DEVELOPMENT OF MUNGBEAN BREEDING LINES WITH IMPROVED RESISTANCE TO CERCOSPORA LEAF SPOT AND POWDERY MILDEW BY MOLECULAR MARKER ASSISTED GENE PYRAMIDING

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การพัฒนาถั่วเขียวสายพันธุ์ปรับปรุงให้ต้านทานโรคใบจุดและราแป้งโดยใช้เครื่องหมายโมเลกุลช่วยรวมยีน

นายวิศรุต เชื้อขุนทด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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วิศรุต เชื้อขุนทด : การพัฒนาถั่วเขียวสายพันธุ์ปรับปรุงให้ต้านทานโรคใบจุดและราแป้งโดยใช้เครื่องหมายโมเลกุลช่วยรวมยีน (DEVELOPMENT OF MUNGBEAN BREEDING LINES WITH IMPROVED RESISTANCE TO CERCOSPORA LEAF SPOT AND POWDERY MILDEW BY MOLECULAR MARKER ASSISTED GENE PYRAMIDING) อาจารย์ที่ปรึกษา : ศาสตราจารย์ ดร.ปิยะดา อลิฌาณ์ ตันตสวัสดิ์, 90 หน้า.

การพัฒนาถั่วเขียวที่มีความต้านทานแบบกว้างต่อโรคใบจุดและราแป้งสามารถทำได้โดยใช้เครื่องหมายโมเลกุลช่วยรวมยีนในการปรับปรุงพันธุ์ด้วยวิธีผสมกลับ งานวิจัยนี้มีวัตถุประสงค์เพื่อ 1) คัดเลือกพันธุ์ให้ที่มียีนต้านทานโรคใบจุดและราแป้งโรคละ 1 ยีนจากสายพันธุ์ต้านทาน V4718 และยีนต้านทานโรคราแป้ง 1 ยีนจากสายพันธุ์ต้านทาน V4785 2) คัดเลือกพันธุ์รับโดยอาศัยความแตกต่างทางพันธุกรรมกับพันธุ์ให้ที่ตำแหน่งซึ่งเชื่อมโยงกับยีนต้านทานโรคใบจุดและราแป้งเหล่านี้ และ 3) รวมยีนต้านทานหลายยีนเข้าสู่พันธุ์ที่ให้ผลผลิตสูงโดยการปรับปรุงพันธุ์ด้วยวิธีผสมกลับร่วมกับการใช้เครื่องหมายโมเลกุลช่วยคัดเลือก การทดลองที่ 1 ทำการคัดเลือกพ่อแม่ที่เหมาะสมโดยการวิเคราะห์ความแตกต่างที่ได้จากเครื่องหมายโมเลกุลจำนวน 6 เครื่องหมาย ได้แก่ เครื่องหมาย inter-simple sequence repeat (ISSR) (I85420) และ ISSR-anchored resistance gene analog (ISSR-RGA) (I42PL222) ซึ่งอยู่ขนาบข้างยีนต้านทานโรคราแป้งในสายพันธุ์ V4718 เครื่องหมาย simple sequence repeat (SSR) (VR393 และ CEDG084) ซึ่งอยู่ขนาบข้างยีนต้านทานโรคใบจุดในสายพันธุ์ V4718 และเครื่องหมาย ISSR-RGA (I27R211 และ I27R565) ซึ่งเชื่อมโยงกับยีนต้านทานโรคราแป้งในสายพันธุ์ V4785 ในการศึกษาครั้งนี้ทำการคัดเลือกพันธุ์ให้จากลูกผสมจำนวน 36 สายพันธุ์ ที่มียีนต้านทานโรคใบจุดจำนวน 1 ยีน (จาก V4718) และยีนต้านทานโรคราแป้งจำนวน 2 ยีน (จาก V4718 และ V4785) ขณะที่การคัดเลือกพันธุ์รับใช้ถั่วเขียวผิวมันจำนวน 23 พันธุ์/สายพันธุ์ พบว่า จากลูกผสมทั้งหมด 36 สายพันธุ์มีเพียง 10 สายพันธุ์ที่มีเครื่องหมายซึ่งเชื่อมโยงกับความต้านทานโรคใบจุดและราแป้งครบทั้ง 6 ตำแหน่ง จึงนำมาใช้เป็นพันธุ์ให้ ส่วนพันธุ์รับในการปรับปรุงพันธุ์ด้วยวิธีผสมกลับร่วมกับการใช้เครื่องหมายโมเลกุลช่วยคัดเลือกครั้งนี้ ได้เลือกใช้พันธุ์รับรองของไทย (มทส 1) ซึ่งพัฒนาโดยมหาวิทยาลัยเทคโนโลยีสุรนารี และพันธุ์นำเข้าที่มีศักยภาพ (KING) จาก World Vegetable Center เนื่องจากมีความแตกต่างของอัลลีลจากเครื่องหมายที่สัมพันธ์กับยีนต้านทานโรคใบจุดและราแป้งจำนวน 6 ตำแหน่งกับพันธุ์ให้ การทดลองที่ 2 ทำการรวมยีนต้านทานโรคใบจุดจำนวน 1 ยีน และยีนต้านทานโรคราแป้งจำนวน 2 ยีน จากพันธุ์ให้จำนวน 10 สายพันธุ์เข้าสู่พันธุ์รับ มทส 1 และ KING โดยใช้เครื่องหมายเหล่านี้คัดเลือกแบบ foreground และคัดเลือกลูกผสมกลับที่มี

พันธุกรรมใกล้เคียงพันธุ์รับด้วยการคัดเลือกแบบ background โดยใช้ชุดเครื่องหมาย SSR, EST-SSR และ ISSR อื่นที่ไม่ได้เชื่อมโยงกับยีนต้านทานเหล่านี้ พบว่า ปริมาณจีโนมของพันธุ์รับในลูกผสมกลับ BC_2F_1 ซึ่งได้จากคู่ผสมที่ใช้ มทส 1 เป็นพันธุ์รับ คือ 87.2-97.6% แต่เมื่อวิเคราะห์ background ในลูกผสมกลับ BC_2F_1 ซึ่งได้จากคู่ผสมที่ใช้ KING เป็นพันธุ์รับ พบปริมาณจีโนมของพันธุ์รับสูงถึง 84.7-100% ลูกผสมกลับ BC_2F_1 ที่มีปริมาณจีโนมเหมือนพันธุ์รับสูงสุดบางต้นแสดงความต้านทานต่อโรคใบจุดสูงในระดับห้องปฏิบัติการ การศึกษาครั้งนี้แสดงให้เห็นถึงประโยชน์ของการใช้เครื่องหมายโมเลกุลช่วยในการคัดเลือกเพื่อเร่งการผสมกลับของลูกผสมกลับที่ได้จากการรวมยีนเหล่านี้ นอกจากนี้สายพันธุ์ปรับปรุงที่ต้านทานโรคใบจุดและราแป้งที่ได้ จะเป็นประโยชน์ต่อการผลิตถั่วเขียวพันธุ์ต้านทานสำหรับใช้ในพื้นที่ที่มีแนวโน้มการระบาดของโรค รวมทั้งในระบบเกษตรอินทรีย์ในอนาคต

สาขาวิชาเทคโนโลยีการผลิตพืช
ปีการศึกษา 2562

ลายมือชื่อนักศึกษา วิศรุต เชื้อขุนทด
ลายมือชื่ออาจารย์ที่ปรึกษา
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม

WITSARUT CHUEAKHUNTHOD : DEVELOPMENT OF MUNGBEAN BREEDING LINES WITH IMPROVED RESISTANCE TO CERCOSPORA LEAF SPOT AND POWDERY MILDEW BY MOLECULAR MARKER ASSISTED GENE PYRAMIDING. THESIS ADVISOR : PROF. PIYADA ALISHA TANTASAWAT, Ph.D., 90 PP.

ISSR/ISSR-RGA/MARKER ASSISTED BACKCROSS BREEDING (MABB)/SSR/ *Vigna radiata* (L.) Wilczek/*Vigna mungo* (L.) Hepper

The development of mungbean with broad spectrum resistance to Cercospora leaf spot (CLS) and powdery mildew (PM) could be achieved by molecular marker assisted gene pyramiding through backcross breeding. The objectives of this study were to 1) select donor parents possessing each of resistance genes for CLS and PM from V4178 and another PM resistance gene from V4785, 2) select recurrent parents based on genetic polymorphism compared with donor parents at marker loci linked to these CLS and PM resistance genes, and 3) pyramid multiple resistance genes into high yielding mungbean varieties through marker assisted backcross breeding (MABB). The first experiment was carried out to select the suitable parents by means of marker polymorphism analysis with 6 marker loci; inter-simple sequence repeat (ISSR) (I85420) and ISSR-anchored resistance gene analog (ISSR-RGA) (I42PL222) markers flanked a PM resistance gene in V4718, simple sequence repeat (SSR) (VR393 and CEDG084) markers flanked a CLS resistance gene in V4718, and ISSR-RGA (I27R211 and I27R565) markers associated with a PM resistance gene in V4785. In this study, 36 hybrids were subjected to selection of donor parents with a CLS resistance gene (from V4718) and 2 PM resistance genes (from V4718 and V4785),

while selection of recurrent parents was based on 23 mungbean varieties/lines. From total of the 36 hybrids, only 10 were present with all 6 marker loci associated with CLS and PM resistance, thereby being used as donor parents. While Thai certified variety (SUT1) developed by Suranaree University of Technology (SUT) and one promising plant introduction derived from the World Vegetable Center (KING) were selected as recurrent parents in MABB because of their distinctive allele polymorphisms among 6 marker loci compared with the donor parents. The second experiment was conducted to pyramid a CLS resistance gene and 2 PM resistance genes from 10 donor parents into SUT1 and KING using foreground selection by those of marker loci. And backcross (BC) progenies having close genetics with recurrent parents were selected by background selection. Background selection was carried out using other SSR, EST-SSR, and ISSR marker loci unlinked to these resistance genes. Recurrent parent genome (RPG) recovery of BC_2F_1 in the crosses that used SUT1 as recurrent parent was 87.2-97.6%. While background analysis in BC_2F_1 from the crosses using another recurrent parent (KING) reached 84.7-100% RPG recovery. Some of the BC_2F_1 plants with the maximum RPG recovery were highly resistant to CLS under laboratory condition. This study revealed the usefulness of marker assisted selection (MAS) to accelerate backcrossing of these BC plants. Moreover, it can be expected that the improved CLS and PM breeding lines of SUT1 and KING will be beneficial for the production of resistant mungbean varieties useful for prone areas with disease epidemics and organic farming systems in the future.

School of Crop production Technology

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LIST OF ABBREVIATIONS

AVRDC	=	Asian Vegetable Research and Development Center
BC	=	Backcross
CLS	=	Cercospora leaf spot
EST-SSR	=	Expressed sequence tag-derived simple sequence repeat
ISSR	=	Inter-simple sequence repeat
ISSR-RGA	=	ISSR-anchored resistance gene analog
MABB	=	Marker assisted backcross breeding
MAS	=	Marker assisted selection
PM	=	Powdery mildew
RPG	=	Recurrent parent genome
SSR	=	Simple sequence repeat

CHAPTER I

INTRODUCTION

1.1 Significance of this study

In the 21st century, global agriculture faces the major challenge of supplying sufficient sustainable food production for a growing population under worsening anthropogenic climate change. This change results in reducing available water and increasing air temperatures. Mungbean (*Vigna radiata* (L.) Wilczek) is one of the most important leguminous staple crops, particularly in Asian countries because of its short life cycle (about 60 days) with wide adaptability, drought tolerance, ability to fix atmospheric nitrogen (N) in its root nodules in symbiosis with rhizobium, as well as its valuable nutritional and health benefits. The global annual mungbean production is 3 million tons of grain from more than 6 million hectares worldwide (Nair et al., 2013). India is the world's largest mungbean producers followed by China, Pakistan, Taiwan, Australia, Myanmar, and Indonesia. Although Thailand is not among the major producers, mungbean is considered as a strategic crop for local and national agribusiness. Nowadays, about 0.11 million tons are internally demanded, however, the current production is only about 0.09 million tons from the cultivated areas of 0.14 million hectares (Office of Agricultural Economics, 2016). Major constraints are the inherently low yielding potential of the current varieties and susceptibility to destructive diseases, particularly several foliar diseases. Among them, Cercospora leaf spot (CLS) caused by *Cercospora canescens* Illis & Martin inflicts significantly seed

yield losses. Under the rainy season coupling with the sufficient number of fungal spores, 68% and 35% yield reduction were recorded in the susceptible variety Uthong1 (UT1) and resistant variety (Pagasu), respectively (Chinsawangwattanakul, 1984). Powdery mildew (PM) caused by another fungus *Sphaerotheca phaseoli* is also an important foliar disease of mungbean, because its outbreak, mainly devastating in the winter season can reduce seed yield more than 50% (Khajudparn et al., 2007) or even 100% at the seedling stage (Reddy et al., 1994). Currently, these major yield losses have been recognized in the varieties of mungbean recommended to farmers in Thailand, i.e. UT1, Kampaeng Saen 1 (KPS1), KPS2, Chai Nat 36 (CN36), CN60, CN72, CN84-1, and Suranaree University of Technology 1 (SUT1). However, these varieties, particularly KPS2, CN36, CN72, and CN84-1 have still been cultivated, along with chemical spraying. Chemical usage increases farmer production costs and causes dramatically negative effects on human health, as well as the environment. Regular chemical spraying results in the evolutionary change of chemical resistance in the pathogens. On the other hand, using resistant varieties with only one resistance gene may face a problem associated with their resistance breakdown by new virulent races according to the classic boom and bust cycles of major gene resistance to plant pathogens. If so, resistant varieties derived from pyramiding of multiple disease resistance genes are the most desirable strategy to provide more durable and broad spectrum resistance in an economical and eco-friendly way.

Despite the significant progress achieved in plant breeding programs, there are still many challenges in order to develop new varieties. Several techniques have been contributed in plant breeding programs by means of conventional breeding methods, mutation breeding, molecular marker assisted selection (MAS), genetic engineering,

or even genome editing. Some of these methods have been successfully used in mungbean breeding (Wongpiyasatid et al., 2000; Chaitieng et al., 2002). Genetic engineering and genome editing are still limited in Thailand, and the difficulty of conventional methods still remains due to the dominance and epistatic effects of genes, time-consuming, high labor, as well as dependence on environmental influences, particularly when pyramiding of multiple genes. For this purpose, molecular markers can be very helpful for pyramiding of multiple desirable genes into the recommended varieties.

Genetic diversity evaluation of plant genetic resources (PGRs) is an important first step in any plant breeding programs. The genetic differences of PGRs have been traditionally analyzed using morphological or physiological traits. However, to minimize the impact from environmental factors, various molecular techniques detecting at the DNA level such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellites or simple sequence repeat (SSR), inter-SSR (ISSR), which are structural markers have been used to assess genetic variation (Bhat et al., 2005; Somta et al., 2009; Sony et al. 2012; Islam et al. 2015; Nath et al., 2017). Recently, sequence databases of DNA, cDNA, and expressed sequence tag (EST) or gene-based SSR (genic SSR) have been generated by next generation sequencing (NGS) for several plants, including mungbean, and have been available for screening SSRs to develop EST-SSRs. EST-SSRs are physically linked to expressed genes in contrast to the traditional genomic SSRs, which are derived from any DNA regions throughout the genome. Moreover, identification of SSRs is more costly and time-consuming than EST-SSRs, and EST-SSRs are also useful for analyzing the

diversity in PGRs, particularly high yielding and resistant varieties. (Varshney et al., 2005). PGRs with outstanding characters and maximum genetic diversity which also have yield stability in induced crosses can be obtained through the estimation using EST-SSR markers.

The identification of resistance genes to CLS and PM in PGRs is helpful for developing resistant varieties. Inheritance of CLS and PM resistance depends on resistance mechanism. For CLS resistance using different resistant sources, the resistance was found to be controlled by either a single dominant gene (Thakur et al., 1980; Chankaew et al., 2009; Singh et al., 2017), a single recessive gene (Mishra et al., 1988), or quantitative genes (AVRDC, 1980; Leabwon and Oupadissakoon, 1984). While PM resistance was controlled by either a single dominant gene (Chaitieng, 2002; Gawande and Patil, 2003; Khajudparn et al., 2007), 2 dominant genes (Reddy, 1994; Reddy, 2009), or quantitative genes (Young et al., 1993; Chaitieng et al., 2002; Kasettranan et al., 2010; Chankaew et al., 2013). Khajudparn et al. (2007) revealed that resistance to PM in each of 3 resistant mungbean lines from Asian Vegetable Research and Development Center: AVRDC (V4718, V4758, and V4785) having high resistance to the disease in Thailand is controlled by a single dominant gene with non-allelic interactions. In addition, Chankaew et al. (2009) also indicated that resistance to CLS in V4718 is controlled by a single dominant gene. More recently, our laboratory found molecular markers linked to the CLS and PM resistance genes in 2 resistant lines (V4718 and V4785). ISSR (I85420) and newly developed ISSR-anchored resistance gene analog (ISSR-RGA) (I42PL229) markers derived from the cross between susceptible variety (CN72) and resistant line (V4718) were identified at the distance of 4 and 9 centimorgan (cM), respectively from a major QTL,

qPMC72V18-1 controlling PM resistance (Poolsawat et al., 2017). Two ISSR-RGA (I27R211 and I27R565) markers were also found to be associated with PM resistance in another resistant line (V4785) (Poolsawat et al., unpublished data). Moreover, SSR (VR393 and CEDG084) markers were localized between the QTLs, *qCLSC72V18* and *qPMC72V18* controlling CLS and PM resistance in V4718, respectively (Arsakit et al., 2017). These markers can be immediately used to pyramid these resistance genes into a recommended variety for durable resistance to CLS and PM through MAS. In addition, MAS for pyramiding desirable genes, along with background selection is potentially helpful for minimizing unlinked regions that negatively affect crop performance from the donor segment and recovering recurrent parent genome (RPG) within the early backcross (BC) generations (Hasan et al., 2015). In mungbean, pyramiding of desirable CLS and PM genes with MAS have not been accomplished. Therefore, this study attempts to use marker assisted backcross breeding (MABB) for pyramiding a CLS resistance gene and 2 PM resistance genes from V4718 and V4785 into the suitable recurrent parents derived from selection based on genetic polymorphisms compared with donor parents and genetic diversity evaluation. The final expected outcomes are the resistant mungbean individuals having all 3 resistance genes, which also resemble their recurrent parents in other characters.

1.2 Research objectives

1.2.1 To select donor parents possessing all 3 resistance genes.

1.2.2 To select recurrent parents based on genetic polymorphisms with donor parents at 6 marker loci linked to CLS and PM resistance genes.

1.2.3 To assess the genetic diversity and relationships of mungbean and blackgram, and to generate their molecular fingerprints using EST-SSR analysis.

1.2.4 To pyramid a CLS resistance gene and 2 PM resistance genes into the genetic background of 2 high yielding mungbean varieties (SUT and KING) through MABB.

1.3 Research hypotheses

1.3.1 MABB can be applicable to facilitate transferring of resistance gene(s) from donor parents to recurrent parents if markers linked to the resistance gene(s) are polymorphic between donor and recurrent parents.

1.3.2 The F_1 and BC progenies selected through markers linked to a CLS resistance gene and 2 PM resistance genes may have higher levels of resistance to both diseases.

1.3.3 Using background selection, more than 90% of RPG similarity of the pyramided line may be identified within the early BC generations, and their phenotypes related to yields are expected to be mostly similar to recurrent parents.

1.4 Scope of study

This study focuses on CLS and PM resistance gene pyramiding into the suitable recurrent parents through MABB. The experiments were divided into 2 parts. The first experiment was carried out to select the suitable donor and recurrent parents from 36 hybrids derived from the crosses between RILs of 3 resistant lines, including V4718, V4758, and V4785 with a susceptible cultivated mungbean variety CN72, and from 22 mungbean varieties/lines, respectively. This experiment was performed with

6 markers linked to CLS and PM resistance genes by means of marker polymorphism analysis. In addition, genetic diversity evaluation of 23 mungbean and 4 backgram varieties/lines using EST-SSR markers was carried out in order to identify their genetic relationships and variability. The second experiment was to pyramid a CLS resistance gene and 2 PM resistance genes from donor parents into the background of recurrent parents through MABB. This experiment included genotyping the progeny from F_1 generation to BC_2F_1 generation using markers linked to all target resistance genes; ISSR (I85420) and ISSR-RGA (I42PL222) markers flanked a PM resistance gene from V4718, SSR (VR393 and CEDG084) markers flanked a CLS resistance gene from V4718, and ISSR-RGA (I27R211 and I27R565) markers associated with a PM resistance gene from V4785. In addition, the BC progenies carrying all of the resistance alleles were verified for their resistance against CLS disease using bioassay under laboratory condition. Background selection was concurrently performed to identify the BC progeny with a high level of genetic similarity to their recurrent parents using SSR, EST-SSR, and ISSR markers.

1.5 Expected outcomes

1.5.1 The suitable donor and recurrent parents derived from analysis of resistance allele polymorphisms will be obtained.

1.5.2 Genetic diversity and relationships of the potential parents will be obtained using EST-SSR analysis.

1.5.3 The F_1 and BC progenies with a CLS resistance gene and 2 PM resistance genes will be obtained using foreground selection and bioassay. The BC progenies with high genetic background similarity to recurrent parents will be obtained

using background selection.

1.5.4 The promising BC progenies with high level of resistance to CLS and PM and resembling recurrent parents on other characters will be obtained which are beneficial for future development of new pyramided CLS and PM resistant mungbean varieties.

CHAPTER II

LITERATURE REVIEWS

2.1 World food situation

Nowadays, the global food crop production is counteracting the major challenges at the same time such as a) 70% more food is required for an additional 2.3 billion people by 2050, b) struggle with poverty and hunger, and c) adaptation towards climate change. Increasing the global grain legume production has the potential to provide food and protein security, but legume crops are ranked behind cereal and oilseed crops (Popelka et al., 2004). At present, there are increasing efforts by the Food and Agriculture Organization of the United Nations (FAO) and other organizations on legume production and genetic improvement (Considine et al., 2017).

2.2 Legume and mungbean importance

Legumes belong to the family Fabaceae or Leguminosae (with about 700 genera and 18,000 species). The crops are rich of nutritious food, feed, and raw materials for human, livestock, and industries, respectively. They also have a symbiotic association with Rhizobium bacteria present in the nodules for N fixation. Legumes have been cultivated in crop rotation with other crops, and their productions, as well as harvested area, particularly in Asia in 2014 to 2015 are shown in Figure 2.1

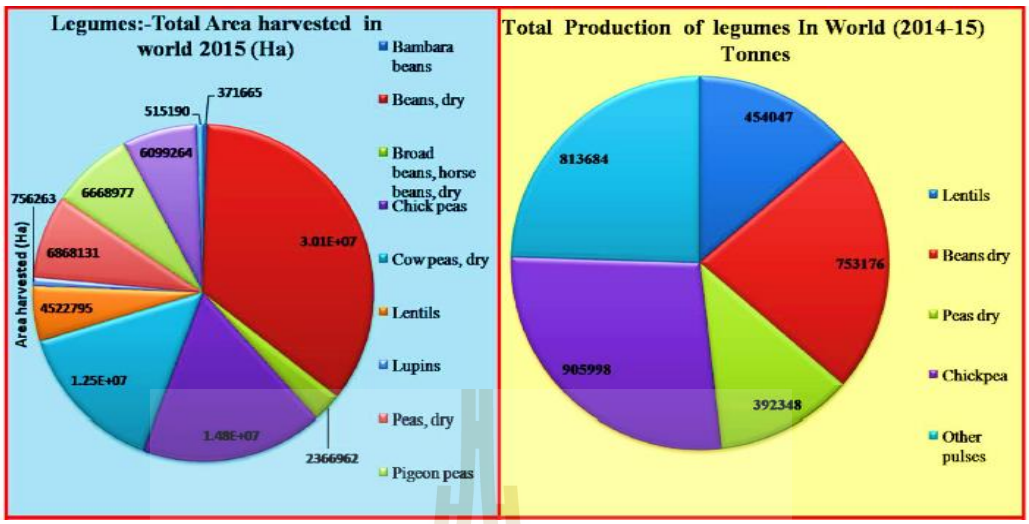

Figure 2.1 Legume production and harvested area modified from FAOSTAT (Sita et al., 2017).

Mungbean (*Vigna radiata* (L.) Wilczek) is an important short life cycle legume crop (about 60 days) of high nutritional values and atmospheric nitrogen fixing ability (Yaqub et al., 2010). It is a self-pollinated diploid leguminous crop with 2n = 2x = 22 chromosomes and a genome size of 579 Mb, which are similar to those of other *Vigna* species. The main cultivated areas are South, East, and Southeast Asia (Kang et al., 2014). Globally, the annual production is 3 million tons of grain from more than 6 million hectares (Nair et al., 2013). India is the largest producer accounting for about 65% of the global annual production (about 6 million tons) allowing it to be the primary center of genetic diversity. Other countries, i.e. China, Pakistan, Taiwan, Australia, Myanmar and Indonesia are considered as major producers behind India. In Thailand, although mungbean is not mainly cultivated, it is an important strategic crop for local and national agribusiness because of its popularity in the direct consumption and processing of several products, including bean sprout, vermicelli,

starch, dessert (green bean soup), as well as medicine. Thus, it is classified as a potential crop in the future. In 2015, the cultivated area is 0.14 million hectares, mainly from lower northern Thailand such as Phetchabun, Nakhon Sawan, Sukhothai, Phitsanulok, Phichit, Uttaradit, Kanchanaburi, and others with total production of over 0.10 million tons. It can be cultivated during 3 seasons throughout the year, particularly the late rainy season between late August and September. However, the current mungbean production is not sufficient for the requirement. In addition, it is constrained by an array of low yielding potential of the current varieties from lack of genetic variability, poor harvest index, and susceptibility to abiotic stresses (drought, calcareous, or saline soil) and biotic stresses (diseases and insect pests) (Tantasawat et al., 2010).

2.3 Major diseases affecting crop production

2.3.1 Mungbean yellow mosaic virus (MYMV)

MYMV, a member of family Geminiviridae, is usually caused by begomoviruses through the vector, the whitefly (*Bemisia tabaci*), which delivers this virus to the phloem cells of host plants. In leaf cells, the virus particles form loose aggregates and scattered throughout. These aggregates sometimes fill the nuclei of infected phloem cells. Then, it causes yellow-coloured spots, mainly on young leaves followed by yellow mosaic pattern. After that, the size of spots gradually increases, leading to complete yellowing of leaves. The yellow leaves slowly dry and wilt. Infected plants possess few flowers and pods with some immature, and deform seeds, resulting in the yield lose by means of qualitatively and quantitatively. Pods are reduced and turn yellow-coloured spots. In severe cases, other plant parts become

completely yellow. Infection can reduce the photosynthetic efficiency, thereby affecting crop yield (Karthikeyan et al., 2014). The economic impact on yield is involved to the plant development and depends on the time of infection. Early infection results in the highest reduction in yield with 100%, particularly when the infection takes place after 3 weeks from planting. However, the losses will be meagre if infection takes place after 8 weeks from planting.

2.3.2 Root rot

Root rot incited by fungus *Rhizoctonia solani* (*Macrophomina phaseolina*) causes pre-and post-emergence rot, resulting in maximum mortality of plants and consequent reduction in yield of seedlings. Its sclerotia, which are tough and brownish-black structures allow it to survive in the soil or infected plant tissue for several years. With regards to the fungus infestation, infected stems start to form brown to reddish brown lesion tissues. Cankers enlarge and girdle the stem restricting absorption of water and nutrients into the plant, consequently resulting in wilting and possible nutrient deficiencies. Seed infection ranges from 2.2 to 15.7%, which causes 10.8% and 12.3% in grain yield and protein losses, respectively.

2.3.3 Cercospora leaf spot (CLS)

Cercospora conescens, *C. cruenta*, *C. kikuchii*, and *C. caracallae* are the causal fungi of the leaf spot disease. Among these, *C. conescens* is the most prevalent species and causes significantly yield losses in several host legumes, i.e. urdbean, hyacinth bean, common bean, yard long bean, as well as mungbean. In rainy season of relatively hot and high humidity, the fungus initially forms water soaked angular spots with brown to grayish centers and reddish margins of leaves. When spots become older and gather together, consequently they will cause enlarged dead

area on the infected leaves. Severe infections can also affect premature defoliation. Sometimes, the leaves may become malformed and wrinkled. Maturity is also delayed, resulting in poor pod formation. Seeds that are developed on severely infected plants are small and immature (Shahbaz et al., 2014). Chinsawangwattanakul et al. (1984) reported that infected mungbean, resulted in 68% and 35% yield reduction of the susceptible variety (UT1) and resistant variety (Pagasu), respectively. Wongpiyasatid et al. (1999) indicated losses in seed yield to 29.60% due to the reduction of pods/plant and seeds/pod, when UT1 was early infected. Kumar et al. (2011) also reported that CLS occurs significantly severe, when the temperatures range 25 to 35^{o}C and relative humidities range 98 to 100% with increasing the number of germtube and conidial germination.

2.3.4 Powdery mildew (PM)

PM is caused by fungus *Sphaerotheca phaseoli*, which requires living hosts, including mungbean to complete its life cycle. In cool-dry season, the fungus causes initial symptoms, which are faint, slightly dark areas, later turning into white powdery spots on the infected leaves with conidia and epiphytic mycelia. In epidemic form, the fungus covers all parts of the plant with white powdery growth, thereby adversely affecting the photosynthetic efficiency of the plant. Particularly, before flowering is infection stage for the maximum damage. Defoliation occurs and pods are formed with bear subnormal seeds or are not formed (Jyothi, 2012). Khajudparn et al. (2007) reported yield losses more than 50%. Reddy et al. (1994) also reported even 100% in yield loses, when the fungus infects at the seedling stage. In addition, Tantanapornkul et al. (2005) reviewed that PM reduced yield, seed weight per plant, seeds per plant, pods per plant, and seed size by 32.42, 24.57, 39.72, 23.17, and 6.82%,

respectively in 3 susceptible varieties; CN36, CN60, and VC3476A.

2.4 Management of diseases and effective ways for crop protection

2.4.1 Field management

Earlier, when other methods were not evolved, cultural practices were the only method, which includes the removal or ploughing-in of infected parts after harvest, management of plant nutrients and water, avoiding overcrowding and shade, pruning, or replacing plant materials to control the plant diseases. For plant nutrient management, the effect of N is quite variable depending on the types of pathogens; facultative vs. obligate parasites. With regards to the facultative parasites, i.e. *C. canescens*, when supplying high N, there is decrease in disease severity. However, when the disease is corresponded by obligate parasites, i.e. MYMV and *S. phaseoli*, high N supply typically increases disease severity. These responses rely on the difference of their nutritional requirements. In the case of obligate parasites, their N requirements promote higher growth rate during the vegetative stage and change the proportion of the young to mature tissue in favor of the young tissues, which are more susceptible. There is also a significant increase in amino acid concentration in the cell wall and on the leaf surface, thereby inducing the germination and growth of conidia. However, the response to the N level of facultative parasites is different. These reports indicate that susceptibility to diseases depends on N supply with pathogen specific cases (Dordas, 2008). Later, additional applications of chemicals are the most common methods for controlling diseases. The effective chemicals are used worldwide to inhibit diseases in different ways such as seed priming, soil drenching, and foliar spray with the susceptible varieties (Table 2.1). Although chemical control

is one of the most effective ways in Thailand, poor farmers cannot meet the expense of pesticides to manage diseases until harvest. In addition, the chemical can definitely cause a variety of serious effects on human health and the environment. The continuous use of chemical pesticides also leads to maximum risk of the pathogens becoming resistant to pesticides. Disease forecasting is the system used to predict the disease incidence with more accuracy. There were many works used this system in various plant diseases, i.e. Stewart's wilt, rice blast, MYMV, leaf spot and PM (Esker et al., 2006; Srivastava et al., 2017; Meti et al., 2017; Smith, 1986, Arafat, 2015).

Table 2.1 Most common pesticides used against diseases in mungbean.

Disease[a]	Fungicide	Mode of action	Reference
MYMV	Imidacloprid	- Interfering the transmission of stimuli in the insect nervous system	Ghosh et al (2009), Karthikeyan et al. (2014)
	Acetamiprid	- Inhibitor of acetylcholine receptor	Karthikeyan et al. (2014)
	Ethion	- Inhibitor acetylcholine esterase	Karthikeyan et al. (2014)
	Triazophos	- Inhibitor of cholinesterase	Karthikeyan et al. (2014)
Root rot	Copper oxychloride	- Interfering the enzyme system of spores and mycelium	Muthomi et al. (2007)
	Bavistin	- Inhibiting germ tubes, appressoria, and mycelia	Kumari et al. (2012)
CLS	Hexaconazole	- Disrupting membrane function - Inhibitor of sterol biosynthesis	Khunti et al. (2005) Ali et al. (2011)
PM	Hexaconazole	- Disrupting membrane function - Inhibitor of sterol biosynthesis	Khunti et al. (2005)
	Karathane	- Uncoupling of oxidative phosphorylation, upsetting the electrochemical balance of fungi cell, and preventing ATP synthesis - Affecting the respiration and cell wall formation in target fungi	Suryawanshi et al. (2009)

[a] MYMV = Mungbean yellow mosaic virus; CLS = Cercospora leaf spot; PM = Powdery mildew.

2.4.2 Use of resistant varieties

Use of resistant varieties to control plant diseases can be regarded as a form of integrated pest management (IPM), and is more sustainable than several other methods. Before the early 1900s, the development of resistant varieties was achieved by selection rather than by breeding. The selection process is only based on natural selection. During the favorable weather conditions, when disease epidemics occur, only the most resistant varieties can survive and provide yields with decreasing pesticide application, thereby subsequently reducing agrochemical pollution in the fields and the cost of production. Therefore, disease control with resistant varieties is much required for farmers and consumers. The levels of resistance have been accumulated in crops over many generations. Local and wild varieties with different levels of resistance have been typically identified and used. However, these varieties often have inherently low yielding potential, i.e. small seed size and short pod length. In addition, the adaptation of highly variable pathogen populations to host resistance renders ineffective resistance in the host. These varieties can be alternatively used as the resistant sources for introgression of resistance genes into cultivated mungbean through breeding programs.

2.5 Breeding for disease resistant varieties

A scientific basis of the plant breeding for resistance to diseases was provided since the early 1900s, when Rowland H. Biffin showed that resistance in wheat to stripe rust was inherited according to Mendel's laws. This finding provides the new strategy to accelerate large-scale breeding programs to incorporate resistance genes into many important crop plants.

2.5.1 Inheritance of CLS and PM resistance

Plant resistance to diseases is often modulated by Mendelian genes, typically known as resistance (R) genes for qualitative resistance, and follows the gene-for-gene relationship between host plants and their pathogens according to H.H. Flor., who studied the rust resistance in flax and proposed since 1956. The gene-for-gene concept provides a valuable model for studying host-pathogen systems. The interaction can also be modulated by polygenes called quantitative trait loci (QTLs) or even modifier genes in some diseases. However, this does not distort the basis of theory. It is most likely to seem that the gene-for-gene relationship is a basal concept in host-pathogen interaction. For more understanding, some researchers use a lock and key analogy to describe the gene-for-gene hypothesis, which also provides a theoretical explanation for boom and bust cycles (Figure 2.2). When a resistant variety is widely used (boom year), the selection pressure of pathogens is continually increased. As a consequence, the virulent pathogens become the prevalent race, and then it successfully infects a resistant variety (bust year), because the resistant variety lacks the protection from resistance gene(s). Breakdown of plant resistance to diseases is largely based on 2 properties of pathogens; their relatively high reproductive rates and great genetic variability, resulting from any mutation events. Taken these together, the properties allow pathogens to overcome the resistance of the resistant variety. Reddy (2007) demonstrated that there were the different combinations of mungbean resistance genes; *Pm-1Pm-1Pm-2Pm-2* (TARM-1), *Pm-1Pm-1pm-2pm-2* (S-158-16), *pm-1pm-1Pm-2Pm-2* (S-2-4-1), and no resistance genes *pm-1pm-1pm-2pm-2* (TPM-1) in the investigation on race identification of the PM pathogen. These mungbean genotypes were challenged with 4 PM isolates. The interactions of resistant

differentials with the Akola isolate differed from the other 3 isolates, and it can be able to only infect all the resistant hosts. Some of the variations in the Akola isolate can be explained as the result of PM mutation by means of the dominant avirulence *Avr-1Avr-1* and *Avr-2Avr-2* genes to virulence *avr-1avr-1* and *avr-2avr-2* genes corresponding to host resistance *Pm-1Pm-1 and Pm-2Pm-2* genes.

Varieties	Resistance "locks"	Pathogenicity "keys"	Disease expression
1		Any, For none needed	Susceptible
2	A	None	Resistant
3	A	aa	Susceptible
4	A B	aa	Resistant
5	A B	bb	Resistant
6	A B	aa bb	Susceptible
7	A B C	aa bb	Resistant
8	A B C	aa bb cc dd ee	Susceptible

Figure 2.2 The gene-for-gene concept by means of resistance gene locks and pathogenicity key (modified from Browning, 1963).

In mungbean, the inheritance of CLS resistance in different resistance sources has been studied through conventional genetic analysis. CLS resistance is differently controlled by a single dominant or recessive gene and quantitative genes. Thakur et al. (1980) evaluated the CLS reaction and inheritance in 9 mungbean varieties/lines under artificial conditions. Among them, EC-27087-2, EC-2627I-3, and ML-I were scored as resistant. F_2 population was segregated into resistant and

susceptible classes in a ratio of 3:1, while segregation of BC progenies was 1:1. These results confirmed that CLS resistance was found to be inherited and controlled by a single dominant gene. Mishra et al. (1988) studied inheritance pattern for *C. canescens* and *C. cruenta* resistance in mungbean crosses of resistant×susceptible, resistant×resistant, and susceptible×susceptible lines. A 3:1 ratio was observed in all the 14 F_2 progenies from resistant×susceptible parents with resistance, being controlled by a single recessive gene. Chankaew et al. (2009) also studied inheritance pattern of CLS resistance in a cross between resistant line (V4718) and susceptible variety (KPS1). The results revealed that the resistance in V4718 is controlled by a single dominant gene. Singh et al. (2017) indicated that the segregation ratio in F_2 population was observed to be 3:1 for resistance and susceptibility, respectively. In addition, the segregation of F_3 population was similar to the F_2 observation, again confirming the control of a single dominant gene for CLS resistance in mungbean.

In addition, the inheritance of PM resistance has also been reported. Reddy et al. (1994) studied the inheritance of the resistance in the F_1, F_2, and F_3 derived from resistant line (RUM) and several susceptible varieties. They indicated that PM resistance in mungbean is controlled by 2 dominant genes designated as *Pm-1* and *Pm-2* conferring specific disease reaction 1 and 2, respectively. However, Chaitieng, (2002) revealed that the resistance to PM in resistant lines (SUT4 and VC1210A) is controlled by a single dominant gene. Gawande and Patil (2003) also concluded that both additive and dominance gene actions were found to be necessary in the inheritance of PM resistance from resistant line (TARM 18) and susceptible genotypes by means of disease incidence, % disease index, and area under disease progress curve with non-allelic interactions. Khajudparn et al. (2007) tested the allelic

relationship using 3 F_1 resistant hybrids; V4718×V4758, V4718×V4785, and V4758×V4785, F_2, and F_1×susceptible variety (CN72). They found that the resistance in the F_1×CN72 and F_2 population segregated in a ratio of 3:1 and 15:1 for resistance and susceptibility, respectively among all 3 crosses. These results confirmed that PM resistance in each resistant line is controlled by a single dominant gene, and these resistance genes are non-allelic. On the other hand, Young et al. (1993) found 3 genomic regions responsible for PM resistance of F_3 derived from a cross between a moderately PM resistant parent (VC3980A) and a susceptible parent (TC1966). Two genomic regions with 64.9% of the total variation from VC3890A were associated with increased PM resistance at 65 days after planting. A third genomic region from TC1966 was the one associated with higher levels of PM resistance at 85 days. Chaitieng et al. (2002) also found a major quantitative trait locus that accounted for 64.9% of the total variation conferring PM resistance in a moderately resistant breeding line (VC1210A). Kasettranan et al. (2010) identified 2 QTLs; *qPMR-1* and *qPMR-2* on different linkage groups (LGs) that accounted for 20.10 and 57.81% of the total variation for PM resistance in a resistant line (VC6468-11-1A), respectively. Chankaew et al. (2013) detected a major QTL on LG9 and 2 minor QTLs on LG4 for the resistance in V4718, as well as 2 major QTLs on LG6 and LG9, and a minor QTL on LG4 for the resistance in another resistant line (RUM5).

2.5.2 Genetic diversity for plant breeding program

Genetic diversity of PGRs or gene pools is helpful for plant breeders to develop new variety with desirable traits through several breeding methods. It can be described as the variation in alleles of genes or in DNA/RNA sequences, resulting from mutation, recombination, selection, genetic drift, and gene flow, or even crop

monoculture. Thus, no living organisms or even maternal twins is exactly similar to each other. The variation in hereditary traits may show in the form of altered morphology, physiological, or biochemical features for survival of plants. Some of the mungbean desirable genes controlling different traits in wild species, related species, breeding stocks, mutant lines etc. are listed in Table 2.2. Usually, these germplasms are derived from the World Vegetable Center (formerly the Asian Vegetable Research and Development Center: AVRDC) that currently holds the world's largest collection of *Vigna* germplasm, consisting of 12,153 accessions (6,742 mungbean accessions) (Sue et al., 2015). Theoretically, the exclusive presence of the greatest genetic diversity within and between crop species allows plant breeders to select superior genotypes either to be directly used as plant introduction or to be used as parents in hybridization programs. Genetic diversity between 2 suitable parents is important to obtain heterosis and transgressive segregants among their progeny.

Table 2.2 Sources for different traits in mungbean.

Germplasm	Traits[a]	Origin	Reference
V4718	CLS and PM resistance	India	Khajudparn et al. (2007), Chankaew et al. (2009)
V4758	PM resistance	India	Khajudparn et al. (2007)
V4785	PM resistance	India	Khajudparn et al. (2007)
EG-MD-6D	General resistance	Philippines	Shanmugasundaram et al. (1988), AVRDC (2017)
KING	Large seed, high yield	Australia	Chueakhunthod et al. (unpublished data)
SUT1	High yield, drought tolerance	Thailand	Khajudparn et al. (2007), Chueakhunthod et al. (unpublished data)
CN84-1	High yield	Thailand	Ngampongsai et al. (2012)

[a] CLS = Cercospora leaf apot; PM = Powdery mildew.

Assessment of genetic diversity and relatedness within and between PGRs can be carried out using various methods based on morphological, physiological, and DNA levels. Initially, morphological traits have been used because of their visually accessible traits. Singh et al. (2014) used 17 morphological traits, i.e. number of branches per plant, peduncle length, number of clusters per plant, and harvest index to estimate diversity across 104 mungbean accessions. They were clustered into various groups, indicating the diversity of genotypes. In addition, seed weight was indicated to be used as criteria for selection in breeding program. Shyamalee et al. (2016) also used 12 quantitative agro-morphological traits, yield, and yield parameters, including days to first flowering, days to 50% flowering, days to first pod maturity, days to maturity, plant height, pod length, number of seeds per pod, pod length, number of seeds per plant, thousand seed weight, total yield, and yield per plant to characterize 61 mungbean accessions. They were classified into 7 groups, and the largest group containing 18 mungbean genotypes was classified as the most diverse. This group can be used as new potential parent with diverse agro-morphological traits for variety development. However, some highly morphological similarity among plant materials can disrupt the assessment. Alternatively, molecular markers, which can detect any variation among genotypes at DNA or RNA level throughout the genome to estimate diversity are very useful for this purpose. Moreover, they are completely independent from the environmental factors. These advantages allow them to be considerably utilized for variety characterization and parental selection (Casassola et al., 2012).

2.5.2.1 Genetic diversity based on molecular markers

Molecular markers have recently become more crucial, and have

almost completely replaced morphological markers and other marker systems. Polymorphisms in the nucleotide sequence can be detected by different techniques. The most commonly used marker systems for genetic diversity in several plants, including mungbean are RFLP, RAPD, AFLP, ISSR, and SSR markers. Islam et al. (2015) estimated diversity using RFLP markers with single restriction enzyme *Eco*RI in 5 mungbean accessions. The RFLP profile revealed a high level of polymorphism (90.90%) with 10 polymorphic bands, which could group accessions into 2 clusters. Sony et al. (2012) tested 20 RAPD markers for genetic diversity in mungbean, and they were found to be 100% polymorphic. A total of 10 unique DNA bands was amplified from the 13 mungbean varieties, which have segregated into 2 major clusters. Bhat et al. (2005) used 12 AFLP markers with each 2 and 3 selective nucleotides to assess the genetic diversity and relationships among 27 mungbean varieties. The polymorphism was more with 3 selective nucleotide AFLP markers (67.40%) than with 2 selective nucleotide AFLP markers (48.26%). However, the dendrograms derived from both types of selective nucleotides and their combinations showed 2 clusters with a high degree of similarity due to the use of limited diversity of plant materials. Nath et al. (2017) revealed that ISSR markers were very effective in detecting polymorphism among accessions because of targeting multiple microsatellite loci distributed throughout the genome. Eighty eight polymorphic bands (81%) clearly generated 4 major clusters. Somta et al. (2009) grouped the parents and 5 varieties derived from AVRDC breeding lines by 48 polymorphic SSR alleles, which could differentiate all the mungbean lines. Clustering pattern was in general agreement with the origin, including pedigree information. The AVRDC elite parental germplasms possessed a greater genetic variability.

2.5.2.2 Analysis of genetic diversity using EST-SSR markers

Several markers revealed above are structural markers, which are largely generated from non-coding regions or gene-coding regions, thereby targeting across these regions of genome, particularly in the larger content of non-coding regions. There are more costly and time-consuming in order to develop these markers from genomic DNA libraries, particularly SSR markers. Therefore, one of the molecular markers that has been replacing structural markers and become popular is known as EST-SSR markers, because they have several some intrinsic advantages over conventional genomic SSR markers, i.e. less costly to identify, being embedded in functional gene sequences, and in direct association with transcribed genes, thereby detecting the variations in both transcribed and known-function genes (Varshney et al., 2005). More recently, an increasing number of EST-SSRs from many crops, including mungbean have been generated in public databases with the advancement of NGS to acquire sequences in most portions of genome. Development and usefulness of EST-SSR markers have been carried out in several studies. Gupta et al. (2013) designed 1,742 SSR loci from 12,596 EST sequences of a mungbean genotype 'Jangan', and performed polymorphism analysis among 20 mungbean genotypes. They provided 78% of polymorphism and the number of alleles ranged from 2 to 6 with an average polymorphism information content (PIC) value of 0.34. Cluster analysis can clearly separate the cultivated and wild genotypes into separate groups. Transferability study showed that 97% EST-SSR markers were transferable to 8 other *Vigna* species, thus expanding their utility. Chen et al. (2015) developed and identified 13,134 EST-SSRs and 66 EST-SSR markers, which showed polymorphism among 31 mungbean accessions derived from diverse locations. Numerous EST-SSRs from this

study were compared with common bean and soybean, whose genomic information is more identified in order to determine the possible functions of these EST-SSRs. Most of which were similar to known or hypothetical protein-encoding genes such as auxin efflux carrier component, dof zinc finger, F-box, gibberellin receptor, helicase, mitogen-activated and leucine-rich repeat extensin-like proteins.

2.5.3 Conventional breeding methods

The use of breeding methods for improving plant resistance to diseases mainly relies on the availability of resistance sources. Breeding strategies can be divided into conventional, mutation, and biotechnological approaches. Several conventional methods for self-pollinated crops include pure line selection, mass selection, pedigree selection, bulk selection, single seed descent, and backcross breeding etc. Examples of the widely used methods are given.

2.5.3.1 Pedigree selection

The pedigree selection is one of the most frequently used methods to select desirable progeny after gathering genetic variability. Segregating populations derived from artificial crosses between parents with maximum genetic distance are highlighted. Selection starts at the early generations based on visual evaluations, and depends on the degree of genetic variability within individuals. Selection in advanced generations (F_6 or later) is generally to obtain adequate uniformity with small proportion of segregating loci expected of individuals. High heritability traits, i.e. height, maturity, and flowering date, as well as qualitative disease resistance that all are easily identified are typically considered. In addition, recording data of genetic relationships among individuals selected can be very helpful to select multiple individuals or to maximize genetic variability among lines retained

during selection by avoiding closely related individuals and by obtaining the maximum expression of other desirable agronomical traits. However, this method requires more land and labor than other methods, and selection for low-heritability traits is still difficult, because the effects of environmental factors on these traits can hamper their expression. Sadiq et al. (1998) developed a mungbean variety (NM92) having high yield potential, large seed size, synchronous maturity with non-shattering pods, and resistance to MYMV using this method. Two thousand five hundred of F_2 plants from each combination cross between 3 large seeded true breeding lines (VC 1560D, VC 2768B, and VC 3726), as well as NM36 were generated from the F_1 plants and selected until F_5 generation. Twenty five true breeding lines were evaluated, and one of these showed the highest grain yield, along with other desirable economic traits, as well as resistance to MYMV in multi-locations. Note that this method takes over 8 years to obtain a new variety ready for general cultivation, indicating that it is very time-consuming for variety improvement.

2.5.3.2 Backcross breeding

Backcross breeding is not considered to create entirely new variety but to just modify existing variety. This method plays an important role in the introgression of a single or a few disease resistance genes into a susceptible high yielding variety. The high yielding variety is recurrent parent, while the resistant variety is donor parent, which may be useless agriculturally, except for its possession of valued gene, i.e. for disease resistance. Recurrent parent (suppose S) is hybridized with donor parent (suppose R). The resultant progeny is called F_1, and contains 50% of the genetic content from each parent. To recover high yielding potential from recurrent parent, S variety is backcrossed with F_1 and successive backcross (BC)

screened progeny having resistance gene in subsequent years until nearly 99% genes from S variety are recovered in the BC_6 generation. The scheme described above assumes, when the allele for disease resistance is dominant. If it is recessive, then it is necessary to alternate backcrossing with selfing of the BC progeny. Backcross breeding has been widely used to develop new varieties for CLS and PM resistance in several cases. KPS1, KPS2, and PSU1 were improved for CLS resistance. These varieties were crossed with a resistant line (VC3689A), and backcrossed to recurrent parents for 4 times. SUT2, SUT3, and SUT4 obtained from this program were the new CLS resistant varieties in Thailand at that time (Chaitieng, 2002). Chaitieng (2002) developed PM resistant mungbean lines through backcross breeding. Two crosses between a recurrent parent (CN36) and 2 donor parents (SUT4 and VC1210A) showed that there was a successful transfer of the resistance genes to F_1 and BC_1 to BC_3 progeny under greenhouse or field selection. The resistant levels in 10 BC_3F_3 lines were higher or comparable to those of their parents, and some of them also produced higher yield. This corroborated that backcross breeding can successfully transfer the resistance gene to susceptible variety. Ngampongsai et al. (2011) improved PM resistance for a susceptible variety (CN72) through backcross breeding for 5 times with resistant lines (VC1163-12-B-1-2-B-6, VC1560D, VC2777-B-1-2-B, and 500181). BC_5F_2 progenies from CN72 and VC1163-12-B-1-2-B-6 cross showed a higher yield and more PM resistance.

2.5.3.3 Gene pyramiding

For horizontal resistance, gene pyramiding is responsible for long-lasting resistance to diseases, and has become the great strategy of plant breeders to develop durable disease resistant varieties against different isolates, races, or

biotypes of pathogens (Ashkani et al., 2015). In other words, if 2 or more genes are pyramided into a single variety, it is less probable for the plant to lose both resistance genes at the same time. Gene pyramiding scheme can be separated into 2 steps. The first step is called a pedigree step, which purposes at cumulating of all resistance genes from founding parents derived from double haploid, RIL etc. in a single genotype called the root genotype. The final step is called the fixation step, which purposes at fixing all the resistance genes into a homozygous state in the resistant genotype called the ideotype from the one single genotype to avoid their segregation in successive generations (Figure 2.3). This ideotype can be used as new resistant variety to be recommended to farmers or as new resistance source for breeding programs (Joshi and Nayak, 2010). The strategy has been used in several plants, i.e. rice, wheat, cotton, pea, chickpea, and soybean (Malav et al., 2016). However, the use of gene pyramiding for any purposes has not been accomplished in mungbean.

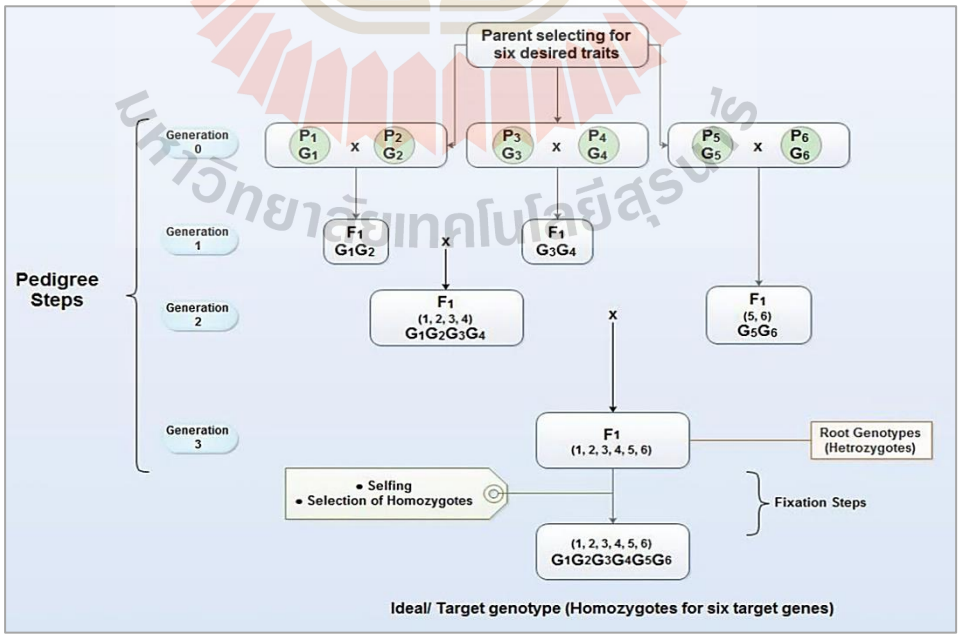

Figure 2.3 Gene pyramiding scheme cumulating 6 resistance genes (G_1 to G_6).

2.5.4 Mutation breeding

Mutation breeding technique is helpful in the development of new resistance alleles that do not exist in germplasm pools through both types of mutagenic agents; physical and chemical mutagens. Resistant mungbean lines including M5-5, M5-1, and M4-2 with the moderate CLS and PM resistance from mutation of susceptible varieties CN36 and KPS1 were achieved. Seeds of both varieties were gamma irradiated at 500 gray or soaked in 1% ethylmethane sulphonate (EMS) with selection in each M_2 to M_4 generation (Wongpiyasatid et al., 2000). Masari et al. (2015) reported that mungbean mutant lines CNMB06-01-40-4, CNMB 06-02-20-5, and CNMB 06-03-60-7 derived from the mutation by gamma radiation at 0, 200, 400, and 600 grays of the susceptible varieties CN36, CN72, KPS2, and SUT1 showed a higher level of PM resistance and yield. However, the limitation of mutation breeding is challenged due to the difficulty in generating the dominant alleles and its less efficiency.

2.5.5 Biotechnologically based breeding methods

Plant breeders face the problems associated with conventional breeding methods, i.e. the longer times required to develop resistant varieties, more effort and labor requirements, resistance breakdown, gene or linkage drag due to transfer of non-desirable genes, along with resistance genes, non-availability of resistant sources and the difficulty of disease screening among segregating populations. Biotechnologically based breeding methods with the advancement of molecular genetic knowledge are needed to overcome these problems.

2.5.5.1 Genetic engineering

Genetic engineering is employed to cut a DNA sequence of the

candidate genes from any organisms, i.e. animals, viruses, bacteria, fungi, or even totally man-made sequences and to introduce into another organism through *Agrobacterium* transformation or biolistic method within a shorter time than through other breeding methods. The candidate genes associated with disease resistance are involved in plant microbe interaction, and limit the virulence of the pathogens, i.e. pathogen cell wall degrading enzymes and toxins, i.e. nucleic acid synthesis inhibitor. Such genes can enhance the production of plant defense molecules, i.e. antimicrobial peptides, phytoalexin, and relative oxygen species (ROS). Enhanced resistance to diseases, particularly MYMV in another *Vigna* species, i.e. blackgram was developed by expression of soybean replication initiation protein (Rep) gene. The results revealed that blackgram co-agroinoculated with infectious constructs of soybean isolate of MYMV, along with antisense Rep gene construct showed relatively high resistance to the virus (Haq et al., 2010). However, it will not replace conventional breeding due to some limitations for commercial uses, particularly in Thailand.

2.5.5.2 Genome editing

Development of new genetically engineered crops without selectable marker genes of antibiotic resistance, genome editing technologies (GETs) have currently emerged. These technologies are the newest methods and faster to edit DNA in a sequence specific manner, that is, susceptibility genes at precise locations through small deletions and insertions for gene silencing, changing gene function, or even introducing functionally targeted genes. GETs rely on the introduction of targeted DNA double-strand breaks through programmable nucleases. The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 (CRISPR-associated protein-9 nuclease) is based on RNA-guided engineered nucleases, and is

widely used among several systems for GETs in order to function according to mechanisms. The successful examples of GETs with CRISPR/Cas9 to improve disease resistance were already attempted in some crop plants, i.e. rice and cucumber. Wang et al. (2016) used CRISPR/Cas9 coupled with sequence-specific nucleases to improve blast resistance by engineering the *OsERF922* in multiple sites of rice genome. The results showed that the blast symptom was significantly decreased in all 6 mutant lines compared with wild-type plants at both the seedling and tillering stages. In addition, there were no significant differences of agronomic traits tested between any of the 6 T_2 mutant lines and the wild-type plants.

2.5.5.3 Gene pyramiding based on molecular markers

Common disease screening techniques, including field testing under natural disease pressure, greenhouse, or growth room screening procedures have been used worldwide. However, using these phenotypic screenings alone for disease resistance are always influenced by the environmental factors, resulting in the variations of disease symptoms. Screening individual plants pyramided multiple resistance genes is rather difficult because of time-consuming and very difficult to test all desirable traits mediated by the genes of interest. Moreover, identifying the plants with either 1, 2 or more resistance genes is difficult to be accomplished by only the phenotypic screenings. Genotypic selection based on molecular markers called MAS which is environmentally independent (heritability = 1) can be helpful to identify the number of genes. Plant breeders can also use it, together with conventional breeding to precisely track the introgression of the resistance genes into the susceptible plants and to screen a large population of plants. Particularly, it is very useful in backcross breeding called MABB to introgress 2 or more resistance genes for durable resistance

(Jain and Brar, 2010).

2.5.5.3.1 Marker assisted selection (MAS)

MAS is an indirect selection based significantly on molecular markers tightly linked (i.e. < 5 cM) to underlying gene rather than on the desirable trait per se, resulting in greater speed and accuracy. Target genotypes for traits, which may be expressed in adult plants can be more effectively selected at the early stage of plant growth allowing breeders to grow more generations per year (for example 3-4 times for mungbean), thereby saving resources. With respect to the linked markers, the number of genes can be easily identified, thus MAS is very helpful for pyramiding combinations of multiple resistance genes or several QTLs that generally show similar phenotypic effects, or some genes mask the expression of other genes. This can be used to clearly distinguish plants carrying all desired genes from those that only carry some of them.

2.5.5.3.2 Foreground selection

MABB involves 3 general levels of selection (Figure 2.4). Typically, plant breeders use foreground selection to select plants with the marker allele according to its segregation in accordance with Mendel's law of donor parent at the target locus in each resistance gene. A heterozygous state with one donor allele and one recurrent parent allele is obtained until the final backcross is completed. The selected plants are then self-pollinated for obtaining homozygous plants for the resistance allele. Markers tightly linked or flanked to the target resistance genes or QTLs can be used. For example, using markers flanked a target gene (i.e. about < 5 cM on either side), double recombination events occurring on both sides of a target locus are extremely rare, and linkage drag that may adversely affect the important agronomic

traits can be minimized (Figure 2.5).

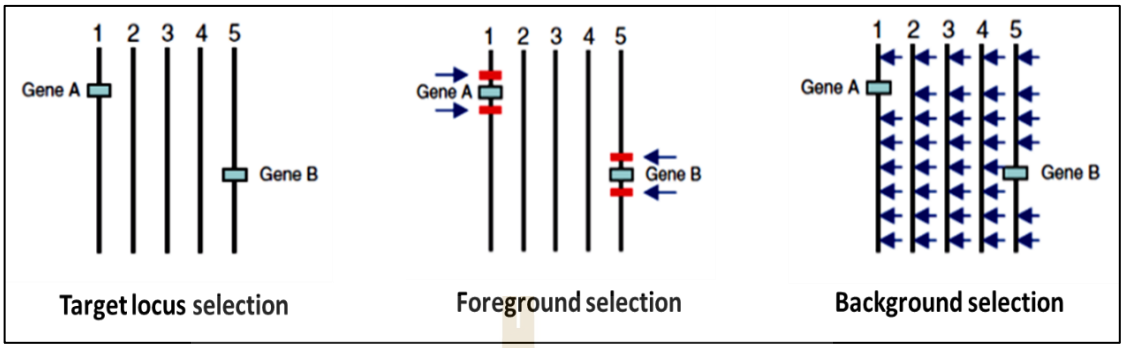

Figure 2.4 Levels of selection during marker assisted backcross breeding (Jain and Brar, 2010).

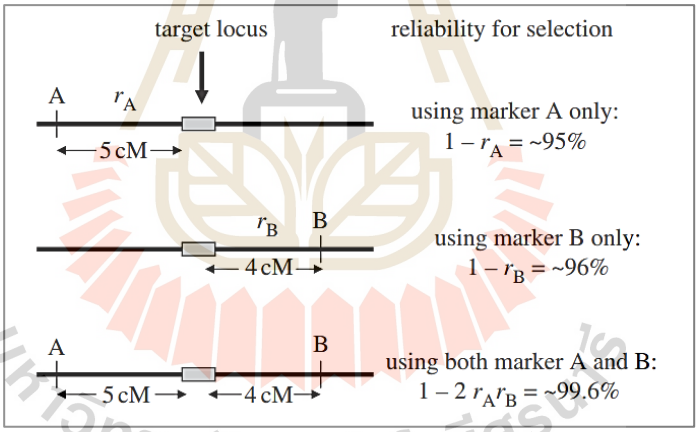

Figure 2.5 The recombination frequency from using single and flanking markers, assuming no crossover interference. Using marker A or B alone is about 5 or 4% (5 or 4 cM), thus recombination may occur between the target locus and marker A or B in about 5 or 4% of the progeny, respectively. The probability of recombination occurring between both markers, i.e. double crossover is much lower than using single markers (about 0.4%) (Tanksley, 1983).

Molecular studies for CLS and PM resistance genes have continually laid the foundation for MAS in mungbean breeding programs. In our laboratory, Poolsawat et al. (2017) developed markers linked to the PM resistance gene from the $F_{2:7}$ and $F_{2:8}$ RIL populations of a cross between CN72 and V4718. ISSR (I85420) and ISSR-RGA (I42PL229) markers were closest to the PM resistance gene with a distance of 9 and 4 cM, respectively, and only 0.72% recombination of both markers with the PM resistance gene will occur in MAS. They also fine-mapped a major QTL, *qPMC72V18-1* controlling PM resistance. This QTL was located at the logarithms of odds (LOD) peaks of 5.89 and 5.04 and between both markers at position 19.5 and 23.5 cM using the 2013 and 2016 data, respectively (Figure 2.6). It explained up to 92.4% of the total phenotypic variance explained (PVE) by the QTL with higher than the major QTL, *qPMV4718-3*, previously reported in a cross between KPS1 and V4718 that explained 22 to 46% (Chankaew et al. 2013). Poolsawat et al. (unpublished data) found 2 ISSR-RGA (I27R211 and I27R565) markers associated with PM resistance in another cross of CN72 and V4785. Arsakit et al. (2017) reported that SSR (VR393 and CEDG084) markers were linked to 2 major QTLs, *qCLSC72V18* and *qPMC72V18* for CLS and PM resistance in a cross of CN72 and V4718, respectively. Both QTLs accounted for 47.76% and 18.72% of the total PVE for CLS and PM, respectively. They concluded that VR393 marker was significantly associated with both CLS and PM resistance, and can be used in MAS (Figure 2.6). These 6 marker loci including I85420, I42PL229, VR393, CEDG084, I27R211, and I27R565 are very useful for pyramiding of CLS and PM resistance genes in mungbean breeding programs.

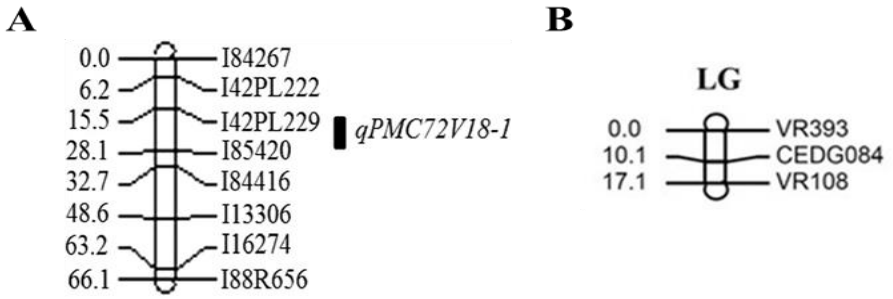

Figure 2.6 Genetic linkage map of $F_{2:7}$ RIL populations derived from a cross between CN72×V4718 showing the position of the major QTL, *qPMC72V18-1* for PM resistance (A) (Poolsawat et al., 2017) and major QTLs, *qCLSC72V18* and *qPMC72V18* for CLS and PM resistance (B) (Arsakit et al., 2017).

2.5.5.3.3 Background selection

To recover recurrent parent genome and to remove unnecessary genes (linkage drag) from donor parent (i.e. > 10 cM), background selection combined with other selections can be used coincidentally, thereby enhancing faster recovery of the recurrent parent genome and breaking linkage drag with MAS within the early BC progeny compared to conventional backcross breeding. In each BC generation, the proportion of the recurrent parent genome recovered based on the formula $1-(1/2)^{n+1}$, where n is the number of BC generations (Table 2.3) (Hasan et al. 2015). Note that recovering the recurrent parent genome on a chromosome carrying the target donor locus is much slower than other chromosomes because of the difficulty for reducing linkage drag associated with the target gene on carrier chromosome, resulting in still segregating the unwanted donor genes after BC_6 generation. By contrast, the unwanted donor genes located on non-carrier

chromosomes in the early BC generations are rapidly removed. Background selection is accomplished using recurrent parent marker alleles that are unlinked to the target locus in order to remove the unwanted donor alleles in the same genomic region (Figure 2.4). Generally, 2 to 4 co-dominant polymorphic markers between parents on a chromosome of 100 cM provide adequate coverage of the genome (Visscher 1996; Servin and Hospital 2002). In mungbean, several QTLs for yield-related traits were constructed by SSR and EST-SSR markers and identified by Isemura et al. (2012). It is interesting to note that their relations to yield performance may be associated with nature "high yielding potential" of recurrent parent. More interestingly, 20 major QTLs (PVE ≥ 20%) associated with several traits, i.e. 100 seed weights, seed length, seed weight, seed thickness, seed dormancy, seeds per pod, pod length, pod width, and others are distributed on 7 out of 11 LGs of mungbean, and they can be used for detecting yield-related regions that are controlled by a few major genes plus some minor genes. These genes are distributed within the narrow regions on a small number of LGs. In addition, a total of 53 QTLs with small effects (PVE < 10%) were also found on each LG. These QTLs associated with pod per plants, the rate of scattered pods, and others can be subsequently considered to be additionally helpful for the same purpose (Table 2.4).

MABB for accelerating the development of new resistant varieties in several plants have been successful. For qualitative resistance, Liu et al. (2017) developed the new Cabbage Fusarium Wilt (CFW) lines through transferring a CFW resistance gene (*FOC1*) from donor parent (D134) into recurrent parent (01-20). Two hundred and forty BC_1 and 280 BC_2 individuals were developed and further screened for foreground selection through 13 SSR markers closely linked

to *FOC1* (0.1 cM). One hundred and twenty three of 240 BC_1 individuals and 134 of 280 BC_2 individuals were resistant according to a Mendelian ratio of 1:1. Subsequently, 100 BC_1 progenies were challenged with the CFW, and 47 individuals showed resistant to the pathogen. For background analysis, 24 polymorphic InDel markers distributed on each polymorphic chromosome segment were used to evaluate the background of 123 BC_1 and 134 BC_2 individuals carrying CFW resistance alleles. Twelve BC_1 individuals were similar to the 01-20 genomes, and were backcrossed for generating BC_2 plants. Eventually, 8 candidate BC_2 individuals with homozygous *FOC1* allele and genomic background, as well as phenotype almost identical to the 01-20 were obtained and used to generate homozygous lines. In BC_2F_2 generation, one of the self-pollinated progenies had the highest resistance level, and had recovered up to 99.8% genetic background of recurrent parent. This plant is subsequently used for breeding of new CFW-resistant cabbage hybrids. For quantitative resistance, Pradhan et al. (2015) pyramided bacterial blight resistance genes *xa5*, *xa13*, and *Xa21* from a resistant line (Swarnafor) with broad-spectrum resistance in deepwater rice variety (Jalmagna). One hundred forty three true F_1 plants were backcrossed to generate 360 BC_1F_1 seeds for further backcrossing. Ninety three, 91 and 116 BC_1F_1 plants showed the presence of *Xa21*, *xa13*, and *xa5* resistance genes, respectively. Thirty one, 42 and 46 BC_1F_1 plants showed the presence of *Xa21* and *xa13*, *Xa21* and *xa5*, and *xa13* and *xa5* resistance genes, respectively. Only 14 plants showed the presence of all 3 resistance genes. Out of these 14 BC_1F_1 progenies, plant showing 77.5% genetic background with recurrent parent based on 60 polymorphic SSR markers was backcrossed. One hundred and twenty two BC_2F_1 progenies were generated. Only 9 plants exhibited the amplification of 3 resistance genes, and hold 88.13 to 91.82%

with an average of 90.95% of recurrent genome content (RGC). In BC_3F_1 generation, 285 plants were obtained by backcrossing the best BC_2F_1 individual with 91.82% of RGC with recurrent parent. Only 14 plants showed the presence of 3 resistance genes with RGC of recurrent parent ranging from 91 to 97% with an average of 92.38%. Two BC_3F_1 candidate plants having the maximum RGC recovery of more than 95% were self-pollinated to generate BC_3F_2 plants with homozygous condition for 3 resistance genes. Finally, 26 plants containing 3 resistances genes were obtained and grown as BC_3F_3. One BC_3F_3 with the highest level of bacterial blight resistance showed the maximum of RGC, and had slightly better yielding than recurrent parent. This plant will be beneficial in the deepwater growing region, where chemical control was less effective. In addition, it is expected to have high yield stability and sustainability of deepwater rice production.

Table 2.3 Expected recovery of RPG comparing MABB and conventional in each BC generations.

Backcross generation	**Number of individuals**	**% Recurrent parent genome recovery**	
		MABB[a]	**Conventional breeding**
BC_1	70	79.0	75.0
BC_2	100	92.2	87.5
BC_3	150	98.0	93.7
BC_4	300	99.0	96.9

[a] MABB = Marker assisted backcross breeding.

Table 2.4 Yield-related QTLs in BC_1F_1 or $BC_1F_{1:2}$ populations (Isemura et al., 2012).

Traits	LG[a]	Intervals	LOD[b]	PVE[c]	Substitution effects
100 seed weight	2	GMES0477-CEDG026a	20.6	16.6	0.69
	8	VM37-CEDG030	28.1	22.2	0.81
	11	GMES3893a-BM149	9.0	5.9	0.41
Seed length	2	GMES0477-CEDG026a	13.1	11.4	0.23
	8	VM37-CEDG030	22.1	20.4	0.30
Seed weight	2	GMES0477-CEDG026a	12.8	14.3	0.19
	8	VM37-CEDG030	14.7	15.1	0.19
Seed thickness	2	GMES0477-CEDG026a	12.7	14.6	0.18
	3	GMES6583-GMES0294a	14.7	14.2	10.47
	8	VM37-CEDG030	20.3	22.7	0.23
Seed per pod	1	CEDG220-GMES4400	5.2	9.1	0.87
	9	CEDG166-GATS11	3.7	7.0	0.76
Pod per plant	2	CEDG096a-GMES0216b	3.7	6.5	-12.69
	4	GMES0216a-GMES1216a	4.4	12.0	-13.82
Pod length	7	CEDG064-CEDG174	22.3	20.5	1.06
Pod width	7	CEDG064-CEDG174	30.1	28.5	0.64
	8	VM37-CEDG030	20.7	19.5	0.53

[a] LG = Linkage group.
[b] LOD = Logarithms of odds.
[c] PVE = Phenotypic variation value.

2.5.6 Next steps towards new CLS and PM resistant mungbean

The CLS and PM resistant mungbean line(s) developed in this study may represent elite materials useful for the breeding of new resistant mungbean varieties. To support Thailand or Agriculture 4.0, one of the important keys is the use of durable disease resistant varieties, which will reduce inefficiencies, pesticides, and other chemicals that dramatically affect environment, animals, and human. Based on innovation, new biotechnological approaches, including MAS are very helpful to speed up the process of getting new disease resistant crops. Highly disease resistant mungbean derived from MAS can be used by farmers or industries in open field plantations or organic farming. Smart technologies, i.e. field sensors, robotic vehicles,

and digital imaging can also be implemented to optimize productivity. The new disease resistant mungbean varieties will be beneficial for farmers as well as consumers in terms of cost reduction and health.

CHAPTER III

MATERIALS AND METHODS

3.1 Plant materials and breeding method

A total of 23 mungbean and 4 blackgram varieties/lines (Table 3.1) reported to have special features, i.e. high yield and disease resistance were evaluated for genetic diversity using EST-SSR markers. Most of these genotypes of mungbean were also used for allele polymorphism analysis at marker loci linked to the CLS and PM resistance genes in order to select the recurrent parent for MABB. Thirty six hybrids derived from crosses between RILs of 3 resistant lines (V4718, V4758, and V4785) and susceptible cultivar (CN72) containing a CLS resistance gene and 3 PM resistance genes were developed by Poolsawat et al. (unpublished data), and were detected for their resistance genes. After all 6 marker loci linked to CLS and PM resistance were evaluated, 2 mungbean varieties with high polymorphisms and putative resistant hybrids carrying a CLS resistance gene and 2 PM resistance genes identified by the linked marker loci were selected and used as recurrent and donor parents, respectively. Two recurrent parents were hybridized with all donor parents, and F_1 plants were backcrossed with their recurrent parents to produce the BC_1F_1, BC_2F_1, and BC_3F_1 progenies. MABB was performed up to BC_2 generation, and approximately more than 100 to 150 progenies in each cross were genotyped each generation. Only individual plants possibly carrying all resistance alleles and a high level of genetic background similarity with each recurrent parent were advanced to the next generation of

backcrossing. The crossing scheme for the development of pyramided lines with multiple CLS and PM resistance genes is presented in Figure 3.1.

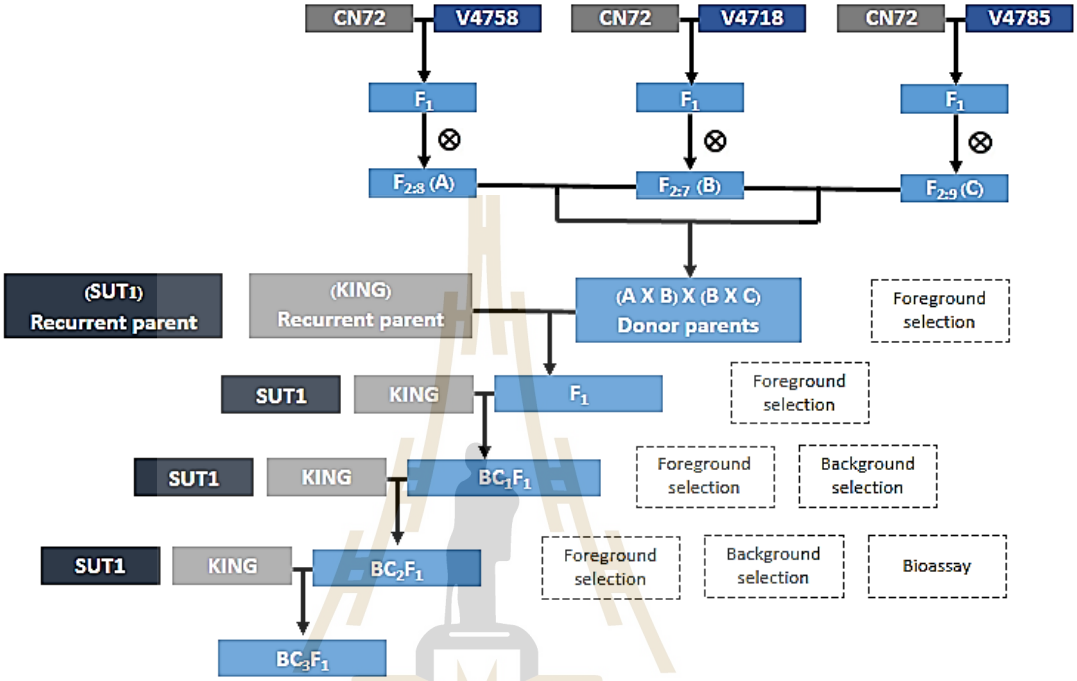

Figure 3.1 Crossing scheme for pyramiding of CLS and PM resistance genes into SUT1 and KING through marker assisted backcross breeding.

Table 3.1 Pedigree and special features of 23 mungbean and 4 blackgram varieties/lines used in this study (Chueakhunthod et al., unpublished data).

Genotypes	Pedigree	Special features	Origin
CN36[a]	PAGASA1×PHLV18	Large seed, uniform maturity, moderate resistance to CLS and PM	Thailand
CN72[a]	Selection from mutated KPS2 [BPI GLABROUS #3×(CES44 × ML-3)] ×CN36	Large seed, high yield, suitability for all conditions	Thailand
CN84-1[a]	Selection from mutated CN36	Large seed, high yield, high percentage of starch	Thailand
KPS1[b]	PAGASA1×EG-MG-16	High yield, moderate resistance to PM and CLS	Thailand
SUT1[c]	UTHONG1×NP-29	High yield, high suitability for harvest, moderate resistance to PM and CLS	Thailand
SUT4[c]	MV1×(MX 4-7 M0317×M0277)	Resistance to PM and CLS	Thailand
V4718 (PLM. 945)[d]	-	High resistance to PM and CLS	India
V4758 (PLM. 994)[d]	-	High resistance to PM	India
V4785 (PLM. 1033)[d]	-	High resistance to PM	India
PUSA-105[d]	(TAINAN-1×ML-6)×(EG-MG-16×ML3)	Moderate resistance to PM, CLS, and MYMV	India
ML-131[d]	ML-1×ML-23	Resistance to PM and MYMV	India
VARA-G[d]	-	Moderate resistance to PM	India
BARI MUNG2[d]	M-7715	Photo-insensitive, moderate resistance to MYMV and CLS	Bangladesh
NM92[d]	LM641×NM36	High yield, resistance to CLS and MYMV	Pakistan
NM94[d]	YEZIN MUNGBEAN 11	Resistance to CLS and MYMV	Myanmar
EG-MD-6D[d]	-	High yield, general resistance to disease	Philippines
CES55[d]	CES14×MG50-10A	High yield	Philippines
MG50-10A (Y)[d]	-	High yield, photo-insensitive, uniform maturity	Philippines
BPI GLABROUS #3[d]	MG50-10A×Ilag S-6A	Large seed, high yield	Philippines
WALET[d]	EG-MG-4×ML6	High yield, moderate resistance to PM and CLS	Indonesia
GELATIK[d]	(CES55×ML3)	Moderate resistance to PM and CLS	Indonesia
KING[d]	EG-MG-7	Large seed, high yield, moderate resistance to PM	Australia
TAINAN SEL#5[d]	-	High yield, moderate resistance to PM and CLS	Taiwan
CN2[e]	KAB4×PLU1131	High yield	Thailand
CN80[e]	PRAJEEN×NBG	High yield, suitability for harvest	Thailand
BR-1[f]	-	Resistance to bruchid	India
PAK40592[f]	-	High yield	Pakistan

[a] The certified mungbean varieties from Chai Nat Field Crops Research Center, Chai Nat, Thailand.
[b] The certified mungbean varieties from Kasetsart University, Thailand.
[c] The certified and improved mungbean varieties from Suranaree University of Technology, Thailand.
[d] The mungbean breeding lines/varieties from AVRDC.
[e] The certified blackgram varieties from Chai Nat Field Crops Research Center, Chai Nat, Thailand.
[f] The blackgram breeding lines/varieties from AVRDC.

3.2 Selection of donor and recurrent parents

3.2.1 DNA extraction

Total genomic DNA was extracted from young leaves of seedlings using the cetyl trimethyl ammonium bromide (CTAB) method as described by Lodhi et al. (1994). The concentration and purity of the DNA samples were determined by ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) at A_{260} and A_{280}, and adjusted to a final concentration of 150 ng μL^{-1} for use in polymerase chain reaction (PCR) amplification analysis.

3.2.2 Evaluation of resistance allele polymorphisms

Three bulked plants of each of the 23 mungbean varieties/lines and 36 putative donor parents were screened using markers linked to CLS and PM resistance developed from RIL populations of the crosses between V4718 and CN72, V4758 and CN72, as well as V4785 and CN72. Six markers loci, including ISSR (I85420) and ISSR-RGA (I42PL222) markers flanked PM resistance gene from V4718, SSR (VR393 and CEDG084) markers flanked CLS resistance gene from V4718, and ISSR-RGA (I27R211 and I27R565) markers associated with PM resistance gene from V4785 (Table 3.2) were used. The PCR reaction for SSR markers was performed in 20 µl containing 150 ng of genomic DNA, 1 Unit of Taq DNA polymerase, 1X buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.1), and 0.01% Triton™ X-100), 2 mM $MgCl_2$, 0.2 mM of each deoxyribonucleotide triphosphate (dNTP), and 0.5 µM of each primer (forward and reverse). Amplification was performed with initial denaturation at 94°C for 2 min, 35 cycles of denaturing at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. PCR for ISSR markers was prepared in a reaction mixture of 20 µL containing 150 ng of DNA

template, 1X buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.1), and 0.01% Triton™ X-100), 3.5 mM $MgCl_2$, 250 µM of each dNTP, 0.4 µM of each ISSR primer, and 1 µM of each RGA primer (P-Loop or RLK for) was added for ISSR-RGA markers. The DNA was amplified according to the following program: initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Amplification was ended with a final extension step at 72 °C for 10 min in an T100™ Thermal Cycler. The PCR products were then separated on 6.0% denaturing polyacrylamide gel electrophoresis (PAGE) at 200 V for 50 and 70 min for SSR and ISSR, ISSR-RGA, respectively. The silver nitrate method according to Sambrook and Russell (2001) was used for gel staining.

Table 3.2 Markers linked to CLS and PM resistance genes.

Resistance genes	Markers	Primer sequences (5'-3')[c]	%R[d]	Reference
CLS (fromV4718)	VR393	F-TGGCACTTTCCATAACGAATAC R-ATCAGCCAAAAGCTCAGAAAAC	0.48	Arsakit et al. (2017)
	CEDG084	F-ATCAACTGAGGAGCATCATCGA R-CAACATTTCAACCTTGGGACAG		
PM (from V4718)	I85420	BHB $(GA)_7$	2.34	Poolsawat et al. (2017)
	I42PL222[a]	$(GA)_8$YG- $(GGI)_2$GTIGGIAAIACIAC		
PM (from V4785)	I27R211[b]	$(AC)_8$G-GAYGTNAARCCIGARAA	5.00	Poolsawat et al. (unpublished data)
	I27R565[b]	$(AC)_8$G-GAYGTNAARCCIGARAA	10.00	Poolsawat et al. (unpublished data)

[a] I42P222 was derived from resistant line (V4718), and was used instead of I42P229, which linked to susceptible allele of susceptible variety (CN72).
[b] I27R211 and I27R565 which located on the same side of PM gene were developed from the CN72×V4785 cross.
[c] B = C, G, T; H = A, C, T; I = inosine; N = A, G, C, T; R = A, G; Y = pyrimidines (C, T).
[d] % recombination in MAS.

3.2.3 Genetic diversity evaluation based on EST-SSR analysis

Eleven EST-SSR markers (Table 3.3) derived from Chen et al. (2015) were chosen for the analysis. These primers were generated from ESTs of 2 mungbean genotypes (ZL1 and V6), and the possible functions were determined compared with

common bean and soybean. These primers also contained several microsatellite repeats anchored at either 3' or 5' end by 2-6 nucleotides. Amplification was performed in 20 µl volume reactions containing 150 ng of genomic DNA, 1 Unit of Taq DNA polymerase, 1X buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.1), and 0.01% Triton™ X-100), 1.5 mM $MgCl_2$, 25 µM of dNTP, and 0.4 µM of each primer (forward and reverse). Microsatellite loci were amplified on a T100™ Thermal Cycler. PCR amplification was performed with the following cycling conditions: one cycle of 4 min at 94°C, 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s. The final extension was performed at 72°C for 10 min. The PCR products were separated on 8.0% PAGE at 200 V for 50 to 70 min depending on the specificity of primers, and were visualized using silver staining as described by Sambrook and Russell (2001). Only reproducible and polymorphic bands were considered for the analysis.

For single locus evaluation of the EST-SSR data, all clearly amplified DNA fragments were scored as allele sizes at each locus. Similarity coefficients between various genotypes, in a pair-wise comparison, were computed using Jaccard's coefficient, and the resulting similarity matrix was further analyzed by unweighted pair-group method arithmetic average (UPGMA) clustering algorithm. The computations were carried out using NTSYSpc version 2.1 (Rohlf, 2000). The goodness of fit of the genotypes to a specific cluster in the UPGMA cluster analysis was determined by the Mantel's correlation test (Mantel, 1967). To measure the informativeness of the markers, polymorphic information content (PIC) for each SSR locus was determined as followed: $PIC = 1- (\sum pi^2)$, where, i is the total number of alleles detected for SSR marker, and pi is the frequency of the i^{th} allele in the set of the

27 varieties/lines investigated.

In addition, EST-SSR markers exhibited polymorphism between both resistant lines (V4718 and V4785) and potential recurrent parents were also used in background selection.

Table 3.3 EST-SSR primers used for estimating genetic diversity.

Primers	Putative function	Repeat motifs	Primer sequences (5'-3')
MB11659	5'-adenylysulfate reductase-like 5-like	$(TCTT)_5$	F-CCCTCACAAACTCGAGACCC
			R-GAAACGAAGGTGGCTGAGGA
MB14180	Protein FRIGIDA-like	$(GGAAGA)_{10}$	F-CAGATTCCAACCCGAAGCCA
			R-GCGAAAGAAGCTCGTCCTCT
MB15686	Glucose-6-phosphate isomerase 1, chlo-	$(GCG)_9$	F-CCCAACCTCTCCGCAAAGAT
	oro plastic -like		R-ACAGCCAATCCACGTACCTC
MB19157	No-hit	$(CCCTAA)_3$	F-AAGGAGGGATTCTCGCCTCT
			R-TGGTACCCGAACTTCTTGGC
MB21347	Xyloglucan galactosyltransferase KA-	$(CCATCA)_3$	F-GCCATCACCAACTACCCCTC
	TAMARI1 homolog		R-AGGGGAGGGCGTAGATGTAG
MB23088	Auxin efflux carrier component 1-like	$(ATATC)_5$	F-GATCGGCCTCATGCTCCTTT
			R-GTGGTGGTGAGAGTGGGAAG
MB24478	Pentatricopeptide repeat-containing	$(TCATCT)_8$	F-TGGCATTCTCCCAATTCCCT
	pro- tein At5g04810, chloroplastic-like		R-TCCTCCTGATTGGACCTCTCA
MB25181	Mediator of RNA polymerase II	$(TGT)_7$	F- ATTTCCCTGTGCGCCCATAA
	transcription subunit 15a-like		R- TCTGTTATGCAGCAGGCTCC
MB27164	Mitochondrial import inner membrane	$(GCCACC)_3$	F-CTCAACAAGTTCCTCAGCGC
	trans- locase subunit TIM 17-2-like		R-CCAGAACCGGTGGAAGTCTC
MB33094	Leucine-rich repeat extensin-like	$(CCAACA)_3$	F-ATTGCCACCCCCATTTCCAT
	protein 4-like		R-AGCAGTCCACCACTCTCTCT
MB64504	Dof zinc finger protein DOF 4.6-like	$(GATGAA)_3$	F-CTCCTGAGGGCACTGAACTG
			R-GCTTCTGCAACGAGTTTCAACT

3.3 Pyramiding of CLS and PM resistance genes in mungbean through marker assisted backcross breeding

3.3.1 Foreground selection

The selection for CLS and PM resistance was performed using markers linked to resistance genes (Table 3.2), which were used for selecting the segregating

plants from F_1 generation to BC_2F_1 generation. The PCR amplification for all 6 markers was performed similar to the section 3.2.2.

3.3.2 Background selection

SSR, EST-SSR linked to yield-related traits (Isemura et al. 2012) (Table 3.4), and ISSR markers developed from the University of British Columbia (Table 3.5), which are not linked to the CLS and PM resistance genes, and possibly distributed well throughout the genome were used for parental polymorphism survey. Only polymorphic markers were subsequently used for background selection of the BC progenies carrying all resistance genes, and DNA fragment identical to recurrent parent and polymorphic to donor parent were counted and used to calculate genetic similarity.

The SSR and EST-SSR reactions were prepared for a 20 µl containing150 ng of genomic DNA, 1 Unit of Taq DNA polymerase, 1X buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.1), and 0.01% Triton™ X-100), 2 mM $MgCl_2$, 0.2 mM of each dNTP, and 0.5 µM of each primer (forward and reverse). The analysis of markers linked to yield-related traits derived from Isemura et al. (2012) was carried out following a bit modified reaction conditions: one cycle of 2 min at 94°C, 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. The final extension was performed at 72°C for 10 min in an T100™ Thermal Cycler. While ISSR amplification and band visualization were carried out using similar PCR conditions as the section 3.2.2. The PCR products of SSR and EST-SSR markers were electrophoresed on 8.0% PAGE at 200 V for 50 to 70 min depending on the specificity of primers, and stained with the silver nitrate method according to Sambrook and Russell (2001). SSR and EST-SSR polymorphic fragments linked to

yield-related traits that showed molecular weights similar to that of Isemura et al. (2012) were classified as set A. Other SSR and EST-SSR fragments unlinked to yield-related traits and EST-SSR fragments derived from the section 3.2.3 were classified as set B. While all ISSR fragments were classified as set C.

Table 3.4 SSR and EST-SSR markers used for background selection.

Primers	CS[a]	Primer sequences (5'-3')	Primers	CS	Primer sequences (5'-3')
CEDG074	1	F-CGAGTGAATGGAAGGGAGTC	GMES5572	5	F-GCAGCAGCACTACATGGGTA
		R-ATTCTCACAGCACGGACCAC			R-AGATGGCATAGGAGGTGGTG
CEDG220	1	F-GGTATTGAAGTCACATGGTCC	CEDG245	6	F-GATAGAGCTTAAACCCTC
		R-GGTTGTTATCTTTGTGCACTCC			R-CTTTTGATGACAAATGCCC
Cp05137	1	F-CCGATTGTAGATGATCCCGATTGT	MBSSR021	6	F-ACATCCGGGAACAAACAAAACG
		R-TGATGATTGCTGTGGGGAAATATG			R-AACTGAGGCTTGAGAAGATGAC
GMES4400	1	F-CCAAACCTCACGTGTGCTAA	CEDG064	7	F-TGTAAGGTCACTTTGGCCTCAAG
		R-GGGCATTTGAGTATGCTTGC			R-TTAAGTTGACTCGTTGCCCTTTG
CEDG026	2	F-TCAGCAATCACTCATGTGGG	CEDG174	7	F-GAGGGATCTCCAAAGTTCAACGG
		R-TGGGACAAACCTCATGGTTG			R-GAAGGCTCCGAAGTTGAAGGTTG
GMES0216	2,4	F-CCGGGACAGGGTTTCTAACT	CEDG030	8	F-TGAGGGAATGGGAGAGAGGC
		R-CCGAAGAAGACGACGAAATC			R-TCCGCAGATAGAGGCTCACG
GMES0477	2	F-ATTCCGACCTCGAAGATTCC	VM37	8	F-TGTCCGCGTTCTATAAATCAGC
		R-CGTCTCTCGAAGAAGGGTTG			R-CGAGGATGAAGTAACAGATGATC
GMES0294	3	F-AAAACGCAACTCCCTTTCCT	CEDG166	9	F-GGTACAACATTCTTCTATTTG
		R-AAACCCTAGCCCAAACCCTA			R-GGCTTATGAGTTTATCTTATC
GMES6583	3	F-CACCCTCTTCTCCTTCCTCC	GATS11	9	F-CACATTGGTGCTAGTGTCGG
		R-CGGCTCTCATGACCTCTCTC			R-GAACCTGCAAAGCAAAGAGC
CEDG085	4	F-AGCATGGAATCTCAGACTGAGACA	CEDG097	10	F-GTAAGCCGCATCCATAATTCCA
		R-AACAAGATCGAAGAAGTCGCTCAC			R-TGCGAAAGAGCCGTTAGTAGAA
GMES1216	4,9	F-GGGATTGACCTCACAACTCC	CEDG150	10	F-GAAGGGAATGAAAATGAAACCC
		R-ACCGTCACCTCATCTCCAAC			R-GTTCAATCCATTCAGTCTCC
MBSSR015	4	F-ATCATCATGACTCCGACACTC	BM149	11	F-CGATGGATGGATGGTTGCAG
		R-GTCGCGTAGCATGTTGGAG			R-GGGCCGACAAGTTACATCAAATTC
CEDG132	5	F-GGGTGTAATCCGTCAGAGGC	GMES3893	11	F-TTACCGGCTGAGGGTTATTG
		R-CTTCCCCCTCTTCCGTTCTC			R-GCAAGAAGGAGAATGAACAGTG

[a] Chromosome

Table 3.5 ISSR markers used for background selection.

Primers	Primer sequences (5'-3')	Primers	Primer sequences (5'-3')
809	$(AG)_8G$	850	$(GT)_8YC$
811	$(GA)_8C$	857	$(AC)_8YG$
830	$(TG)_8G$	864	$(ATG)_6$
834	$(AG)_8YT$	884	HBH $(AG)_7$
835	$(AG)_8YC$	887	DVD $(TC)_7$
841c	$(GA)_8CC$	890	VHV $(GT)_7$

[a] B = C, G, T; D = A, G, T; H = A, C, T; V = A, C, G; Y = pyrimidines (C, T).

3.4 Bioassay for CLS resistance

After foreground selection, screening for CLS resistance using detached leaf assay was carried out with BC_2F_1 generation. CLS inoculum was prepared using the infected leaves collected from SUT farm (Tharapreuksapong et al., unpublished data). Trifoliate leaves with about 5 cm of petioles of each pyramided BC progeny carrying all resistance loci, along with their parents were kept above the plastic sheet, which was placed over the trays filled with tap water. After 24 h, the leaves were separately inoculated by spraying with the CLS mixed spore suspensions (2,000 and 4,000 spore/ml at 1 day and 7 days, respectively). Disease incidence was recorded at 14 days after inoculation. The percentage of leaves challenged by CLS was evaluated using an arbitrary scale 1-5 as described in Iqbal et al. (2004). This scale is as follows: 1 = no visual disease infection, 2 = 1-25% infection, 3 = 26-50% infection, 4 = 51-75% infection, and 5 = 76-100% infection.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Selection of donor and recurrent parents

Parental selection based on molecular markers is very essential before starting MABB for pyramiding of CLS and PM resistance genes from donor parents into recurrent parents. To select donor parents as potentially important resistance sources, 36 putative resistant hybrids derived from the crosses between RILs of 3 resistant lines (V4718, V4758, and V4785) and a susceptible variety (CN72) were screened by 6 marker loci linked to one each resistance gene for CLS and PM in the resistant line (V4178) and another PM resistance gene from the resistant line (V4785) (Table 3.2). The co-dominant SSR markers (VR393 and CEDG084) were capable of identifying homozygote and heterozygote of CLS resistance gene from V4718. While for PM resistance, homozygote and heterozygote carrying the resistance allele could not be differentiated by dominant ISSR marker (I85420) and ISSR-RGA markers (I42PL222 and I42PL229). Note that I42PL222 and I42PL229 markers were linked to PM resistance and susceptibility, respectively (Poolsawat et al., 2017) and are useful for identifying homozygote in later generations. Similarly, homozygote and heterozygote of the allele linked to PM resistance from V4785 could not be distinguished by ISSR-RGA (I27R211 and I27R565). When 6 marker loci were identified in all 36 hybrids, 10 of them, including (14B×19C)×(67A×5B)-2, (55A×5B)×(71B×19C)-3, (55A×5B) ×(71B×19C)-4, (55A×12B)×(71B×182C)-4, (67A×27B)×(71B×14C)-2, (67A×27B

×(71B×14C)-3, (181A×5B)×(68B×14C)-1, (181A×5B)×(68B×14C)-2, (181A×5B) ×(68B×14C)-3, and (181A×5B)×(68B×14C)-4 exhibited all DNA fragments linked to CLS and PM resistance genes similar to the resistance checks (V4718 and 4785) (Table 4.1). These promising hybrids will be used as donor parents in MABB. To select the suitable recurrent parent, varieties without these resistance alleles were considered. The elite varieties, i.e. CN36, CN72, CN84-1, SUT1, SUT4, NM92, EG-MD-6D, CES55, MG50-10A (Y), BPI GLABROUS #3, and KING displayed distinct polymorphisms with all this set of markers tested (Table 4.2), indicating that their genetic backgrounds could be manipulated better by adding the CLS and 2 PM resistance alleles through these marker loci. In the present study, SUT1 developed at Suranaree University of Technology and KING derived from AVRDC collection, which did not only exhibit high resistance allele polymorphisms, but could also have better field performance (Chueakhunthod et al., unpublished data) were chosen as recurrent parents for improving CLS and PM resistance through MABB.

Table 4.1 Polymorphisms in 36 hybrids derived from double crosses of RILs between 2 resistant lines (V4718 and V4785) and susceptible variety (CN72).

Varieties/lines	CLS from V4718		PM from V4718		PM from V4785	
	VR393[b]	CEDG084[b]	I85420[d]	I42PL222 and I42PL229[e]	I27R211[f]	I27R565[f]
V4718 (Resistant line)	B_1B_1	B_2B_2	$B_3B\_$	B_4B_4	NA	NA
V4785 (Resistant line)	NA[c]	NA	NA	NA	$C_1C\_$	$C_2C\_$
CN72 (Susceptible variety)	bb	bb	bb	bb	cc	cc
(14B×19C)×(67A × 5B)-1[a]	B_1B_1	B_2b	$B_3B\_$	B_4B_4	cc	$C_2C\_$
(14B×19C)×(67A × 5B)-2	B_1b	B_2b	$B_3B\_$	B_4b	$C_1C\_$	$C_2C\_$
(14B×19C)×(67A × 5B)-3	B_1B_1	B_2b	bb	B_4b	$C_1C\_$	$C_2C\_$
(14B×19C)×(67A × 5B)-4	B_1b	B_2b	$B_3B\_$	B_4b	$C_1C\_$	cc
(55A×5B)×(71B×19C)-1	B_1B_1	B_2b	bb	B_4b	$C_1C\_$	$C_2C\_$
(55A×5B)×(71B×19C)-2	B_1B_1	bb	bb	B_4b	$C_1C\_$	$C_2C\_$
(55A×5B)×(71B×19C)-3	B_1b	B_2B_2	$B_3B\_$	B_4b	$C_1C\_$	$C_2C\_$
(55A×5B)×(71B×19C)-4	B_1b	B_2B_2	$B_3B\_$	B_4b	$C_1C\_$	$C_2C\_$
(55A×12B)×(71B×182C)-1	bb	B_2b	$B_3B\_$	bb	cc	$C_2C\_$
(55A×12B)×(71B×182C)-2	bb	B_2b	bb	B_4b	$C_1C\_$	$C_2C\_$
(55A×12B)×(71B×182C)-3	B_1b	B_2b	bb	B_4b	$C_1C\_$	cc
(55A×12B)×(71B×182C)-4	B_1b	B_2b	$B_3B\_$	B_4b	$C_1C\_$	$C_2C\_$
(67A×27B)×(71B×14C)-1	bb	B_2b	$B_3B\_$	bb	$C_1C\_$	$C_2C\_$
(67A×27B)×(71B×14C)-2	B_1B_1	B_2b	$B_3B\_$	B_4b	$C_1C\_$	$C_2C\_$
(67A×27B)×(71B×14C)-3	B_1b	B_2b	$B_3B\_$	B_4b	$C_1C\_$	$C_2C\_$
(67A×27B)×(71B×14C)-4	bb	B_2b	$B_3B\_$	bb	$C_1C\_$	$C_2C\_$
(181A×5B)×(68B×14C)-1	B_1b	B_2b	$B_3B\_$	B_4b	$C_1C\_$	$C_2C\_$
(181A×5B)×(68B×14C)-2	B_1B_1	B_2b	$B_3B\_$	B_4b	$C_1C\_$	$C_2C\_$
(181A×5B)×(68B×14C)-3	B_1B_1	B_2b	$B_3B\_$	B_4b	$C_1C\_$	$C_2C\_$
(181A×5B)×(68B×14C)-4	B_1B_1	B_2b	$B_3B\_$	B_4b	$C_1C\_$	$C_2C\_$
(55A×19C)×(27B×14C)-1	B_1B_1	bb	bb	bb	$C_1C\_$	$C_2C\_$
(55A×19C)×(27B×14C)-2	B_1b	bb	$B_3B\_$	B_4b	$C_1C\_$	cc
(55A×19C)×(27B×14C)-3	B_1b	bb	$B_3B\_$	B_4b	$C_1C\_$	cc
(55A×19C)×(27B×14C)-4	B_1b	bb	$B_3B\_$	bb	$C_1C\_$	$C_2C\_$
(5B×41C)×(181A×35C)-1	B_1b	B_2b	bb	bb	$C_1C\_$	cc
(5B×41C)×(181A×35C)-2	bb	B_2b	$B_3B\_$	bb	$C_1C\_$	$C_2C\_$
(5B×41C)×(181A×35C)-3	B_1b	B_2b	bb	B_4B_4	$C_1C\_$	$C_2C\_$
(5B×41C)×(181A×35C)-4	bb	bb	$B_3B\_$	bb	$C_1C\_$	cc
(27B×182C)×(181A×35C)-1	B_1b	bb	bb	B_4b	$C_1C\_$	$C_2C\_$
(27B×182C)×(181A×35C)-2	bb	B_2b	bb	B_4b	$C_1C\_$	cc

Table 4.1 Polymorphisms in 36 hybrids derived from double crosses of RILs between 2 resistant lines (V4718 and V4785) and susceptible variety (CN72) (Continued).

Varieties/lines	CLS from V4718		PM from V4718		PM from V4785	
	VR393[b]	CEDG084[b]	I85420[d]	I42PL222 and I42PL229[e]	I27R211[f]	I27R565[f]
V4718 (Resistant line)	B_1B_1	B_2B_2	B_3B_-	B_4B_4	NA	NA
V4785 (Resistant line)	NA[c]	NA	NA	NA	C_1C_-	C_2C_-
CN72 (Susceptible variety)	bb	bb	bb	bb	cc	cc
(27B×182C)×(181A×35C)-3[a]	B_1b	bb	B_3B_-	bb	C_1C_-	C_2C_-
(27B×182C)×(181A×35C)-4	B_1b	bb	B_3B_-	B_4b	C_1C_-	C_2C_-
(68B×19C)×(181A×35C)-1	bb	bb	bb	B_4b	C_1C_-	cc
(68B×19C)×(181A×35C)-2	B_1b	bb	bb	B_4b	C_1C_-	C_2C_-
(68B×19C)×(181A×35C)-3	B_1b	bb	bb	B_4b	C_1C_-	C_2C_-
(68B×19C)×(181A×35C)-4	B_1b	bb	bb	B_4B_4	C_1C_-	cc

[a] A = RILs of V4758×CN72, B = RILs of V4718×CN72, C = RILs of V4785×CN72.
[b] SSR markers (VR393 and CEDG084) showing homozygous dominant (B_1B_1 and B_2B_2, respectively), heterozygous (B_1b and B_2b, respectively), and homozygous recessive (bb) alleles of CLS resistance from V4718.
[c] NA = Not available (These markers were not identified for the resistance genes in the resistance lines.).
[d] ISSR marker (I8 5 4 2 0) showing either homozygous dominant or heterozygous (B_3_) and homozygous recessive (bb) of PM resistance from V4718.
[e] ISSR-RGA markers (I42PL222 and I42PL229 linked to PM resistance and susceptibility of V4718 and CN72, respectively) showing homozygous dominant (B_4B_4), heterozygous (B_4b), and homozygous recessive (bb).
[f] ISSR-RGA markers (I27R211 and I27R565) showing either homozygous or heterozygous alleles (C_1_ and C_2_, respectively) and homozygous recessive (cc) of PM resistance from V4785.

Table 4.2 Polymorphisms in 22 mungbean varieties/lines.

Varieties/lines	CLS from V4718		PM from V4718		PM from V4785	
	VR393[a]	CEDG084[a]	I85420[c]	I42PL222 and I42PL229[d]	I27R211[e]	I27R565[e]
CN36	bb	bb	bb	bb	cc	cc
CN72	bb	bb	bb	bb	cc	cc
CN84-1	bb	bb	bb	bb	cc	cc
KPS-1	B_1b	bb	bb	bb	cc	cc
SUT1	bb	bb	bb	bb	cc	cc
SUT4	bb	bb	bb	bb	cc	cc
V4718	B_1B_1	B_2B_2	$B_3B\_$	B_4B_4	NA	NA
V4785	NA[b]	NA	NA	NA	$C_1C\_$	$C_2C\_$
PUSA-105	B_1B_1	B_2B_2	$B_3B\_$	B_4B_4	cc	$C_2C\_$
ML-131	B_1B_1	B_2b	$B_3B\_$	B_4B_4	cc	$C_2C\_$
VAR A-G	B_1B_1	bb	$B_3B\_$	B_4B_4	$C_1C\_$	cc
BARI MUNG2	B_1B_1	bb	$B_3B\_$	B_4B_4	cc	$C_2C\_$
NM92	bb	bb	bb	bb	cc	cc
NM94	B_1B_1	B_2B_2	bb	bb	cc	cc
EG-MD-6D	bb	bb	bb	bb	cc	cc
CES55	bb	bb	bb	bb	cc	cc
MG50-10A (Y)	bb	bb	bb	bb	cc	cc
BPI GLABROUS #3	bb	bb	bb	bb	cc	cc
GELATIK	B_1b	bb	bb	B_4B_4	cc	cc
WALET	bb	bb	bb	bb	$C_1C\_$	$C_2C\_$
KING	bb	bb	bb	bb	cc	cc
TAINAN SEL #5	B_1b	B_2b	bb	B_4B_4	$C_1C\_$	$C_2C\_$

[a] SSR markers (VR393 and CEDG084) showing homozygous dominant (B_1B_1 and B_2B_2, respectively), heterozygous (B_1b and B_2b, respectively), and homozygous recessive (bb) alleles of CLS resistance from V4718.

[b] NA = Not available (These markers were not identified for the resistance genes in the resistance lines.).

[c] ISSR marker (I8 5 4 2 0) showing either homozygous dominant or heterozygous ($B_3\_$) and homozygous recessive (bb) of PM resistance from V4718.

[d] ISSR-RGA markers (I42PL222 and I42PL229 linked to PM resistance and susceptibility of V4718 and CN72, respectively) showing homozygous dominant (B_4B_4), heterozygous (B_4b), and homozygous recessive (bb).

[e] ISSR-RGA markers (I27R211 and I27R565) showing either homozygous or heterozygous alleles ($C_1\_$ and $C_2\_$, respectively) and homozygous recessive (cc) of PM resistance from V4785.

4.2 Genetic diversity evaluation based on EST-SSR analysis

4.2.1 Levels of polymorphisms

11 EST-SSR markers were amplified and were highly polymorphic (96.60%) among 23 mungbean and 4 blackgram varieties/lines, when compared with other previous studies for mungbean (10 from 15 EST-SSR markers found polymorphic (Gupta et al., 2013)) and blackgram (32 from 55 EST-SSR markers found polymorphic (Souframanien and Reddy, 2015)). The high polymorphisms found in this study may stem from types and number of genotypes used, which included 2 *Vigna* species (*V. radiata* and *V. mungo)*. Their genetic backgrounds were completely distinct, particularly those identified by 7 EST-SSR markers that produced some species-specific alleles in blackgram varieties/lines ranging from 1 (MB11659, MB14180, MB15686, and MB23088) to 4 (MB21347) alleles as shown in Table 4.3. These results are consistent with Tantasawat et al. (2010) , who stated that the differences between these 2 species, i.e. seeds/pod, terminal leaf width, pod length, seed color, or seed luster involve a substantial portion of the genome, while other areas of the genome are shared together. Furthermore, wide geographical origin of the materials listed in Table 3.1 might contribute to these differences. Gupta et al. (2013) reported that EST-SSR markers had higher polymorphisms compared to genomic SSR markers, because the EST-SSR polymorphisms associated with transcribed genes of the primers represent the functional variation. In total, 56 alleles ranging from 2 (MB23088) to 9 (MB21347) alleles with an average of 5 alleles per locus were found (Table 4.3). This is in association with the highly conserved nature of the primer sequences flanking the SSR region. The allele numbers were higher than those previously reported in some legume species such as *Phaseolus vulgaris* (Garcia et al.,

2011). The amplified fragment sizes ranged from approximately 85 (MB14180) to 256 (MB33094) bp. The PIC values ranged from 0.22 (MB23088) to 0.80 (MB14180) with an average of 0.60. Nine EST-SSR markers were classified as having a high degree of polymorphism (> 0.50). Highly informative primers, particularly MB14180, MB15686, and MB21347, which amplified EST-SSRs containing GGAAGA, GCG, and CCATCA repeat motifs, respectively produced many alleles possibly due to their association with the conserved function of different genes. Primer MB21347 with the highest allele number derived from ESTs of xyloglucan galactosyltransferase KATAMARI1 homolog, which involves in the pathway of protein modification for cell elongation. In addition, these repeat motifs have been successfully used in mungbean (Gupta et al., 2013). Thus, they were most valuable for studies of genetic diversity in mungbean and blackgram. Furthermore, the significantly positive correlation was found between the PIC values and the number of alleles (r = 0. 775, $p < 0.05$), an indication that the number of alleles can be used to estimate diversity.

Table 4.3 Informativeness of EST-SSR loci following amplification from 23 mungbean and 4 blackgram varieties/lines.

EST-SSR primers	Putative function	Repeat motifs	Primer sequences (5'-3')	Size (bp)	Na[a]	Nb[b]	PIC
MB11659	5'-adenylysulfate reductase-like 5-like	$(TCTT)_5$	F-CCCTCACAAACTCGAGACCC R-GAAACGAAGGTGGCTGAGGA	153-243	4	1	0.62
MB14180[d]	Protein FRIGIDA-like	$(GGAAGA)_{10}$	F-CAGATTCCAACCCGAAGCCA R-GCGAAAGAAGCTCGTCCTCT	85-152	7	1	0.80
MB15686[d]	Glucose-6-phosphate isomerase 1, chloroplastic-like	$(GCG)_9$	F-CCCAACCTCTCCGCAAAGAT R-ACAGCCAATCCACGTACCTC	129-180	6	1	0.76
MB19157	No-hit	$(CCCTAA)_3$	F-AAGGAGGGATTCTCGCCTCT R-TGGTACCCGAACTTCTTGGC	154-244	4	0	0.66
MB21347[d]	Xyloglucan galactosyltransferase KATAMARI1 homolog	$(CCATCA)_3$	F-GCCATCACCAACTACCCCTC R-AGGGGAGGGCGTAGATGTAG	158-245	9	4	0.75
MB23088	Auxin efflux carrier component 1-like	$(ATATC)_5$	F-GATCGGCCTCATGCTCCTTT R-GTGGTGGTGAGAGTGGGAAG	152-242	2	1	0.22
MB24478[d]	Pentatricopeptide repeat-containing protein At5g048 10, chloroplastic-like	$(TCATCT)_8$	F-TGGCATTCTCCCAATTCCCT R-TCCTCCTGATTGGACCTCTCA	156-249	6	2	0.67
MB25181	Mediator of RNA polymerase II transcription subunit 15a-like	$(TGT)_7$	F- ATTTCCCTGTGCGCCCATAA R- TCTGTTATGCAGCAGGCTCC	147-202	7	3	0.66
MB27164	Mitochondrial import inner membrane translocase subunit TIM17-2-like	$(GCCACC)_3$	F-CTCAACAAGTTCCTCAGCGC R-CCAGAACCGGTGGAAGTCTC	133-167	3	0	0.29
MB33094	Leucine-rich repeat extensin-like protein 4-like	$(CCAACA)_3$	F-ATTGCCACCCCCATTTCCAT R-AGCAGTCCACCACTCTCTCT	155-256	4	0	0.56
MB64504[d]	Dof zinc finger protein DOF4.6-like	$(GATGAA)_3$	F-CTCCTGAGGGCACTGAACTG R-GCTTCTGCAACGAGTTTCAACT	96-156	4	0	0.62
Average					5	1	0.60

[a] Number of total alleles.
[b] Number of blackgram specific alleles.
[c] PIC: Polymorphic information content.
[d] Primers showing polymorphism between SUT1 and KING with V4718 and V4785 for background selection in MABB.

4.2.2 Genetic diversity and relationships among mungbean and blackgram varieties/lines

Genotyping data obtained from all 54 polymorphic alleles were used to evaluate pair-wise similarity comparisons among materials studied. Jaccard's similarity coefficients were calculated in order to assess the genetic resemblances, and the similarity coefficients matrix was used for UPGMA cluster analysis. The similarity coefficient values of the phenogram ranged from 0.61 (V4718 versus CN2 and CN80) to 1.00 (CN72 versus CN84-1 and KING versus BPI GLABROUS #3) with an average of 0.82. The genetic distance between mungbean and blackgram groups was 0.28, again indicating that their genetic backgrounds were distant. These results are consistent with Tantasawat et al. (2010), who found that the genetic distance between both species was 0.27. Although both species share the same chromosome number (2n = 20), and are partially cross-compatible, the cluster analysis showed completely separated clusters of mungbean (cluster I) and blackgram (cluster II) varieties/lines (Figure 4.1). The Mantel's test with cophenetic correlation coefficient value of 0.89 ($p < 0.01$) indicated that data in the similarity matrix was well represented by the dendrogram (Table 4.4). Within the mungbean cluster, varieties/lines from Thailand and AVRDC were clustered quite together in a large group (> 83% similarity). Among them, CN72 and CN84-1, as well as KING and BPI GLABROUS #3 had similar genetic backgrounds with 100% similarity, thus the discrimination of their genetic relationships requires additional molecular markers. By contrast, V4758 that was the most distantly related to other mungbean genotypes possessed the minimum genetic similarity (69.60%) with CN72 and CN84-1. Noted that V4758 was originated from India, while CN72 and CN84-1 were developed in

Thailand. CN72 and CN84-1 also share similar pedigree relationships, particularly the relatedness from CN36. In addition, the different special features presented were considerably noticed, i.e. resistance to diseases (V4758) and high yielding potential (CN72 and CN84-1), as indicated in Table 3.1. The cluster corresponding to mungbean was divided into 2 sub-clusters between IA and IB. Sub-cluster IA accommodated V4718, V4758, V4785, NM94, and ML131, all of which were reported to have resistance to PM, CLS and/or MYMV. On the other hand, sub-cluster IB held quite a larger group of mungbean varieties/lines comprising of the ones with high yielding potential, particularly a subgroup of CN36, CN72, and CN84-1 and a sub-group of SUT1, KING, BPI GLABROUS #3, and CES55, which formed close relationships between one another with more than 97% and 94% genetic similarity, respectively. Thus, it should be noteworthy that there were clearly distinct genetic profiles between these 2 sub-clusters. When using resistance loci to evaluate the genetic diversity and relationships of these mungbean genotypes, separation according to their special features between resistance to diseases and high yielding potential into 2 sub-clusters was also observed (Chueakhunthod, unpublished data). With respect to blackgram varieties/lines, all 4 formed a relatively tight group (> 95% similarity), despite their different pedigrees, particularly CN2 and CN80. Based on all of these results carried out by EST-SSR markers, it suggests the potential utilization of this marker system for elucidating genetic diversity and relatedness of 2 *Vigna* species of mungbean and blackgram.

The genetic relationships among these mungbean and blackgram varieties/lines may fulfill the parental selection of breeders in the breeding programs. More interestingly, most mungbean varieties/lines in the sub-cluster IB, particularly

CN84-1, SUT1, KING, BPI GLABROUS #3, and CES55, which were more distally related to the disease resistant varieties/lines in the sub-cluster IA may be used as parents for developing the disease resistant varieties, and will be beneficial for the future development of mungbean varieties because these genetic diversity and relationship information can be used to maximize the level of variation of many desirable characters in segregating populations by crossing the genotypes between sub cluster IA and IB with greater genetic distance. However, selecting potential parental lines based only on genetic diversity using molecular markers is often insufficient to ensure the presence of superior genotypes in their progeny. It is necessary that varieties/lines used in this study should also be evaluated for their phenotypic responses, including yielding potential under field conditions. Unpublished results revealed that some mungbean varieties, i.e. SUT1 and KING, which were allocated in the sub-cluster IB of high yielding potential had a high capacity to produce higher yield based on several physiological and photosynthetic characters compared to other Thai certified varieties, i.e. CN36 or CN72, and also have high adaptability (Chueakhunthod et al., unpublished data). SUT1 was developed by Suranaree University of Technology by crossing between susceptible variety UT1 and leaf spot resistant line VCI 1560D. This developed variety has high yield, synchronous maturity and the pod located above the canopy allowing farmers to easily harvest, as well as moderate resistance to leaf spot. KING originated from Australia was derived from selection of mungbean variety EG-MG-7. This variety is noted for its very large seed size (78.9 g/1,000 seed (AVRDC, 2017)). In addition, it was reported to be excellent for seed quality (high protein and total digestible nutrients yields) (Abd El-Salam et al., 2013). Thus, both of them showing the characters probably preferred by farmers

were chosen to be used as the promising recurrent parents for improving the resistance in MABB.

This study also suggests that several varieties/lines with high yielding potential, including CN36, CN72, CN84- 1, SUT1, KING, BPI GLABROUS #3, and CES55, which were genetically distinct from disease resistant varieties/lines, including V4758, V4718, V4785, NM94, and ML-131 can be immediately used as germplasms for the improvement of mungbean for disease resistance.

The genetic relationships among these mungbean and blackgram varieties/lines may corroborate the selection of parents in MABB. Most mungbean varieties/lines in the sub-cluster IB, particularly CN36, CN72, CN84-1, SUT1, KING, BPI GLABROUS #3, and CES55, which lacked all CLS and PM resistance alleles identified by marker loci as shown in Table 4.2, and were classified in the sub-cluster IB of several varieties/lines with high yielding potential may be the promising parents for developing the disease resistant varieties. The results also confirm that genetic backgrounds of SUT1 and KING, which are classified as the high yielding varieties can be used to be introduced CLS and PM resistance genes to improve the resistance using MABB.

Table 4.4 Similarity matrix of 23 mungbean and 4 blackgram varieties/lines

Genotypes	CN36	CN72	CN84-1	KPS1	SUT1	SUT4	V4718	V4758	V4785	PUSA-105	ML-131	VARA-G	BARI MUNG2	NM92
CN36	1.000													
CN72	0.957	1.000												
CN84-1	0.957	1.000	1.000											
KPS1	0.870	0.913	0.913	1.000										
SUT1	0.796	0.839	0.839	0.857	1.000									
SUT4	0.839	0.882	0.882	0.923	0.891	1.000								
V4718	0.731	0.731	0.731	0.769	0.848	0.761	1.000							
V4758	0.717	0.696	0.696	0.711	0.747	0.747	0.879	1.000						
V4785	0.761	0.739	0.739	0.800	0.769	0.791	0.857	0.822	1.000					
PUSA-105	0.739	0.783	0.783	0.778	0.835	0.857	0.743	0.756	0.711	1.000				
ML-131	0.710	0.731	0.731	0.791	0.804	0.783	0.826	0.813	0.879	0.791	1.000			
VARA-G	0.710	0.753	0.753	0.813	0.783	0.848	0.804	0.791	0.769	0.813	0.891	1.000		
BARI MUNG2	0.761	0.804	0.804	0.778	0.879	0.857	0.769	0.778	0.689	0.911	0.813	0.835	1.000	
NM92	0.787	0.809	0.809	0.874	0.814	0.909	0.773	0.805	0.828	0.851	0.795	0.805	0.805	1.000
NM94	0.731	0.753	0.753	0.791	0.804	0.783	0.891	0.813	0.923	0.703	0.913	0.769	0.769	0.818
EG-MD-6D	0.817	0.839	0.839	0.813	0.826	0.826	0.826	0.791	0.813	0.725	0.804	0.739	0.769	0.841
CES55	0.796	0.839	0.839	0.791	0.913	0.848	0.826	0.769	0.703	0.791	0.783	0.804	0.879	0.818
MG50-10A (Y)	0.796	0.817	0.817	0.791	0.870	0.804	0.826	0.791	0.791	0.747	0.826	0.761	0.791	0.818
BPI GLABROUS #3	0.774	0.817	0.817	0.857	0.957	0.891	0.848	0.791	0.769	0.835	0.848	0.826	0.879	0.841
WALET	0.817	0.860	0.860	0.879	0.826	0.826	0.761	0.747	0.747	0.769	0.717	0.795	0.769	0.795
GELATIK	0.804	0.826	0.826	0.822	0.857	0.879	0.813	0.800	0.800	0.778	0.747	0.804	0.778	0.886
KING	0.774	0.817	0.817	0.857	0.957	0.891	0.848	0.791	0.769	0.835	0.848	0.826	0.879	0.841
TAINAN SEL#5	0.882	0.925	0.925	0.857	0.804	0.870	0.739	0.769	0.725	0.813	0.761	0.783	0.857	0.841
CN2	0.697	0.697	0.697	0.690	0.705	0.727	0.614	0.667	0.667	0.736	0.682	0.636	0.736	0.729
CN80	0.697	0.697	0.697	0.690	0.705	0.727	0.614	0.667	0.667	0.736	0.682	0.636	0.736	0.729
BR-1	0.689	0.689	0.689	0.682	0.697	0.719	0.652	0.682	0.705	0.727	0.674	0.629	0.727	0.744
PAK40592	0.689	0.689	0.689	0.682	0.697	0.719	0.652	0.705	0.682	0.727	0.674	0.629	0.727	0.767

Table 4.4 Similarity matrix of 23 mungbean and 4 blackgram varieties/lines (Continued).

Genotypes	NM94	EG-MD-6D	CES55	MG50-10A (Y)	BPI GLA BROUS #3	WALET	GELATIK	KING	TAINAN SEL#5	CN2	CN80	BR-1	PAK40592
NM94	1.000												
EG-MD-6D	0.848	1.000											
CES55	0.783	0.870	1.000										
MG50-10A (Y)	0.826	0.957	0.913	1.000									
BPI GLABROUS #3	0.804	0.870	0.913	0.913	1.000								
WALET	0.739	0.783	0.761	0.804	0.826	1.000							
GELATIK	0.791	0.923	0.879	0.901	0.857	0.813	1.000						
KING	0.804	0.870	0.913	0.913	1.000	0.826	0.857	1.000					
TAINAN SEL#5	0.761	0.870	0.891	0.848	0.848	0.804	0.857	0.848	1.000				
CN2	0.659	0.682	0.682	0.682	0.727	0.682	0.690	0.727	0.727	1.000			
CN80	0.659	0.682	0.682	0.682	0.727	0.682	0.690	0.727	0.727	0.952	1.000		
BR-1	0.697	0.719	0.674	0.719	0.719	0.719	0.727	0.719	0.719	0.894	0.941	1.000	
PAK40592	0.674	0.697	0.674	0.697	0.719	0.697	0.705	0.719	0.719	0.918	0.894	0.907	1.000

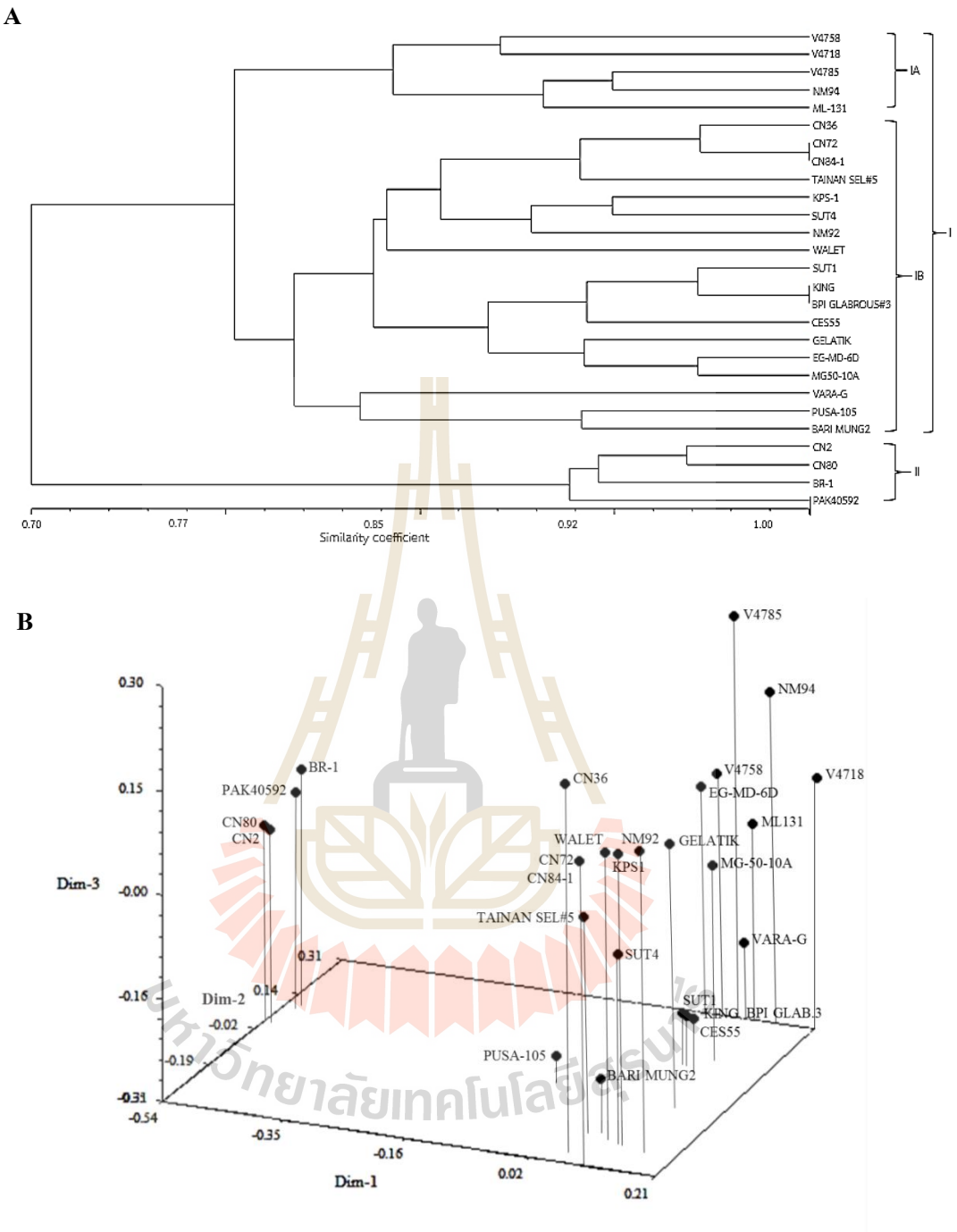

Figure 4.1 EST-SSR markers derived from dendrograms generated by UPGMA (A) and 3-dimentional plot based on the first three principal coordinates from PCA analysis (B) of 23 mungbean and 4 blackgram varieties/lines.

4.3 Pyramiding of CLS and PM resistance genes into mungbean through MABB

4.3.1 Genotyping F_1 generation

To pyramid a CLS resistance gene and 2 PM resistance genes through MABB, 10 promising donor parents were hybridized with the selected recurrent parents (SUT1 and KING) to generate F_1 seeds. The crosses using SUT1 and KING as the recurrent parents were referred as cross I and II, respectively. Selection by all 6 marker loci linked to these resistance genes derived from Poolsawat et al. (2017), Arsakit et al. (2017), and Poolsawat et al. (unpublished data) (Table 3.2) was concurrently carried out to identify the promising plants in each generation throughout MABB. Twenty-nine and 25 F_1 plants from cross I and II, respectively were produced and identified for carrying all resistance genes in a heterozygous form by the presence of these marker loci by means of foreground selection (Table 4.5). As a result, 4 and 3 plants, which contained detectable heterozygous alleles based on all 6 marker loci in cross I and II, respectively were used as resistant source to produce several BC_1F_1 crosses. However, only the crosses with the maximum number of cross-hybridized seeds were subjected to foreground selection.

4.3.2 Marker-assisted foreground and background selection in BC_1F_1

The maximum number of cross-hybridized seeds were obtained from 2 F_1 plants, including SUT1×[(14B×19C)×(67A×5B)] and KING×[(67A×27B)×(71B×14C)] compared with other F_1 plants (data not shown) in cross I and II, respectively, and 149 and 114 BC_1F_1 seeds were planted. When foreground selection was carried out, 3 plants in cross I were heterozygous for all marker loci used, except for

CEDG084 marker, while 4 promising plants in cross II were heterozygous and still detected by 6 marker loci (Table 3.2).

Background selection of the putative resistant plants carrying these resistance genes in this generation to select the plants with the highest recurrent parent genome (RPG) recovery and to hybridize with recurrent parent for next backcrossing was carried out with 3 different sets of markers, which exhibited polymorphisms between parental genomes in each cross. SSR and EST-SSR markers linked to several yield-related traits in mungbean (set A) were selected from Isemura et al. (2012). This purposed to recover the genetic backgrounds, particularly of the traits associated with recurrent parent performance in the pyramided BC progeny. Ten selected markers as given in Table 3.4, including CEDG132, CEDG150, CEDG174, CEDG220, CEDG245, GATS11, GMES0477, GMES3893, MBSSR015, and VM37, as well as some of these, including CEDG150, CEDG174, CEDG220, MBSSR015, and VM37 were found to be polymorphic between parents in cross I and II, respectively. With regards to some markers of set A, all SSR and EST-SSR markers linked to pod length, pod width, seed length, seed width, 100 seed weights, and seeds/pod on linkage group 1, 7, 8, and 11 of mungbean genome revealed high recurrent parent similarity in BC_1F_1-SUT66 and BC_1F_1-SUT82 plants from cross I and most plants from cross II (Table 4.6). These results suggest that some proportions of genome related to agronomically important characters with high yielding potential that are influenced by these QTLs may be presumably recovered. Set B with 11 and 10 polymorphic markers in cross I and II, respectively was obtained from EST-SSR markers used in determining genetic diversity of mungbean and backgram, as well as EST-SSR and SSR markers unlinked to yield-related traits from Isemura et al. 2012. In addition, to

cover throughout the genome, 12 ISSR markers designated as set C with 52 and 34 polymorphic loci in cross I and II, respectively that presumably randomly distribute on the genome were also included. In BC_1F_1 generation, the percentage of RPG recovery based on all sets of markers ranging from 81.8 to 100.0% was observed among 2 crosses, indicating a high level of genetic background similarity between the pyramided lines and their recurrent parents (Table 4.7). The plants with high RPG recovery ranging from 86.4 to 90.9% and 82.1 to 100.0% were BC_1F_1-SUT1 and BC_1F_1-SUT82, as well as BC_1F_1-KING34 and BC_1F_1-KING65 plants in cross I and II, respectively. They also produced higher BC_2F_1 seeds within their population. Taken together, they were subjected to foreground and background selection to drive successive BC generation(s) because of the general theory describing that if the target allele is revealed in BC_1 generation with a high RPG recovery, then the best promising pyramided plants can be considered in priority for generating BC_2 based on the other non-target locus recovery performance associated with recurrent parents (Semagn et al., 2006). However, due to the relatively low number of such BC_1F_1 pyramided plants in each cross, there may have been a drawback for background selection. Thus, in BC_2F_1 generation, large population sizes for recurrent parent genome recovery using backcrossing are required.

4.3.3 Marker-assisted foreground and background selection in BC_2F_1

In this generation, there were 4 populations, including 2 from each cross I and II with 4 pyramided BC_1F_1 plants (BC_1F_1-SUT1 and BC_1F_1-SUT82, BC_1F_1-KING34, and BC_1F_1-KING65) as resistance source. From cross I, 145 and 135 BC_2F_1 plants from SUT1×BC_1F_1-SUT82 designated as cross A and SUT1×BC_1F_1-SUT1 designated as cross B were produced, respectively, of which 12 and 15 plants

exhibited common heterozygotes for all resistance alleles based on all marker loci, except for CEDG084. Regarding other populations that used KING as recurrent parent, KING×BC_1F_1-KING65 designated as cross C produced 141 BC_2F_1 plants, of which 9 heterozygous plants were found based on all marker loci, except for CEDG084, while KING×BC_2F_1-KING34 designated as cross D produced 65 BC_2F_1 plants, of which 8 plants were found to be heterozygous with 6 marker loci (Table 4.5). All of these putative resistant plants were subjected to CLS bioassay, as well as background selection using the similar sets of polymorphic markers, respectively.

On the basis of 3 sets of polymorphic markers for genotyping background analysis, together with the pyramided BC_3F_1 seeds generated after hybridization among 4 populations, the CLS resistant BC_2F_1 plants, which were confirmed for their resistance to CLS under laboratory condition showed background recovery from 84.7 to 100.0%. Within cross I that used BC_1F_1-SUT82 and BC_1F_1-SUT1 as resistance source, more than 90% RPG recovery were observed with most plants. BC_2F_1-SUTA125 and BC_2F_1-SUTA59 in cross A, as well as BC_2F_1-SUTB57 and BC_2F_1-SUTB43 in cross B showed high RPG recovery ranging from 90.0 to 97.6% and high number of pyramided BC_3F_1 seeds. In addition, high RPG recovery ranging from 84.7 to 100.0% was observed with CLS resistant plants (BC_2F_1-KINGC8 and BC_2F_1-KINGC27, as well as BC_2F_1-KINGD50 in cross C and D, respectively). Note that these plants were presumably recovered for their pod length, pod width, seed length, seed width, 100 seed weights, and seeds/pod according to SSR and EST-SSR markers linked to these characters (Table 4.6) and generated higher number of seeds after cross hybridization (Table 4.7). Based on all of these results, the promising pyramided plants may be possibly repeatedly backcrossed only 3-4 times for

recovering the parental genome compared to when using conventional backcrossing that practically takes 6-7 times. Thus, it can considerably save times and costs. These results are consistent with many studies, some of which was reported by Ahmed et al. (2016), who also found high percentage of RPG recovery with 78.79 to 95.9% in BC_2F_1 generation of rice. Consequently, they proposed the optimum number of backcross generations as 2 to 3. However, the number of backcross generations may differ and depend on not only background selection in early backcross generations but also breeder preference and genetic distance between the recurrent and donor parents (Miah et al. 2015). When considering genetic relationships based on EST-SSR markers (Table 4.4), genetic distance between recurrent parents (SUT1 and KING) and donor parents may be possibly not large. Genetic background of donor parents used ((14B×19C)×(67A×5B) and (67A×27B)×(71B×14C); A = V4758, B = V4718, and C = V4785) is largely derived from V4718, whose genetic was approximately 15.2% distant from SUT1 and KING, whereas higher genetic dissimilarities of more than 21% were observed with V4758 and 4785. These results speculate that many areas of the genome, which may include the target genes are possibly shared in common. Thus, these portions of genetic background may be transferred, together with the target genes into the recurrent parents, and result in increased performance of other traits.

4.3.4 Bioassays

The resistance and susceptibility reaction to CLS among the pyramided BC_2F_1 derivatives of all crosses, donor and recurrent parents were evaluated under detached leaf condition using mixed spores for the confirmation of previous results. The resistance check V4718 was highly resistant, while recurrent parents SUT1 and KING and the susceptible check CN72 were susceptible to CLS (Table 4.8 and 4.9).

From cross A and B using SUT1 as recurrent parent, 13 from 27 plants were highly resistant. While from cross C and D using KING as recurrent parent, only 3 from 17 plants were resistant. The resistant plants in cross A and B showed lower disease scores than cross C and D according to reasonably lower susceptibility to disease of SUT1 than KING.

It is evident from this study that MABB, together with phenotypic confirmation can be capable of saving both time and cost of the experiment. If the total number of polymorphic markers used in backcross breeding is large, then the backcrossing will be very effective, however, in this study it is impossible to achieve a high percentage of marker polymorphism between the parents using MAS for the background selection because of the limited number of markers. In this case, using recurrent and donor parents with close genetic relatedness for the RPG recovery is a feasible option for accelerating RPG recovery of backcrossing.

Table 4.5 Number of triple resistant gene heterozygote plant.

Generation	**# of triple heterozygous plants**			
	Cross I	**Cross II**	**Cross I**	**Cross II**
F_1	29	25	4	3
BC_1F_1	149	114	3	4
BC_2F_1[a]	145	141	12	9
BC_2F_1[b]	135	65	15	8

[a] BC_2F_1 plant derived from the pyramided BC_1F_1 plant with the first maximum RPG recovery.
[b] BC_2F_1 plant derived from the pyramided BC_1F_1 plant with the second maximum RPG recovery.

Table 4.6 Background profiling of the pyramided BC plants based on some markers linked to yield-related traits.

Cross	Triple heterozygote BC plants	Markers linked to yield-related traits			
		CEDG174[a]	CEDG220[c]	GMES3893[d]	VM37[e]
Cross I	BC_1F_1				
	BC_1F_1-SUT1	AB[b]	BB	BB	BB
	BC_1F_1-SUT66	BB	BB	BB	BB
	BC_1F_1-SUT82	AB	BB	BB	BB
	BC_2F_1				
Cross A	BC_2F_1-SUTA59	AB	BB	BB	BB
	BC_2F_1-SUTA64	AB	BB	BB	BB
	BC_2F_1-SUTA65	BB	BB	BB	BB
	BC_2F_1-SUTA78	BB	BB	BB	BB
	BC_2F_1-SUTA117	AB	BB	BB	BB
	BC_2F_1-SUTA125	AB	BB	BB	BB
Cross B	BC_2F_1-SUTB24	AB	BB	BB	BB
	BC_2F_1-SUTB43	BB	BB	BB	BB
	BC_2F_1-SUTB45	BB	BB	BB	BB
	BC_2F_1-SUTB46	AB	BB	BB	BB
	BC_2F_1-SUTB57	BB	BB	BB	BB
	BC_2F_1-SUTB111	AB	BB	BB	BB
	BC_2F_1-SUTB146	BB	BB	BB	BB
Cross II	BC_1F_1				
	BC1F1-KING34	BB	BB	-	BB
	BC_1F_1-KING46	BB	BB	-	BB
	BC_1F_1-KING65	BB	BB	-	BB
	BC_1F_1-KING106	BB	BB	-	BB
	BC_2F_1				
Cross C	BC_2F_1-KINGC8	BB	BB	-	BB
	BC_2F_1-KINGC27	BB	BB	-	BB
Cross D	BC_2F_1-KINGD50	BB	BB	-	BB

[a] Marker linked to pod length, seed length, seed width, and number of twists along the length of scattered pod.
[b] A = similar to donor parent and B = similar to recurrent parent.
[c] Marker linked to seeds/pod and seed length.
[d] Marker linked to 100 seed weights, seed thickness, and pod width.
[e] Marker linked to 100 seed weights, seed length, seed width, seed thickness, and pod width.

Table 4.7 Estimation of recurrent parent genome recovery and yield contribution in BC_1F_1 and BC_2F_1 plants.

Cross	Triple heterozygous BC plants	Estimated maximum % contribution of recurrent parent genome to selected backcross plants			# of seeds
		A[a]	B[b]	C[c]	
Cross I	BC_1F_1				
	BC_1F_1-SUT1	90.0%	86.4%	87.8%	326
	BC_1F_1-SUT66	95.0%	81.8%	84.7%	62
	BC_1F_1-SUT82	90.0%	90.9%	90.0%	370
	BC_2F_1				
Cross A	BC_2F_1-SUTA59	90.0%	90.9%	97.6%	82
	BC_2F_1-SUTA64	90.0%	90.9%	90.0%	29
	BC_2F_1-SUTA65	95.0%	90.9%	93.7%	63
	BC_2F_1-SUTA78	95.0%	90.9%	87.2%	165
	BC_2F_1-SUTA117	90.0%	90.9%	91.1%	85
	BC_2F_1-SUTA125	90.0%	90.9%	93.6%	176
Cross B	BC_2F_1-SUTB24	90.0%	90.9%	91.1%	71
	BC_2F_1-SUTB43	95.0%	90.9%	90.7%	74
	BC_2F_1-SUTB45	95.0%	90.9%	91.7%	17
	BC_2F_1-SUTB46	90.0%	90.9%	92.3%	53
	BC_2F_1-SUTB57	95.0%	90.9%	94.9%	110
	BC_2F_1-SUTB111	90.0%	90.9%	88.8%	70
	BC_2F_1-SUTB146	95.0%	90.9%	90.9%	23
Cross II	BC_1F_1				
	BC_1F_1-KING34	100.0%	83.3%	82.1%	77
	BC_1F_1-KING46	83.3%	85.0%	84.2%	110
	BC_1F_1-KING65	100.0%	85.0%	90.0%	191
	BC_1F_1-KING106	91.7%	85.0%	82.1%	84
	BC_2F_1				
Cross C	BC_2F_1-KINGC8	100.0%	95.0%	84.7%	15
	BC_2F_1-KINGC27	100.0%	87.2%	89.8%	30
Cross D	BC_2F_1-KINGD50	100.0%	87.2%	85.7%	8

[a] SSR and EST-SSR markers linked to yield-related traits derived from Isemura et al. 2012 (10 and 6 polymorphic markers used in cross I and II, respectively).
[b] EST-SSR markers derived from Chueakhunthod et al. (2018) and SSR and EST-SSR markers unlinked to yield-related traits derived from Isemura et al. (2012) (11 and 10 polymorphic markers used in cross I and II, respectively).
[c] ISSR markers developed from the University of British Columbia (52 and 34 polymorphic loci markers used in cross I and II, respectively).

Table 4.8 Cercospora leaf spot score of parental and BC_2F_1 pyramided plants from the cross used SUT1 as recurrent parent.

Lines/ varieties		Disease score	Lines/ varieties		Disease score
V4718		1.00	BC_2F_1-SUTA117[a]		1.00
V4758		1.00	BC_2F_1-SUTA125		1.00
V4785		2.33	BC_2F_1-SUTB24[b]		1.33
CN72		4.00	BC_2F_1-SUTB43		1.00
SUT1		2.67	BC_2F_1-SUTB45[b]		1.33
BC_2F_1-SUTA59[a]		1.00	BC_2F_1-SUTB46		1.33
BC_2F_1-SUTA64		1.00	BC_2F_1-SUTB57		1.33
BC_2F_1-SUTA65		1.00	BC_2F_1-SUTB111		1.33
BC_2F_1-SUTA78		1.33	BC_2F_1-SUTB146[b]		1.00

[a] BC_2F_1 progenies derived from cross A used the BC_1F_1 progeny with the first highest recurrent parent genome recovery as resistant parent and SUT as recurrent parent.

[b] BC_2F_1 progenies derived from cross B used the BC_1F_1 progeny with the second highest recurrent parent genome recovery as resistant parent and SUT as recurrent parent.

Table 4.9 Cercospora leaf spot score of parental and BC_2F_1 pyramided plants from the cross used KING as recurrent parent.

Lines/ varieties		Disease score	Lines/ varieties		Disease score
V4718		1.00	KING		4.33
V4758		1.00	BC_2F_1-KINGC8[a]		1.67
V4785		2.33	BC_2F_1-KINGC27		2.00
CN72		4.00	BC_2F_1-KINGD50[b]		2.00

[a] BC_2F_1 progenies derived from cross C used the BC_1F_1 progeny with the first highest recurrent parent genome recovery as resistant parent and KING as recurrent parent.

[b] BC_2F_1 progenies derived from cross D used the BC_1F_1 progeny with the second highest recurrent parent genome recovery as resistant parent and KING as recurrent parent.

CHAPTER V

CONCLUSION

In the beginning of MABB for gene pyramiding, distinct polymorphisms were revealed with all 6 marker loci linked to CLS and PM resistance genes between donor parents V4718 and V4785 and recurrent parents SUT1 and KING. Interestingly, when evaluating genetic relationships with EST-SSR analysis, these recurrent parents were classified into the group containing several varieties/lines with high yielding potential, which were distally genetically related to resistant cultivars/lines. Taken together, they would be able to be used as potential parents in this MABB. In addition to parental selection, this study exposes the effectiveness of EST-SSR markers for elucidating genetic diversity and relatedness of 2 *Vigna* species of mungbean and blackgram. Within the mungbean group, the high yielding potential and the resistance to diseases genotypes studied were clearly differentiated. EST-SSR markers proved to be applicable for functional diversity analysis of mungbean and blackgram, which are not only useful for genotyping and genetic diversity and relatedness evaluation, but might also be used for MAS in future breeding programs. Interestingly, this study achieved the introgression of CLS and PM resistance genes from the hybrids of 3 resistant lines, including V4718, V4758, and V4785 crossed with a susceptible variety CN72 into high yielding potential varieties SUT1 and KING through MABB technique within the early generations. This method can be used within a short time frame due to the exclusive presence of high RGP recovery with approximately 85 to 100% in

pyramided BC_2F_1 plants among all different populations, and some of these with the relative maximum recovery based on 3 different sets of background markers, i.e. BC_2F_1-SUTA59 (90.0 to 97.6%) and BC_2F_1-KINGC8 (84.7 to 100.0%) were also highly resistant to CLS under laboratory condition. As a result, MAS is recommended as a strategy to accelerate backcrossing of these pyramided BC_2F_1 plants with recovering from 85 to 100% of RPG. Moreover, it was expected that the recently improved CLS and PM lines of SUT1 and KING will be beneficial for the production of resistant mungbean varieties useful for prone areas with disease epidemics, as well as organic farming systems in the future.

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