PYRAMIDING OF SUBMERGENCE TOLERANCE AND BROWN PLANTHOPPER RESISTANCE GENES INTO PUTRA-2 RICE VARIETY THROUGH MARKER-ASSISTED BACKCROSSING

By

SANI HALIRU BELLO

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

August 2022

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DEDICATIONS

This thesis is dedicated to my late beloved grand father (Alhaji Haliru Alkammawa) for his utmost love, care, and assistance, who could not live up to this moment to witness this tremendous achievement of my peak academic carrier. May Allah (SWT) forgive him all his shortcomings and grant him Jannatul Firdaus, amin. I also dedicated it to my grandmother, Hajiya Amina who had shown keen interest on my education at an early stage of my childhood development. Furthermore, I dedicated it to my beloved late grand mothers (Hajiya Maryam and Hajiya Khadijatu) for their love and care for me throughout their life time. May Allah (SWT) forgive them all their mistakes and grant them Jannatul Firdaus, amin. Finally, I dedicated it to my parent and entire members of my family.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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August 2022

Chairman : Professor Mohd Rafii bin Yusop, PhD
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Rice is a main food crop for majority of the world's population. It is cultivated in over 100 countries on about 164 million hectares, mostly in the tropical and subtropical regions, and predominantly in Asia. Submergence stress and brown planthopper (BPH) infestation are the two main production constraints of rice in Asia. Gene pyramiding is a strategy whereby two or more genes are combined into a single variety to confer resistance to two or more biotic/abiotic production constraints of rice. The marker-assisted backcrossing (MABC) was used to incorporate BPH resistant genes from Rathu Heenati rice variety (Donor parent) into the genome of Putra-2 (Recurrent parent, high yielding and submergence tolerant (SUBT), but BPH susceptible). The main objective of this research was to develop high yielding, SUBT and BPH resistant rice variety for commercial cultivation in Malaysia. The specific objectives were; to identify polymorphic simple sequence repeat (SSR) markers linked to submergence tolerance and BPH resistance genes/QTLs, to identify polymorphic SSR markers for recurrent parent genome recovery, to introgress BPH resistance genes/QTLs from Rathu Heenati to Putra-2 rice variety, to quantify the percentage genome recovery of Putra-2 carrying BPH resistance genes/QTLs in BC_1F_1, BC_2F_1 and BC_2F_2 populations, and to validate the presence of submergence tolerance and BPH resistance genes/QTLs in backcross progenies with polymorphic linked markers and phenotypic selection. Out of 316 SSR markers screened, 79 (25%) markers exhibited polymorphism between the two parents. These markers were applied to determine the recovery of recurrent parent genome (RPG) in the BC_1F_1, BC_2F_1 and BC_2F_2 generations. The mean RPG recovery of the four chosen BC_1F_1 progenies was 80.3%. These progenies acquired the genes/QTLs of the foreground markers (RM8300, RM545 and RM544) and were used to develop the BC_2F_1 population. Due to two repeated generations of backcrossing and one cycle of selfing, the RPG recovery increased from 80.3% in the BC_1F_1 to 97.2% in BC_2F_2 selected lines. The marker genotypic segregation analyses using Chi-square test in the BC_1F_1 (1:1), BC_2F_1 (1:1) and BC_2F_2 (1:2:1) populations for the observed and expected proportions, showed non-significance probability at 5% level for both SUBT and BPH resistant loci, thus conforming to Mendelian single dominant gene inheritance model. Similarly, the phenotypic segregation for the observed and expected ratios (3:1) in BC_2F_2 population for submergence tolerance and BPH resistance loci, using Chi-square analysis, indicated non-significance probability at 5% level, and thus obeying Mendelian single gene inheritance model. On the other hand, phenotypic segregation for observed and expected fractions for two gene inheritance model (9:3:3:1) and epistatic effect (15:1) for SUBT and BPH resistance, had revealed significant difference ($P<0.05$) between the ratios using Chi-square analysis. Hence, indicating absent of two gene inheritance model and epistatic effect, in the present population. Validation experiments for SUBT and BPH resistance in improved pyramided lines indicated that all the selected improved lines had significantly ($P<0.05$) differed with negative checks, but however, statistically similar with positive checks. Mean comparison of yield and agro-morphological traits between Putra-2 variety and the selected improved lines showed no significant difference using independent t-test at 5% level of probability, and thus revealing that all the traits were recovered. In conclusion, ten improved pyramided rice lines were developed, namely: BC_1-P_1-P_{15}-P_6, BC_1-P_1-P_{15}-P_{13}, BC_1-P_1-P_{15}-P_{16}, BC_1-P_3-P_{13}-P_9, BC_1-P_3-P_{13}-P_{19}, BC_1-P_7-P_9-P_1, BC_1-P_7-P_9-P_{11}, BC_1-P_7-P_9-P_{17}, BC_1-P_{19}-P_1-P_3 and BC_1-P_{19}-P_6-P_{10}. The ten improved lines are recommended for further large scale evaluation and multi-location trials at rice granary areas to identify superior lines with high yield, submergence tolerance and brown planthopper resistance for commercial cultivation in Malaysia.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PIRAMIDAN GEN TOLERAN SUBMERGEN DAN KERINTANGAN BENA PERANG KE VARIETI PADI PUTRA-2 MELALUI KACUKBALIK BANTUAN PENANDA MOLEKUL

Oleh

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Padi merupakan tanaman makanan utama bagi majoriti penduduk dunia. Ia ditanam di lebih 100 negara dengan keluasan lebih 164 juta hektar, kebanyakannya di kawasan tropika dan subtropika, dan terutamanya di Asia. Masalah biotik dan abiotik adalah kekangan pengeluaran utama padi di seluruh dunia. Tekanan submergen adalah salah satu kekangan abiotik pengeluaran padi, terutamanya di tanah pamah tadah hujan di Asia. Antara perosak serangga padi, bena perang merupakan serangga perosak utama yang menjadi masalah dalam pengeluaran padi di Asia. Serangan teruk oleh bena perang boleh mengakibatkan kehilangan hasil secara langsung dan tidak langsung sebanyak 20 hingga 80% dalam kultivar padi yang rentang. Dalam situasi sebegini, pembiakbakaan varieti padi berhasil tinggi, tahan submergen (SUBT) dan rintang bena perang (BPH) merupakan pendekatan yang berkesan untuk menguruskan masalah ini secara paling mudah bagi pesawah padi di Asia. Piramidan gen dengan bantuan penanda ialah strategi di mana dua atau lebih gen digabungkan kedalam satu variety untuk memberikan kerintangan terhadap dua atau lebih tekanan biotik/abiotik dalam pengeluaran padi. Penggunaan penanda DNA dalam pembiakbakaan kacukbalik dan pemilihan memainkan peranan penting untuk mengatasi kelemahan utama, seperti penambahan segmen genom induk penderma yang tidak diingini (seretan pautan) serta tempoh yang lebih lama yang diperlukan dalam strategi pembiakbakaan tradisional. Kacukbalik bantuan penanda (MABC) telah digunakan untuk menggabungkan gen kerintangan BPH daripada varieti padi Rathu Heenati (induk penderma) ke dalam genom Putra-2 (induk berulang dan varieti padi berhasil tinggi dan toleran submergen tetapi rentang BPH). Objektif utama penyelidikan ini adalah untuk membangunkan varieti padi berhasil tinggi, toleran submergen serta rintang bena perang untuk penanaman komersial di Malaysia. Objektif khusus adalah; untuk mengenal pasti penanda jujukan berulang mudah (SSR) yang polimorfik yang dikaitkan dengan gen toleran submergen dan kerintangan BPH, untuk mengenalpasti penanda SSR polimorfik untuk pemulihan genom induk berulang, untuk mengintrogresi gen kerintangan BPH daripada Rathu Heenati kepada varieti padi Putra-2, untuk menentukan peratusan pemulihan genom Putra-2 yang membawa gen kerintangan BPH dalam populasi BC_1F_1, BC_2F_1 dan BC_2F_2, dan untuk mengesahkan kehadiran gen toleran/kerintangan SUBT dan BPH dalam progeni kacukbalik dengan penanda berkait polimorfik dan pemilihan fenotip. Daripada 316 penanda SSR yang disaring, 79 (25%) penanda mempamerkan polimorfisme antara kedua induk. Penanda ini digunakan untuk menentukan pemulihan genom induk berulang (RPG) dalam generasi BC_1F_1, BC_2F_1 dan BC_2F_2. Purata pemulihan RPG bagi empat progeny dari BC_1F_1 yang terpilih ialah 80.3%, yang mana telah digunakan untuk membangunkan populasi BC_2F_1. Pokok terbaik dalam generasi BC_1F_1 yang memiliki pemulihan RPG yang maksimum (84.9%) dan memperoleh gen yang berkait rapat dengan penanda RM8300, RM544 dan RM545 ialah BC_1-P_7. Disebabkan oleh dua generasi kacukbalik dan satu generasi swa-kacuk, pemulihan genom induk penerima meningkat daripada 80.3% dalam BC_1F_1 kepada 97.2% dalam titisan terpilih BC_2F_2. Analisis segregasi genotip penanda menggunakan ujian khi kuasa dua ke atas populasi BC_1F_1 (1:1), BC_2F_1 (1:1) dan BC_2F_2 (1:2:1) untuk nisbah dicerap dan dijangka, menunjukkan kebarangkalian adalah tidak signifikan pada tahap kebarangkalian 5% untuk kedua-dua lokus SUBT dan BPH, dengan itu mematuhi padanan yang baik dengan model pewarisan gen dominan tunggal Mendelian. Begitu juga, segregasi fenotip untuk nisbah yang diperhatikan dan jangkaan (3:1) dalam populasi BC_2F_2 untuk lokus toleran submergen dan kerintangan BPH, menggunakan analisis ujian khi kuasa dua, menunjukkan kebarangkalian tidak signifikan pada tahap 5%, dan oleh itu mematuhi model pewarisan Mendel untuk gen utama tunggal. Sebaliknya, kedua-dua segregasi fenotip untuk nisbah yang diperhatikan dan dijangka untuk dua model pewarisan gen (9:3:3:1) dan kesan epistatik (15:1) untuk kerintangan SUBT dan BPH, menunjukkan perbezaan yang ketara antara nisbah tersebut pada 5% tahap kebarangkalian menggunakan analisis khi kuasa dua. Oleh itu, menunjukkan tidak wujud model pewarisan dua gen dan kesan epistatik dalam populasi ini. Eksperimen validasi untuk kerintangan SUBT dan BPH dalam titisan piramida maju menunjukkan bahawa semua titisan maju terpilih mempunyai perbezaan yang signifikan ($P<0.05$) dengan kawalan negatif, tetapi sebaliknya, sama secara statistik dengan kawalan positif. Oleh itu, ianya menandakan kehadiran gen kerintangan *Sub1* dan BPH dalam titisan piramida maju. Sepuluh titisan piramida padi maju telah dipilih, iaitu: BC_1-P_1-P_{15}-P_6, BC_1-P_1-P_{15}-P_{13}, BC_1-P_1-P_{15}-P_{16}, BC_1-P_3-P_{13}-P_9, BC_1-P_3-P_{13}-P_{19}, BC_1-P_7-P_9-P_1, BC_1-P_7-P_9-P_{11}, BC_1-P_7-P_9-P_{17}, BC_1-P_{19}-P_1-P_3 dan BC_1-P_{19}-P_6-P_{10}. Perbandingan min bagi ciri-ciri hasil dan agro-morfologi antara varieti padi Putra-2 dan titisan piramida maju tersebut menunjukkan tiada perbezaan yang signifikan menggunakan ujian-t bebas dan dengan ini membuktikan bahawa kesemua ciri telah dipulihkan. Sepuluh titisan maju ini selanjutnya disyorkan untuk penilaian berskala besar dan percubaan pelbagai lokasi di kawasan jelapang padi untuk mengenal pasti titisan unggul dengan hasil tinggi, toleran submergen dan rintang bena perang untuk penanaman komersial di Malaysia.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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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.

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LIST OF ABBREVIATIONS

BC	Backcross
BP	Base pairs
BPH	Brown planthopper
BR	Brassinosteroids
CTAB	Cetyl trimethyl-ammonium bromide
DNA	Dioxyribonucleic acid
ERF	Ethylene responsive factors
GA	Gibberelic acid
MABC	Marker-assisted backcrossing
MAGP	Marker-assisted gene pyramiding
MAS	Marker-assisted selection
mRNA	Messenger ribonucleic acid
PCR	Polymerase chain reaction
SSR	Simple sequence repeats
SUBT	Submergence tolerance

CHAPTER 1

INTRODUCTION

1.1 General Introduction

In the last few decades, agricultural ecological system is substantially influenced by flooding as result of climate change (Isma'il et al., 2013). Currently, rice is the most flood affected crop especially in rainfed low lying areas of Asian countries where rice serves as major source of nourishment (Bailey-Serres et al., 2012; Oladosu et al., 2020). Rainfed lowland occupies 25% (38 million hectares) of the global rice cultivable area and 25 million hectares falls within rainfed lowland in Asia (Fahim, 2015). With current pattern of the world's climate change, specifically global warming, agricultural ecosystems have been severely influenced. Malaysia has been affected by adverse effect of climate change and the country's agro-ecosystems are confronted by multiple environmental stresses (Fahim, 2015).

Among the many rice biotic production constraints, insect pests are of great significance (Heong and Hardy, 2009). There are over hundred insect pest species of rice, 20 of them are of great economic benefit (Prakash et al., 2007). However, brown planthopper (BPH, *Nilaparvata lugens* Stal), constitute a serious problem to rice production in Asia (Normile, 2008; Prasannakumar et al., 2013). It is a typical vascular feeder that derives its nourishment from phloem sap, which result to characteristic symptoms known as 'hopperburn' (PhilRice, 2010; Vanitha et al., 2011). Both adults and nymphs BPH suck sap from down part of the plants, which subsequently leads to reduction in plant height and tillering, which resulted to unfilled grains. Increase feeding results to decrease in leaves' protein, chlorophyll content, drymatter accumulation, nitrogen level of leaves and stem as well as photosynthetic rate in cultivars that are susceptible (Horgan, 2009).

To further understand the mechanism of BPH resistance in rice, some genes that administer resistance to BPH have been successfully cloned and characterised. The genes include *Bph6*, *Bph9*, *Bph14*, *Bph17*, *Bph18*, *Bph26*, *Bph29* and *Bph32* (Du et al., 2009; Tamura et al., 2014; Liu et al. 2015; Wang et al., 2015; Zhao et al., 2016; Ji et al., 2016; Ren et al., 2016; Guo et al., 2018). The *Bph6* resistance gene, produces an unspecific protein that is associated to exocysts and its smaller units. Its expression increases exocytosis and administer rigidity to the cell wall and maintenance (Guo et al., 2018). The BPH9 gene, confer both antixenosis and antibiosis to the BPH. It synthesises rarely occurring nucleotide-binding and leucine-rich (NLR)-containing protein, available on the inner membrane system, which results to cell death phenotypes (Zhao et al., 2016). The *Bph14* manufactures a coiled-coil, nucleotide-binding and leucine-rich repeat (CC-NB-LRR) protein, which hinders BPH attack at young and maturity growth stages (Du et al., 2009). The *Bph17* gene is a group (cluster) of repeated genes that are arranged in sequence. The genes produce putative lectin receptor kinases, referred to as *Oryza sativa Lectin Receptor Kinase* (*OsLecRK*), and the cluster includes *OsLecRK1*, *OsLecRK2*, *OsLecRK3* and *OsLecRK4*. Additional investigation indicated that *OsLecRK1*-*OsLecRK3* is the main cluster that confer strong resistance to BPH (Liu et al., 2015). The

Bph18 proteins are majorly confined to membrane-bound organelles within the cells such as endoplasmic reticulum, golgi body, endoplasmic reticulum etc., hence revealing that *Bph18* may perceive BPH attack at endomembrane level in phloem cells. The gene, produces the CC-NBS-NBS-LRR protein, possessing double domains of NBS, which differ with majority of rice resistance genes that bear only one NBS domain (Ji et al., 2016). The *Bph26* gene, bears CC-NBS-LRR protein, which expresses antibiosis to BPH through sucking inhibition in the phloem sieve cells (Tamura et al., 2014). The *Bph29* gene, synthesises a B3 DNA-binding protein domain restricted to xylem and phloem tissues, where BPHs invade. The gene regulates salicylic acid movement and prevent ethylene/jasmonic acid-regulated pathways in reaction to BPH attack, thereby causing accumulation of callose in the phloem cells, resulting to BPH resistant of the rice plant (Wang et al., 2015). The *Bph32* gene, manufactures an unperceived short consensus protein that resists BPH attack through antibiosis. It mainly found in the leaf sheath of rice plant where BPH attack begins (Ren et al., 2016).

Gene pyramiding refers to combination of two or more resistance genes into single variety to provide resistance to two or more biotic/abiotic problems of a crop plant (Joshi and Nayak, 2010; Ikmal et al., 2021). In recent years, this approach is receiving attention due to the fact that two or more genes are involved, and therefore makes it very difficult for the insect pests to overcome the resistance of many genes, simultaneously (Ferrater, 2015). In gene pyramiding, it is possible to combine two or more genes of diverse origin into single variety (Ikmal et al., 2021). In otherwords, it is possible to combine gene that confer submergence tolerance (SUBT) and BPH resistance into single variety (Korinsak et al., 2016). Nowadays, with application of molecular markers in varietal development, it is possible to combine two or more genes into single variety (Hu et al., 2016). Gene pyramiding approach, has been used to enhance submergence and salt stresses tolerance in IR64 rice variety for increased yield and higher adaptability (De Ocampo et al., 2013). Combination of two major genes (*Grh2* and *Grh4*) that control rice green leafhopper resistance (*Nephotettix cincticeps* Ulher), had resulted to a greater degree of antibiosis against different strain of rice green leafhoppers compared to a line possessing single gene for resistance (Fujita et al., 2006). Korinsak et al. (2016) used two major QTLs, *Sub1* and *Qbph12* to improve submergence tolerance and BPH resistance of KDML105 (Thai jasmine rice cultivar) through marker-assisted gene pyramiding approach. Their results revealed that pyramided lines significantly increased tolerance to submergence and resistance to BPH than the original parental line, KDML105.

1.2 Justification

Biotic and abiotic constraints have been regarded as the major production problems of rice in Asia (Korinsak et al., 2016). Submergence stress is one of the abiotic constraints of rice production, especially in the rain-fed lowlands of Asia (Septininsih et al., 2009). Recently, the adverse effects of change in climate such as severe water shortage, global warming and unpredictable flash flood particularly during the monsoon season have posed serious threat to rice farmers in Asia (Septiningsih et al., 2009). Local cultivars that are peculiar to the flood vulnerable areas are poor yielding as a result of poor tillering capacity, sensitive to lodging, low grain quality and long droppy leaves (Ahmed et al., 2016). In addition, the performance of present new varieties under flooding environment is not encouraging due to poor adaptability (Ahmed et al., 2016). To overcome this problem, development of new varieties that can combine submergence tolerance with better adaptability and high grain yield characteristics are desirable (Ismail et al., 2013). Among the rice insect pests, the BPH has been described as the most damaging rice pest in Asia (Jena et al., 2006; Jena and Kim, 2010; Suh et al., 2011; Krishnaiah and Varma, 2011). Heavy infestation by BPH can result to direct and indirect yield losses of 20-80% in sensitive rice cultivars (Hitendra et al., 2012; Rashid et al., 2017; Wei et al., 2019). The BPH also serves as a vector for viral diseases such as grassy, ragged and wilt stunt viruses (Jena and Kim, 2010), which can result to more severe yield losses (Ram et al., 2010; Li et al., 2010; Zhang et al., 2010). In Malaysia, BPH cases have been on the increase and more hopperburn cases have been recorded (Maisara and Habibudddin, 2018), and there is no high yielding resistant variety available to date (Shabanimofrad et al., 2015b). Previous investigations conducted have identified control measures such as pesticide use, planting of early maturing varieties, host plant resistance, split application of fertilizer, wider spacing and biological control (PhilRice, 2010). However, Development of resistant cultivars is the most environmentally safe and economically viable alternative method for BPH control (Song et al., 2002; Botrell and Schoenly, 2012).

Several researches have been conducted on gene pyramiding in Asian rice cultivars (Sharma et al., 2004; Neeraja et al., 2007; Myint et al., 2012; Hu et al., 2012; Korinsak et al., 2016), however, there is no work done on pyramiding of submergence tolerance and BPH resistance genes into Putra-2 rice variety through MABC. Based on this background, this research was carried out with view to develop new high yielding, submergence tolerant and BPH resistant rice variety to improve rice production specifically in Malaysia, and Asia at large. This research is in line with Malaysian government development plan to reduce rice importation and maintain self-sufficiency of not less than 70% from 2011 to 2020 (Chee-Wan and Meng-Chang, 2012). This is obvious and more so at this critical period, when the production of rice need to be increased by more than 40%, to meet the requirement of the world's population by the year 2030 (Kush, 2005).

1.3 Research Hypothesis

The study hypothesised that, incorporation BPH resistance genes into Putra-2 rice variety (BPH susceptible) can produce BPH resistant rice lines that will contribute to the yield improvement and performance of main agronomic traits in this study.

1.4 Objectives of the Study

1.4.1 Main Objective

The main objective of the study was to develop high yielding, submergence tolerant and BPH resistant rice variety for commercial cultivation in Malaysia.

The specific objectives of the study were:

i. To validate polymorphic SSR markers linked to submergence tolerant and BPH resistance genes/QTLs.

ii. To introgress BPH resistance genes/QTLs from Rathu Heenati to Putra-2 rice variety

iii. To identify polymorphic SSR markers for recurrent parent genome recovery

iv. To quantify the percentage recovery of Putra-2 carrying BPH resistance genes/QTLs in BC_1F_1, BC_2F_1 and BC_2F_2 populations.

v. To validate the presence of submergence tolerance and BPH resistance genes/QTLs in BC_2F_2 progenies with polymorphic linked markers and phenotypic selection.

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