markers were screened, out of them 72 markers were found to be polymorphic between the parental lines and used for background recovery of the recurrent parent (MR219) in each backcross population. The inheritance patterns and identification of microsatellite markers linked to the rice blast resistance were observed in BC₂F₁ and BC₂F₂ generations. The recurrent parent MR219 showed susceptibility with lesion 5 to 7 score, and donor parent PS2 showed resistivity with lesion 0 to 2 while challenging to pathotype P7.2 fungus inoculum under control conditions. In
BC$_2$F$_1$ generation, 320 plants were inoculated with pathotype P7.2 and 154 plants showed the resistance mechanism while another 166 plants showed susceptible reaction to blast. Chi-square test ($\chi^2$) for the single-gene model was applied for testing goodness of fit of observed frequencies. The two linked markers RM208 ($\chi^2=1.5130; p=0.2188$) and RM206 ($\chi^2=0.6130; p=0.4338$) for blast resistance to pathotype P7.2 showed good fit with expected test cross ratio (1:1) for single-gene model analysis. The markers RM208 and RM206 found suitable for marker-assisted selection of $Pi$-$b$ and $Pi$-$kh$ blast resistance genes conferring resistance against the blast pathotype P7.2. Phenotypically BC$_2$F$_2$ population segregated into 3:1 ratio. The genotypic segregation of the BC$_2$F$_2$ population segregated into 1:2:1 ratio. The background selection analysis for the recovery of MR219 variety among the best improved lines ranged from 73 to 94% in BC$_1$F$_1$, 79.4 to 96.1 in the BC$_2$F$_1$ and 94 to 97.5% in BC$_3$F$_2$ generations. The average proportions of the recurrent parent genome in the selected 15 improved lines of BC$_3$F$_2$ were 96.17%, explaining that very close phenotypic resemblance to the recurrent parent MR219. The 15 homozygous lines carrying blast resistant genes with similar background to MR219 were selected for the development of improved blast resistant rice variety. The agro-morphological traits of the improved lines and recurrent parent showed no significant difference between those lines. In conclusion, from the present rice breeding program, 15 homozygous advanced blast resistant rice lines were developed with a high potential to be released as a new variety for commercial cultivation.
Abstrak tesis dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan ijazah Doktor Falsafah

PEMBANGUNAN VARIETI PADI RINTANG KARAH MENERUSI KACUKAN ANTARA MR219 DAN PONGSU SERIBU 2 MELALUI PEMBIAKBAKAAN KACUKBALIK BANTUAN PENANDA

Oleh

TANWEER FATAH

November 2015

Pengerusi : Profesor Mohd Rafii Yusop, PhD
Fakulti : Pertanian

Karah padi adalah disebabkan oleh fungi Magnaporthe oryzae, merupakan satu penyakit utama padi yang mengakibatkan penurunan hasil yang ketara di seluruh dunia serta di Malaysia. Penanaman padi rintang karah merupakan pendekatan yang paling berkesan, ekonomik dan praktikal untuk mencegah penyakit karah ini. Pembibakbakaan kacukbalik bantuan penanda menunjukkan impak yang berkesan dan penting dalam memindahkan gen kerintangan karah ke varieti padi berhasil tinggi yang rentan penyakit.

Dalam kajian ini dua gen kerintangan karah (putatif Pi-b dan Pi-kh) telah dikenalpasti dari varieti padi Malaysia, Pongsu Seribu 2 dan didapati ianya mengandungi domain Nucleotide Binding Site-Leucine Rich Repeat (NBS-LRR). Gen kerintangan (Pi-b dan Pi-kh) telah diintrogresikan dari varieti padi Pongsu Seribu 2, dengan menggunakan strategi pembibakbakaan kacukbalik bantuan penanda, ke dalam satu varieti padi berhasil tinggi yang rentan karah, MR219. Kacukkan telah dilakukan antara MR219 sebagai induk penerima dan Pongsu Seribu 2 sebagai induk penderma untuk memindahkan gen kerintangan karah dari Pongsu Seribu 2 ke MR219 tanpa kehilangan kualiti dan prestasi hasil induk penerima. Sebelas penanda molekul SSR yang berkait rapat dengan gen kerintangan karah padi memberikan polimorfik diantara kedua induk tersebut dan telah digunakan untuk menentukan potensinya sebagai penanda untuk gen kerintangan karah dalam populasi kacukbalik yang sedang dibangunkan ini. Dari 11 penanda polimorfik tersebut, hanya dua SSR penanda iaitu RM208 (terletak pada kromosom 2 dikaitkan dengan gen Pi-b) dan RM206 (terletak pada kromosom 11 dikaitkan dengan gen Pi-kh) menunjukkan kerintangan karah telah dikenalpasti dan telah digunakan dalam generasi F1, BC1F1, BC2F1 dan BC2F2 terhadap patotip fungi karah karah padi Malaysia M. oryzae yang virulen, P7.2.

Daripada 300-penanda SSR yang telah diuji, didapati 72 penanda polimorfik antara kedua-dua induk, dan penanda tersebut telah digunakan untuk pemulihan genom induk penerima (MR219) dalam setiap populasi kacukbalik. Pewarisan gen kerintangan dicerap dalam generasi BC1F1 dan BC2F2. Induk penerima, MR219 menunjukkan
kerentanan dengan skor lesion 5 hingga 7, dan induk penderma, PS2 menunjukkan kerintangan dengan lesion 0 hingga 2 terhadap fungi patotip P7.2 yang diinokulasi di bawah keadaan terkawal. Dalam generasi BC$_2$F$_1$, 320 pokok telah diinokulasi dengan patotip P7.2, dan 154 pokok telah menunjukkan mekanisme kerintangan, manakala 166 pokok lagi menunjukkan reaksi rentan terhadap karah. Ujian Chi-NXDVGXD$X$QWXN model gen tunggal telah dijalankan ujian padanan yang baik dengan frekuensi yang GLFHUDS `XDSHQDGDWHUHHE$W50$ S GDQ50$ 0,6130; p = 0,4338) untuk kerintangan kara bagi patotip P7.2 menunjukkan padanan yang tepat dengan nisbah dijangka (1:1) untuk analisis model gen tunggal. Kedua-dua penanda tersebut didapati sesuai untuk pemilihan bantuan penanda kerintangan gen karah, $Pi-b$ dan $Pi-kh$ yang memberikan rintangan terhadap patotip P7.2. Secara fenotipik populasi BC$_2$F$_2$ memberikan kadar segregasi 3:1 terhadap karah patotip P7.2. Segregasi genotip populasi BC$_2$F$_2$ adalah dengan nisbah 1:2:1. Analisis pemulihan genom bagi varieti MR219 dikalangan titisan maju adalah dari 73 hingga 94% dalam generasi BC$_1$F$_1$, 79.4 hingga 96.1% dalam BC$_2$F$_1$ dan 94 hingga 97.5% dalam BC$_2$F$_2$. Purata peratusan genom induk penerima dalam 15 titisan maju terpilih adalah 96.17%, yang menunjukkan persamaan fenotip sangat rapat dengan induk penerima, MR219. Lima belas titisan homozaigus yang mempunyai gen kerintangan serta dengan kesamaan genom MR219 yang tinggi telah dipilih untuk pembangunan varieti padi yang rintang penyakit karah. Ciri agro-morfologi diantara titisan maju tersebut dengan induk penerima MR219 menunjukkan tidak terdapat perbezaan yang bererti. Kesimpulannya, dari program pembiakbakaan ini, 15 titisan maju homozaigus rintang karah telah dihasilkan dan ianya sangat berpotensi untuk menghasilkan varieti padi baru bagi penanaman secara komersial.
ACKNOWLEDGEMENTS

First, I wish to extend my profound gratitude to Almighty $\text{ALLAH}$ for His spiritual guidance and encouragement during my study. Deepest gratitude and many thanks to Professor Dr. Mohd Rafii Yusop for his valuable time, constructive advice, continuous support, guidance, encouragement, patience and wisdom throughout my PhD. I would also like to extend my appreciate thankfulness to my co-supervisor Associate Prof. Dr. Kamaruzaman Sijam, Dr. Abdul Rahim Harun and Dr. Mohammad Abdul Latif for their fruitful criticism, valuable guidance, patience and support. I was fortunate to have such a prodigious supervisory committee.

My gratitude goes to the management of Sindh Agriculture University, Tandojam, Sindh, Pakistan for granting a scholarship to pursue a Ph.D study at Universiti Putra Malaysia (UPM), Malaysia. I also acknowledge the Ministry of Education (MOE), Malaysia for the Long Term Research Grant Scheme LRGS (Food Security) and Universiti Putra Malaysia for funding this research project and providing the technical support. I am grateful to the staff and all the students who have been in the Plant Breeding and Genetic laboratory, Faculty of Agriculture, UPM. I wish to thank my lab colleague Fahim Ahmed and Welland for their help, courage and moral support.

I am also thankful to my friends Arfan Ahmed Gilal, Asmatullah Kaka, Atique Ahmed Behan, Abdul Raheem Channa, Ubedullah Kaka, Shafiq Ahmed and Mujtaba Brohi for their support throughout my stay in Malaysia. I wish to extend my sincere thanks to Dr. Ali Gohar Abro for their sincere help and encouragement to accomplish my PhD degree.

I am also grateful to Muhammad Zubair, Arsalan Abbasi, Asad Rajput, Ahsan Khanzada, and Kamran Arain, whose blessings and encouragement have helped me to accomplish this task. I wish to pay special thanks and the deepest appreciation to Muhammad Umer Memon for his moral and social encouragement, support and help from the beginning of the process of application till the end of my study who remained my well-wisher and acted as best friend during my study and throughout my life.

Last but certainly not the least, my deepest gratitude goes to my family, my father, Abdul Fatah, my mother Sakina, my brother Muneer, his wife Saima and daughter Tayaba and my sister Firdous for their blessings and prayers from far away and support needed to accomplish this goal. Most importantly, my sincere gratitude goes to my wife Anika and beloved son Azmeer for their understanding, patience and unconditional love and support all the time. Finally, I am thankful to all who helped me directly or indirectly during the period of my study.
I certify that a Thesis Examination Committee has met on 17 November 2015 to conduct the final examination of Tanweer Fatah on his thesis entitled "Development of Blast Resistant Rice Variety Derived from Crossing between MR219 and Pongsu Seribu 2 Through Marker-Assisted Backcross Breeding" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

**Faridah binti Qamaruz Zaman, PhD**
Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Chairman)

**Datin Siti Nor Akmar binti Abdullah, PhD**
Professor
Institute of Tropical Agriculture
Universiti Putra Malaysia
(Internal Examiner)

**Mohd. Puad bin Abdullah, PhD**
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

**Bing Yang, PhD**
Associate Professor
Iowa State University
United States of America
(External Examiner)

\[Signature\]

**ZULKARNAIN ZAINAL, PhD**
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 15 December 2015
This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the supervisory committee were as follows:

**Mohd Rafii Yusop, PhD**  
Professor  
Institute of Tropical Agriculture  
Universiti Putra Malaysia  
(Chairman)

**Kamaruzaman Sijam, PhD**  
Associate Professor  
Faculty of Agriculture  
Universiti Putra Malaysia  
(Member)

**Dr. Abdul Rahim Harun**  
Agrotechnology and Bioscience Division  
Malaysian Nuclear Agency  
(Member)

**Md. Abdul Latif, PhD**  
Principal Scientific Officer  
Plant Pathology Division  
Bangladesh Rice Research Institute  
(Member)

__________________________________________________________

**BUJANG BIN KIM HUAT, PhD**  
Professor and Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date:
Declaration by graduate student

I hereby confirm that:

- this thesis is my original work;
- quotations, illustrations and citations have been duly referenced;
- this thesis has not been submitted previously or concurrently for any other agree at any other institution
- intellectual property from the thesis and copyright of thesis are fully-owned by Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012;
- Written permission must be obtained from the supervisor and the office of Deputy Vice Chancellor (Research and Innovation) before the thesis is published (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writings, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other material as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- There is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software.

Signature: ____________________________  Date: __________________

Name and Matric No.: Tanweer Fatah, GS32718
Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

Signature: ____________________________  Signature: ____________________________
Name of Chairman of Supervisory Committee: ____________________________  Name of Member of Supervisory Committee: ____________________________
Prof. Dr. Mohd Rafii Yusop  Dr. Abdul Rahim Harun
Head of Food Crops Laboratory  Kepala Kumpulan Agro Pengurusan
Institute of Tropical Agriculture  Bahagian Agroteknologi & Biosains
Universiti Putra Malaysia  Agensi Nuklear Malaysia

Signature: ____________________________  Signature: ____________________________
Name of Member of Supervisory Committee: ____________________________  Name of Senior Fellow Researcher:
Dr. Kamaruzaman Bin Sijam  Dr. Mohammad Abdul Latif
Prof. Madya  Department of Crop Science
Jabatan Perlindungan Tumbuhan  Faculty of Agriculture
Universiti Putra Malaysia  Universiti Putra Malaysia

Selangor Darul Ehsan
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>v</td>
</tr>
<tr>
<td>APPROVAL</td>
<td>vi</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xvii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xix</td>
</tr>
</tbody>
</table>

## CHAPTER

### 1 INTRODUCTION

1.1 General introduction 1

1.2 Significance of the study 2

1.3 Problem statement 2

1.4 Research Objectives 2

### 2 REVIEW OF LITERATURE

#### 2.1 Rice

2.1.1 Rice production in the world 4

2.1.2 Rice production in Malaysia 5

2.1.3 Cultivation of MR219 and Pongsu Seribu 2 rice varieties in Malaysia 6

2.1.4 MR219 Variety 6

2.1.5 Pongsu Seribu 2 (PS2) Variety 6

2.1.6 Rice as model plant 7

#### 2.2 Rice diseases

2.2.1 Rice blast 8

2.2.2 Mechanism of pathogenesis of *Magnaporthe oryzae* 9

2.2.3 Symptoms of blast disease 10

2.2.4 Variation among pathotype of *Magnaporthe oryzae* 11

2.2.5 Strategies to manage blast disease 12

2.2.6 Classification of blast resistance 12

#### 2.3 Overview of blast resistance genes in rice

2.3.1 Blast resistance *Pi-b* and *Pi-kh* genes in rice 18

2.3.2 Putative location of blast resistance genes in rice 18

2.3.3 Cloning of blast resistance genes 20

#### 2.4 Molecular markers

2.4.1 SSR markers for rice breeding 24

2.4.1.1 Advantages and disadvantages of SSR markers 24

2.4.1.2 Application of SSR markers 25

2.4.1.3 SSR markers for tracking blast resistance genes 25

#### 2.5 Conventional breeding

27
2.6 Marker-assisted backcrossing (MABC) 28
   2.6.1 Foreground selection 29
   2.6.2 Recombinant selection 29
   2.6.3 Background selection 30
   2.6.4 Advantages and disadvantages of marker-assisted backcrossing 31
   2.6.5 Application of marker-assisted backcrossing 32
   2.6.6 Marker-assisted backcrossing for the blast resistance 32

3 CLONING AND SEQUENCING OF BLAST RESISTANCE GENES FROM RESISTANT RICE VARIETY PONGSU SERIBU 2 36
   3.1 Introduction 36
   3.2 Material and methods 37
      3.2.1 Plant materials 37
      3.2.2 Pathological activities (media and inoculum preparation) 37
      3.2.3 Disease reaction, Scoring and pathogenecity assay 38
      3.2.4 Genomic DNA extraction 39
      3.2.5 PCR amplification 40
      3.2.6 Gel electrophoresis 40
      3.2.7 Designing of primers 40
      3.2.8 Cloning and sequence of PCR amplified products 41
         3.2.8.1 Preparation of competent cells 41
         3.2.8.2 Ligation and transformation 41
         3.2.8.3 Recombinant cell screening 42
         3.2.8.4 Clone storage 43
         3.2.8.5 Plasmid DNA isolation 43
         3.2.8.6 Analysis of DNA sequence 43
   3.3 Results 43
      3.3.1 Disease reaction of M. oryzae pathotype P7.2 against Pongsu Seribu 2 and MR219 43
      3.3.2 Cloning of Pi-b and Pi-kh fragment from Pongsu Seribu 2 44
      3.3.3 Characterization of transcript product of Pi-b and Pi-kh blast resistance genes 45
      3.3.4 Multiple sequence alignment with known R-genes 46
      3.3.5 Searching for identical protein sequence by using BLASTp tool in NCBI database 47
      3.3.6 Phylogenetic analysis 48
   3.4 Discussion 49
   3.5 Conclusion 51
IDENTIFICATION OF SUITABLE SEGREGATING SSR MARKERS FOR BLAST RESISTANCE IN RICE USING INHERITANCE AND DISEASE REACTION ANALYSIS IN BACKCROSS POPULATION

4

4.1 Introduction 52
4.2 Material and methods 53
4.2.1 Plant material 53
4.2.2 Leaf sample collection 53
4.2.3 Backcrossing scheme and breeding strategy 53
4.2.4 Primers for SSR amplification 55
4.2.5 DNA extraction, PCR amplification and Gel electrophoresis 58
4.2.6 Marker genotyping 58
4.2.7 Raising of seedling for pathogenicity assay 58
4.2.8 Preparation of Inoculum 58
4.2.9 Blast disease scoring 58
4.2.10 Statistical analysis 59

4.3 Results 59
4.3.1 Checking quality and quantity of DNA 59
4.3.2 Polymorphic SSR markers between parental lines 60
4.3.3 SSR marker tightly linked with blast resistance genes 63
4.3.4 Analysis of SSR marker segregation in BC$_2$F$_1$ population 65
4.3.5 Inheritance of blast resistance 66
4.3.6 Distribution of Trait Frequency 68

4.4 Discussion 68

4.5 Conclusion 70

5 ESTIMATION OF RECOVERY OF RECURRENT PARENT GENOME IN MARKER-ASSISTED BACKCROSS POPULATIONS BY CROSSING MR219 AND PONGSU SERIBU 2

5.1 Introduction 71
5.2 Material and Methods 72
5.2.1 Plant material and leaf Sample collection 72
5.2.2 Backcrossing scheme and breeding strategy 72
5.2.3 Analysis of molecular markers 72
5.2.3.1 Foreground selection 72
5.2.3.2 Background selection 72
5.2.4 DNA Extraction, PCR (Polymerase Chain Reaction), Gel Electrophoresis 72
5.2.5 Analysis of Data 73

5.3 Results 73
5.3.1 Foreground and background polymorphic SSR markers 73
5.3.2 Genotypic survey of BC$_1$F$_1$ generation plants 73
5.3.2.1 Foreground selection of blast resistance genes 73
6 EVALUATION, VERIFICATION AND SELECTION OF SELECTED ADVANCE IMPROVED BLAST RESISTANT LINES FROM BC$_2$F$_2$ POPULATION

6.1 Introduction 88
6.2 Material and Methods 88
   6.2.1 Plant materials and leaf sample collection 88
   6.2.2 Developing blast resistant lines 89
   6.2.3 Microsatellite analysis 89
   6.2.3.1 Markers for foreground selection 89
   6.2.3.2 Markers for background selection 89
   6.2.4 Molecular marker data analysis 89
   6.2.5 DNA extraction, PCR amplification, gel electrophoresis 89
   6.2.6 Phenotypic screening of plant against *Magnaporthe oryzae* pathotype P7.2 89
   6.2.7 Agronomic performance of selected best lines of BC$_2$F$_2$ generation 90
   6.2.8 Statistical analysis 90
6.3 Results 92
   6.3.1 Marker assisted foreground selection 92
   6.3.2 Screening against blast disease in improved blast resistant lines of BC$_2$F$_2$ population 93
   6.3.3 Assessment of phenotypic segregation of blast resistant versus susceptible plants 95
   6.3.4 Marker-Trait association 96
   6.3.5 Variation and correlation among trait 96
   6.3.6 Recovery of recurrent parent genome in selected improved homozygous lines 97
   6.3.7 Comparison of agro-morphological performance of improved lines versus recurrent parent MR219 102
6.4 Discussion 103
6.5 Conclusion 105

7 SUMMARY, GENERAL CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH

7.1 Summary 106
7.2 Conclusion 108
7.3 Recommendation for future research 109
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>REFERENCES</td>
<td>111</td>
</tr>
<tr>
<td>APPENDICES</td>
<td>130</td>
</tr>
<tr>
<td>BIODATA OF STUDENT</td>
<td>139</td>
</tr>
<tr>
<td>LIST OF PUBLICATIONS</td>
<td>140</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>General agro-morphological characteristics of MR219 and Pongsu Seribu 2 rice varieties</td>
<td>7</td>
</tr>
<tr>
<td>2.2</td>
<td>Classification of blast resistance in rice</td>
<td>13</td>
</tr>
<tr>
<td>2.3</td>
<td>Blast resistance genes (Pi) identified from different rice cultivar</td>
<td>15</td>
</tr>
<tr>
<td>2.4</td>
<td>List of cloned blast resistance genes from different rice cultivars</td>
<td>21</td>
</tr>
<tr>
<td>2.5</td>
<td>Common features of mostly used molecular markers and their comparison based on their utility</td>
<td>23</td>
</tr>
<tr>
<td>2.6</td>
<td>Microsatellite markers linked to rice blast disease resistance gene</td>
<td>26</td>
</tr>
<tr>
<td>2.7</td>
<td>Successful example of conventional breeding for blast resistance in rice</td>
<td>27</td>
</tr>
<tr>
<td>2.8</td>
<td>Comparison of conventional and marker assisted backcross breeding for recovery of recurrent parent genome in subsequent generations</td>
<td>31</td>
</tr>
<tr>
<td>2.9</td>
<td>Successful example of marker-assisted selection for disease resistance in rice</td>
<td>33</td>
</tr>
<tr>
<td>3.1</td>
<td>Standard evaluation system for blast lesion degree in rice</td>
<td>39</td>
</tr>
<tr>
<td>3.2</td>
<td>Ingredients of PCR amplification</td>
<td>40</td>
</tr>
<tr>
<td>3.3</td>
<td>List of primers used for amplification designed from flanking sequence of Pi-b and Pi-kh blast genes</td>
<td>41</td>
</tr>
<tr>
<td>3.4</td>
<td>Result of searching similarity between Pi-kh and Pi-b blast resistance gene deduced amino acid sequence with other identified sequences by using BLASTp algorithm</td>
<td>48</td>
</tr>
<tr>
<td>4.1</td>
<td>Polymorphic SSR markers tightly linked to blast resistance genes in MR219 x Pongsu Seribu 2</td>
<td>57</td>
</tr>
<tr>
<td>4.2</td>
<td>Information of seventy two polymorphic SSR markers between MR219×Pongsu Seribu 2</td>
<td>61</td>
</tr>
<tr>
<td>4.3</td>
<td>Marker segregation analysis in BC_2F1 lines derived from the cross between rice varieties MR219 × Pongsu Seribu 2</td>
<td>66</td>
</tr>
<tr>
<td>4.4</td>
<td>Phenotypic segregation of BC_2F1 lines against blast resistance after artificial inoculation with pathotype P7.2 of Magnaporthe oryzae</td>
<td>67</td>
</tr>
<tr>
<td>4.5</td>
<td>Chi-square test for two-gene model (1:1:1:1) and epistatic interaction (15:1) for blast resistance in BC_2F1 population</td>
<td>68</td>
</tr>
<tr>
<td>5.1</td>
<td>Analysis of background and introgressed segment in selected best lines of BC_1F1 population</td>
<td>76</td>
</tr>
<tr>
<td>5.2</td>
<td>Calculation of resistant and susceptible plants in BC_1F1 and BC_2F1 generation</td>
<td>79</td>
</tr>
<tr>
<td>5.3</td>
<td>Background recovery and introgressed segment analysis in selected best lines of BC_2F1 population</td>
<td>82</td>
</tr>
<tr>
<td>6.1</td>
<td>Description of the Agro-morphological traits measured in parental lines along with BC_2F2 generation plants</td>
<td>91</td>
</tr>
<tr>
<td>6.2</td>
<td>Allele size of the foreground markers linked to blast resistant genes (Putative Pi-b and Pi-kh) in susceptible (MR219) and resistant (Pongsu Seribu 2) parents</td>
<td>92</td>
</tr>
<tr>
<td>6.3</td>
<td>Analysis of markers in BC_3F2 segregating population</td>
<td>92</td>
</tr>
<tr>
<td>6.4</td>
<td>Phenotypic segregation ratio of observed and expected number of resistant and susceptible plants in the BC_3F2 population inoculated with highly virulent pathotype P7.2 of Magnaporthe oryzae</td>
<td>95</td>
</tr>
</tbody>
</table>
6.5 Chi-square test for independent gene model (9:3:3:1) and epistatic effect (15:1) for blast resistance in BC$_2$F$_2$ population inoculated with pathotype P7.2 of Magnaporthe oryzae.

6.6 Association between the marker and trait in the BC$_2$F$_2$ population analysed by regression analysis

6.7 Trait variation for selected pathotype P7.2 of Magnaporthe oryzae inoculated in BC$_2$F$_2$ population

6.8 Correlation coefficient between BLD, BLT, and %DLA for pathotype P7.2 in BC$_2$F$_2$ population

6.9 Introgressed and background recovery analysis in selected improved lines

6.10 Performance of major agronomic traits of BC$_2$F$_2$ improved lines carrying blast resistant (Putative Pi-b and Pi-kh) genes
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Paddy Rice production in the world by countries</td>
<td>5</td>
</tr>
<tr>
<td>2.2</td>
<td>Trend of area under production and yield of rice in Malaysia</td>
<td>6</td>
</tr>
<tr>
<td>2.3</td>
<td>Mechanism of Pathogenesis of <em>Magnaporthe oryzae</em></td>
<td>10</td>
</tr>
<tr>
<td>2.4</td>
<td>Typical symptoms of blast disease and types of lesion appeared on the young and old leaves causing leaf blast and collar blast</td>
<td>11</td>
</tr>
<tr>
<td>2.5</td>
<td>Distribution of blast resistance genes among 12 chromosomes of rice</td>
<td>19</td>
</tr>
<tr>
<td>2.6</td>
<td>Putative location of the blast resistance genes</td>
<td>19</td>
</tr>
<tr>
<td>2.7</td>
<td>Level of selection used in marker assisted backcrossing</td>
<td>30</td>
</tr>
<tr>
<td>3.1</td>
<td>Pathological activities for preparation of inoculum and spraying conidial suspension</td>
<td>37</td>
</tr>
<tr>
<td>3.2</td>
<td>pGEM-T Easy vector with multiple cloning sites</td>
<td>42</td>
</tr>
<tr>
<td>3.3</td>
<td>Frequency of leaf blast disease reaction between PS2 and MR219 cultivar</td>
<td>44</td>
</tr>
<tr>
<td>3.4</td>
<td>Amplification of cloned segment of <em>Pi-b</em> and <em>Pi-kh</em> blast resistance gene</td>
<td>45</td>
</tr>
<tr>
<td>3.5</td>
<td>Presence of LRR domain in the cloned fragment of <em>Pi-kh</em> blast resistance gene</td>
<td>46</td>
</tr>
<tr>
<td>3.6</td>
<td>Presence of Zinc finger protein domain in the cloned fragment of <em>Pi-b</em> blast resistance gene</td>
<td>46</td>
</tr>
<tr>
<td>3.7</td>
<td>Alignment of <em>Pi-kh</em> putative conserved domain with other known blast resistance gene</td>
<td>47</td>
</tr>
<tr>
<td>3.8</td>
<td>Alignment of <em>Pi-b</em> putative conserved domain with other known blast resistance genes</td>
<td>47</td>
</tr>
<tr>
<td>3.9</td>
<td>Average distance tree using % identity showing relationship of <em>Pi-b</em> and <em>Pi-kh</em> blast resistance genes with other cloned blast resistance genes NBS analogs with NBS-LRR class of R-genes</td>
<td>49</td>
</tr>
<tr>
<td>4.1</td>
<td>Diagrammatic representation of rice varieties MR219 and Pongsu Seribu 2</td>
<td>54</td>
</tr>
<tr>
<td>4.2</td>
<td>General Steps for the producing crossed seed</td>
<td>54</td>
</tr>
<tr>
<td>4.3</td>
<td>Procedure of marker-assisted backcrossing scheme for the development of different generations</td>
<td>56</td>
</tr>
<tr>
<td>4.4</td>
<td>Nano-drop technologies for DNA quantity and quality</td>
<td>60</td>
</tr>
<tr>
<td>4.5</td>
<td>Genotyping with SSR marker RM208 and RM206 linked to blast resistance in F1 progenies of rice population derived from MR219 × Pongsu Seribu 2 (PS2)</td>
<td>64</td>
</tr>
<tr>
<td>4.6</td>
<td>Genotyping with SSR marker RM208 and RM206 linked to blast resistance in BC2F1 progenies of rice population derived from MR219 × Pongsu Seribu 2 (PS2)</td>
<td>64</td>
</tr>
<tr>
<td>4.7</td>
<td>The position of RM208 and RM206 marker on rice chromosome 2 and 11</td>
<td>65</td>
</tr>
<tr>
<td>4.8</td>
<td>Rice cultivar (a) MR219 highly susceptible (b) Pongsu Seribu 2 (PS2) highly resistant</td>
<td>67</td>
</tr>
</tbody>
</table>
4.9 Distribution of frequency of the blast lesion degree (BLD) in the BC_2F_1 population against pathotype P7.2

5.1 Screening of resistant and susceptible plants using RM208 marker in BC_1F_1 generation

5.2 Banding pattern of background marker RM258 in BC_1F_1 generation

5.3 Banding pattern of background marker RM1337 in BC_1F_1 generation

5.4 Frequency distribution of the recurrent parent genome (RPG) recovery in BC_1F_1 generation population derived from cross between MR219 and Pongsu Seribu 2

5.5 Chromosome-wise recovery of recurrent parent genome in selected six best plants of BC_1F_1 generation

5.6 Chromosome-wise highest recovery of recurrent parent genome of the best plant no. 5-1 in BC_1F_1 generation

5.7 Screening of resistant and susceptible plant using RM208 and RM206 marker in the BC_2F_1 generation

5.8 Banding pattern of background marker RM242 in BC_2F_1 generation

5.9 Banding pattern of background marker RM5961 in BC_2F_1 generation

5.10 Banding pattern of background marker RM515 in BC_2F_1 generation

5.11 Frequency distribution of the percentage of recurrent parent genome in the BC_2F_1 population derived from cross between MR219 and Pongsu Seribu 2

5.12 Proportion of recovery of recurrent parent genome of selected best plants covering all 12 rice chromosome of BC_2F_1 generation

5.13 Chromosome-wise highest recovery of recurrent parent genome of the plant no. 5-3-7 in BC_2F_1 generation

6.1 Blast resistant improved homozygous line genotyping using linked marker RM208

6.2 Blast resistant improved homozygous line genotyping using linked marker RM206

6.3 Blast disease reaction of blast inoculated plants with the virulent pathotype P7.2 of *Magnaporthe oryzae*

6.4 Calculation of distribution of blast lesion length after inoculation with *Magnaporthe oryzae* pathotype P7.2 in parental lines along with improved introgressed blast resistant genes lines of BC_2F_2 population

6.5 Graphical genotype of selected improved lines with introgressed *Pi* genes along with MR219 background

6.6 Graphical genotype of the improved lined with lowest recovery among the best 15 improved lines

6.7 Graphical genotype of the improved lined with highest recovery among the best 15 improved lines
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>QTL</td>
<td>Quantitative trait loci</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>MAS</td>
<td>Marker-assisted selection</td>
</tr>
<tr>
<td>MABB</td>
<td>Marker-assisted backcross breeding</td>
</tr>
<tr>
<td>MABC</td>
<td>Marker-assisted backcrossing</td>
</tr>
<tr>
<td>SSR</td>
<td>Simple sequence repeat</td>
</tr>
<tr>
<td>MT</td>
<td>Million tons</td>
</tr>
<tr>
<td>Mb</td>
<td>Mega base</td>
</tr>
<tr>
<td>NBS-LRR</td>
<td>Nucleotide binding site- Leucine rich repeat</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>YAC</td>
<td>Yeast artificial chromosome</td>
</tr>
<tr>
<td>AFLP</td>
<td>Amplified fragment length polymorphism</td>
</tr>
<tr>
<td>STS</td>
<td>Sequence tag site</td>
</tr>
<tr>
<td>RAPD</td>
<td>Randomly Amplified Polymorphic DNA</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>RIL</td>
<td>Recombinant inbred line</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethylammonium bromide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine Tetraacetic Acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>SOB</td>
<td>Super Optimal Broth</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-triphosphate</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>Taq</td>
<td>Thermus aquaticus</td>
</tr>
<tr>
<td>RGA</td>
<td>Resistance gene analogue</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed sequence tag</td>
</tr>
<tr>
<td>RPG</td>
<td>Recurrent parent genome</td>
</tr>
<tr>
<td>RIL</td>
<td>Recombinant inbred line</td>
</tr>
<tr>
<td>RH</td>
<td>Relative humidity</td>
</tr>
<tr>
<td>SMA</td>
<td>Single marker analysis</td>
</tr>
<tr>
<td>SES</td>
<td>Standard evaluation score</td>
</tr>
<tr>
<td>TE</td>
<td>Tris/EDTA</td>
</tr>
<tr>
<td>V</td>
<td>volt</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
</tr>
<tr>
<td>- ME</td>
<td>-mercaptoethanol</td>
</tr>
<tr>
<td>ddH2O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>BLD</td>
<td>Blast lesion degree</td>
</tr>
<tr>
<td>BLT</td>
<td>Blast lesion type</td>
</tr>
<tr>
<td>%DLA</td>
<td>Percent diseased leaf area</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 General introduction

Rice (Oryza sativa L.) is the most widely consumed staple dietary food and largest cereal crops throughout the world, especially in Asia (Khush, 2005). Rice is not only used for human consumption, but it is also used for animal feeding and provides the major source of income for rural people (Datta, 2004). Rice provides nutrition for six billion people worldwide (Maclean et al., 2002). Considering its nutritional value, along with carbohydrates rice also provides other essential nutrients such as protein, iron, calcium, thiamine, riboflavin, niacin and vitamin E, to the human body (Akinbile et al., 2011).

In Malaysia, rice is mostly grown in eight granary areas of Peninsular Malaysia, Sabah and Sarawak, mainly under flooded conditions (Akinbile et al., 2011). Malaysia is ranked 24th in terms of rice production in the world with annual production of 2.750 million tons (FAO, 2014). Total area under rice cultivation in Malaysia in 2014 was 692,340 ha, out of which 43,353 ha are situated in Sabah and 121,921 in Sarawak (DOA, 2014). Presently, Muda Agricultural Development Authority (MADA) and Kemudu Agriculture Development Authority (KADA) are two main rice growing areas in Malaysia. At present Malaysia in term of rice production is not self-sufficient. The main factor in limiting the rice production includes various biotic and abiotic stresses.

Among biotic stresses, diseases are one of the main factor that reduces rice production. The only best way to minimize the yield losses is the production of high-yielding resistant rice varieties. Blast caused by the fungal pathogen Magnaporthe oryzae is one of the most serious diseases because the yield loss caused by blast range from 1 to 50 % worldwide including Malaysia. On the other hand Malaysia has targeted to achieve 80% rice-sufficiency by 2020 (FAO, 2014). There is an urgent need to expand research in rice in order to assure rice sufficiency by controlling disease and increasing yield potential. Recently, several techniques have been adopted to control the blast disease, including chemical control, disease forecasting, cultivation practice, but these control measures are not very effective (Srinivasachary et al., 2002). The recent advent of DNA marker technology, gene cloning, development of numerous markers, molecular breeding approaches helped plant breeders to produce rice varieties with blast resistance and higher yield potential. The cloning and characterization of blast resistant genes facilitated the exploration of mechanism of host-pathogen interaction. DNA markers also acted as a valuable resource to localize the genes/QTL controlling important traits such as plant disease resistance (Yano and Sasaki, 1997; Paran and Zamir, 2003). Currently SSR markers are widely used to screen the rice population for blast resistance. Several SSR markers tightly linked to blast resistant genes have been identified; therefore tagging of blast resistant genes until several generations are possible (Miah et al., 2013a). Furthermore the application of marker-assisted selection
(MAS) and marker-assisted backcross breeding (MABB) could greatly improve the potential of modern rice varieties through introgression of novel resistant genes.

1.2 Significance of the study

Achieving stable resistance to blast is perhaps the most important goal in managing blast disease. Plant breeding has been very successful in improving blast susceptible varieties using marker-assisted selection (Zhao et al., 2010). In Malaysia several upland and traditional rice varieties are available conferring strong resistance against the blast disease. Among them Pongsu Seribu 2 is one of blast resistant rice variety conferring broad spectrum resistance against the different blast pathotypes. Another Malaysia rice variety MR219 remained very popular among farmers for a long time because of high yield, long grain and good eating quality (Alias, 2002). At present MR219 is highly susceptible to blast disease. MR219 rice cultivar can be improved by the introduction of blast resistant genes from Pongsu Seribu 2 variety. Considering the above discussed advancement of marker-assisted selection the aim of this research was to identify the blast resistant genes from Pongsu Seribu 2 and tightly linked SSR marker associated with blast resistance against the most virulent pathotype P7.2 of M. oryzae. In the present study marker-assisted backcross breeding approach was also used to incorporate blast resistant genes into MR219 rice variety. The introgression of blast resistant genes in MR219 will retain its high yield and will be widely adopted again by local rice farmers of Malaysia.

1.3 Problem statement

In Malaysia, rice blast has become noticeable disease due to occurrence in main and off-season across the rice growing regions. Several outbreaks occur in different areas of peninsular Malaysia and about 3000 farmers lost RM5 million (DOA, 2014). The major outbreak was in the year of 2006 at KADA, Pasir Puteh, and Kelantan where 60% of 4,000 ha land cultivated with rice was affected due to blast disease and caused heavy economic loss (Ashkani et al., 2011). In the recent years the frequency of blast occurrence has increased with invasion into new areas. Moreover, there is not cultivated blast resistant rice variety in Malaysia. Research for developing highly blast resistant rice varieties are urgently needed to reduce the yield losses and supporting the poor farmers by combating blast disease.

1.4 Research objectives

The main objective of the study was to develop blast resistant, high yielding rice variety suitable to local environments through marker-assisted backcrossing. Accordingly, the specific objectives were:

1. To clone and sequence the blast resistant genes from resistant rice variety Pongsu Seribu 2.
2. To identify suitable segregating SSR markers for blast resistance using inheritance and disease reaction analysis in BC$_2$F$_1$ population.
3. To estimate the recovery of recurrent parent genome (MR219) contribution in marker-assisted backcross populations for introgression of blast resistant genes.
4. To evaluate, verify and select the advanced blast resistant lines from BC$_2$F$_2$ population.
REFERENCES


