PYRAMIDING MULTIPLE CERCOSPORA LEAF SPOT AND POWDERY MILDEW RESISTANCE GENES IN SUT1 AND KING MUNGBEAN VARIETIES USING MARKER-ASSISTED SELECTION

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การรวมยีนต้านทานโรคใบจุดและราแป้งในถั่วเขียวพันธุ์ มทส1 และคิง โดยใช้เครื่องหมายโมเลกุลช่วยคัดเลือก

นายพิชชากร พาพันธ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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พิชชากร พาพันธ์ : การรวมยีนต้านทานโรคใบจุดและราแป้งในถั่วเขียวพันธุ์ มทส1 และคิง โดยใช้เครื่องหมายโมเลกุลช่วยคัดเลือก (PYRAMIDING MULTIPLE CERCOSPORA LEAF SPOT AND POWDERY MILDEW RESISTANCE GENES IN SUT1 AND KING MUNGBEAN VARIETIES USING MARKER-ASSISTED SELECTION) อาจารย์ที่ปรึกษา : ศาสตราจารย์ ดร.ปิยะดา อลิฌาณ์ ตันตสวัสดิ์, 179 หน้า.

การปรับปรุงพันธุ์ถั่วเขียวให้มีความต้านทานต่อโรคและแมลงเป็นสิ่งจำเป็นสำหรับการผลิตถั่วเขียว การศึกษานี้มีวัตถุประสงค์เพื่อ 1) บ่งชี้ยีนควบคุมลักษณะต้านทานโรคใบจุดในคู่ผสมระหว่างพันธุ์ชัยนาท72 (CN72) และ V4718 2) รวมยีนต้านทานโรคใบจุดและราแป้งเข้าสู่พันธุ์ที่ให้ผลผลิตสูง ได้แก่ พันธุ์มทส1 (SUT1) และคิง (KING) โดยการใช้เครื่องหมายโมเลกุลช่วยคัดเลือกในการผสมกลับ (marker-assisted backcrossing; MABC) 3) ประเมินความต้านทานโรคใบจุดและราแป้ง และลักษณะทางพืชไร่ของสายพันธุ์ที่ได้จากการผสมกลับ (backcross; BC) ในสภาพแปลงทดลอง และ 4) ยืนยันความสัมพันธ์ทางพันธุกรรมระหว่างสายพันธุ์ที่ได้จากการผสมกลับและสายพันธุ์พ่อแม่ โดยใช้ลักษณะทางพืชไร่และการสังเคราะห์ด้วยแสง การทดลองที่ 1 ใช้เครื่องหมายที่คาดว่ามีความสัมพันธ์กับความต้านทานโรคใบจุดที่พบในการศึกษาครั้งนี้จำนวน 5 เครื่องหมาย ร่วมกับเครื่องหมายที่มีการรายงานมาก่อนหน้านี้ คือเครื่องหมาย VR393 และ CEDG084 เพื่อหาตำแหน่ง QTL ของยีนควบคุมความต้านทานโรคใบจุด ผลการทดลอง พบ QTL 1 ตำแหน่ง (*qCLSC72V18-1*) ซึ่งสามารถอธิบายความแปรปรวนของคะแนนความรุนแรงของการเกิดโรคใบจุดได้ 32.86 ถึง 41.56% ขึ้นอยู่กับปีและอยู่ระหว่างเครื่องหมาย I16274 และ VrTAF5_Indel ซึ่งห่างจากตำแหน่ง QTL 4.0 และ 5.0 cM ตามลำดับ ในการทดลองที่ 2 ทำการถ่ายยีนต้านทานโรคใบจุดและราแป้งเข้าสู่พันธุ์รับมทส1 และคิง ในคู่ผสมที่ใช้พันธุ์รับมทส1 ใช้เครื่องหมายที่เชื่อมโยงกับยีนต้านทานโรคใบจุดและราแป้งจำนวน 4 เครื่องหมาย สำหรับการคัดเลือกแบบ foreground ในขณะที่การคัดเลือกแบบ background ใช้เครื่องหมายที่สามารถแยกความแตกต่าง จำนวน 72 ตำแหน่ง จากการทดลอง ได้พัฒนาสายพันธุ์ถั่วเขียวที่ได้จากการผสมกลับเพื่อรวมยีนชั่วที่ 4 (BC_4) จากพันธุ์รับมทส1 จำนวน 6 สายพันธุ์ (A1, B1, B2, D2, D5 และ G1) ที่มีจีโนมเหมือนพันธุ์รับมทส 1 98.8-100.0% และมีตำแหน่งของเครื่องหมายที่เชื่อมโยงกับยีนต้านทานในรูปแบบ homozygosity ทุกตำแหน่ง ในคู่ผสมที่ใช้พันธุ์รับคิง ใช้เครื่องหมายที่เชื่อมโยงกับยีนต้านทาน สำหรับคัดเลือกแบบ foreground จำนวน 5 เครื่องหมาย และเครื่องหมายที่ให้ความแตกต่างจำนวน 49 ตำแหน่ง สำหรับคัดเลือกแบบ background เช่นเดียวกัน จากผลการทดลอง พบว่า สายพันธุ์ที่ได้จากการผสมกลับเพื่อรวมยีนชั่วที่ 4 จากพันธุ์รับคิง จำนวน 2 สายพันธุ์ (H3 และ H4) มีเครื่องหมายคัดเลือกแบบ

foreground ครบทั้ง 5 ตำแหน่ง และทั้งสองสายพันธุ์ที่ได้จากการผสมกลับเพื่อรวมยีนชั่วที่ 4 มีจีโนมเหมือนพันธุ์รับคิง เท่ากับ 94.4-100.0% ต่อจากนั้น ทำการประเมินความต้านทานต่อโรคใบจุดและราแป้ง รวมถึงลักษณะทางพืชไร่ ของสายพันธุ์ที่ได้จากการผสมกลับชั่วที่ 4 จากทั้ง 2 คู่ผสม เพื่อเปรียบเทียบกับสายพันธุ์พ่อแม่และพันธุ์เปรียบเทียบในหลายฤดูกาล ปี และสถานที่ ในระหว่างการประเมินในสภาพแปลงทดลอง พบว่า สายพันธุ์ที่ได้จากการผสมกลับเพื่อรวมยีนชั่วที่ 4 ทุกสายพันธุ์มีความต้านทานต่อโรคราแป้งในระดับปานกลางถึงสูง เมื่อเปรียบเทียบกับพันธุ์รับมทส1 และคิง บางสายพันธุ์ (H3, H4 และ B2) มีความต้านทานต่อโรคใบจุดในระดับปานกลางหรือระดับสูงเช่นกัน สิ่งที่น่าสนใจคือ เมื่อมีการระบาดของโรคใบจุดและราแป้ง สายพันธุ์ที่ได้จากการผสมกลับ A1, B1, B2 และ D5 จากพันธุ์รับมทส1 และ H3 จากพันธุ์รับคิง มีลักษณะทางพืชไร่ส่วนใหญ่เหมือนหรือดีกว่าพันธุ์รับ และยังมีแนวโน้มให้ผลผลิตสูงกว่าพันธุ์รับมทส1 3.5-31.0% และสูงกว่าพันธุ์รับคิง 18.0-32.0% ตามลำดับ สายพันธุ์ที่ได้จากการผสมกลับชั่วที่ 4 และมทส1 มีความสัมพันธ์ทางพันธุกรรมใกล้ชิดกัน โดยเฉพาะ B2 และ D2 เมื่อประเมินจากลักษณะทางพืชไร่และการสังเคราะห์ด้วยแสง ดังนั้นจะเห็นได้ว่าสายพันธุ์ที่ได้จากการผสมกลับเพื่อรวมยีนเหล่านี้มีศักยภาพที่จะพัฒนาเป็นถั่วเขียวพันธุ์ต้านทานใหม่ได้ในอนาคต

สาขาวิชาเทคโนโลยีการผลิตพืช ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2563 ลายมือชื่ออาจารย์ที่ปรึกษา_____

PITCHAKON PAPAN : PYRAMIDING MULTIPLE CERCOSPORA LEAF SPOT AND POWDERY MILDEW RESISTANCE GENES IN SUT1 AND KING MUNGBEAN VARIETIES USING MARKER-ASSISTED SELECTION. THESIS ADVISOR : PROF. PIYADA ALISHA TANTASAWAT, Ph.D., 179 PP.

MOLECULAR MARKER/QUANTITATIVE TRAIT LOCI (QTL)/MARKER-ASSISTED BACKCROSSING/MARKER-ASSISTED GENE PYRAMIDING

The improvement of mungbean varieties for resistance to diseases and pests is required for mungbean production. The objectives of this study were to 1) identify the Cercospora leaf spot (CLS) resistance gene in a cross between CN72 and V4718, 2) pyramid CLS and PM resistance genes into high yielding mungbean varieties, SUT1 and KING, through marker-assisted backcrossing (MABC), 3) evaluate backcross (BC) lines for CLS and PM resistance as well as agronomic traits in the field conditions, and 4) confirm genetic relationship among BC lines and their parents based on agronomic and photosynthetic characters. The first experiment used five putative markers linked to CLS resistance discovered in this study together with markers VR393 and CEDG084 from a previous report to refine quantitative trait loci (QTL) mapping of a gene conferring CLS resistance. As a result, a major QTL (*qCLSC72V18-1*) accounted for 32.86 to 41.56% of phenotypic variation in CLS disease severity score depending on years was identified and flanked between I16274 and VrTAF5_Indel markers at a distance of 4.0 and 5.0 cM, respectively. In the second experiment, the CLS and PM resistance genes were transferred into the recurrent parents SUT1 and KING. In the cross with the recurrent parent SUT1, four markers associated with CLS and PM

resistance genes were used for foreground selection, while 72 polymorphic marker loci were used for background selection. Six pyramided BC_4 lines (A1, B1, B2, D2, D5, and G1) from recurrent parent SUT1 with 98.8-100.0% recurrent parent genome (RPG) recovery that possessed all resistance gene linked marker loci in homozygosity were developed. Five markers linked to resistance genes for foreground selection and 49 polymorphic marker loci for background selection were also used in the cross with recurrent parent KING. The results revealed two pyramided BC_4 lines from the recurrent parent KING, carrying all five foreground marker loci, H3 and H4. The RPG recovery of these pyramided BC_4 lines was 94.4-100.0%. Subsequently, these pyramided BC_4 lines of both crosses were further evaluated for CLS and PM resistance along with agronomic traits compared to their parents and check varieties in various seasons, years and locations. During field evaluation, a moderate to high level of PM resistance was observed in all pyramided BC lines compared to the recurrent parents SUT1 and KING. Some of these lines (H3, H4, and B2) were also moderate resistant and resistant to CLS. Interestingly, A1, B1, B2, and D5 from SUT1 and H3 from KING had most agronomic traits similar or superior to the recurrent parents and their yields tended to be 3.5-31.0% higher than SUT1 and 18.0-32.0% higher than KING, respectively under CLS and PM outbreaks. The close relationship among BC_4 lines and SUT1 was found especially in B2 and D2 based on agronomic and photosynthetic performances. Therefore, these pyramided BC lines had potential to be developed into new resistant varieties of mungbean in the future.

School of Crop production Technology Student's Signature Pitchakon Papan

Academic Year 2020 Advisor' Signature Pimdao Alisha Tantut

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LIST OF ABBREVIATIONS

AVRDC	=	Asian Vegetable Research and Development Center
BC	=	Backcross
BSA	=	Bulk segregant analysis
CLS	=	Cercospora leaf spot
EST-SSR	=	Expressed sequence tag-derived simple sequence repeat
ICIM	=	Inclusive composite interval mapping
ISSR	=	Inter simple sequence repeat
ISSR-RGA	=	ISSR-anchored resistance gene analog
LG	=	Linkage group
LOD	=	Logarithms of odds
MABC	=	Marker-assisted backcrossing
MAGP	=	Marker-assisted gene pyramiding
MAS	=	Marker-assisted selection
PM	=	Powdery mildew
QTL	=	Quantitative trait loci
RIL	=	Recombinant inbred line
RPG	=	Recurrent parent genome
SSR	=	Simple sequence repeat
TAF5	=	TATA-binding-protein-associated factor 5

CHAPTER I

INTRODUCTION

1.1 Significance of this study

Mungbean [*Vigna radiata* (L.) Wilczek] is one of the most important grain legume crops. It is rich in digestible proteins (240 g/kg of seed), carbohydrates (630 g/kg of seed), and minerals (iron and zinc) (Nair, 2020). It is popular among farmers due to its short life cycle (60-65 days), drought tolerance, and ability to fix nitrogen in the soil. Globally, mungbean production is 5.3 million tons of grain yield with an average 0.72 ton/ha from over 7.0 million hectares worldwide. Asia is the world's largest producer, mainly in India, Myanmar, China, Thailand, and Indonesia (Nair and Schreinemachers, 2020). In Thailand, mungbean production is approximately 0.092 million tons with an average of 0.72 ton/ha, obtained from the cultivated area of 0.13 million hectares. Although the average of mungbean production in Thailand is not different from worldwide standards, the productivity is still insufficient to meet the domestic demand of more than 0.11 million tons per year (Office of Agricultural Economics, 2019). The low productivity is because of the abiotic and biotic constraints, poor crop management practices, and the lack of new varieties with higher yield potential to farmers (Chauhan et al., 2010; Pratap et al., 2019). Among them, Cercospora leaf spot (CLS) and powdery mildew (PM) are the most serious diseases in Thailand.

Cercospora leaf spot (CLS), a foliar disease, is caused by *Cercospora canescens*

Ellis & Martin (Chand et al., 2015). This disease spreads in warm-wet growing season, and can lead to 50- 97% yield losses if there is no protection (AVRDC, 1984; Iqbal et al., 1995; Chand et al., 2012; Nair et al., 2019). Meanwhile, powdery mildew (PM) is caused by the biotrophic fungus *Sphaerotheca phaseoli.* The spread of PM can reduce yield more than 50% in cool-dry growing season (Khajudparn et al., 2010). The resistance source of CLS in the V4718 line is controlled by a single dominant gene (Chankaew et al., 2011; Arsakit et al., 2017). Meanwhile, PM resistance in the V4718, V4758 and V4785 is also controlled by a single non-allelic dominant gene (Khajudparn et al., 2010, Poolsawat et al., 2017), which are useful for plant breeders in developing new resistant varieties. However, the selection for CLS and PM resistant genotypes still faces obstacles mainly because CLS occurs only in rainy season while PM occurs in winter season. Therefore, selection can be performed only once per year for each disease, resulting in slow progress of the breeding program. Conventional breeding programs are based mainly on morphological selection which is seasonal dependent. The difficulty of conventional breeding is mainly due to the environmental effects on some traits of interest such as yield related traits which were controlled by several genes, time-consuming processes, and high labor cost. Moreover, pyramiding multiple resistance genes into a single genotype through conventional breeding is very difficult because it cannot differentiate the resistant plants which possess different number of resistance genes.

Molecular markers associated with the genes controlling resistance are becoming an effective tool for resolving the limitations of conventional breeding method. They can help selection for a trait of interest at all stages of plant growth and in all environmental conditions, allowing year-round selection. This strategy is called

marker-assisted selection (MAS) of which a phenotype is selected based on the genotype of marker(s) either directly or indirectly. In addition, the application of MAS through marker-assisted backcrossing (MABC) and marker-assisted gene pyramiding (MAGP) are wildly used in molecular plant breeding. When MAGP was used through MABC, it allows combining several genes into an elite variety and differentiating plants with different genes simultaneously, while improved lines still have similar genetic background to the elite variety (recurrent parent). The procedure of MABC consists of foreground and background selection (Hospital, 2003). Foreground selection is the selection of desirable plants by using markers linked to desirable traits e.g., disease resistance. While background selection is carried out by using markers unlinked with the traits of interest to estimate the recovery of the recurrent parent genome (RPG). Using both foreground and background selection, plants with foreground marker (s) and the highest RPG recovery can be selected for the next backcross generation. Therefore, breeders can reduce backcross generations, saving time and cost. Success of accelerated backcrossing was achieved since early generations i.e., BC_2 (Krishna et al., 2017; Sagare et al., 2019), BC_3 (Divya et al., 2014; Pradhan et al., 2015; Baliyan et al., 2018), and BC_4 (Ragot et al., 1995) generations. However, the efficiency of MABC is affected by several factors such as the population size of each backcross generation, the genetic background of the recurrent parent, undesirable linkage drags, and the distance between the closest markers and target genes/QTLs (Jiang, 2013). Particularly, the development of closest markers is one of the prerequisites to enhance the efficiency of MABC.

Quantitative trait loci (QTL) analysis is one of the most important approaches for identifying QTL controlling a trait of interest. It is used to verify the correlation between markers and desirable traits. However, the markers identified in preliminary genetic

mapping should be validated in independent populations and different genetic background with known target phenotype for testing their effectiveness (Collard et al., 2005). Once tightly linked markers to genes or QTLs of interest have been identified and verified, the use of flanking markers linked to target genes/QTLs helps in transferring a desirable trait into an elite variety. Recently, the identification of CLS and PM resistance genes using different molecular marker systems has been reported in mungbean. Chankaew et al. (2011) identified a major QTL controlling resistance to CLS using SSR markers in a KPS1 × V4718 cross. This QTL was flanked between the markers CEDG117 and VR393. Using this marker system, a major QTL associated with CLS resistance in the CN72 × V4718 cross was also identified (Arsakit et al., 2017). A QTL was located between the markers VR393 and CEDG084 with the distance of 4.0 and 6.0 cM, respectively. Therefore, finding additional markers closer to the gene is crucial for efficient selection. In addition, Poolsawat et al. (2017) also reported a major QTL controlling PM resistance in a cross between CN72 (susceptible) and V4718 (resistant) using ISSR and ISSR-RGA markers. This QTL was flanked between I42PL229 and I85420 markers at the distance of 4.0 and 9.0 cM, respectively. In addition, I41tP379 and I27R565 markers were also identified to be linked to PM resistance genes obtained from CN72 × V4758 and CN72 × V4785 crosses, respectively (Tantasawat et al., 2021). These markers linked to CLS and PM resistance genes can be directly used to pyramid these resistance genes into high yielding mungbean variety for durable resistance or broad-spectrum resistance to CLS and PM through MAS.

Genetic relationship is essential for helping plant breeders find suitable parental lines or selecting desirable progenies in any breeding program. A high genetic distance between parents is important to obtain heterosis and segregants among their progeny,

allowing plant breeders to select superior genotypes. Recently, several mungbean genotypes originated in India, Bangladesh, Pakistan, Philippines, Myanmar, Indonesia, Australia, Taiwan, and Thailand have been used to evaluate genetic relationship based on agronomic traits, morphological traits, photosynthetic performance as well as molecular markers (ISSR, EST-SSR and SRAP) (Tantasawat et al., 2010; Chueakhunthod, 2019; Chueakhunthod et al., 2020). Interestingly, some genotypes i.e., WALET, SUT1, SUT4, EG-MD-6D, CN84-1, CN72, CN36, MG50-10A (Y), BPI GLABROUS #3, KING, CES55, and KPS1 with high yielding potential were grouped together, separating from the distantly related resistant genotypes (V4718, V4758, V4785). Therefore, they were used to find potential recurrent parents based on genetic polymorphism with resistant donor parents at 6 markers linked to CLS and PM resistance genes. The elite varieties i.e., SUT1, SUT4, EG-MD-6D, CN84-1, CN72, CN36, MG50-10A (Y), BPI GLABROUS #3, KING, and CES55 exhibited polymorphisms at these marker loci, therefore, they were promising parents for developing new resistant varieties via MAS. Among these, SUT1 developed by Suranaree University of technology has high yield, large seed, synchronous maturity, and the pod located above the canopy, as well as moderate resistance to CLS and PM. KING is originated in Australia and has larger seed than Thai certified varieties i.e., CN72 and CN84-1 (Chueakhunthod et al., 2020). In addition, its seed also contains high protein and total digestible nutrient yields (Abd El-Salam et al., 2013). Therefore, SUT1 and KING possessing the outstanding characters which are probably preferred by farmers have been used as the recurrent parents in MABC up to BC_2F_1 generation (Chueakhunthod, 2019). In this study, we continued the work until BC_4F_7 generations and evaluated their potential based on CLS and PM responses and agronomic traits in

multiple locations, seasons, and years.

1.2 Research objectives

1.2.1 To explore DNA polymorphisms and identify markers putatively associated with CLS resistance with bulk segregant analysis (BSA) in recombinant inbred line (RIL) population derived from a cross between a susceptible cultivated variety (Chai Nat 72; CN72) and a resistant line (V4718) using ISSR, ISSR-RGA, SSR and InDel markers.

1.2.2 To identify the CLS resistance gene in a CN72 × V4718 cross using markers putatively associated with CLS resistance from bulk segregant analysis (BSA) and obtain markers closest to the CLS resistance gene to be used in marker-assisted selection (MAS).

1.2.3 To pyramid a CLS resistance gene and 2 PM resistance genes into two high yielding mungbean varieties i.e., SUT1, and KING through MABC.

1.2.4 To evaluate the CLS and PM resistance as well as agronomic traits of pyramided backcross (BC) lines compared to parents and check varieties in different locations, seasons, and years.

1.2.5 To confirm genetic relationship among BC lines and their parents based on agronomic and photosynthetic characters.

1.3 Research hypotheses

1.3.1 The ISSR, ISSR-RGA, and SSR markers are spread throughout the plant genome providing great opportunity to detect QTL for CLS resistance in $F_{2:7}$ RIL

population of the CN72 × V4718 cross. Particularly, ISSR-RGA which uses the RGA primers complementary to RGA sequences may locate near resistance gene clusters.

1.3.2 InDel marker (VrTAF5_Indel) was designed from a candidate gene in the resistant line V4718 that is suggested to be responsible for CLS resistance in the KPS1 × V4718 cross. This marker may also be used to identify CLS resistance in different crosses using V4718 as resistance source, especially in the CN72 × V4718 cross.

1.3.3 Polymorphic markers between parents can be used to find putative association with the CLS resistance gene using BSA.

1.3.4 The markers linked to CLS and PM resistance genes may be used in MAS for improving resistance to CLS and PM diseases.

1.3.5 BC progenies selected through markers linked to the CLS resistance gene and 2 PM resistance genes may show higher levels of disease resistance to both diseases or exhibit board-spectrum resistance.

1.3.6 BC_4 progenies selected by background selection to have high RPG recovery may have similar yield to their recurrent parents in multiple environments.

1.3.7 A close genetic relationship can be observed between BC_4 progenies and their recurrent parent.

1.4 Research scope

This experiment was divided into 2 parts. The first experiment focused on identification of a gene conferring CLS resistance in RIL population derived from a cross between CN72 (susceptible cultivar) and V4718 (resistant line). The evaluation of CLS resistance in RIL population was performed at the field level in rainy season (May to August) in 2016 and 2018 at Suranaree University of Technology Farm,

Nakhon Ratchasima province, Thailand. Meanwhile, a total of 156 primers/primer pairs were used to detect polymorphisms with BSA consisting of 68 ISSR primers, 48 ISSR-RGA primer pairs, 39 SSR primer pairs, and 1 InDel primer pairs.

The second experiment focused on pyramiding a CLS resistance gene and 2 PM resistance genes into the recurrent parents i.e., SUT1 and KING through MABC. This experiment was continued from the work of Chueakhunthod (2019). The obtained BC_3F_1 (SUT1) and BC_2F_1 (KING) seeds were used to generate BC_4F_1 generation and were selfed to produce BC_4F_2 to BC_4F_7 generations. The evaluation of CLS and PM resistance and their agronomic traits was performed in BC_4F_4 to BC_4F_7 generations. In addition, in each generation five markers linked to all target resistance genes (foreground selection), ISSR (I85420) and ISSR-RGA (I42PL222) markers flanked the PM gene from V4718, SSR (VR393 and CEDG084) markers flanked the CLS gene from V4718 and ISSR-RGA (I27R565) marker associated with the PM gene from V4785 were used for foreground selection. Moreover, markers linked to CLS resistance obtained from the first experiment were also used for the foreground selection. Meanwhile, background selection was performed to identify BC progenies with a high level of genetic background similarity to their recurrent parents using SSR, EST-SSR, and ISSR markers. Moreover, genetic relationship among BC progenies and their parents was studied based on agronomic and photosynthetic characters.

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CHAPTER II

LITERATURE REVIEWS

2.1 Mungbean importance

Mungbean [*Vigna radiata* (L.) Wilczek] is known as moong bean, golden gram, and green gram. It is classified into genus Vigna, subgenus *Ceratotropis* and has a diploid chromosome number of 2n = 2x =22. It is an economically important pulse crop in South and Southeast Asia. Mungbean is grown as a monocrop and as a component crop in many cropping systems. Mungbean seeds are rich in several sources of nutrients such as digestible proteins, minerals, vitamins, and amino acids, etc., and are used in industrial foods (vermicelli and starch) and cosmetics. In addition, sprouts and young pods are eaten as vegetables. The plant parts (leaves and stems) are used as forage and green manure. Approximately, up to 90% of cultivated area and mungbean production in the world are located in Asia, mainly in India, Myanmar, China, Thailand, and Indonesia. Mungbean production ranging from 450-1,920 kg/ha (72-307 kg/rai) with an average of 721 kg/ha or 115 kg/rai (Nair and Schreinemachers, 2020). In Thailand, yield potential is in the range of 719-825 kg/ha or 115-132 kg/rai. Moreover, mungbean cultivated area and their production tended to decrease in 2018 and 2019 (Figure 2.1) (Office of Agricultural Economics, 2019). In addition, several Thai certified cultivars consisting of Chai Nat 84-1 (CN84- 1), Chai Nat 72 (CN72), Chai Nat 36 (CN36), Chai Nat 60 (CN60), Suranaree University of Technology 1 (SUT1), and Kamphaeng Saen 1 (KPS1) are frequently used by Thai

Farmers, which have high yield potential but are susceptible to diseases (Chaitieng, 2002; Khajudparn et al., 2010; Chai Nat Field Crops Research Center, 2018). However, the limitations of mungbean production are environments, insects, diseases. The lack of varieties having high yield is also constrained to mungbean production.

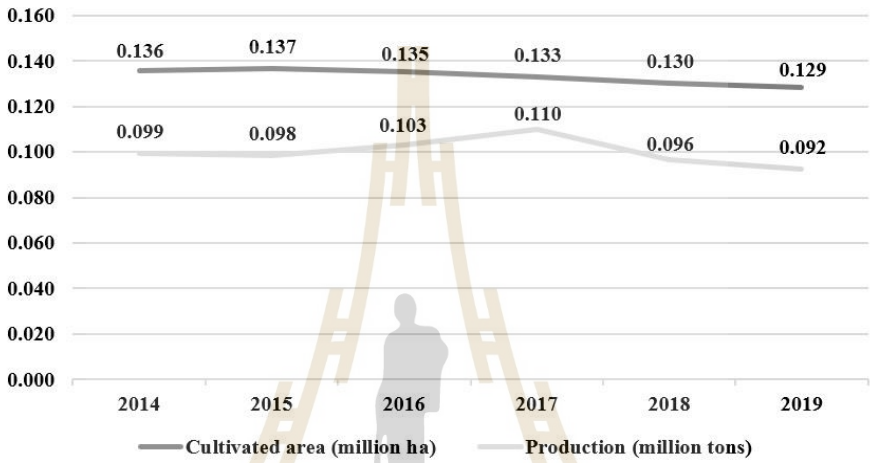

Figure 2.1 Mungbean cultivated area and production in Thailand (Office of Agricultural Economics, 2019).

2.2 Mungbean diseases

Among the important diseases of mungbean, mungbean yellow mosaic virus (MYMV), Cercospora leaf spot (CLS), and powdery mildew (PM) are the major foliar diseases.

2.2.1 Mungbean yellow mosaic virus (MYMV)

MYMV is a serious viral disease of mungbean. It is caused by several begomoviruses, which are transmitted by whitefly (*Bemisia tabaci*). The most conspicuous symptom on the foliage begins as small yellow spots along the veinlets and spreads over the lamina; the pods become thin and curl upward. This disease can

lead to a yield reduction of 85% (Karthikeyan et al., 2014) and up to 100% when infected at early growth stages (Kitsanachandee et al., 2013).

2.2.2 Cercospora leaf spot (CLS)

CLS disease is caused by the hemibiotrophic fungus *Cercospora canescens* Ellis & Martin (Chand et al., 2015). The CLS can reduce mungbean seed yield, leading to 50-97% yield loss (AVRDC, 1984; Iqbal et al., 1995; Chand et al., 2012; Nair et al., 2019). Symptoms of CLS include spotting on mungbean leaves and the spots is increasing during flowering until pod-filling stage in warm-wet growing season, resulting in a reduction in the size of pods and seeds (Grewal et al., 1980; Chankaew et al., 2011).

2.2.3 Powdery mildew (PM)

PM disease is caused by the biotrophic fungus *Sphaerotheca phaseoli*. Symptoms of PM include white spotting on both sides of leaf surface and stems. The fungus covers all parts of the plant with white powdery growth. Therefore, it adversely affects the photosynthetic efficiency of the plant, especially before flowering, resulting in the maximum damage. PM is widely distributed in cool-dry seasons, causing yield losses of 50% (Khajudparn et al., 2010) and 100% at the seedling stage (Reddy et al., 1994).

2.3 Sources of CLS and PM resistances

Genetic resistance sources of CLS and PM are also important to mungbean improvement. Mungbean genotypes resistant to CLS or PM are presented in Table 2.1. Some of these genotypes such as V4718 and M5-25 have been reported to be resistant to both diseases. In addition, the resistance source of PM in V4718, V4758,

and V4785 is controlled by a single dominant gene, and they are non-allelic (Khajudparn et al., 2010), which is useful for plant breeders in developing new resistant varieties. Meanwhile, the resistance source of CLS in the V4718 line is also controlled by a single dominant gene (Chankaew et al., 2011; Tantasawat et al., 2020). Therefore, these resistance sources are suitable to be used for improving mungbean varieties resistant to CLS and PM.

Table 2.1 Sources of CLS and PM resistance in several countries.

Countries	Disease resistance	Genotypes/accession number	References
India	PM	Phule M-2003-3, Phule M- 2002-13, Phule M-2002-17, Phule M- 2001-3, Phule M-2001-5, LGG-460, Vaibhav, BPMR-145, TARM-18, TARM-1, S-158-16, S-2-4-1, Mulamarada, TARM-1, TJM-3, TM-96-2, and TMB-37	Mandhare and Suryawanshi (2008); Reddy (2007); Reddy (2009)
	CLS	LGG-460, GM-02-08, GM-02-13, GM-03-03, C2/94-4-42, CO-3, 98-cmg-003, 98cmg-018, NM-1, NM-2, NM-98, BRM-188, Basanti, BARI Mung-2, PDM-11, and VC3960-88	Haque et al. (1997)
Taiwan	PM	V1104, V4631, V4658, V4662, V4717, and V4883	Hartman et al. (1993)
	CLS	V1471, V2757, V2773, V4718, and V5036	Hartman et al. (1993)
Thailand	PM	M5-10, M5-25, V4718, V4758, and V4785	Wongpiyasatid et al. (1999); Khajudparn et al. (2010)
	CLS	M5-22, M5-25, and V4718	Wongpiyasatid et al. (1999); Chankaew et al. (2011)
Pakistan	CLS	C2/94-4-42, CO-3, 98-cmg-003, 98cmg-018, NM-1, NM-2, NM-98, BRM-188, Basanti, BARI Mung-2, PDM-11, and VC3960-88	Iqbal et al. (2004)

2.4 Breeding of mungbeans

2.4.1 Conventional breeding

Chai Nat Field Crops Research Center (CNFCRC), Chai Nat aims to improve mungbean cultivars for pest and disease resistance and to develop high-yielding varieties. In 1976, U-Thong 1 variety was released by DOA. This variety was introduced from Philippines. The selection of this variety was based on even maturity,

high yield and large seed when compared with local varieties. Subsequently in 1986, Kamphaeng Saen 1 (KPS1) and Kamphaeng Saen 2 (KPS2) were released by Kasetsart University. They gave high yield and more resistant to CLS and PM. These 2 cultivars were selected by using mass selection of breeding lines VC1973A and VC2778A from Taiwan, respectively (Srinives, 1994). In 1997, Suranaree University of Technology 1 (SUT1) was developed from a cross between U-Thong 1 and NP-29 and selected by using single seed descent program. SUT1 variety was reported as moderately resistant to CLS and PM (Laosuwan, 1999). Later, SUT2, SUT3 and SUT4 varieties were also developed by Suranaree University of Technology. All of these were reported as resistant to CLS. These 3 varieties were obtained from different crosses, 'VC3689A × KPS1' cross for SUT2, 'KPS2 × VC3689A' cross for SUT3 and 'VC3689A × PSU1' cross for SUT4 and backcrossed to recurrent parents (KPS1, KPS2 and PSU1) for 4 times (Chaitieng, 2002). However, the limitations of conventional breeding are the number of genes that control a trait, time-consuming and environmental effects. Therefore, the use of marker-assisted selection (MAS) may accelerate plant breeding procedure.

2.4.2 Molecular assisted breeding (MAB)

Molecular markers are useful alternative tools for selecting a trait of interest based on genotyping at the DNA level. It can be used to detect the presence of allelic variation in the genes. The use of DNA makers to assist in plant selection is called marker-assisted selection (MAS). MAS is a method whereby a phenotype is selected based on the genotype of marker(s), either directly or indirectly. There are five broad areas of MAS consisting of gene pyramiding, marker assisted backcrossing (MABC), early generation selection, combined marker assisted selection, and marker

assisted evaluation of breeding materials (Collard and Mackill, 2008). Molecular markers are not controlled by environment and conditions in which the plants are grown and are detected in all stages of plant growth. It can also reduce the time of the breeding process. The application of MAS that were used for plant breeding are given.

2.4.3 Marker-assisted backcrossing (MABC)

Backcrossing is the most commonly used to transfer one or a few genes from donor parent into an elite variety or recurrent parent. In traditional backcrossing breeding, the genetic content from donor parent is eliminated when backcrossed to recurrent parent many times (e.g., 6 times or until BC_6 generation). However, size of the donor chromosome segment or size of the introgression from donor parent that contain in recurrent parent is important to performance of plants because some donor fragments may be linked to the target gene of negative effects. It is called linkage drag (Brumlop and Finckh, 2011). Therefore, the use of DNA markers that flank a target gene (less than 5 cM on either side) in backcrossing greatly increases the efficiency of selection and can minimize linkage drag. Jiang (2013) summarized that the efficiency of MABC is affected by several factors i.e., the distance between the closest marker and *R* gene, the population size of each backcross generation, the genetic background of the recurrent parent, and undesirable linkage drag. At present, MABC is the most widely and successfully used method in practical molecular breeding. The selection of MABC program can be divided into 2 types consisting of foreground and background selection (Hospital, 2003).

In foreground selection, plant breeders can use markers to select for the presence of the target gene or quantitative trait loci (QTL) of donor parent. The BC progenies are selected in heterozygous alleles until final backcrossing is completed. It

may be particularly useful for traits that have time-consuming phenotypic screening procedures. In addition, the plants that possess recessive alleles can be selected and eliminated, which is easier than conventional methods. Collard and Mackill (2008) suggested that the distance between the tightly linked marker and target gene/QTL should be less than 5.0 cM. Furthermore, the use of flanking markers is more powerful than a single marker. Closest markers linked to the target gene/QTL could be developed from different approaches; design from the target genes directly known as gene-specific markers, from gene functions known as functional markers, and obtained from QTL analysis known as markers tightly linked to the QTLs.

In background selection, the selection of BC progenies based on markers of recurrent parent in all genome region of desirable traits except the target locus and eliminate the undesirable genome of donor parent. Besides, BC progenies are selected in homozygous alleles of the recurrent parent. However, the progress in recovery of the recurrent parent genome (RPG) depends on the number of markers used in background selection. Servin and Hospital (2002) recommend that two to four markers placed on each chromosome with the length of 100 cM are sufficient for background selection. The highest efficiency will occur if the markers are optimally located along the chromosomes. Particularly, co-dominant markers are wildly used in this technique. These markers displayed the distinction between homozygous and heterozygous DNA banding patterns. Interestingly, using SSR and EST-SSR markers, Isemura et al. (2012) and Kajonphol et al. (2012) identified several QTLs for agronomic traits distributed among 7 of 11 linkage groups of mungbean. They are related to several traits such as 100-seed weight, seeds per pod, pod length, pod width, seed length, seed weight, days to flowering, days to maturity, days to harvest, etc.

Therefore, the use of flanking markers of those QTLs may be associated with a nature of high yielding recurrent parent. Using the background selection method, the expected RPG recovery in each backcross generation when using marker-assisted background selection and non-selection is showed in Table 2.2. In a practical genetic background, three or four times backcrossing to the recurrent parent is sufficient to increase RPG recovery more than 99 %, with selection is similar in BC_6 generation without marker-assisted background selection (Hospital, 2003).

Table 2.2 Expected results of a MABC scheme modified from Hospital (2003).

generation	population size	Recurrent parent genome (%)	
		MABC	Conventional backcross
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

2.4.4 Marker-assisted gene pyramiding (MAGP)

Gene pyramiding is one of the most important objectives of MAS in plant breeding which is used to combine several genes into a single genotype. Introgression of multiple genes/QTLs may be applied though different approaches such as backcrossing, using multiple parent crossing or complex crossing. It depends on number of genes/QTLs required for improvement of desirable traits. For example, pyramiding of three or four genes/QTLs from three or four parents can be crossed by double cross, three-way cross or four-way cross (Jiang, 2013). However, it is usually difficult to identify the plants that possess more than one gene when using conventional approach. Therefore, using DNA markers can facilitate selection because it can be evaluated at DNA level without the need for phenotyping (Collard and Mackill,

2008). MAGP is wildly used together with MABC method. The efficiency of MAGP technique depends on the distance between the closest markers and target gene/QTL (less than 5.0 cM) (Collard and Mackill, 2008). The most frequently used strategy of pyramiding is combining multiple resistance genes which is purposed to enhance broad-spectrum resistance to diseases or pests. Some examples of MABC and MAGP in several crop species are presented in Table 2.3.

These reviews concluded that MABC with foreground and background selection, and MAGP were successfully utilized for crop improvement. MABC can reduce the time of BC generation, the genetic background of improved lines is similar to recurrent parent as well as carrying some traits from the donor parent. Meanwhile, MAGP can enhance broad-spectrum resistance to pests or diseases. Although molecular markers are very useful in the breeding process, marker development is subjected to several specific procedures to verify the correlation of markers and the desirable traits. The most common procedure is quantitative trait loci (QTL) analysis.

2.5 Quantitative trait loci (QTL) analysis

QTL analysis is a statistical method that associate between phenotypic trait values and genotypes of markers. It is a tool for identifying QTL controlling a trait of interest which known as QTL mapping or genetic mapping (also gene or genome mapping). Closest markers linked to agronomically important genes or resistant genes that are identified by this technique may be used as molecular tools for marker-assisted selection (MAS) in plant breeding (Ribaut and Hoisington, 1998). The procedure of QTL analysis is similar to linkage map construction as follows: 1. creation of mapping population, 2. identification of polymorphism, and 3. linkage analysis of markers (Figure 2.2).

Table 2.3 Examples of marker-assisted backcrossing (MABC) and marker-assisted gene pyramiding (MAGP) in several crop species.

Species	Trait(s)	Gene(s)/QTL(s)	Marker(s) used		Range of RPG recovery (%)	selected generation	Remarks	References
			Foreground	Background				
Rice (*Orzyza sativar*)	Blast resistance	*Pi1*, *Pi2*, *Pi33*	SSR	48 polymorphic SSR	75.5-94.9	BC_3F_1	MAS applied for MAGP through MABC (Target variety: ADT43)	Divya et al. (2014)
	Blast resistance	*Pi*-gene	SSR	70 polymorphic SSR	92.7-97.7	BC_2F_2	MAS applied for MABC (Target variety: MR219)	Miah et al. (2015)
	Bacterial blight resistance	*xa5*, *xa13*, *Xa21*	STS	120 polymorphic SSR	92.5-97.0	BC_3F_1	MAS applied for MAGP through MABC (Target variety: Jalmagna)	Pradhan et al. (2015)
	Submergence tolerance	*sub1*	SSR	88 polymorphic SSR	93.8-96.3	BC_2F_2	MAS applied for MABC (Target variety: MR219)	Ahmed et al. (2016)
	Bacterial blight and blast resistance	*Xa21*, *xa13*, *Pi54*	Functional marker	83 polymorphic SSR	82.0-92.0	Intercross F_2(ICF$_2$)	MAS applied for MAGP through MABC (Target variety: MTU1010)	Arunakumari et al. (2016)
	Blast resistance	*Pi46*, *Pita*	SSR, gene-specific marker	26 polymorphic SSR	92.3-100.0	BC_3F_2	MAS applied for MAGP through MABC (Target variety: HH179)	Xiao et al. (2016)
	Bacterial blight	*Xa21*, *xa13*, *xa5*	STS	131 polymorphic SSR	72.9-97.1	BC_3F_1	MAS applied for MAGP through MABC (Target variety: Basmati rice)	Baliyan et al. (2018)
	Bacterial blight and blast resistance	*Xa21*, *xa13*, *Pi54*	Gene-specific marker	136 polymorphic SSR	82.0-92.3	Intercross F_2(ICF$_2$)	MAS applied for MAGP through MABC (Target variety: JGL1798)	Swathi et al. (2019)
	Bacterial blight and blast resistance	*Xa21*, *xa13*, *Pi54*, *Pi1*	SSR, gene-specific marker	60 polymorphic SSR	89.1-95.6	Intercross F_2(ICF$_2$)	MAS applied for MAGP through MABC (Target variety: Tellahamsa)	Jamaloddin et al. (2020)
	Brown planthopper resistance	*Bph3*, *Bph14*, *Bph18*, *Bph32*	InDel	Morphological traits	-[a]	Quasi-BC_2F_2 (qBC_2F_2)	MAS applied for MAGP through MABC (Target variety: Guang 8B)	He et al. (2020)
Wheat (*Triticum aestivum* L.)	Leaf rust resistance	*Lr24*, *Lr28*	SSR, SCAR	42 polymorphic SSR	67.5-94.7	BC_2F_2	MAS applied for MAGP through MABC (Target variety: DWR162)	Yadawad et al. (2017)
	Grain quality and rust resistance	*Yr70/Lr76*, *Lr37/Yr17 /Sr38*, *Gpc-B1/Yr36*, *QPhs.ccsu-3A.1*, *QGw. ccsu-1A.3*, *Lr24/Sr24*, *Glu-A1-1/Glu-A1-2*	Gene-specific marker, SDS-PAGE analysis	-[a]	-[a]	F_5	MAS applied for MAGP (Target variety: PBW343)	Gautam et al. (2020)

Table 2.3 Examples of marker-assisted backcrossing (MABC) and marker-assisted gene pyramiding (MAGP) in several crop species (continued).

Species	Trait(s)	Gene(s)/QTL(s)	Marker(s) used		Range of RPG recovery (%)	selected generation	Remarks	References
			Foreground	Background				
Wheat (*Triticum aestivum* L.)	Grain protein content (GPC)	*Gpc-B1*	SSR	106 polymorphic SSR	88.4-92.3	BC_2F_3	MAS applied for MABC (Target variety: HUW468)	Vishwakarma et al. (2014)
Maize (*Zea mays* L.)	Quality protein maize (QPM)	*opaque-2*	Gene-specific marker	160 polymorphic SSR	80-92.85	BC_2F_1	MAS applied for MABC (Target variety: BML-6)	Krishna et al. (2017)
	Quality protein maize (QPM)	*opaque-2, opaque-16*	Gene-specific marker, SSR	138-152 polymorphic SSR	81.3-95.7	BC_1F_2, BC_2F_2	MAS applied for MAGP through MABC (Target varieties: HKI161, HKI163, HKI193-1, and HKI193-2)	Sarika et al. (2018)
	β-carotene	*crtRB1*	SSR, functional marker	82, 161 polymorphic SSR	88.3-93.3, 88.0-91.1	BC_2F_1	MAS applied for MABC (Target varieties: CBML6 and CBML7)	Sagare et al. (2019)
Soybean (*Glycine max* L. Merr.)	Soybean mosaic virus resistance	R_{SC4}, R_{SC8}, R_{SC14Q}	SSR	-[a]	-[a]	F_6-F_7	MAS applied for MAGP (Target variety: Nannong 1138-2)	Wang et al. (2017)
	β-conglycinin	*cgy-2*	SDS-PAGE analysis	98 polymorphic SSR	91.3-99.5	BC_3F_2	MAS applied for MABC (Target variety: Dongnong47)	Song et al. (2014)
	Null allele of Kunitz trypsin inhibitor (KTI)	*kti*	SSR	93, 81 polymorphic SSR	93.0-98.9, 83.3-91.7	BC_2F_1	MAS applied for MABC (Target varieties: DS9712 and DS9814)	Maranna et al. (2016)
Common bean (*Phaseolus vulgaris* L.)	White mold resistance	*Phs-QTL*	SCAR	25 polymorphic SSR	83-95.2	BC_2F_1	MAS applied for MABC (Target variety: M20)	Carneiro et al. (2010)
	anthracnose resistance	*Co-5, Co-4*[2]	SCAR	-[a]	-[a]	$BC_1F_{1:2}$	MAS applied for MABC (Target variety: Andean Climbing Beans)	Garzón et al. (2008)
Tomato (*Solanum lycopersicum* L.)	Multiple disease resistance	*Ph-2, Ph-3, Bwr-12, Ty-2, Ty-3, I2, Sm, Tm2*[2]	CAPS, SCAR	-[a]	-[a]	$F_{7:8}$	MAS applied for MAGP (Target variety:fresh market tomato)	Hanson et al. (2016)
Chickpea (*Cicer arietinum* L.)	Fusarium Wilt and Ascochyta Blight	*foc1, ABQTL-I, ABQTL-II*	SSR	40, 43 polymorphic SSR	90.0-98.0, 80.0-90.0	BC_3F_1	MAS applied for MABC (Target variety: C 214)	Varshney et al. (2014)

[a] There is no description.

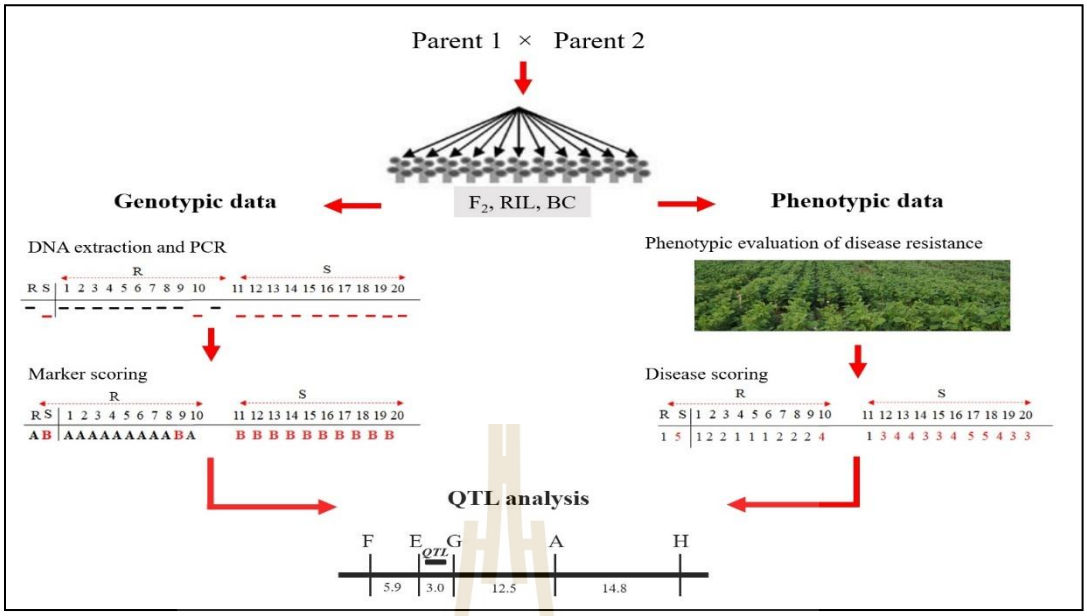

Figure 2.2 Construction of a QTL analysis for disease resistance modified from Collard et al. (2005).

2.5.1 Mapping populations

The segregation of plants with traits of interest in a population is required for linkage map construction and QTL mapping. The parents selected for generating segregating populations need to be polymorphic. The population sizes used in genetic mapping studies range from 50 to 250 individuals (Mohan et al., 1997). Several types of populations can be used for mapping construction i.e., F_2, backcross (BC), recombinant inbred line (RIL), and doubled haploid (DH) populations. Each type of population is generated by different procedures (Figure 2.3). F_2 and BC populations are the simplest types, easy to construct and required only a short time to generate. F_2 population which derived from selfing of F_1 hybrids while BC population derived by a cross between F_1 and one of the parents. RIL population derived from the selfing of F_2 several times (6-8 generations) which consist of a series of homozygous lines, and

containing a unique combination of chromosomal segments from the parents. DH population generated by the induction of chromosome doubling from pollen grains and regenerating plants. The advantages of RIL and DH populations are that they generate homozygous plant which can be multiplied as well as reproduced without genetic change occurring. This provides opportunity for conducting replicated trials in different locations and years (Collard et al., 2005). Three of these population types have been used in mungbean studies for different traits and crosses including F_2, RIL and BC populations (Fatokun et al., 1992; Young et al., 1992; Kaga and Ishimoto, 1998; Lambrides et al., 2000; Chaitieng et al., 2002; Humphry et al., 2005; Mei et al., 2009; Kasettranan et al., 2010; Chankaew et al., 2011; Chotechung et al., 2011; Kajonphol et al., 2012; Isemura et al., 2012; Chankaew et al., 2013; Chen et al., 2013; Dhole and Reddy, 2013; Kitsanachandee et al., 2013; Arsakit et al., 2017; Poolsawat et al., 2017).

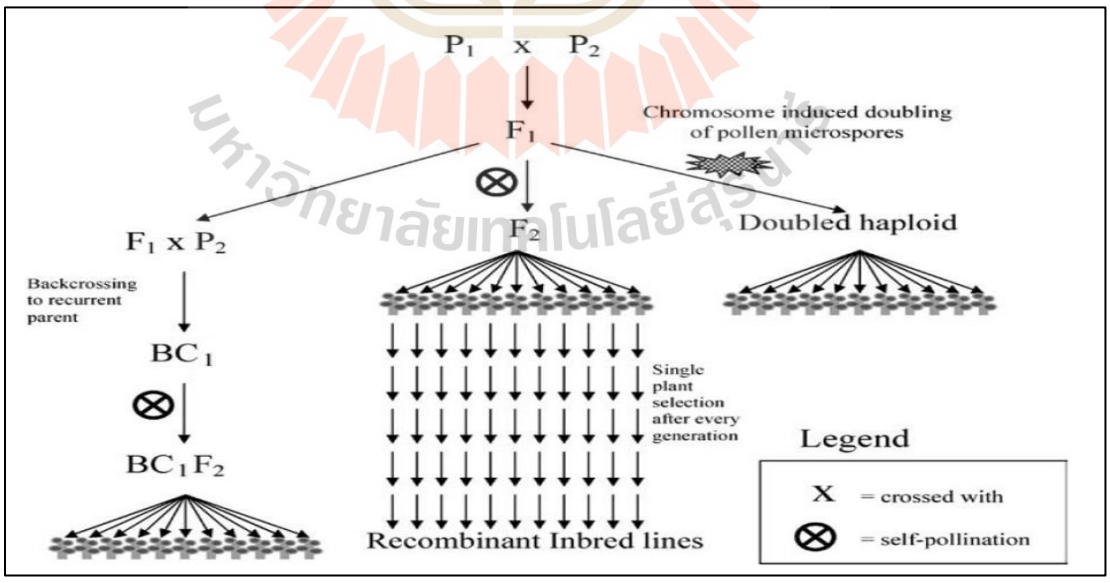

Figure 2.3 Diagram of main types of mapping populations for self-pollinating species (Collard et al., 2005).

2.5.2 Identification of DNA polymorphism

Markers are used for separation of different genotypes based on the presence or absence of a marker locus for dominant markers and based on similarity of DNA fragments between parents and progenies for co-dominant markers. Polymorphisms arose from insertion, deletion, errors in replication of tandem repeated DNA, substitution mutations or changes in number of tandem repeats. The southern hybridization technique and polymerase chain reaction (PCR) technique are basic methods to detect the polymorphisms. The polymorphic markers are widely used for QTL analysis. However, the number of polymorphic markers depends on genetic variation between parents, marker types, and the number of primers used. Several types of DNA markers have been used to detect the polymorphisms in mungbean studies, especially markers based on PCR such as SSR, ISSR, and ISSR-RGA markers.

Simple sequence repeat (SSR) markers or microsatellites are tandem repeats of short nucleotide motifs (1-6 bp) which are distributed throughout the genome of plants. The SSR primers were designed from the nucleotide sequence flanking regions. These primers are very useful for accurate detection of polymorphic loci in specific locus. SSR markers are characterized as co-dominant markers, having multiple alleles and high reproducibility. In previous studies, Chankaew et al. (2011) reported the polymorphisms when 753 SSR primer pairs were screened. Among these, 496 (65.87%) primer pairs amplified DNA bands of mungbean parents (KPS1 and V4718), 69 out of 496 (13.91%) primer pairs showed polymorphisms. In addition, when Chankaew et al. (2013) used 375 SSR primer pairs that were obtained from previous report, only 76 (20.3%) showed polymorphisms between two mungbean cul-

tivars CN60 and RUM5. Moreover, when a total of 582 SSR primer pairs were screened, 466 out of 582 (80.1%) SSR primer pairs amplified DNA bands of mungbean parents (JP211874 and JP22096 cv. Sukhothai). Among these 220 (47.2%) SSR primer pairs detected polymorphisms (Isemura et al., 2012).

Inter-simple sequence repeat (ISSR) markers used primers that are complementary to tandem repeats and/or contain 1-4 base anchors at 3', 5' or both ends. The amplification of ISSR marker used only one primer to amplify DNA with PCR technique. ISSR markers are characterized as dominant, multi locus markers that are highly reproducible. ISSR-anchored resistance gene analog (ISSR-RGA) marker is based on combination between ISSR and RGA primers to amplify target DNA with PCR technique. The characterization of ISSR-RGA is similar with that of ISSR markers. RGA primers were designed from conserved domain of resistance genes such as leucine-rich repeat (LRR), nucleotide-binding site (NBS), protein kinase. In recent study, Poolsawat et al. (2017) reported the screening of 92 ISSR and 40 ISSR-RGA primers on CN72 and V4718. Seventy five out of 92 (81.52%) ISSR amplified clear bands and produced a total of 1,297 scorable DNA bands. Among these 48 (64%) ISSR primers showed polymorphisms. Meanwhile, all of ISSR-RGA primers successfully amplified DNA bands, but only 8 (20%) ISSR-RGA primers showed polymorphisms and produced new 52 ISSR-RGA DNA bands.

2.5.3 Linkage analysis of markers

Linkage analysis provides a framework for detecting marker that associated with a trait and for choosing markers to use in MAS. Markers linked to the resistance gene can be preliminary identified by bulk segregant analysis (BSA), a method used to determine the association between markers and the traits of interest.

Bulk of DNA was separated into two groups depending on a trait and each bulk DNA contained 5-10 individual plants from a segregating population. These two bulks should differ for a trait of interest such as disease resistance (resistant and susceptible). BSA was performed using DNA of both parents and two bulks. Once polymorphic markers are detected between parents and bulks that may associate with a QTL of interest, they will be used to screen individual plants.

The genetic linkage map can be constructed by MAPMAKER/EXR 3.0b program (Lincoln et al., 1993), JoinMap 3.0 (Van Ooijen and Voorrips, 2001), and QTL IciMapping (Meng et al., 2015). In addition, the main methods for detecting QTLs are single marker analysis, simple interval mapping (SIM), composite interval mapping (CIM), multiple inteval mapping (MIM), and inclusive composite interval mapping (ICIM) (Tanksley, 1993; Liu, 1998; Kao et al., 1999; Li et al., 2007). Single marker analysis is the easiest method for detecting QTL with single marker using simple linear regression (R^2). R^2 is used for explaining the variation between markers and phenotypes. The software WinQTL Cartographer 2.5 program can be used to analyze QTL associated with a trait of interest by SIM, CIM and MIM. While ICIM function can be analyzed by using QTL IciMapping 4.1 software (Meng et al., 2015). This function was more efficient than CIM for background control through a two-step mapping strategy.

2.5.4 Quantitative trait loci (QTL) mapping in mungbean

QTL mapping of several traits of mungbean were constructed using various types of DNA markers in different crosses including agronomic traits, insect resistance and disease resistance. They are of importance for mungbean improvement. QTL associated with agronomic traits such as seed weight, 100-seed weight, pod length,

days to flowering, days to maturity, days to harvest, etc. have been identified in several populations. the QTL and their associated markers are showed in Table 2.4. QTL mapping of these traits is located in different linkage groups depending on mapping populations. For disease resistance, three major diseases consisting of PM, CLS, and MYMV have been mapped (Table 2.5). These three diseases are of the most important factors affecting mungbean production. In addition, QTL for insect resistance and seed damage, caused by a group of pests of the genus *Callosobruchus*, especially azuki bean weevils (*C. chinensis* L.) and cowpea weevils (*C. maculatus* F.), known as bruchids were identified. While bean bug (*Riptortus clavatus* Thunberg) damages young pod and seed in the field condition. These insects are serious pests during reproductive stages to seed storage. QTL associated with bruchid and bean bug was also identified (Table 2.6). These linked markers can be used for selecting of desirable traits in MAS based on populations or parents. MAS allows selection based on polymorphisms of DNA markers to assist phenotypic selection. The advantages of MAS are more efficient, reliable, and cost effective compared to conventional breeding because selection can be performed at the DNA level without confounding effects from the environments.

Table 2.4 QTL mapping for agronomically important traits in mungbean.

Traits	Cross combinations	Populations	Marker type(s)	Number of QTLs	linkage group (LG)	Linked markers	References
Seed weight	VC3890 × TC1966	F_2	RFLP	1	2	pM182-pA124	Fatokun et al. (1992)
	Berken × ACC41	RIL	RFLP	9	1, 2, 4, 5, 9, 10, 11	VrCS207-mgQ062-2, VrCS282-2-VrCS23-2, LpCS304-VrCS225, VrCS215-2-VrCS123, VrCS164-VrCS53, VrCS84-1-mc017-3, mgM213-VrCS161, VrCS375-VrCS323-1, LpCS82-VrCS65	Humphry et al. (2005)
	JP211874 × Sukhothai	BC_1F_1, $BC_1F_{1:2}$	SSR, EST-SSR	2	2, 8	GMES0477-CEDG026a, VM37-CEDG030	Isemura et al. (2012)
	BM1 × BM6	F_2	SSR	4	1, 6, 8, 9	CEDG141-MB-SSR179, VES0987-cp01225, cp01225-CEDG248, cp00228-MBSSR-008, CEDG286-cp00228, VR354-CEDG238	Alam et al. (2014)
100-seed weight	V1725GB × AusTRCF321925	F_2	SSR	5	1, 2, 8, 9, 10	CP1713-CP5137, VR078-CEDG136, CP2470-CLM871, VRSSR010-MB36, CEDG097-CP2142	Sompong et al. (2012)
	KUML29-1-3 × W021	F_2	SSR	6	2, 4, 8, 9, 11	VR078-CEDG065, VR17-VR0200, VR03660-VR035, VR-SSR031-VR0225, CEDG259-CEDG166, MB-SSR104-VR-SSR011	Kaionphol et al. (2012)
	JP211874 × Sukhothai	BC_1F_1, $BC_1F_{1:2}$	SSR, EST-SSR	3	2, 8, 11	GMES0477-CEDG026a, VM37-CEDG030, GMES3893a-BM149	Isemura et al. (2012)
	NM92 × TC1966	RIL	RAPD, AFLP, SCAR, CAP, SSR	3	1, 3, 9	m9pag242, m1pcg351, w02s8	Chen et al. (2013)
Pod length	JP211874 × Sukhothai	BC_1F_1, $BC_1F_{1:2}$	SSR, EST-SSR	1	7	CEDG064-CEDG174	Isemura et al. (2012)
	KUML29-1-3 × W021	F_2	SSR	2	7, 8	CEDG111-VR0126, VR-SSR005-VR-SSR031	Kaionphol et al. (2012)

Table 2.4 QTL mapping for agronomically important traits in mungbean (continued).

Traits	Cross combinations	Populations	Marker type(s)	Number of QTLs	linkage group (LG)	Linked markers	References
Seeds per pod	JP211874 × Sukhothai	BC_1F_1, $BC_1F_{1:2}$	SSR, EST-SSR	2	1, 9	CEDG220-GMES4400, CEDG166-GATS11	Isemura et al. (2012)
	KUML29-1-3 × W021	F_2	SSR	2	1	VR-SSR015-VR-SSR018, VR0194-VR0198	Kaionphol et al. (2012)
Pods per plant	JP211874 × Sukhothai	BC_1F_1, $BC_1F_{1:2}$	SSR, EST-SSR	2	2, 4	CEDG096a-GMES0216b, GMES0216a-GMES1216a	Isemura et al. (2012)
Pod width	JP211874 × Sukhothai	BC_1F_1, $BC_1F_{1:2}$	SSR, EST-SSR	2	7, 8	CEDG064-CEDG174, VM37-CEDG030	Isemura et al. (2012)
Seed length	JP211874 × Sukhothai	BC_1F_1, $BC_1F_{1:2}$	SSR, EST-SSR	2	2, 8	GMES0477-CEDG026a, VM37-CEDG030	Isemura et al. (2012)
Seed thickness	JP211874 × Sukhothai	BC_1F_1, $BC_1F_{1:2}$	SSR, EST-SSR	3	2, 3, 8	GMES0477-CEDG026a, GMES6583-GMES0294a, VM37-CEDG030	Isemura et al. (2012)
Hard seededness	Berken × ACC41	RIL	RFLP	2	1, 11	cgO103-VrCS364, VrCS65-VrCS73	Humphry et al. (2005)
Days to flowering	KUML29-1-3 × W021	F_2	SSR	4	2, 4, 11	VR0364, CEDG241-VR-SSR019, DMB-SSR199-CEDG107, VR0216-CEDG168	Kaionphol et al. (2012)
Days to maturity	KUML29-1-3 × W021	F_2	SSR	3	2, 4	VR0364, CEDG241-VR-SSR019, DMB-SSR199-CEDG107	Kaionphol et al. (2012)
Days to harvest	KUML29-1-3 × W021	F_2	SSR	3	2, 4	VR0364, CEDG241-VR-SSR019, VR0313	Kaionphol et al. (2012)
Seed germination rate	NM92 × TC1966	RIL	RAPD, AFLP, SCAR, CAP, SSR	3	7, 9	mg1pga193, w02s8, m3pca314	Chen et al. (2013)

Table 2.5 QTL mapping for disease resistance in mungbean.

Traits	Cross combinations	Populations	Marker type(s)	Number of QTLs	linkage group (LG)	Linked markers	References
PM	TC1966 × VC3980A	F_2	RFLP	3	3, 7, 8	sgK472, mgM208, mgQ39	Young et al. (1993)
	TC1966 × VC1210A	F_2	RFLP, AFLP	1	-[a]	Mac71a-Mac114	Chaitieng et al. (2002)
	Berken × ATF3640	RIL	RFLP	1	9	VrCS65-VrCS296	Humphry et al. (2003)
	KPS1 × VC6468-11-1A	RIL	SSR	2	1, 2	CEDG282-CEDG191, MBSSR238-CEDG166	Kasettranan et al. (2010)
	KPS1 × V4718	F_2	SSR	3	4,9	CEDC055-DMBSSR199, CEDG232-DMBSSR167, VRSSR010-DMBSSR130	Chankaew et al. (2013)
	CN 60 × RUM5	F_2, BC_1F_1	SSR	3	4, 6, 9	CEDC055-CEDG154, CEDG159-CEDG121, CEDG070-CEDG259	Chankaew et al. (2013)
	CN 72 × V4718	RIL	ISSR, ISSR-RGA	1	-[a]	I85420-I42PL229	Poolsawat et al. (2017)
	CN 72 × V4785	RIL	ISSR, ISSR-RGA	1	-[a]	I27R211 and I27R565	Tantasawat et al. (2021)
CLS	KPS1 × V4718	F_2, BC_1F_1	SSR	1	3	VR393-CEDG117	Chankaew et al. (2011)
	CN72 × V4718	RIL	SSR	1	3	VR393-CEDG084	Arsakit et al. (2017)
	CN72 × V4718	RIL	SSR, ISSR, ISSR-RGA	1	3	VR393-I16274	Tantasawat et al. (2020)
	KPS1 × V4718	F_2, BC_8F_2	SSR, InDel	1	3	Vr6gCLS085-VrTAF5_indel	Yundaeng et al. (2020)
MYMV	NM92 × TC1966	RIL	RAPD, AFLP, SCAR, CAP, SSR	4	7,8,9	v02a7, mg3pat423, m4pcc585, 9DMB158	Chen et al. (2013)
	KPS2 × NM10-12-1	RIL	AFLP, SSR	5	2, 4, 6, 9A	CEDG100-cp02662, DMB-SSR008-VR113, CEDG121-CEDG191, CEDG166-CEDG304	Kitsanachandee et al. (2013)
	BM1 × BM6	F_2, BC_1F_1	RGA, SCAR, SSR	2	2, 7	CEDG275-CEDG006, CEDG041-VES503	Alam et al. (2014)

[a] There is no description.

Table 2.6 QTL mapping for insect resistance in mungbean.

Traits	Cross combinations	Populations	Marker type(s)	Number of QTLs	linkage group (LG)	Linked markers	References
Bruchid	NM92 × TC1966	RIL	RAPD, AFLP, SCAR, CAP, SSR	3	7,9	mg7pgc325, mg3pat361, 9DMB158	Chen et al. (2013)
	Sunhwa × Jangan	F_2	RAPD, CAP, SSR, STS	2	-[a]	RP-COPU06, MB87-COPU06	Hong et al. (2015)
	TC1966 × NM92	RIL	SNP	1	5	5:5,178,332-5:6,944,902	Schafleitner et al. (2016)
	Jilyu7 × V1128	F_2	SSR, EST-SSR, STS, InDel	1	5	DMB158-VRBR-SSR033	Liu et al. (2018)
Bean bug	Sunhwa × Jangan	F_2	RAPD, CAP, SSR, STS	1	-[a]	RP-COPU06	Hong et al. (2015)

[a] There is no description.

2.6 References

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CHAPTER III

IDENTIFICATION AND VALIDATION OF MOLECULAR MARKERS LINKED TO CERCOSPORA LEAF SPOT DISEASE RESISTANCE IN MUNGBEAN

3.1 Abstract

Cercospora leaf spot (CLS) is widespread and causes significant economic loss for mungbean production in Thailand. In this study, we verified DNA polymorphism in bulk segregant analysis (BSA) and identified CLS resistance gene in mungbean using ISSR, ISSR-RGA, SSR, and InDel markers, a total of 156 primers/primer pairs were used to detect polymorphisms with BSA, consisting of 68 ISSR primers, 48 ISSR-RGA primer pairs, 39 SSR primer pairs, and 1 InDel primer pair. Among these, twenty-eight markers (14 ISSR, 7 ISSR-RGA, 6 SSR, and 1 InDel) were found to be putatively associated with the CLS resistance in BSA. Simple linear regression confirmed that 5 out of 28 markers (I16274, I88656, I35P716, CEDG008, and VrTAF5_Indel) were significantly associated with the CLS resistance gene with a LOD score of more than 3 in both 2016 and 2018. When these five markers together with markers VR393 and CEDG084 were used for quantitative trait loci (QTL) analysis, inclusive composite interval mapping (ICIM) identified a major QTL (*qCLSC72V18-1*), accounting for 32.86% to 41.56% of the phenotypic variation depending on years and flanked between I16274 and VrTAF5_Indel markers at the distance of 4.0 and 5.0 cM from the QTL, respectively. In addition, when using both I16274 and VrTAF5_Indel markers

to assist selection of CLS resistance in MAS, only 0.40% recombination of both markers with the CLS resistance gene will occur. These markers could be useful in future breeding for CLS resistance in mungbean.

3.2 Introduction

Mungbean [*Vigna radiata* (L.) Wilczek] is an economically important legume crop in Asian countries where over 90% of it in the world is cultivated, mainly in India, China, Myanmar, Indonesia, Pakistan, Bangladesh, and Thailand (Ruanpanun and Somta, 2021). It is utilized in several ways; for example, mungbean seeds containing starch, minerals, vitamins, and amino acids are used in industrial foods (vermicelli and starch) and cosmetics, whereas sprouts and young pods are eaten as vegetables. The leaves and stems are used as forage and green manure. However, production is dramatically constrained by various factors, i.e., susceptibility to pests and diseases or weakness to environments.

CLS, a serious foliar disease capable of inciting leaf spot and defoliation, is caused by *Cercospora canescens* Ellis & Martin (Chand et al., 2015). The disease spreads to mungbean fields, particularly in the warm-wet growing season, and often leads to 50% economic losses if there is no protection (AVRDC, 1984). Deployment of resistant varieties is the most efficient and durable strategy for integrated disease management. By screening of mungbean genotypes resistant to CLS from several countries, Nair et al. (2019) reviewed that there were several genotypes showing resistance to CLS i.e., NM-1, NM-2, NM-98, BRM-188, BARIMung-2, C2/94-4-42, 98-cmg-003, 98cmg-018, Basanti, PDM-11, CO-3, and VC3960-88 (Iqbal et al., 2004), M5-22, and M5-25 (Wongpiyasatid et al., 1999), and V1471, V2773, V2757, V5036,

and V4718 (Hartman et al., 1993), etc. However, only a few resources have been identified to provide high and stable resistance to CLS, including V4718 (Hartman et al. 1993). Moreover, our previous studies also found this resistance pattern in V4718 (Arsakit et al., 2017). Genetic inheritance of CLS resistance has been reported elsewhere, which is either controlled by qualitative (Mishra et al., 1988; Chankaew et al., 2011) or quantitative genes (AVRDC, 1980; Leabwon and Oupadissakoon, 1984), depending on resistance source. Identification of the CLS resistance has been reported using various mapping populations. Chankaew et al. (2011) identified a major QTL controlling resistance to CLS using simple sequence repeat (SSR) markers in a cross between KPS1 (susceptible variety) and V4718 (resistant line). This QTL was flanked between CEDG117 and VR393 markers. Using this marker system, our previous report revealed a major QTL associated with CLS resistance in another cross generated by hybridizing CN72 (susceptible variety) and V4718 which was located between VR393 and CEDG084 markers (Arsakit et al., 2017). Both markers flanked the gene at a distance of 4-6 cM. Therefore, finding additional markers closer to the gene is crucial for efficient selection. Recently, genome/transcriptome sequencing throughout the genome has evolved which influence a trend away from structural markers to functional markers (Poczai et al., 2013). Functional markers located on or near any genes are very useful for the selection. Using this technique in the KPS1 × V4718 cross, Yundaeng et al. (2020) identified TATA-binding-protein-associated factor 5 (TAF5), a subunit of transcription initiation factor IID and Spt-Ada-Gcn5 acetyltransferase complexes, which is encoded by a candidate gene responsible for CLS resistance (*VrTAF5*). Moreover, they also found two markers, InDel (VrTAF5_indel) and SSR (Vr6CLS085) markers, that flanked the functional gene *VrTAF5*, and the distance of these

VrTAF5_Indel and Vr6CLS085 markers was only 12 and 13 Kb from the *VrTAF5* which was closer than those previously identified flanking markers CEDG117 and VR393. The markers associated with the CLS resistance can be used to accelerate the development of a new resistant variety(s) by year-round selection through MAS.

In this study, we verified DNA polymorphism in bulk segregant analysis (BSA) and refined QTL mapping of a gene conferring resistance to CLS in recombinant inbred line (RIL) population of mungbean derived from a cross between a susceptible cultivated variety (Chai Nat 72; CN72) and a resistant line (V4718).

3.3 Materials and Methods

3.3.1 Plant materials and DNA extraction

The population of 143 $F_{2:9}$ and $F_{2:10}$ RILs of the CN72 × V4718 cross was obtained from Khajudparn (2009), who developed the RIL population via single seed descent method. Chai Nat 72 (CN72) is a susceptible cultivated variety with a high yield in Thailand, while V4718 is a resistant line which was obtained from the World Vegetable Center. Genomic DNA of parents and each RIL was extracted from fresh young leaves of 2-week-old seedlings by using a modified CTAB extraction protocol of Lodhi et al. (1994). The concentration and purity of DNA were quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA).

3.3.2 Evaluation for Cercospora leaf spot (CLS) resistance

The $F_{2:9}$ and $F_{2:10}$ RILs of the CN72 × V4718 cross and their parents were grown during the rainy season (May to August) in 2016 and 2018 for evaluating their resistance to CLS in a field at Suranaree University of Technology, Nakhon Ratchasima, Thailand. It was conducted in a randomized complete block design

(RCBD) with three replications. In each block, seeds of each RIL were sown in a single row of 2 m long with a spacing of 20 and 50 cm for inter-row and intra-row, respectively. Two plants per hill (ca. 20 plants per row) were kept. The variety CN72 was sown around the experimental blocks as a source of CLS inoculum. All RIL and their parents were observed for disease severity at 65 days after planting (DAP) using a scoring system described by Chankaew et al. (2011) as follow: 1 = no visual disease infection, 2 = 1-25% leaf area infected, 3 = 26-50% leaf area infected, 4 = 51-75% leaf area infected and 5 = 76-100% leaf area infected (Figure A.1). The scale of disease severity was divided into two categories (resistant with a score rating 1-2.9 and susceptible with a score rating 3-5). The CLS scores from the field evaluation were transformed with $(X+1)^{1/2}$ formula. The significant difference of mean was evaluated by Duncan's Multiple Range Test (DMRT) to compare means of disease severity score by using SPSS version 14.0 (Levesque and SPSS Inc., 2006). The distribution of CLS resistance gene in RIL population was determined by chi-square (χ^2) test.

3.3.3 DNA marker analysis

3.3.3.1 ISSR and ISSR-RGA analysis

A total of 68 ISSR primers and 48 ISSR-RGA primer pairs were used to initially screen with bulk segregant analysis (BSA) technique. It was carried out using DNA of resistant (V4718; R) and susceptible (CN72; S) parents and resistant bulk (RB) and susceptible bulk (SB). The same concentration and volume of DNA pooled from 10 $F_{2:7}$ plants showing the highest resistance and susceptibility to CLS was used to constitute RB and SB, respectively. In addition, the representative of both resistant bulk and susceptible bulk was selected by using disease scores at field conditions. Among them, 68 ISSR primers were designed from the complementary of SSR repeats

or microsatellite with added anchor nucleotides. They were developed from University of British Columbia (UBC), Canada (Table 3.2). In addition, forty-eight ISSR-RGA primer pairs were developed by a combination of 4 RGA primers with 12 ISSR primers (Table 3.3). Four RGA primers were designed from the complementary sequences of the conserved protein domains of RGAs consisting of kinase domain (Pto kin 1 [Chen et al., 1998] and RLK for [Feuillet et al., 1997]) and NBS (GLPLAL 1 and P-Loop [Mahanil, 2007]). Polymerase chain reaction (PCR) for ISSR and ISSR-RGA analysis was performed in 20 µl reaction mix containing 150 ng of genomic DNA template, 1× buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.1, 0.01% Triton^{TM} X-100), 3.5 mM $MgCl_2$, 250 µM of each dNTPs, 1 unit of *Taq* DNA polymerase, 0.4 µM of each ISSR primer and 10 µM of each RGA primer (only ISSR-RGA). PCR amplification was performed in a T100^{TM} Thermal Cycler (Bio-Rad Laboratories, Inc., California, USA) programmed as follow: initial denaturation at 95^{o}C for 5 min; 35 cycles of denaturing at 95^{o}C for 1 min, annealing at 50^{o}C for 1 min, extension at 72^{o}C for 1 min; and final extension at 72^{o}C for 10 min.

3.3.3.2 SSR and InDel analysis

Thirty-nine SSR and one InDel primer pairs were also screened with BSA. For SSR primers, there were obtained from azuki bean [*Vigna angularis* (Willd.) Ohwi & Ohashi], mungbean [*V. radiata* (L.) Wilczek] and cowpea [*V. unguiculata* (L.) Walp.] (Wang et al., 2004; Han et al., 2005; Somta et al., 2009; Tangphatsornruang et al., 2009; Seehalak et al., 2009; Kongjaimun et al., 2012; Yundaeng et al., 2020) (Table 3.4). In addition, one InDel primer pair specific to 24 bp deletion which was designed from intron 7 of *VrTAF5 (LOC106765332)* in V4718 line was derived from Yundaeng et al. (2020). PCR for SSR and InDel analysis was

performed in 20 µl reaction mix containing 2 ng of genomic DNA, 1× buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.1, 0.01% Triton^{TM} X-100), 2 mM $MgCl_2$, 0.2 mM of each dNTPs, 1 unit of *Taq* DNA polymerase, and 0.5 µM each of forward and reverse primers. PCR amplification was performed in a T100^{TM} Thermal Cycler (Bio-Rad Laboratories, Inc., California, USA) programmed as follow: initial denaturation at 94^{o}C for 2 min; 35 cycles of denaturing at 94^{o}C for 30 s, annealing at 50-65^{o}C for 30 s, extension at 72^{o}C for 1 min and final extension at 72^{o}C for 10 min.

3.3.4 PCR product detection

The PCR products were separated on 6% denaturing polyacrylamide gel and detected by silver nitrate staining according to Sambrook and Russell (2001). The size of the DNA bands was estimated by using 100 bp DNA Ladder (Invitrogen, CA, USA). The DNA patterns between parents (R and S) and DNA bulk (RB and SB) of each primer were observed to identify the polymorphic loci. In addition, number of polymorphic bands and linked loci of ISSR and ISSR-RGA markers were recorded. These putative markers were then used to analyze individual RIL to verify the linkage with CLS resistance. The DNA band of ISSR and ISSR-RGA was scored with the presence of DNA products as "1" and the absence as "0", while SSR marker was divided into three categories consisting of 1 = DNA fragment is homozygous for resistant parent (V4718) allele, 2 = DNA fragment is heterozygous, and 3 = DNA fragment is homozygous for susceptible parent (CN72) allele.

3.3.5 Linkage and QTL analysis

DNA band scoring of each marker was used for estimation of the correlation between marker and CLS score using 2016 and 2018 data by simple linear regression, recombination calculation, chi-square test, and logarithm of odd (LOD)

analysis. In previous study, the *qCLSC72V18* conferring CLS resistance in V4718 line was flanked between VR393 and CEDG084 (Arsakit et al., 2017). Thus, we also used these two markers to construct a genetic linkage map in this study. Markers associated with CLS resistance were used for constructing the genetic linkage map using the software QTL IciMapping 4.1 (Meng et al., 2015). They were assigned to the linkage group (LG) with a minimum LOD of 3.0 and ordered markers based on their positions on the reference genome. The calculation of genetic distance was performed using the Kosambi mapping function (Kosambi, 1994). Location of the QTL associated with CLS resistance was determined by using QTL IciMapping 4.1 software with an inclusive composite interval mapping (ICIM) function (Li et al., 2007). The permutation tests were run 10,000 times at $P = 0.01$ to determine the LOD score threshold for the QTL. In addition, LG map and QTL in this study were compared to previous studies.

3.4 Results

3.4.1 Evaluation of Cercospora leaf spot resistance

A total of 143 $F_{2:9}$ and $F_{2:10}$ RILs of the CN72 × V4718 cross and their parents were evaluated for CLS resistance at 65 days after planting in 2016 and 2018, respectively. In 2016, the CLS resistant line V4718 had a mean disease score of 1.67 while the susceptible variety CN72 had a score of 4.00. In 2018, the CLS resistant line V4718 had a mean disease score of 1.00 while the susceptible variety CN72 had a score of 4.00. The disease scores were significantly different between the parents and 143 $F_{2:10}$ RILs ($P < 0.01$). The correlation coefficient (r) between the CLS disease scores of both years was 0.479 ($P < 0.01$), suggesting that they were significantly correlated. The difference may be affected by environment. Frequency distribution of $F_{2:9}$ and $F_{2:10}$

RILs progenies from the cross deviated from normality with skewness toward the parents (Figure 3.1). From Table 3.1, 80 were resistant and 63 were susceptible in 2016, and 83 were resistant and 60 were susceptible in 2018. The segregation ratio of 1:1 (resistant: susceptible) in both years (χ^2 = 2.02 and 3.70, respectively, $P_{0.05}$ = 3.84) was observed for this population. The results indicated that the resistance to CLS conferred by V4718 in this cross was controlled by a single dominant gene.

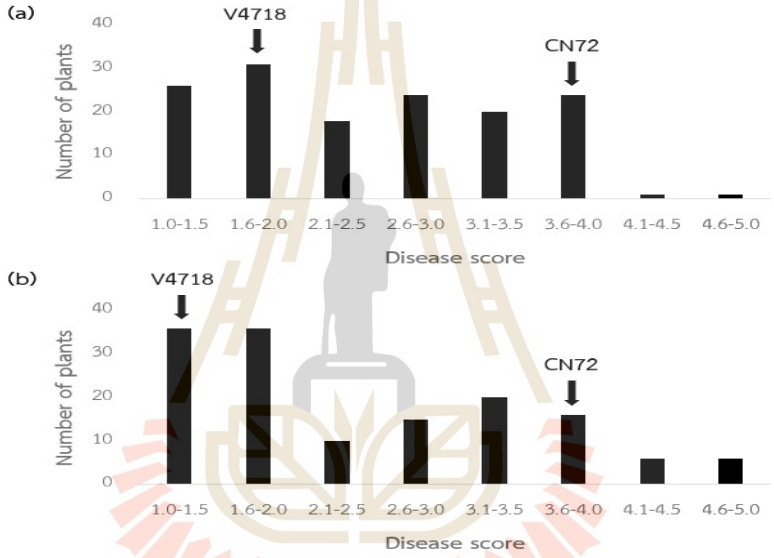

Figure 3.1 Frequency distribution of disease scores for response to Cercospora leaf spot of mungbean in CN72 × V4718 cross, (a) the $F_{2:9}$ RILs population evaluated in 2016, (b) the $F_{2:10}$ RILs population evaluated in 2018.

Table 3.1 Segregation in reaction to Cercospora leaf spot in $F_{2:9}$ and $F_{2:10}$ RILs populations derived from CN72 × V4718 cross.

Populations	Year	No. of lines	No. of resistant: susceptible lines	Chi-square test		
				Expected ratio	χ^2 value[a]	$P = 0.05$[b]
$F_{2:9}$	2016	143	80:63	1:1	2.02	3.84
$F_{2:10}$	2018	143	83:60	1:1	3.70	3.84

[a] The χ^2 value was tested for goodness of fit against 1:1 ratio for RILs.
[b] $P = 0.05$ the differential levels of chi-square test for the resistance/susceptibility ratios with the probabilities of 95%.

3.4.2 ISSR and ISSR-RGA analysis

A total of 68 ISSR and 48 ISSR-RGA primers/primer pairs were used in BSA to identify polymorphic markers between CN72 and V4718, CLS resistant bulk and CLS susceptible bulk. All of 68 ISSR primers produced reproducibly clear bands by using the annealing temperature of 50°C. They amplified a total of 1344 scorable DNA bands ranging from 11 (ISSR 815, 819 and 843) to 38 (ISSR 841) bands per primer with an average of 20 bands per primer. Among these, 40 (58.8%) primers showed polymorphisms between CN72 and V4718 and amplified a total of 68 polymorphic DNA bands ranging from one to six bands with a mean of 1.0 polymorphic bands per primer, and of these bands, 14 bands were putatively associated with CLS resistance with a mean of 0.21 bands per primer (Table 3.2). These 14 bands putatively associated with CLS resistance were amplified by 9 ISSR primers (ISSR 814, 816, 818, 827, 830, 835, 841, 884, and 888).

Table 3.2 Primer sequences, range of amplified products, number of scorable DNA bands, number of polymorphic bands between parents, number of loci associated with Cercospora leaf spot (CLS) resistance of 68 ISSR primers in BSA obtained from a cross between CN72 and V4718.

Primers	Primer sequences[a]	Range of amplified products (bp)	Number of scorable DNA bands	Number of polymorphic bands (male-female parents)	I-linked[b]
807	$(AG)_8T$	200-1500	28	1	0
808	$(AG)_8C$	200-1500	26	0	0
809	$(AG)_8G$	200-1500	27	2	0
810	$(GA)_8T$	200-1500	30	1	0
811	$(GA)_8C$	200-1000	22	3	0
812	$(GA)_8A$	200-1200	19	1	0
813	$(CT)_8T$	300-2072	16	1	0
814	$(CT)_8A$	200-1200	12	1	1
815	$(CT)_8G$	250-1200	11	0	0
816	$(CA)_8T$	200-1500	23	2	1
817	$(CA)_8A$	250-1500	16	1	0
818	$(CA)_8G$	200-1500	20	5	2
819	$(GT)_8A$	500-1400	11	0	0
820	$(GT)_8C$	200-2072	17	0	0

Table 3.2 Primer sequences, range of amplified products, number of scorable DNA bands, number of polymorphic bands between parents, number of loci associated with Cercospora leaf spot (CLS) resistance of 68 ISSR primers in BSA obtained from a cross between CN72 and V4718 (continued).

Primers	Primer sequences[a]	Range of amplified products (bp)	Number of scorable DNA bands	Number of polymorphic bands (male-female parents)	I-linked[b]
821	$(GT)_8T$	300-1500	14	1	0
823	$(TC)_8C$	250-2072	15	0	0
824	$(TC)_8G$	200-2072	13	0	0
825	$(AC)_8T$	250-1500	26	2	0
826	$(AC)_8C$	200-1200	19	0	0
827	$(AC)_8G$	200-1500	22	2	1
828	$(TG)_8A$	300-1200	12	1	0
829	$(TG)_8C$	300-1200	13	0	0
830	$(TG)_8G$	200-1500	27	2	1
834	$(AG)_8YT$	200-1200	24	1	0
835	$(AG)_8YC$	150-1000	27	4	2
836	$(AG)_8YA$	200-1000	23	1	0
841	$(GA)_8YC$	200-1500	38	2	1
841c	$(GA)_8CC$	200-1000	23	2	0
841t	$(GA)_8TC$	250-1200	18	0	0
842	$(GA)_8YG$	200-1300	32	0	0
843	$(CT)_8RA$	200-2072	11	1	0
844	$(CT)_8RC$	300-1200	14	0	0
846	$(CA)_8AT$	200-1500	14	0	0
847	$(CA)_8RC$	300-1200	15	1	0
848	$(CA)_8RG$	400-1200	14	0	0
849	$(GT)_8YA$	150-2072	14	2	0
850	$(GT)_8YC$	250-1000	19	0	0
851	$(GT)_8YG$	200-1000	19	0	0
853	$(TC)_8RT$	200-1200	12	1	0
854	$(TC)_8RG$	200-2072	12	0	0
855	$(AC)_8YT$	350-1200	15	0	0
856	$(AC)_8YA$	250-1500	24	0	0
857	$(AC)_8YG$	200-1500	25	1	0
858	$(TG)_8RT$	200-1500	20	1	0
859	$(TG)_8RC$	200-1200	19	1	0
860	$(TG)_8RA$	200-2072	29	1	0
861	$(ACC)_6$	100-2072	18	0	0
862	$(AGC)_6$	150-1000	12	0	0
864	$(ATG)_6$	200-1000	20	1	0
865	$(CCG)_6$	200-2072	15	0	0
866	$(CTC)_6$	200-1300	15	2	0
867	$(GGC)_6$	100-1000	12	0	0
868	$(GAA)_6$	200-1500	24	1	0
869	$(GTT)_6$	200-2072	17	0	0
873	$(GACA)_4$	350-1200	15	0	0
876	$(GATA)_2$ $(GACA)_2$	400-1200	14	2	0
878	$(GGAT)_4$	300-1500	16	1	0
880	$(GGAGA)_3$	200-1200	24	2	0
881	$(GGGTG)_3$	200-2072	20	0	0
884	HBH $(AG)_7$	200-2072	31	6	2
885	BHB $(GA)_7$	200-1000	23	1	0
886	VDV $(CT)_7$	250-2072	25	2	0
887	DVD $(TC)_7$	250-2072	26	1	0
888	BDB $(CA)_7$	200-1000	23	3	3

Table 3.2 Primer sequences, range of amplified products, number of scorable DNA bands, number of polymorphic bands between parents, number of loci associated with Cercospora leaf spot (CLS) resistance of 68 ISSR primers in BSA obtained from a cross between CN72 and V4718 (continued).

Primers	Primer sequences[a]	Range of amplified products (bp)	Number of scorable DNA bands	Number of polymorphic bands (male-female parents)	I-linked[b]
889	DBD $(AC)_7$	200-1200	16	0	0
890	VHV $(GT)_7$	200-1500	29	1	0
891	HVH $(TG)_7$	200-1000	24	0	0
900	ACTTCCC$(CA)_2$GGT TA$(CA)_2$	200-2072	25	0	0
Total	68 primers		1344	68	14
Average			20	1.00	0.21

[a] B = C, G, T; D = A, G, T; H = A, C, T; N = A, G, C, T; R = purines (A, G); V = A, C, G; Y = pyrimidines (C, T).
[b] I-linked = the DNA pattern between resistant parent (R) and resistant bulk (RB) as well as susceptible parent (S) and susceptible bulk (SB) are similar.

Meanwhile, a similar approach was also used with 48 ISSR-RGA primer combinations between 12 ISSR primers and 4 RGA primers. The results are presented in Table 3.3. We found that these 12 ISSR primers and 48 ISSR-RGA primer combinations amplified a total of 1206 and 1158 scorable DNA bands, respectively. When all 48 ISSR-RGA primer combinations were evaluated, it was found that the number of amplified ISSR-RGA bands depends on their combinations of ISSR and RGA primers. Of these 1158 scorable DNA bands, 84 ISSR-RGA loci were found as new genomic loci (not found when using ISSR alone) which were amplified by 36 ISSR-RGA combinations. The remaining 12 ISSR-RGA combinations did not produce new genomic loci. The largest number of new ISSR-RGA loci (6 loci) was amplified by ISSR 808+Pto kin 1 and ISSR 810+Pto kin 1 combinations. In addition, out of 84 ISSR-RGA loci, 20 ISSR-RGA loci (23.8%) were polymorphic. These polymorphic loci were amplified by 14 primer combinations; ISSR 808+GLPLAL 1, ISSR 808+Pto

kin 1, ISSR 809+GLPLAL 1, ISSR 809+RLK for, ISSR 810+P-Loop, ISSR 810+Pto kin 1, ISSR 827+P-Loop, ISSR 827+Pto kin 1, ISSR 835+Pto kin 1, ISSR 835+RLK for, ISSR 841c+GLPLAL 1, ISSR 856+Pto kin 1, ISSR 888+Pto kin 1, and ISSR 891+GLPLAL 1. BSA identified a total of 7 new genomic loci of ISSR-RGA as possibly associated with CLS resistance. These were amplified using five primer combinations of ISSR 808+GLPLAL 1, ISSR 827+P-Loop, ISSR 835+Pto kin 1, ISSR 835+RLK for, and ISSR 856+Pto kin 1 (Table 3.3).

3.4.2 SSR and InDel analysis

A total of 39 SSR markers and 1 InDel marker were also used in BSA. The optimal annealing temperatures of all primers were presented in Table 3.4. Among these, three SSR markers (CEDAAG004, CEDG063, and CEDG294) failed to produce consistent results and were omitted from further experiment. Fourteen SSR markers and one InDel marker, including CEDC055, CEDG002, CEDG006, CEDG008, CEDG014, CEDG024, CEDG044, CEDG051, CEDG056, CEDG070, CEDG154, CEDG304, Vr6gCLS037, Vr6gCLS133, and VrTAF5_Indel were found polymorphic between parents. These six SSR markers (CEDC055, CEDG008, CEDG051, CEDG070, Vr6gCLS037, and Vr6gCLS133) and one InDel marker (VrTAF5_Indel) were identified as possibly associated with CLS resistance in BSA. The remaining 22 primer pairs were found to be monomorphic.

Table 3.3 Characteristics of ISSR and ISSR-RGA loci amplified for identifying Cercospora leaf spot resistance gene in mungbean.

ISSR	Sequences[a]	Primers and sequences		Range of amplified products (bp)	Origin of loci amplified				
	5'-3'	RGA	Sequences 5'-3'		I[b]	I-R[c]	I-R new[d]	Polymorphic	I-R linked[e]
807	$(AG)_8T$	GLPLAL 1	IAGIGCIAGIGGIAGICC	200-1500	28	24	-	-	-
		P-Loop	$(GGI)_2$GTIGGIAAIACIAC	200-1500	28	19	1	-	-
		Pto kin 1	GCATTGGAACAAGGTGAA	200-1500	28	19	-	-	-
		RLK for	GAYGTNAARCCIGARAA	200-1500	28	23	-	-	-
808	$(AG)_8C$	GLPLAL 1	IAGIGCIAGIGGIAGICC	200-1500	26	26	3	1	1
		P-Loop	$(GGI)_2$GTIGGIAAIACIAC	200-1500	26	21	1	-	-
		Pto kin 1	GCATTGGAACAAGGTGAA	200-1500	26	30	6	2	-
		RLK for	GAYGTNAARCCIGARAA	200-1500	26	25	3	-	-
809	$(AG)_8G$	GLPLAL 1	IAGIGCIAGIGGIAGICC	200-1500	27	29	3	2	-
		P-Loop	$(GGI)_2$GTIGGIAAIACIAC	200-1500	27	20	-	-	-
		Pto kin 1	GCATTGGAACAAGGTGAA	200-1200	25	29	4	-	-
		RLK for	GAYGTNAARCCIGARAA	200-1500	27	24	2	1	-
810	$(GA)_8T$	GLPLAL 1	IAGIGCIAGIGGIAGICC	200-1500	30	24	-	-	-
		P-Loop	$(GGI)_2$GTIGGIAAIACIAC	200-1500	30	31	3	2	-
		Pto kin 1	GCATTGGAACAAGGTGAA	200-1500	30	29	6	1	-
		RLK for	GAYGTNAARCCIGARAA	200-1500	30	28	2	-	-
827	$(AC)_8G$	GLPLAL 1	IAGIGCIAGIGGIAGICC	200-1500	22	18	1	-	-
		P-Loop	$(GGI)_2$GTIGGIAAIACIAC	200-1500	22	22	1	1	1
		Pto kin 1	GCATTGGAACAAGGTGAA	200-1500	22	21	3	1	-
		RLK for	GAYGTNAARCCIGARAA	200-1500	22	17	1	-	-
835	$(AG)_8YC$	GLPLAL 1	IAGIGCIAGIGGIAGICC	150-1000	27	25	2	-	-
		P-Loop	$(GGI)_2$GTIGGIAAIACIAC	150-1000	27	24	1	-	-
		Pto kin 1	GCATTGGAACAAGGTGAA	150-1000	27	20	3	2	2
		RLK for	GAYGTNAARCCIGARAA	150-1000	27	18	1	1	1
836	$(AG)_8YA$	GLPLAL 1	IAGIGCIAGIGGIAGICC	200-1000	23	23	1	-	-
		P-Loop	$(GGI)_2$GTIGGIAAIACIAC	200-1000	23	23	1	-	-
		Pto kin 1	GCATTGGAACAAGGTGAA	200-1000	23	16	-	-	-
		RLK for	GAYGTNAARCCIGARAA	200-1000	23	23	1	-	-

Table 3.3 Characteristics of ISSR and ISSR-RGA loci amplified for identifying Cercospora leaf spot resistance gene in mungbean (continued).

ISSR	Sequences[a]	Primers and sequences		Range of amplified products (bp)	Origin of loci amplified				
	5'-3'	RGA	Sequences 5'-3'		I[b]	I-R[c]	I-R new[d]	Polymorphic	I-R linked[e]
841c	$(GA)_8CC$	GLPLAL 1	IAGIGCIAGIGGIAGICC	200-1000	23	25	5	2	-
		P-Loop	$(GGI)_2$GTIGGIAAIACIAC	200-1000	23	21	-	-	-
		Pto kin 1	GCATTGGAACAAGGTGAA	200-1000	23	23	2	-	-
		RLK for	GAYGTNAARCCIGARAA	200-1000	23	20	2	-	-
856	$(AC)_8YA$	GLPLAL 1	IAGIGCIAGIGGIAGICC	250-1500	24	17	-	-	-
		P-Loop	$(GGI)_2$GTIGGIAAIACIAC	250-1500	24	21	2	-	-
		Pto kin 1	GCATTGGAACAAGGTGAA	250-1500	24	23	3	2	2
		RLK for	GAYGTNAARCCIGARAA	250-1500	24	21	-	-	-
857	$(AC)_8YG$	GLPLAL 1	IAGIGCIAGIGGIAGICC	200-1500	25	22	2	-	-
		P-Loop	$(GGI)_2$GTIGGIAAIACIAC	200-1500	25	24	5	-	-
		Pto kin 1	GCATTGGAACAAGGTGAA	200-1500	25	24	3	-	-
		RLK for	GAYGTNAARCCIGARAA	200-1500	25	22	3	-	-
888	BDB $(CA)_7$	GLPLAL 1	IAGIGCIAGIGGIAGICC	200-1000	23	20	1	-	-
		P-Loop	$(GGI)_2$GTIGGIAAIACIAC	200-1000	23	16	1	-	-
		Pto kin 1	GCATTGGAACAAGGTGAA	200-1000	23	19	3	1	-
		RLK for	GAYGTNAARCCIGARAA	200-1000	23	24	1	-	-
891	HVH $(TG)_7$	GLPLAL 1	IAGIGCIAGIGGIAGICC	200-1000	24	22	1	1	-
		P-Loop	$(GGI)_2$GTIGGIAAIACIAC	200-1000	24	19	-	-	-
		Pto kin 1	GCATTGGAACAAGGTGAA	200-1000	24	19	-	-	-
		RLK for	GAYGTNAARCCIGARAA	200-1000	24	21	-	-	-
				Total loci amplified	1206	1074	84	20	7

[a] B = C, G, T; D = A, G, T; H = A, C, T; I = inosine; N = A, G, C, T; R = A, G; Y = C, T.
[b] I = ISSR loci.
[c] I-R = "like-ISSR loci" in ISSR-RGA profiles.
[d] I-R new = new ISSR-RGA loci.
[e] I-R linked = ISSR-RGA loci associated with Cercospora leaf spot resistance.

Table 3.4 Suitable annealing temperature for InDel and each SSR primer pair.

Markers	Primers	Sequences (5' - 3')	LG[a]	Annealing temperature (°C)
InDel	VrTAF5_indel	F: CTCATGAAACCTGGAGAACT R: CCCAGTGTACTCAGTTTGACTT	3	55
SSR	CEDAAG004	F: GGAGGAGAAGTCTCGGACC R: GAGCGTTTTGCACAGTGTTCAC	3	60
	CEDC008	F: GGAATTAGAGATGATTGGAC R: CACCACTTCATTATGTATGG	3	55
	CEDC055	F: CAAACACTTTTGTAACTCCC R: GCTTCTAACCTTGATCCTTC	4	55
	CEDG002	F: AACTGGACCTGTACCACTGG R: TACAGCCTTCTTGCACCATG	11	60
	CEDG006	F: AATTGCTCTCGAACCAGCTC R: GGTGTACAAGTGTGTGCAAG	2	60
	CEDG008	F: AGGCGAGGTTTCGTTTCAAG R: GCCCATATTTTTACGCCCAC	5	60
	CEDG010	F: TGGGCTACCAACTTTTCCTC R: TGAGCGACATCTTCAACACG	3	60
	CEDG014	F: GCTTGCATCACCCATGATTC R: AAGTGATACGGTCTGGTTCC	5	60
	CEDG021	F: GCAGAATTTTAGCCACCGAG R: AAAGGATGCGAGAGTGTAGC	10	60
	CEDG024	F: CATCTTCCTCACCTGCATTC R: TTTGGTGAAGATGACAGCCC	9	60
	CEDG040	F: CGGGGTATAACTTTAGCAGC R: TAACTCAGGCAAAGGTAGCC	8	60
	CEDG043	F: AGGATTGTGGTTGGTGCATG R: ACTATTTCCAACCTGCTGGG	3	60
	CEDG044	F: TCAGCAACCTTGCATTGCAG R: TTTCCCGTCACTCTTCTAGG	11	60
	CEDG051	F: AAACATACCCCTGGCAGTTCC R: TTCTGACCTAAGAAAGAGCCTGG	1	60
	CEDG056	F: TTCCATCTATAGGGGAAGGGAG R: GCTATGATGGAAGAGGGCATGG	9	60
	CEDG059	F: AGAAAAGGGTGGCCTCGTTG R: GCAGGCATTTCCATCGCAG	8	60
	CEDG063	F: TTGGAAACAATTATTGGAGGTGC R: GGTGCTCTTGGACGGCTGG	3	55
	CEDG070	F: CCGATCAAACTCTCCATGCTCG R: TAATTTCATTGCTTTTCCCTCC	-[b]	55
	CEDG121	F: CTTTCAAAATAATGTTGAGGCATA R: CAATACATAAATAACCTTTTCTGC	6	50
	CEDG154	F: GTCCTTGTTTTCCTCTCCATGG R: CATCAGCTGTTCAACACCCTGTG	4	55
	CEDG169	F: CAATGCGCGTCTGCAAGTTG R: CAAGGAACTTGTGGGTCC	-[b]	55
	CEDG176	F: GGTAACACGGGTTCAGATGCC R: CAAGGTGGAGGACAAGATCGG	3	50
	CEDG186	F: GGATGGGAGAGTAAGAAG R: GCATGGCATGATGACTTG	3	60
	CEDG205	F: GTGGTGGTGACAGTAGCAGTAG R: CAGCCACCACAAGACAACCTC	3	55
	CEDG259	F: GATCATCGGACAGAGCTTCC R: CACTCTCTGCGAACTCAATCG	9	55
	CEDG287	F: CCTTATACTAAAGATGTTGGTGG R: GTGATACGCATATAGGTTCAC	11	50
	CEDG290	F: GACACTCTTTGTTTGTAGG R: CAGTGATCACTCTGGTTG	7,9,11	60
	CEDG294	F: CACCTTCTTAATCTCTTCACC R: GGGTTTCTCTTAATTCATTGAGTC	3	55

Table 3.4 Suitable annealing temperature for InDel and each SSR primer pair (continued).

Markers	Primers	Sequences (5' - 3')	LG[a]	Annealing temperature (°C)
	CEDG295	F: CAAAGGTTAGATCCAACATCG R: GGTTAGTCATCAACAACTCC	3,7	60
	CEDG304	F: ACCACTTCATAATCCCTGAG R: GTTGCATGCTATATTTTGGTTCAC	9	50
	CEDGAT008	F: GGATGTGAAAGACTTAACTTC R: GAGGAATCTAAGTAAAACGAG	3	55
	DMB-SSR 59	F: TGCCAGATTTGAGAAGAAAGGT R: CATGCATGTGGATAAGAATTCAG	-[b]	55
	DMB-SSR 167	F: TGGGACTCAAACCACACTTTC R: GAACTATGAAGGTTTCACAGAAATCA	4	55
	DMB-SSR 199	F: AGAAATTAAATCCCCGTCTGCT R: AGAGACAGAAGCTCTGGATGTTTT	4	55
	VR010	F: GAAAGGCTATGACCAAATCCAA R: CGGGAAGAGAACATAAGGGAAT	-[b]	55
	cp03802	F: ATGTTATGAATGCACCTGCACGAT R: CTGCAAGAAGCTAGTGTTGCTCCA	9	55
	Vr6gCLS037	F: GGTTTCGGATAGTTGGAGAG R: TGAAGGTGAATCCAGGACAG	3	55
	Vr6gCLS085	F: AGATCTGTCACACCCATCTG R: GTTGGGAGACACAAAACTCC	3	55
	Vr6gCLS133	F: TCCTCCCAGTGTACTCAGTTTG R: TGCTTGATAATGAGGAAACTAATCC	3	55

[a] Linkage group.
[b] There is no description.

3.4.2 Linkage and QTL analysis

When, 14 ISSR, 7 ISSR-RGA, 6 SSR, and 1 InDel markers that were putatively associated with CLS resistance from BSA were chosen for evaluation of the correlation between markers and CLS score from field evaluation by simple linear regression. An initial screening of 10 resistant and 10 susceptible RILs individually found that these 7 ISSR markers (I14749, I16274, I18363, I41203, I88656, I88302, and I88305), 4 ISSR-RGA markers (I27PL177, I35P716, I56P166, and I56P169), 4 SSR markers (CEDG008, CEDG051, Vr6gCLS037, and Vr6gCLS133) as well as a InDel marker (VrTAF5_Indel) were found to be significantly associated with CLS resistance ($P < 0.01$). Then, these 16 markers were further analyzed with $F_{2:9}$ and $F_{2:10}$ RIL populations to verify the linkage with CLS resistance using simple linear regression,

recombination and LOD analysis. Among these, only 5 markers were segregated at the ratio of 1:1 when testing with chi-square as expected, indicating that they are useful for CLS mapping. The simple linear regression analysis reported a coefficient of determination (R^2) of 0.220 (CEDG008) to 0.619 (I16274) in 2016 and 0.207 (I88656) to 0.416 (I16274) in 2018 with LOD score more than 3 in both years (Table 3.5). Moreover, I16274 and VrTAF5_Indel markers were nearest to the location of CLS resistance gene at distance of 4 and 5 cM, respectively. If we use both I16274 and VrTAF5_Indel markers, which are closely linked and flank the CLS resistance gene, to assist selection of CLS resistance in MAS, only 0.40% recombination between both markers and the CLS resistance gene will be observed.

Table 3.5 Association of markers with CLS resistance in a cross between CN72 and V4718.

Years	Markers	Beta	*t* value	*P* value	R^{2a}	LOD[b]
2016	CEDG008	0.469	4.630	0.000	0.220	5.720
	I88656	-0.545	-5.624	0.000	0.297	7.324
	I16274	-0.787	-11.038	0.000	0.619	15.142
	VrTAF5_Indel	0.693	8.047	0.000	0.481	9.893
	I35P716	-0.603	-6.465	0.000	0.364	6.899
2018	CEDG008	0.494	4.956	0.000	0.244	3.748
	I88656	-0.455	-4.428	0.000	0.207	3.585
	I16274	-0.645	-7.310	0.000	0.416	9.465
	VrTAF5_Indel	0.573	5.848	0.000	0.328	5.111
	I35P716	-0.520	-5.201	0.000	0.270	5.694

[a] Correlation between marker and QTL associated with CLS resistance using simple linear regression.
[b] LOD score explained by the marker.

For CLS mapping, comparative linkage maps comparing the locations of the QTL controlling CLS resistance detected in this study, and in the study of Chankaew et al. (2011) and Yundaeng et al. (2020) are displayed in Figure 3.2. The first QTL mapping for CLS resistance in KPS1 × V4718 cross was constructed by Chankaew et

al. (2011) who found the *qCLS* flanked between VR393 and CEDG117 markers (Figure 3.2a). Later, Yundaeng et al. (2020) mapped closer markers to the *qCLS*, flanked between VrTAF5_Indel /Vr6gCLS133 and Vr6gCLS085 markers (Figure 3.2b). In this study, five putative markers together with VR393 and CEDG084 were used to construct a genetic linkage map for the $F_{2:9}$ and $F_{2:10}$ RIL populations of CN72 × V4718 cross. These seven markers were grouped in the same linkage group and spanned a chromosome length of 78.81 cM (Figure 3.2c). In addition, some markers such as CEDG084, VR393, and VrTAF5_indel markers reported by Chankaew et al. (2011) and Yundaeng et al. (2020, were also found in this study in different positions which may stem from the differences of the maternal parents.

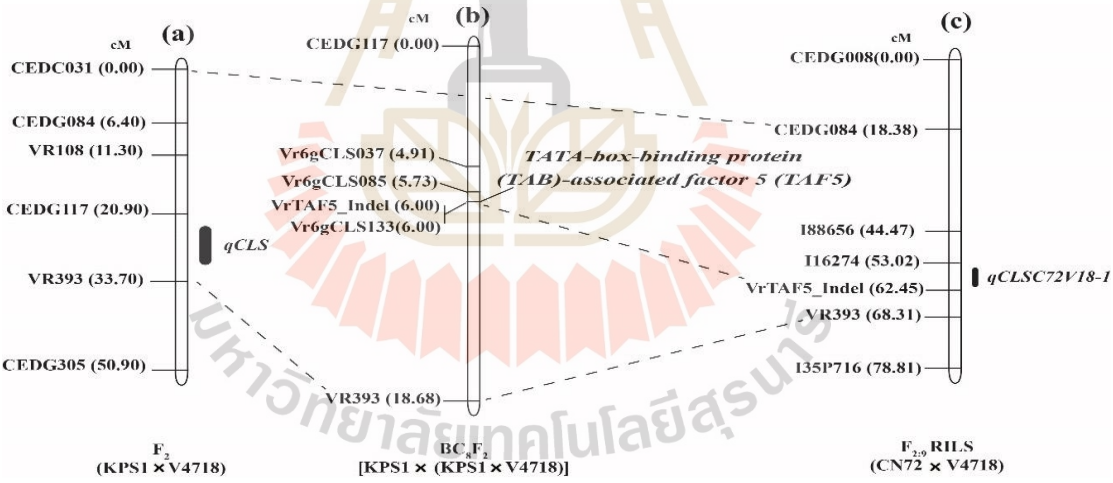

Figure 3.2 Illustration of linkage map reported by Chankaew et al. (2011) (a) and detected candidate gene (TAF5) for CLS resistance reported by Yundaeng et al. (2020) (b) and identified by this study (c).

QTL analysis by ICIM function in both RIL populations using CLS scores in 2016 and 2018 found that the *qCLSC72V18-1* for CLS resistance was flanked

between the I16274 and VrTAF5_Indel markers. This QTL was located at 57.0 cM which accounted for 32.86% of variation in disease responses in 2016 with the additive effect of -0.55. While the higher phenotypic variation of 41.56% was observed in 2018 and the QTL was detected at 56.0 cM with an additive effect of -0.85 (Table 3.6). These flanking markers will be applicable for improving cultivated mungbean varieties for resistance to CLS in the future.

Table 3.6 Quantitative trait loci (QTL) detected for Cercospora leaf spot (CLS) resistance in $F_{2:9}$ and $F_{2:10}$ RILs populations derived from CN72 × V4718 cross, using inclusive composite interval mapping (ICIM) function.

Population	Year	QTL name	Marker interval	Position (cM)[a]	LOD score[b]	PVE (%)[c]	Additive effect
$F_{2:9}$	2016	*qCLSC72V18-1*	I16274-VrTAF5_Indel	57.0	9.54	32.86	-0.55
$F_{2:10}$	2018	*qCLSC72V18-1*	I16274-VrTAF5_Indel	56.0	10.10	41.56	-0.85

[a] Position on the linkage group.
[b] LOD score explained by the QTL.
[c] Percentage of phenotypic variance explained by the QTL.

The I16274 and VrTAF5_Indel markers were also tested on an additional 21 mungbean varieties/lines with known CLS reactions (Table 3.7). The dominant I16274 marker amplified a 274 bp amplicon linked to CLS resistance (A_) in V4718, V4785, SUPER 5, TAINAN SEL#5, ML-131, VAR A-G, BARI MUNG 2 and WALET. While a linked 209 bp amplicon to CLS resistance amplified by the co-dominant VrTAF5_Indel marker was observed in V4718 and SUPER 5 (BB), and in V4785, ML-131 and GELETIK (Bb). The remaining 14 genotypes including V4758, CN36, CN72, CN84-1, SUT1, SUT4, PUSA-105, NM92, NM94, EG-MD-6D, CES55, MG50-10A (Y), BPI GLABROUS #3, and KING did not have either of the amplicons (aabb). Some of these genotypes with aabb alleles from both markers such as CN36,

CN72, CN84-1, SUT1, and EG-MD-6D were reported to possess high-yielding potential under field conditions in Thailand (Chueakhunthod et al., 2020) and can be introduced the CLS resistance gene through MAS using these two tightly linked markers.

Table 3.7 Genotypic and phenotypic analysis of mungbean varieties/lines using markers I16274 and VrTAF5_Indel.

Varieties/lines	Phenotypes[a]	Genotypes[b]	
		I16274	VrTAF5_InDel
V4718	R	A_	BB
V4785	R	A_	Bb
SUPER 5	R	A_	BB
V4758	MR	aa	bb
TAINAN SEL#5	MR	A_	bb
CN36	S	aa	bb
CN72	S	aa	bb
CN84-1	S	aa	bb
SUT1	S	aa	bb
SUT4	S	aa	bb
PUSA-105	S	aa	bb
ML-131	S	A_	Bb
VAR A-G	S	A_	bb
BARI MUNG 2	S	A_	bb
NM92	S	aa	bb
NM94	S	aa	bb
EG-MD-6D	S	aa	bb
CES55	S	aa	bb
MG50-10A (Y)	S	aa	bb
BPI GLABROUS #3	S	aa	bb
WALET	S	A_	bb
GELATIK	S	aa	Bb
KING	S	aa	bb

[a] CLS responses were evaluated in 2018 and 2020, and classified into three classes: resistance (R) = 1-2.5, moderate resistance (MR) = 2.6-3.4, and susceptibility (S) = 3.5-5.0.

[b] For I16274 marker, A_: presence of 274 bp amplicon; aa: absence of 274 bp amplicon. For VrTAF5_Indel marker, BB: presence of 209 bp amplicon; bb: presence of 234 bp amplicon; Bb: presence of both 209 and 234 bp amplicons.

3.5 Discussion

Cercospora leaf spot (CLS) is a foliar disease of mungbean spreading in warm-wet growing season. A few studies have been conducted to understand genetic of the CLS resistance. Mishara et al. (1988) revealed that the CLS resistance is controlled by a single recessive gene; the segregation ratio of 3:1 for susceptible and resistant progenies was found in all 14 F_2 crosses. In contrast, Chankaew et al. (2011) and Singh et al. (2017) demonstrated that a gene conferring resistance to CLS is controlled by a single dominant gene; the ratio of 3:1 and 1:1 (resistant: susceptible) were observed in F_2 and BC_1F_1 populations. In this study, we revealed that the segregation ratio in $F_{2:9}$ and $F_{2:10}$ RIL population of the CN72 × V4718 cross was 1:1 for resistant and susceptible progenies, confirming that the CLS resistance gene in V4718 line is controlled by a single dominant gene. In addition, we found that mungbean accession V4718 showed stable resistance to CLS in both years. Similar results were reported by Hartman et al. (1993) and Chankaew et al. (2011), who found that V4718 line was resistant to CLS in different years and seasons in Taiwan and Thailand, respectively. Therefore, the CLS resistance gene can be transferred from V4718 to susceptible cultivated variety for developing new CLS resistant varieties through several conventional breeding methods such as pedigree selection, bulk selection, single seed descent or backcross method.

When a total of 156 primers/primer pairs were used to detect polymorphisms with BSA. The percentages of polymorphisms were 58.8, 29.2, and 35.9% for ISSR, ISSR-RGA and SSR markers, respectively. This is in agreement with Poolsawat et al. (2017), who found that ISSR and ISSR-RGA markers were effective for identification of DNA polymorphisms in the CN72 × V4718 cross. They found that 64.0% of ISSR and 20.0%

of ISSR-RGA were polymorphic. Additionally, 13.9% to 47.2% of polymorphism were detected when using SSR maker (Chankaew et al., 2011; Isemura et al., 2012). Our results confirm that ISSR, ISSR-RGA and SSR markers are efficient for detecting the DNA polymorphisms in mungbean.

Bulked segregant analysis (BSA) technique, a rapid, simple and cost effective preliminary method was used to identify the markers linked to specific genes. BSA successfully identified markers linked to powdery mildew (PM) and mungbean yellow mosaic virus (MYMV) as well as bean bug and bruchid in mungbean (Selvi et al., 2006; Chen et al., 2007; Dhole and Reddy, 2013; Hong et al., 2015; Poolsawat et al., 2017). In this study, BSA identified five markers (2 ISSR, 1 ISSR-RGA, 1 SSR, and 1 InDel markers) which were significantly associated with CLS resistance gene ($P < 0.001$). Recently, Poolsawat et al. (2017) identified five ISSR and three ISSR-RGA markers linked to PM resistance gene by using BSA and confirmed these markers in large population. They found I85420 and I42PL229 markers closely linked to PM resistance gene.

We have previously identified a QTL, *qCLSC72V18-1*, between markers VR393 and CEDG084 in the CN72 × V4718 cross (Arsakit et al., 2017). Recently, Yundaeng et al. (2020) successfully identified a candidate gene, LOC106765332 (*VrTAF5*), at the *qCLS* of V4718 in the KPS1 × V4718 cross and was able to develop gene-specific markers including VrTAF5_indel and Vr6gCLS133 markers tightly linked to the *qCLS*. Therefore, in order to identify the closer markers linked to CLS resistance gene in our CN72 × V4718 cross, those markers linked to *VrTAF5* identified by Yundaeng et al. (2020) in the KPS1 × V4718 cross as well as our previously identified markers in this current cross were simultaneously characterized. The ISSR marker I16274 and InDel

marker VrTAF5_indel were found to be tightly linked to the *qCLSC72V18-1* with calculated genetic distances of 4 and 5 cM, respectively, giving only 0.40% recombination between both markers and the CLS resistance gene for MAS. The newly identified VrTAF5_Indel marker appeared to be located closer to the *qCLSC72V18-1* than VR393 which was identified in our previous study (Arsakit et al., 2017), making it more efficient for MAS. The genetic distance of VrTAF5_indel marker from the CLS resistance gene in V4718 found in our study (5 cM) differed from that of Yundaeng et al. (2020) who found only 0.1 cM. This may be due to the maternal parents in the two populations being different (Arsakit et al., 2017). When we verified these markers on 21 mungbean genotypes/lines with known CLS reactions. Among these, 14 genotypes/lines with susceptible to CLS did not have either of the resistance amplicons, indicating that they are polymorphic at these loci (difference from V4718). Therefore, we can transfer CLS resistance gene from the resistant line V4718 into these 14 genotypes/lines through MAS.

3.6 Conclusion

We successfully screened the DNA polymorphism with BSA in the RILs of CN72 × V4718 cross when using ISSR and ISSR-RGA as well as SSR and InDel markers. Our study identified a major QTL (*qCLSC72V18-1*), accounting for 32.86% to 41.56% of the CLS disease score variation in 2016 and 2018 and flanked between I16274 and VrTAF5_Indel markers with the distance of 4 and 5 cM from the CLS resistance gene, respectively. The markers closely linked to CLS resistance will be useful for the development of new mungbean cultivar(s) resistant to CLS through MAS.

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CHAPTER IV

IMPROVEMENT OF CERCOSPORA LEAF SPOT AND POWDERY MILDEW RESISTANCE OF MUNGBEAN VARIETY KING THROUGH MARKER-ASSISTED SELECTION

4.1 Abstract

Development of resistant mungbean varieties is one of the most efficient strategies to control major diseases such as Cercospora leaf spot (CLS) and powdery mildew (PM). Moreover, the application of marker-assisted selection (MAS) can be used as an effective tool for overcoming the limitations of the conventional breeding method. It can be carried out at all developmental stages and in all environmental conditions which allows a year-round selection. The objectives of this study were to pyramid a CLS resistance gene and 2 PM resistance genes from the donor parent D2 into a susceptible variety KING through marker-assisted backcrossing (MABC) and to evaluate their agronomic traits and disease resistance under field conditions. Five markers linked to the resistance genes were used for foreground selection while three marker sets (Set A containing 6 markers linked to domestication related traits; Set B containing 9 markers related to other putative protein functions and other fragments with unknown functions; Set C containing 34 polymorphic ISSR loci) were also used for background selection. Two pyramided BC lines, namely H3 and H4, were homo-

zygous at all five marker loci when confirmed in BC_4F_4 and BC_4F_5 generations. Their recurrent parent genome recovery ranged from 94.4 to 100.0%, depending on the marker sets, suggesting that effectiveness of background selection for accelerated backcrossing. During field evaluation, a moderate to high level of CLS and PM resistance was observed in both BC lines compared to the susceptible recurrent parent KING. One of these BC lines (H3) had most agronomic traits similar or superior to KING, and had higher yield than KING (18-32%) under CLS and PM outbreaks. This line has the potential to be developed into a new resistant mungbean variety in Thailand in the future. These results substantiate the usefulness of MABC for transferring multiple resistance genes into an elite variety.

4.2 Introduction

Approximately 7.0 million hectares of mungbean [*Vigna radiata* (L.) Wilczek] are cultivated worldwide with the production of 5.3 million tonnes, which is mainly grown in South and Southeast Asia. Particularly, India is the largest producer, followed by Myanmar, and Thailand (Nair and Schreinemachers, 2020). Mungbean is an important pulse crop. It is rich in essential sources of nutrients for diets. For example, it provides easily digestible proteins, carbohydrates, fatty acids, vitamins, iron, and zinc. In Thailand, the potential mungbean yield is in the range of 719-825 kg/ha or 115-132 kg/rai (Office of Agricultural Economics, 2019). However, mungbean production is constrained by various factors as well as susceptibility to pests and diseases or weakness to environments. Of the diseases, Cercospora leaf spot (CLS) and powdery mildew (PM) are the most serious in Thailand.

CLS, a foliar disease, is caused by *Cercospora canescens* Ellis & Martin (Chand

et al., 2015). The outbreak of this disease during the rainy season can lead to losses of 50-97% of the yield if there is no protection (AVRDC, 1984; Iqbal et al., 1995; Chand et al., 2012; Nair et al., 2019). PM is caused by the biotrophic fungus *Sphaerotheca phaseoli*. The spread of PM can reduce yields by more than 50% in the cool-dry growing season (Khajudparn et al., 2010). In Thailand, the mungbean genotypes which are resistant to CLS or PM have been identified e.g., M5-22 and M5-25 are resistant to CLS and M5-10, V4718, V4758, and V4785 are resistant to PM (Wongpiyasatid et al., 1999; Khajudparn et al., 2010). Some of these genotypes such as V4718 have been reported to be resistant to both diseases. In addition, the source of resistance to CLS in the V4718 line is controlled by a single dominant gene (Chankaew et al., 2011; Tantasawat et al., 2020). The source of resistance to PM in V4718, V4758, and V4785 is also controlled by a single dominant gene, and they are non-allelic (Khajudparn et al., 2010), which is useful for plant breeders in developing new resistant varieties.

The combination of marker-assisted backcrossing (MABC) and marker-assisted gene pyramiding are widely used in molecular plant breeding. When marker-assisted gene pyramiding is used through MABC, it allows several genes to combine into an elite variety simultaneously, and improved lines still have a similar genetic background to this elite variety. Therefore, improved varieties have a broad-spectrum resistance to pests or diseases and their phenotypic characters are similar to those of the recurrent parent, especially as they can reduce the time required for breeding programs (Collard and Mackill, 2008). In addition, tightly linked markers have been identified for CLS and PM resistance genes using quantitative trait loci (QTL) analysis. Yundaeng et al. (2020) developed one functional marker from a candidate gene for CLS

resistance (TATA-binding-protein-associated factor 5) in the resistant line V4718. The closest markers linked to the CLS resistance gene were also identified in a cross between CN72 and this resistant line (Arsakit et al., 2017; Papan et al., unpublished data). Moreover, Poolsawat et al. (2017) identified markers linked to a major QTL conferring PM resistance in the CN72 × V4718 cross. The marker associated with PM resistance in a cross between CN72 and V4785 was also found (Tantasawat et al., 2021). These markers linked to resistance genes are useful for the improvement of mungbean varieties resistant to CLS and PM. Furthermore, if CLS and PM resistance genes are pyramided into cultivated mungbean varieties, they may enhance resistance to both diseases simultaneously, providing broad-spectrum and durable resistance.

In this study, we aimed to transfer a CLS resistance gene and 2 PM resistance genes into a mungbean variety KING using MABC, and to evaluate CLS and PM resistance and agronomic traits of the pyramided BC lines for potential commercialization in the future.

4.3 Materials and Methods

4.3.1 Plant materials and breeding scheme

The recurrent parent, namely KING, is a high yielding mungbean variety obtained from Australia with large seeds and resistance to PM, but it is susceptible to PM and CLS when grown in Thailand (Chueakhunthod et al., 2020). While D2 (67A × 27B) × (71B × 14C)-2 is a donor parent which was derived from double crosses between recombinant inbred lines (RILs) of three populations [CN72 × V4758 (A), CN72 × V4718 (B) and CN72 × V4785 (C)] contains a CLS resistance gene and 2 PM resistance genes. It was developed by Poolsawat et al. (unpublished data). In addition,

three resistant lines (V4718, V4758, and V4785) were obtained from the World Vegetable Center (WORLDVEG) in Taiwan. These resistant lines have high yields and seeds per pod, but their seed size is small.

For the MABC scheme, KING was crossed with D2 to generate F_1 seeds, and the F_1 plants were backcrossed to the recurrent parent KING. Marker-assisted backcrossing was followed up to BC_4F_1 generation. Only desirable plants with all the resistance loci (1 CLS resistance gene and 2 PM resistance genes based on linked markers) were advanced to the next generation. In brief, foreground selection was used to select F_1 until BC_4F_5 to identify heterozygous plants in F_1 to BC_4F_1 generations and homozygous plants in BC_4F_2 to BC_4F_5 generations for all three target resistance genes. In contrast, background selection was used in BC_1F_1 to BC_4F_2 generations to achieve high recurrent parent genome recovery (Figure 4.1). In this study, F_1 to BC_2F_1 generations were performed by Chueakhunthod (2019). In continuation of this work, desirable BC_2F_1 plants were backcrossed to KING twice to generate BC_4F_1 generation followed by selfing to produce BC_4F_2 to BC_4F_7 generations. In addition, the non- segregation of markers in BC_4F_4 - BC_4F_5 was also confirmed with foreground selection to ensure that the BC lines had all 3 resistance genes in homozygosity.

4.3.2 DNA extraction and PCR amplification

Genomic DNA of each plant was extracted by using a modified CTAB extraction protocol described by Lodhi et al. (1994). The PCR reaction mixture of ISSR and ISSR-RGA markers was performed in a 20 µl reaction mix containing 150 ng of genomic DNA template, 1× buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.1, 0.01% TritonTM X-100), 3.5 mM $MgCl_2$, 250 µM of each dNTPs, 1 unit of Taq DNA

polymerase, 0.4 µM of ISSR primer, and 1 µM of RGA primer (only ISSR-RGA marker). At the same time, the reaction mix of SSR and EST-SSR markers contained 150 ng of genomic DNA template, 1× buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.1, 0.01% Triton^{TM} X-100), 2 mM $MgCl_2$, 0.2 mM of each dNTPs, 1 unit of *Taq* DNA polymerase, and 0.5 µM each of forward and reverse primers in a volume of 20 µl. Details of the primer sequence used for marker-assisted foreground and background selection are presented in Tables 4.1 to 4.3. PCR amplification and detection of PCR products were performed as described by Poolsawat et al. (2017) for ISSR and ISSR-RGA markers, Arsakit et al. (2017) for SSR marker, and Chen et al. (2015) for EST-SSR marker.

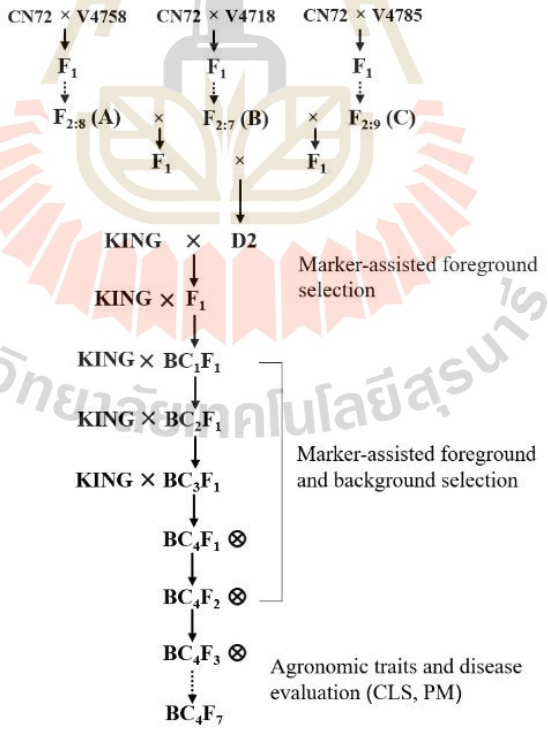

Figure 4.1 Schematic workflow for pyramiding CLS and PM resistance genes into mungbean variety KING through MABC.

4.3.3 Foreground and background selection

Marker-assisted foreground and background selection were used to pyramid a CLS resistance gene and 2 PM resistance genes into a mungbean variety KING. Five markers linked to target resistance genes were used for foreground selection (Table 4.1). Among these, two SSR markers (VR393 and CEDG084) were linked to a CLS resistance gene in V4718 (Arsakit et al., 2017). Other ISSR and ISSR-RGA markers, I85420 and I42PL222, and I27R656 were linked to PM resistance genes in V4718 and V4785, respectively (Poolsawat et al., 2017; Tantasawat et al., 2021). The donor parent D2 containing a CLS resistance gene and 2 PM resistance genes from V4718 and V4785 possessed similar DNA banding patterns as those of the resistant lines V4718 and V4785 which are sources of CLS and PM resistance. All of these were polymorphic between KING and D2 (Chueakhunthod, 2019). Therefore, these five markers were used for foreground selection in each generation. For background selection, a total of 37 SSR and EST-SSR markers associated with domestication related traits and other putative protein functions or unknown functions in mungbean, i.e., days to first flowering, hypocotyl plus epicotyl length, pod width, seed width, 100-seed weight, stem length, protein FRIGIDA-like, mitochondrial import inner membrane translocase subunit TIM17-2-like, leucine-rich repeat extensin-like protein 4-like etc. (Isemura et al., 2012; Chen et al., 2015). In addition, 11 ISSR primers developed from the University of British Columbia which produced ISSR markers that are well distributed throughout the genome, were used for a parental polymorphism survey. Fifteen SSR and EST-SSR were found to be polymorphic between parents (Table 4.2). Among these, 6 polymorphic SSR markers gave a similar allele size to those reported by Isemura et al. (2012) and were assigned

as Set A. EST-SSR related to other putative protein functions and other SSR and EST-SSR fragments with unknown functions were classified as Set B. While the 34 polymorphic fragments amplified by 11 ISSR primers were classified as Set C (Table 4.3). For data analysis, all clearly amplified DNA bands were scored as allele sizes at each locus for SSR and EST-SSR markers, while polymorphic ISSR markers were scored as present/absent in the DNA bands. Similarity coefficients between BC progenies and their parents, in a pair-wise comparison, were computed using Jaccard's coefficient, and the resulting similarity matrix was analyzed by an 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 Mantel's correlation test (Mantel, 1967).

Table 4.1 Markers used for foreground selection of a CLS resistance gene and 2 PM resistance genes in marker-assisted backcrossing.

Resistance genes	QTL/genes	Marker types	Markers	Primer sequences (5'-3')[c]	Expected size (bp)	Reference
CLS (from V4718)	*qCLSC72V18-1*	SSR	CEDG084	F: ATCAACTGAGGAGCATCATCGA R: CAACATTTCAACCTTGGGACAG	168/170 (S/R)	Arsakit et al. (2017)
		SSR	VR393	F: TGGCACTTTCCATAACGAATAC R: ATCAGCCAAAAGCTCAGAAAAC	158/160 (S/R)	
PM (from V4718)	*qPMC72V18-1*	ISSR	I85420	BHB $(GA)_7$	420 (R)	Poolsawat et al. (2017)
		ISSR-RGA	I42PL222[a]	$(GA)_8$YG and $(GGI)_2$GTIGGIAAIACIAC	222 (R)	
PM (from V4785)	*qPMC72V85*	ISSR-RGA	I27R565[b]	$(AC)_8$G and GAYGTNAARCCIGARAA	565 (R)	Tantasawat et al. (2021)

[a] I42P222 was derived from the resistant line (V4718) and was used instead of I42P229, which linked to a susceptible allele of a susceptible variety (CN72).
[b] I27R565 which linked to PM resistance 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).

Table 4.2 Polymorphic SSR and EST-SSR markers used for background selection.

Marker sets	Primer name	Primer sequences (5'-3')	LG[c]	Traits/ putative functions[d]
Set A	CEDG150[a]	F: GAAGGGAATGAAAATGAAACCC	10	HECL, ITL, STL
		R: GTTCAATCCATTCAGTCTCC		
	CEDG174[a]	F: GAGGGATCTCCAAAGTTCAACGG	7	SDL, SDW, PDL
		R: GAAGGCTCCGAAGTTGAAGGTTG		
	CEDG220[a]	F: GGTATTGAAGTCACATGGTCC	1	SDL, SDNPPD
		R: GGTTGTTATCTTTGTGCACTCC		
	CEDG245[a]	F: GATAGAGCTTAAACCCTC	6	RSP
		R: CTTTTGATGACAAATGCCC		
	MBSSR015[a]	F: ATCATCATGACTCCGACACTC	4	FLD, PDDM
		R: GTCGCGTAGCATGTTGGAG		
	VM37[a]	F: TGTCCGCGTTCTATAAATCAGC	8	SD100WT, SDW, SDL, SDT, PDW
		R: CGAGGATGAAGTAACAGATGATC		
Set B	MB14180[b]	F: CAGATTCCAACCCGAAGCCA	-	Protein FRIGIDA-like
		R: GCGAAAGAAGCTCGTCCTCT		
	MB15686[b]	F: CCCAACCTCTCCGCAAAGAT	-	Glucose-6-phosphate isomerase 1,
		R: ACAGCCAATCCACGTACCTC		chloroplastic-like
	MB21347[b]	F: GCCATCACCAACTACCCCTC	-	Xyloglucan galactosyltransferase
		R: AGGGGAGGGCGTAGATGTAG		KATAMARI1 homolog
	cp05137[a]	F: CCGATTGTAGATGATCCCGATTGT	1	-
		R: TGATGATTGCTGTGGGGAAATATG		
	GMES0216[b]	F: CCGGGACAGGGTTTCTAACT	2,4	-
		R: CCGAAGAAGACGACGAAATC		
	MB27164[b]	F: CTCAACAAGTTCCTCAGCGC	-	Mitochondrial import inner membrane
		R: CCAGAACCGGTGGAAGTCTC		trans- locase subunit TIM17-2-like
	MB64504[b]	F: CTCCTGAGGGCACTGAACTG	-	Dof zinc finger protein DOF4.6-
		R: GCTTCTGCAACGAGTTTCAACT		like
	GMES5572[b]	F: GCAGCAGCACTACATGGGTA	5	-
		R: AGATGGCATAGGAGGTGGTG		
	MB33094[b]	F-ATTGCCACCCCCATTTCCAT	-	Leucine-rich repeat extensin-like
		R-AGCAGTCCACCACTCTCTCT		protein 4-like

Note: Set A = polymorphic SSR markers linked to domestication related traits; Set B = polymorphic SSR and EST-SSR markers related to other putative protein functions and other fragments with unknown functions.

[a] SSR markers

[b] EST-SSR markers

[c] Linkage group

[d] FLD = Days to first flower, HECL = Hypocotyl plus epicotyl length, ITL = Internode length, PDL = Pod length, PDDM = Days to maturity of first pod, PDW = Pod width, RSP = Rate of shattered pods, SDL = Seed length, SDW = Seed width, SDNPPD = Number of seeds per pod, SD100WT = 100-seed weight, SDT = Seed thickness, STL = Stem length.

Table 4.3 ISSR markers used for background selection.

Primers	Primer sequences (5'-3')[a]	Number of scorable DNA bands	Number of polymorphic bands (male-female parents)
809	$(AG)_8G$	22	5
830	$(TG)_8G$	20	2
834	$(AG)_8YT$	30	4
835	$(AG)_8YC$	25	3
841c	$(GA)_8CC$	23	4
850	$(GT)_8YC$	22	2
857	$(AC)_8YG$	22	5
864	$(ATG)_6$	23	2
884	HBH $(AG)_7$	36	1
887	DVD $(TC)_7$	20	1
890	VHV $(GT)_7$	23	5
Total		266	34
Average		24.2	3.1

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

4.3.4 Evaluation of CLS and PM resistance and agronomic traits under field conditions

In order to evaluate resistance levels to CLS and PM and agronomic traits in the field, the BC_4F_4 to BC_4F_7 pyramided lines, parental lines (KING, V4718, V4758, and V4785), and check cv. KPS1 and SUT1 were grown in several seasons and locations during 2019-2021. The experiment was conducted under a randomized incomplete block design with two to four replications depending on seasons and locations. Briefly, in a growing season without any disease outbreak, they were grown in December 2019-February 2020 at Suranaree University of Technology (SUT) Farm, Muang district, Nakhon Ratchasima province. Each genotype was sown in a single row 2 m long with a spacing of 0.2 and 0.5 m intra-row and inter-row, respectively. Three plants per hill were maintained (ca. 30 plants per row) and also in December 2020-March 2021 at Pak Thong Chai District, Nakhon Ratchasima province. Each line was sown in two rows 6 m long with a spacing of 0.2 and 0.5 m intra-row and

inter-row, respectively. Three plants per hill were maintained (ca. 90 plants per row).

Genotypes were grown during the wet season in July 2020-September 2020 at SUT Farm, Muang district, Nakhon Ratchasima province, and Chai Nat Field Crops Research Center, Chai Nat province, which were subject to a CLS outbreak. Each genotype was planted in two rows of 6 m long with a spacing of 0.2 and 0.5 m intra-row and inter-row, respectively, and three plants per hill were maintained (ca. 90 plants per row). CLS severity was observed at 65 days after planting (DAP) using a scoring system described by Chankaew et al. (2011). The scale of CLS severity was divided into three categories (resistance = 1.0-2.5, moderate resistance = 2.6-3.4 and susceptibility = 3.5-5.0). The CLS symptoms was presented in Appendix (Figure A.1). In a growing season with a PM outbreak, the genotypes were grown during the cool-dry season in November 2020-February 2021 at SUT farm. Each genotype was grown in two rows of 6 m long with a spacing of 0.2 m intra-row and 0.5 m inter-row, and three plants per hill were maintained (ca. 90 plants per row). The observation of PM severity was scored at 65 DAP using a scoring system described by Khajudparn et al. (2010). The observations of resistance levels were divided into four categories (resistance = 1.0-3.0, moderate resistance = 3.1-4.5, moderate susceptibility = 4.6-6.0 and susceptibility = 6.1-9.0) (Figure A.2). In addition, the susceptible variety CN72 was sown around the experimental blocks as a source of CLS and PM inoculums.

With regard to agronomic traits, nine traits consisting of the number of days to flowering, days to maturity, plant height, clusters per plant, pods per plant, pod length, seeds per pod, 100-seed weight, and yield per plant were recorded from 10 randomly selected plants from the middle row of each block. The techniques for agronomic trait measurement were described by Chai Nat Field Crops Research

Center (2018) and Chueakhunthod et al. (2020) (Table A.1). Data analysis was performed using SPSS version 14.0 (Levesque and SPSS Inc., 2006). The significant difference of means was conducted by Duncan's Multiple Range Test (DMRT).

4.4 Results

4.4.1 Foreground and background selection

All five markers that were reported to be linked to a CLS and 2 PM resistance genes were validated for parental polymorphism between the donor and recurrent parents to be used in MAS for pyramiding CLS and PM resistance genes into the mungbean variety KING (Table 4.1). All markers were polymorphic between KING and D2 (the donor parent). Therefore, these markers were selected in MABC from F_1 to BC_4F_5 generations. Moreover, the genetic backgrounds of the putative resistant plants were evaluated with 3 different sets of markers (Tables 4.2, 4.3) in BC_1F_1 to BC_4F_2 generations. In each generation, the plants having marker loci linked to the three target genes with high recurrent parent genome (RPG) recovery were advanced to the next generation. The results are presented in Table 4.4.

In brief, twenty-five F_1 plants were produced by crossing KING and D2 and were further confirmed using five markers linked to CLS and PM resistance gene loci (Figure 4.2). Three out of 25 plants contained detectable heterozygous alleles based on all marker loci. These marker positive plants were backcrossed to KING to produce the BC_1F_1 seeds.

In BC_1F_1 generation, a total of 114 BC_1F_1 progenies were screened, and 2 BC_1F_1 plants were found to be heterozygous for all marker loci (1.8%). When a background selection was carried out, the percentages of RPG recovery of the two

marker positive plants based on all sets of markers were 82.1 to 91.7% with an average of 87.5, 85.0, and 83.2% for Sets A, B, and C, respectively. RPG recovery of both BC_1F_1 plants was higher than expected (75.0%). These marker positive plants were backcrossed to the recurrent parent to produce BC_2F_1 seeds.

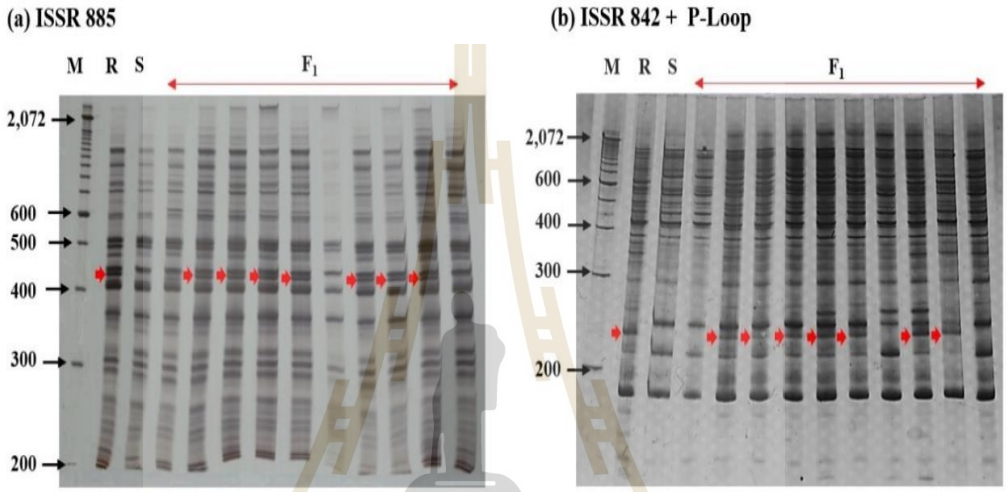

Figure 4.2 Marker-assisted foreground selection for PM resistance gene in F_1 generation using ISSR 885 primer (I85420 marker) (a) and ISSR 842+P-Loop primers (I42PL222 marker) (b). M = 100 bp DNA ladder, R = donor resistant parent (V4718), S = susceptible recurrent parent (KING). An arrow shows the markers linked to a PM resistance gene.

In BC_2F_1 generation, a total of 130 BC_2F_1 progenies were generated, of which 4 BC_2F_1 plants (3.1%) showed the presence of all target markers in heterozygous conditions. These four BC_2F_1 plants were subjected to background selection. They showed the presence of 86.8 to 100.0% of RPG recovery with an average of 95.9, 94.5, and 91.2% for Sets A, B, and C, respectively, which was higher

than expected (87.5%). Of these 4 plants, 3 BC_2F_1 plants that showed high RPG recovery were backcrossed to the recurrent parent to produce BC_3F_1 seeds.

In BC_3F_1 generation, out of 95 BC_3F_1 plants, 6 BC_3F_1 plants (6.3%) were heterozygous for all marker loci. When background selection was performed with three marker sets, the RPG recovery ranged from 88.9 to 100.0% with an average of 100.0, 95.4, and 96.7% for Sets A, B, and C, respectively. Among these, five BC_3F_1 plants had a higher RPG recovery than expected (93.7%), and these were backcrossed to the recurrent parent to produce BC_4F_1 seeds.

In BC_4F_1 generation, five out of 46 BC_4F_1 progenies (10.9%) were identified to be heterozygotes for all marker loci. These selected plants were then subjected to background selection. RPG recovery based on all sets of markers ranged from 94.4 to 100.0% with an average of 100.0, 96.6, and 97.5% for Sets A, B, and C, respectively. Two out of five plants were found to have the maximum recovery of RPG (up to 98.2-100.0%), which was higher than expected (96.9%). All of these marker positive plants were selfed to produce BC_4F_2 seeds.

In BC_4F_2 generation, when 156 BC_4F_2 progenies were screened, it was found that 14 BC_4F_2 plants (9.0%) possessed homozygous alleles for SSR markers and heterozygous/homozygous alleles for ISSR and ISSR-RGA markers. The background selection of these 14 BC_4F_2 progenies exhibited the presence of 94.4 to 100.0% RPG recovery with an average of 100.0, 98.0, and 99.0% for Sets A, B, and C, respectively. In addition, seven out of 14 BC_4F_2 progenies possessed the maximum RPG recovery (100.0% in all three marker sets) while the expected RPG recovery was only 96.9%. However, all 14 promising plants were still selected to produce BC_4F_3 seeds.

Table 4.4 Number of triple resistant gene heterozygotes or homozygotes identified and estimation of recurrent parent genome (RPG) contribution.

Generations	# of plants	# of plants that are triple resistant gene heterozygotes/homozygotes	Estimated contribution of RPG (%) to selected backcross progenies						Expected % contribution of RPG to backcross progenies
			Set A[b]		Set B[c]		Set C[d]		
			Range	Average	Range	Average	Range	Average	
F_1 [a]	25	3 (12.0%)	-		-		-		50
BC_1F_1 [a]	114	2 (1.8%)	83.3-91.7	87.5	85.0	85.0	82.1-84.2	83.2	75
BC_2F_1	130	4 (3.1%)	91.7-100.0	95.9	88.9-100.0	94.5	86.8-96.4	91.2	87.5
BC_3F_1	95	6 (6.3%)	100.0	100.0	88.9-100.0	95.4	96.2-98.2	96.7	93.7
BC_4F_1	46	5 (10.9%)	100.0	100.0	94.4-100.0	96.6	96.4-100.0	97.5	96.9
BC_4F_2	156	14 (9.0%)	100.0	100.0	94.4-100.0	98.0	96.4-100.0	99.0	96.9

[a] F_1 to BC_1F_1 generations were reported by Chueakhunthod (2019).
[b] SSR marker linked to domestication related traits derived from Isemura et al. (2012) (6 polymorphic SSR markers).
[c] EST-SSR markers related to other putative protein functions derived from Chen et al. (2015) and other SSR and EST-SSR markers with unknown functions derived from Isemura et al. (2012) (9 polymorphic markers).
[d] ISSR markers developed from the University of British Columbia (34 polymorphic markers).

4.4.2 Confirmation of homozygosity for all marker loci linked to the three target genes

In BC_4F_3 generation, a total of 14 BC_4F_3 lines were grown in a plant to row without foreground selection, and each plant was harvested individually with a total of 33 BC_4F_4 lines. Of these, 15 BC_4F_4 lines that had showed a good performance in the field condition were confirmed with foreground selection by randomly selected 20 plants per BC line. Only one out of 15 lines, namely H3, possessed all five marker loci in all 20 plants (not segregated). Moreover, 2 additional BC lines (H4 and J3) with all five marker loci were found in some of the 20 plants and these were also selected. These three BC lines were again confirmed for the presence of 5 markers in BC_4F_5 generation. Only H3 and H4 possessed all marker loci in all 20 plants (not segregated), confirming that these BC lines had homozygous alleles for all marker loci. When J3 was found to have segregated at some marker loci, this line was not used for any further experiments. These H3 and H4 lines were evaluated for CLS and PM resistance and agronomic traits in the field conditions together with their parents and check varieties in different locations, seasons, and years.

4.4.3 Comparison of agronomic traits and disease resistance under field conditions

4.4.3.1 Under no disease outbreak

In a growing season without any disease outbreak, two different generations (BC_4F_4 and BC_4F_7) of pyramided BC lines, their parents, and check varieties, KPS1 and SUT1 were evaluated for yield performances and agronomic traits during the winter for two years at two locations. BC_4F_4 generation was evaluated in December 2019-February 2020 at SUT Farm while BC_4F_7 generation was evaluated in

December 2020-March 2021 at Pak Thong Chai. The analysis of yield performance was found to be significantly different ($P < 0.05$ or $P < 0.01$) among pyramided BC lines, their parents, and check varieties in both environments. In December 2019-February 2020 at SUT Farm, the highest yield per plant was observed in H3 line which was higher than two donor parents V4718 and V4758, but was not different from the recurrent parent KING, donor parent V4785, as well as check varieties. Meanwhile, both pyramided BC lines were not significantly different from KING, V4718, and V4785 when evaluated in December 2020-March 2021 at Pak Thong Chai, but yield per plant of H4 line was lower than those of V4758 and check varieties (Figure 4.3).

A significant variation was found between the pyramided BC lines, their parents and check varieties in days to maturity, pods per plant, pod length, seeds per pod, and 100-seed weight in both environments ($P < 0.05$ or $P < 0.01$). While differences in clusters per plant and plant height were only observed in December 2019-February 2020 at SUT Farm and in December 2020-March 2021 at Pak Thong Chai ($P < 0.05$ or $P < 0.01$), respectively (Table 4.5). Only days to flowering was not significantly different in either environment ($P > 0.05$). Pod length and 100-seed weight of pyramided BC lines and recurrent parent (KING) were significantly higher than the three donor parents in both environments. While three donor parents had higher seeds per pod than pyramided BC lines and KING in both environments. However, H3 line tended to have greater pod length and 100-seed weight than check varieties in both environments. In addition, both BC lines had most agronomic traits similar to KING, especially H3 which had all agronomic traits similar to KING in December 2019-February 2020 at SUT Farm while both BC lines

had lower 100-seed weight than KING in December 2020-March 2021 at Pak Thong Chai (Table 4.5).

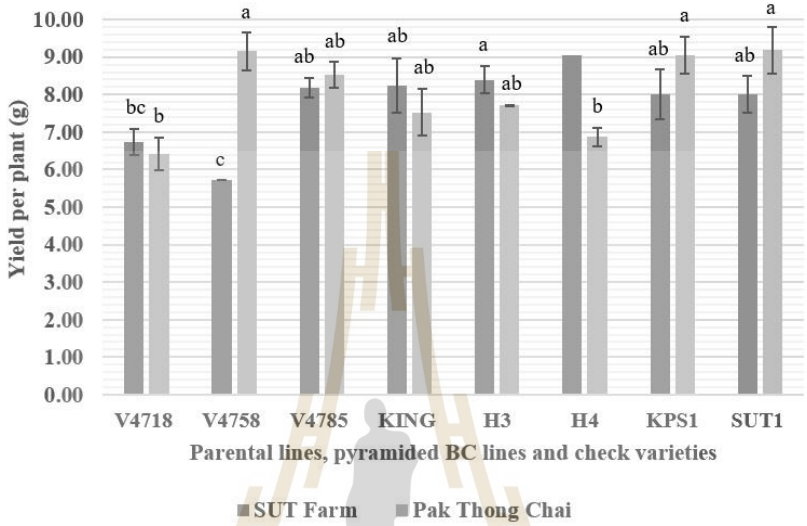

Figure 4.3 Yield performance of parental lines, check varieties and pyramided BC lines when growing without disease outbreak. They were grown in December 2019-February 2020 at SUT Farm and in December 2020-March 2021 at Pak Thong Chai, Nakhon Ratchasima. Different letters are significantly different ($P < 0.05$) based on DMRT. V4785, V4758, V4718, and KING are parental lines; KPS1 and SUT1 are check varieties; H3 and H4 are pyramided BC lines, respectively. H4 line at SUT Farm had only one replication.

Table 4.5 Comparison of eight agronomic traits between parental lines and pyramided BC lines as well as check varieties under field conditions without any disease outbreak.

Locations	Lines/varieties/ BC lines	Days to flowering	Days to maturity	Plant height (cm)	Clusters/plant	Pods/plant	Pod length (cm)	Seeds/pod	100 seed weight (g)
SUT	V4718	46.50 ± 1.80	62.83 ± 1.17 bc[a]	40.85 ± 4.54	6.74 ± 0.84 ab	20.85 ± 3.13 a	6.85 ± 0.18 c	10.83 ± 0.13 a	3.68 ± 0.23 c
Farm	V4758	47.50 ± 3.50	62.00 ± 2.00 c	32.49 ± 2.79	6.05 ± 1.45 abc	17.49 ± 1.89 ab	6.95 ± 0.12 c	11.32 ± 0.14 a	3.55 ± 0.05 c
	V4785	47.00 ± 1.26	65.17 ± 0.60 ab	38.15 ± 1.81	7.86 ± 0.22 a	21.13 ± 0.50 a	7.12 ± 0.17 c	11.34 ± 0.04 a	3.86 ± 0.08 c
	KING	46.50 ± 1.00	66.00 ± 0.50 a	41.40 ± 0.55	5.14 ± 0.07 bc	12.22 ± 0.86 b	9.60 ± 0.05 a	9.28 ± 0.30 b	7.90 ± 0.16 a
	H3 (BC_4F_4)	47.33 ± 0.33	65.67 ± 0.88 a	40.31 ± 2.56	4.29 ± 0.04 c	11.71 ± 0.04 b	9.50 ± 0.31 ab	9.55 ± 0.52 b	7.34 ± 0.38 ab
	H4 (BC_4F_4)[b]	47.00	64.00	36.70	5.33	14.00	9.31	10.25	6.98
	KPS1	45.89 ± 0.93	64.22 ± 0.89 abc	44.12 ± 3.45	5.51 ± 0.19 bc	15.08 ± 1.12 ab	8.89 ± 0.19 b	9.68 ± 0.03 b	6.41 ± 0.22 b
	SUT1	46.31 ± 0.60	65.25 ± 0.90 ab	38.05 ± 0.72	5.54 ± 0.33 bc	16.40 ± 0.72 ab	9.06 ± 0.12 ab	9.63 ± 0.23 b	6.10 ± 0.70 b
	F-test	ns	*	ns	*	*	**	**	**
Pak	V4718	45.00 ± 0.00	64.67 ± 0.33 c	38.94 ± 3.25 c	7.78 ± 0.98	27.23 ± 2.54 a	6.04 ± 0.10 d	11.78 ± 0.22 a	2.31 ± 0.05 f
Thong	V4758	45.00 ± 0.00	66.00 ± 0.58 bc	51.73 ± 1.33 ab	8.54 ± 0.83	24.51 ± 1.40 a	7.84 ± 0.15 c	12.44 ± 0.29 a	3.36 ± 0.01 d
Chai	V4785	45.00 ± 0.00	67.50 ± 1.50 bc	48.84 ± 0.41 bc	7.71 ± 1.29	27.13 ± 3.13 a	7.16 ± 0.26 d	12.22 ± 0.09 a	3.11 ± 0.19 e
	KING	47.00 ± 1.15	69.67 ± 1.45 a	54.36 ± 5.42 ab	5.83 ± 0.43	13.10 ± 1.07 c	9.65 ± 0.18 ab	9.44 ± 0.57 b	6.80 ± 0.11 a
	H3 (BC_4F_7)	45.00 ± 2.00	67.50 ± 0.50 ab	56.96 ± 5.82 ab	6.05 ± 0.38	14.67 ± 0.33 bc	10.07 ± 0.76 a	9.40 ± 0.11 b	6.27 ± 0.19 b
	H4 (BC_4F_7)	50.00 ± 2.65	70.00 ± 1.53 a	54.69 ± 3.04 ab	6.88 ± 0.56	14.32 ± 1.12 c	9.00 ± 0.05 b	9.42 ± 0.46 b	5.72 ± 0.04 c
	KPS1	48.33 ± 1.76	70.00 ± 0.58 a	58.31 ± 1.32 a	7.69 ± 0.48	18.84 ± 0.77 b	9.55 ± 0.21 ab	10.45 ± 0.45 b	5.64 ± 0.07 c
	SUT1	45.67 ± 1.76	68.67 ± 0.88 ab	53.82 ± 3.69 ab	7.11 ± 0.12	19.03 ± 1.01 b	8.73 ± 0.22 b	9.43 ± 0.18 b	5.67 ± 0.06 c
	F-test	ns	**	*	ns	**	**	**	**

Note: At SUT Farm, they were grown in December 2019-February 2020. At Pak Thong Chai, they were grown in December 2020-March 2021.
[a] Means ± SE in the same column with different letters are significantly different ($P < 0.05$) based on DMRT.
[b] BC line that had only one replication.

4.4.3.2 Under warm-wet growing season for CLS evaluation

In a growing season with a CLS outbreak, two different generations (BC_4F_5 and BC_4F_6) of pyramided BC lines, their parents and check varieties, KPS1 and SUT1, were evaluated for yield performances, agronomic traits, and CLS response in July 2020-September 2020 at two different locations. In this season, H3 line was evaluated in BC_4F_5 and BC_4F_6 generations at SUT Farm and Chai Nat Field Crops Research Center, respectively. Meanwhile, H4 line was evaluated in BC_4F_6 generation at both locations. The CLS response was found to be significantly different among mungbean genotypes ($P < 0.05$) at both locations. At SUT Farm, we found that H3 and H4 lines were resistant and moderately resistant to CLS with a score of 2.50 and 2.75, respectively. The disease scores of these BC lines were comparable to that of V4718 which was the donor of the CLS resistance gene (2.50). While KING was identified as susceptible to CLS with a score of 3.63 and had the highest CLS symptoms on their leaves when compared with V4718 and BC lines (Figure 4.4, 4.5a). Check cv. SUT1 and KPS1 were susceptible and moderately resistant to CLS, respectively at this location. At Chai Nat Field Crops Research Center where a CLS outbreak was more severe, all genotypes were found to be susceptible to CLS including V4718, except for V4785 which was moderately resistant to CLS.

The analysis of variance showed significant differences ($P < 0.05$ or $P < 0.01$) for days to flowering, days to maturity, plant height, clusters per plant, pods per plant, pod length, seeds per pod and 100-seed weight at both locations (Table 4.6) while yield per plant was found to be significant only at SUT Farm (Figure 4.5b). The highest yield per plant at SUT Farm was observed in H3 line (6.88 g) which was

higher than parental lines (27-33%) and check varieties (23-44%). Meanwhile, yield per plant of H4 line (4.77 g) was not significantly different from parental lines and check varieties (Figure 4.5b). When comparison of yield performance between without any disease outbreak and CLS outbreak, yield performance of pyramided BC lines, their parents, and check varieties was decreased up to 15-55% and 30-54% at SUT Farm and Chai Nat Field Crops Research Center, respectively. In addition, KING and pyramided BC lines had higher pod length and 100-seed weight, but lower pods per plant and seeds per pod than those of donor parents at both locations, except H3 which had similarly high pods per plant and seeds per pod as V4718 and V4758 at SUT Farm. Two pyramided BC lines and KING had earlier days to flowering than some donor parents (V4758 and V4785) at both locations. Moreover, both BC lines had most agronomic traits similar to KING, especially H4, which had all agronomic traits similar to KING while H3 had higher pods per plant than KING when evaluated at SUT Farm. However, at Chai Nat Field Crops Research Center, H3 had slightly lower pod length and 100-seed weight than KING while H4 also had lower 100-seed weight than KING. We also found that H3 line not only had a higher pods per plant and seeds per pod than check cv KPS1 but also had a higher pods per plant and a tendency for higher clusters per plant and seeds per pod than the recurrent parent KING at SUT Farm (Table 4.6).

Figure 4.4 CLS symptoms of pyramided BC lines with parents at SUT Farm. V4718 is a donor resistant parent; KING is a susceptible recurrent parent; H3 and H4 are pyramided BC lines.

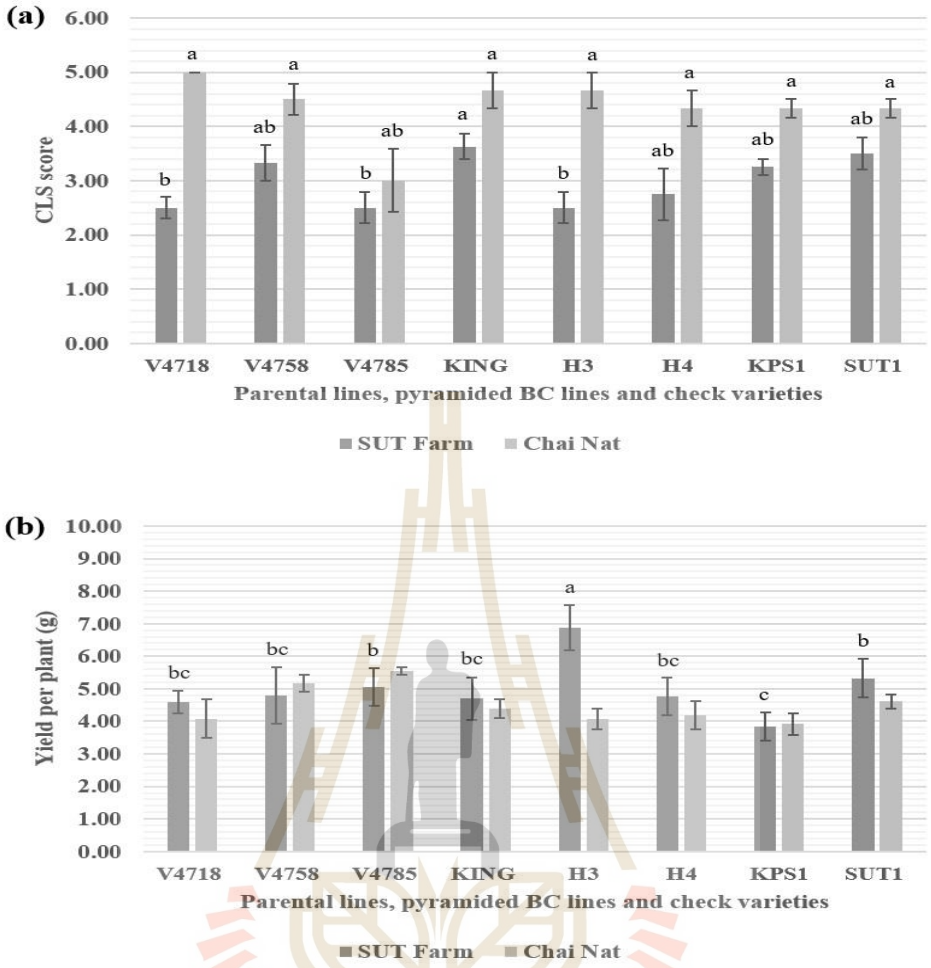

Figure 4.5 Comparison of CLS response (a) and yield performance (b) between the parental lines, check varieties and pyramided BC lines. They were grown in July 2020-September 2020 at SUT Farm, Nakhon Ratchasima, and Chai Nat Field Crops Research Center, Chai Nat. Different letters are significantly different ($P < 0.05$) while no letter is not significantly different ($P > 0.05$) based on DMRT. V4785, V4758, V4718, and KING are parental lines; KPS1 and SUT1 are check varieties; H3 and H4 are pyramided BC lines, respectively.

Table 4.6 Comparison of eight agronomic traits between parental lines and pyramided BC lines as well as check varieties under field conditions with a CLS outbreak.

Locations	Lines/varieties/ BC lines	Days to flowering	Days to maturity	Plant height (cm)	Clusters/plant	Pods/plant	Pod length (cm)	Seeds/pod	100 seed weight (g)
SUT	V4718	36.88 ± 0.66 c[a]	50.50 ± 0.29 c	48.70 ± 4.03 bc	5.26 ± 0.12 bc	17.05 ± 1.27 ab	6.15 ± 0.10 c	10.82 ± 0.39 ab	3.01 ± 0.04 c
Farm	V4758	43.83 ± 0.60 a	61.50 ± 0.76 a	68.40 ± 2.79 ab	7.12 ± 0.67 b	17.90 ± 2.96 abc	7.17 ± 0.04 b	11.18 ± 0.55 ab	3.29 ± 0.07 b
	V4785	44.50 ± 0.76 a	63.17 ± 2.32 a	65.81 ± 8.54 a	8.04 ± 0.68 a	18.56 ± 1.99 a	6.98 ± 0.10 c	11.46 ± 0.70 a	3.35 ± 0.06 bc
	KING	39.63 ± 0.80 b	56.88 ± 0.77 b	47.76 ± 4.94 bc	3.98 ± 0.34 cd	8.61 ± 1.45 e	9.28 ± 0.07 a	9.22 ± 0.08 cd	6.76 ± 0.11 a
	H3 (BC_4F_5)	39.00 ± 0.58 b	54.67 ± 0.88 b	57.29 ± 1.12 ab	5.05 ± 0.19 bcd	13.56 ± 1.92 bcd	9.41 ± 0.03 a	9.99 ± 0.09 bc	6.29 ± 0.06 a
	H4 (BC_4F_6)	39.25 ± 0.25 b	55.00 ± 0.58 b	41.28 ± 2.00 c	3.81 ± 0.16 d	9.51 ± 1.58 de	8.70 ± 0.04 a	8.97 ± 0.10 d	6.29 ± 0.12 a
	KPS1	39.50 ± 0.65 b	56.38 ± 1.14 b	60.37 ± 4.67 ab	4.57 ± 0.36 cd	9.09 ± 0.81 e	8.89 ± 0.41 a	8.73 ± 0.21 d	5.96 ± 0.19 a
	SUT1	40.25 ± 0.95 b	57.25 ± 1.64 b	48.63 ± 2.63 bc	4.28 ± 0.27 cd	11.54 ± 1.87 cde	8.57 ± 0.11 ab	9.46 ± 0.18 cd	5.97 ± 0.07 a
	F-test	**	**	**	**	**	**	**	**
Chai	V4718	38.17 ± 0.17 d	55.33 ± 0.17 c	76.91 ± 1.84 c	7.06 ± 0.75 ab	13.91 ± 1.32 b	6.40 ± 0.04 d	11.41 ± 0.03 a	3.01 ± 0.06 e
Nat	V4758	48.17 ± 0.17 a	64.00 ± 2.50 ab	104.23 ± 1.51 a	6.76 ± 0.42 ab	19.07 ± 0.93 a	7.18 ± 0.10 c	12.28 ± 0.63 a	2.68 ± 0.05 e
	V4785	46.00 ± 0.76 a	65.50 ± 2.57 a	104.04 ± 2.53 a	7.55 ± 0.94 a	17.96 ± 0.79 a	7.15 ± 0.19 c	12.12 ± 0.20 a	3.08 ± 0.08 e
	KING	41.00 ± 1.53 bc	60.00 ± 1.00 abc	90.78 ± 0.64 b	4.12 ± 0.21 c	7.38 ± 0.35 c	9.62 ± 0.22 a	9.74 ± 0.51 b	6.59 ± 0.18 a
	H3 (BC_4F_6)	40.00 ± 0.00 cd	59.33 ± 2.19 bc	89.99 ± 2.62 b	4.53 ± 0.45 c	8.26 ± 0.57 c	8.92 ± 0.45 b	9.12 ± 0.62 b	6.14 ± 0.08 b
	H4 (BC_4F_6)	43.00 ± 2.08 b	64.00 ± 2.00 ab	87.40 ± 1.68 b	4.65 ± 0.35 c	8.06 ± 1.07 c	9.22 ± 0.04 ab	9.61 ± 0.12 b	6.08 ± 0.22 b
	KPS1	42.33 ± 0.88 bc	63.17 ± 1.96 ab	105.83 ± 4.70 a	6.51 ± 0.77 ab	9.06 ± 1.00 c	8.58 ± 0.08 b	10.06 ± 0.04 b	4.94 ± 0.30 d
	SUT1	39.83 ± 0.33 cd	58.50 ± 1.00 bc	90.44 ± 2.86 b	5.38 ± 0.02 bc	9.04 ± 0.55 c	8.69 ± 0.05 b	10.07 ± 0.15 b	5.64 ± 0.09 c
	F-test	**	*	**	**	**	**	**	**

Note: They were grown in July 2020-September 2020 at SUT Farm, Nakhon Ratchasima, and Chai Nat Field Crops Research Center, Chai Nat.
[a] Means ± SE in the same column with different letters are significantly different ($P < 0.05$) based on DMRT.

4.4.3.3 Under cool-dry growing season for PM evaluation

In a growing season with a PM outbreak, two different generations (BC_4F_6 for H3 and BC_4F_7 for H4) of pyramided BC lines, their parents, and check cv. SUT1 were evaluated for yield performance, agronomic traits, and PM response during November 2020-February 2021 at SUT Farm. PM response was found to be significantly different among mungbean genotypes ($P < 0.01$) as presented in Figure 4.6a. We found that H3 and H4 lines were moderately resistant to PM with a score of 3.50 and 4.00, respectively. The disease scores of PM resistance donor parents were resistant and moderately resistant to PM with a score of 2.00 and 4.00 for V4718 and V4785, respectively. While KING was identified as susceptible to PM with a score of 6.33. Check cv. SUT1 was moderately susceptible to PM (5.67) (Figure 4.6a).

When we analyzed the variations in yield performance and agronomic traits, the results showed highly significant differences ($P < 0.01$) for clusters per plant, pods per plant, pod length, seeds per pod, 100-seed weight, and yield per plant (Table 4.7, Figure 4.6b), whereas days to flowering, days to maturity and plant height were not found to be significantly different ($P > 0.05$). The yield per plant of both BC lines was 5.30 and 3.91 g for H3 and H4, respectively, which were not significantly different from those of the recurrent parent KING (4.35 g). However, H3 tended to have a higher yield than KING (18.0%). In this season, yield performance of most mungbean genotypes was reduced up to 15-51% when compared to under no disease outbreak. In addition, pyramided BC lines and KING had greater pod length and 100-seed weight but lower pods per plant and seeds per pod than those of the donor parents. They also had lower clusters per plant than V4718 and V4785. Moreover, H3 and H4 lines not

only had all agronomic traits similar to KING but also had a higher 100-seed weight than check cv. SUT1. One of which (H3) had a higher tendency for clusters per plant and pods per plant than the recurrent parent KING similar to during CLS outbreak.

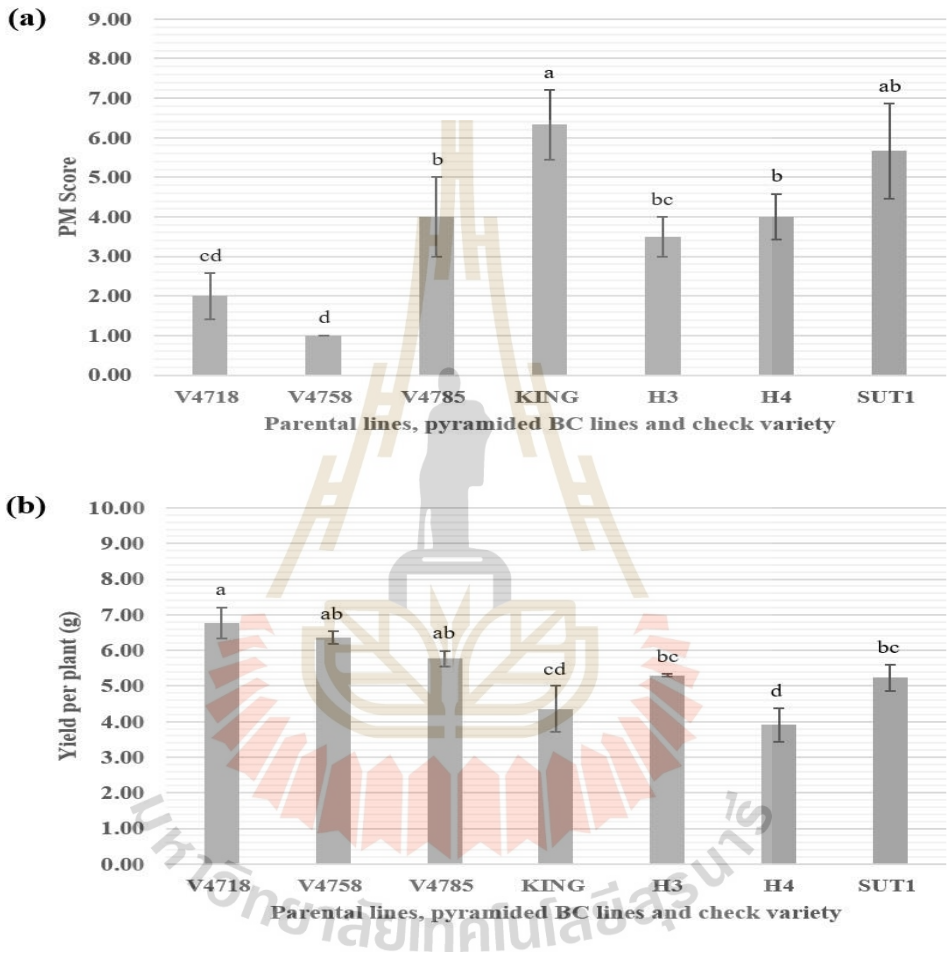

Figure 4.6 Comparison of PM response (a) and yield performance (b) between the parental lines, check varieties and pyramided BC lines. They were grown in November 2020-February 2021 at SUT Farm, Nakhon Ratchasima. Different letters are significantly different ($P < 0.05$) based on DMRT. V4785, V4758, V4718, and KING are parental lines; SUT1 is a check variety; H3 and H4 are pyramided BC lines, respectively.

Table 4.7 Comparison of eight agronomic traits between parental lines and pyramided BC lines as well as check varieties under field conditions with a PM outbreak.

Lines/varieties/ BC lines	Days to flowering	Days to maturity	Plant height (cm)	Clusters/plant	Pods/plant	Pod length (cm)	Seeds/pod	100 seed weight (g)
V4718	43.00 ± 2.08	59.00 ± 2.08	38.28 ± 8.32	7.40 ± 0.48 a[a]	23.19 ± 1.95 a	6.28 ± 0.20 c	10.68 ± 0.45 a	3.18 ± 0.14 d
V4758	44.67 ± 3.28	62.67 ± 3.18	43.97 ± 5.55	6.40 ± 0.79 ab	18.50 ± 1.19 b	7.24 ± 0.13 b	10.42 ± 0.18 a	3.77 ± 0.03 c
V4785	46.33 ± 2.19	65.67 ± 0.88	46.31 ± 6.56	8.15 ± 1.20 a	20.14 ± 0.79 ab	6.75 ± 0.14 bc	9.88 ± 0.31 a	3.60 ± 0.13 cd
KING	46.00 ± 1.53	66.33 ± 0.33	39.90 ± 3.81	3.67 ± 0.59 c	8.05 ± 0.90 c	8.42 ± 0.20 a	7.53 ± 0.11 c	7.48 ± 0.23 a
H3 (BC_4F_6)	47.00 ± 0.00	65.50 ± 0.50	42.24 ± 4.38	4.14 ± 0.14 bc	9.79 ± 0.08 c	8.62 ± 0.02 a	7.70 ± 0.39 bc	7.40 ± 0.44 a
H4 (BC_4F_7)	47.33 ± 0.33	65.33 ± 0.33	32.67 ± 4.38	3.86 ± 1.01 c	7.60 ± 1.32 c	8.50 ± 0.20 a	8.00 ± 0.42 bc	6.97 ± 0.17 a
SUT1	47.00 ± 1.53	64.00 ± 1.15	44.10 ± 5.04	4.71 ± 0.22 bc	10.63 ± 0.66 c	8.23 ± 0.21 a	8.66 ± 0.27 b	6.05 ± 0.01 b
F-test	ns	ns	ns	**	**	**	**	**

Note: They were grown in November 2020-February 2021 at SUT Farm, Nakhon Ratchasima.
[a] Means ± SE in the same column with different letters are significantly different ($P < 0.05$) based on DMRT.

4.5 Discussion

In breeding programs, pyramiding multiple resistance genes into a single genotype can provide broad-spectrum and durable resistance. However, using conventional breeding to pyramid multiple genes and select for several traits simultaneously can be difficult, especially for disease resistance of which the presence of 1, 2, or multiple resistance genes cannot be differentiated. Recently, MAS has become a critical element for the conventional breeding method by helping plant breeders select multiple desirable traits without the confounding effects of environment. When applied to backcrossing, it is called marker-assisted backcrossing (MABC) which allows simultaneous selection for desirable traits (foreground selection) and fast recovery of recurrent genome (background selection). In this study, we used this strategy to pyramid a CLS resistance gene and two PM resistance genes into a high yielding mungbean variety KING so that selection for both CLS and PM resistance can be accomplished year-round without the requirement of suitable environments for disease outbreaks. Besides, it also allows pyramiding 2 PM resistance genes from different sources into the same variety, which may provide broad-spectrum and/or more durable resistance. Similarly, this strategy has also been successfully used to transfer gene (s)/QTL for disease resistance in several legume crops including common bean, chickpea, and soybean (Garzón et al., 2008; Carneiro et al., 2010; Varshney et al., 2014; Maranna et al., 2016).

When five markers associated with CLS and PM resistance were verified for DNA polymorphisms between recurrent parent (KING) and donor parent (D2), it was found that all of them were polymorphic, and that they could be used for foreground selection. Although these 5 markers were identified in different crosses (CN72 ×

V4718 or CN72 × V4785), they can be used successfully in the KING × D2 cross because D2 was the double cross of RILs from the crosses between CN72 and V4718, V4758, and V4785, thereby the CLS and PM resistance genes were the same. Our results confirmed the usefulness of utilizing marker (s) linked to desirable trait (s) in crosses with different recurrent parents if polymorphisms exist. Among these five markers, two were codominant SSR marker (VR393 and CEDG084), flanking the QTL controlling CLS resistance in the CN72 × V4718 cross, two were dominant ISSR and ISSR-RGA markers (I85420 and I42PL222), flanking the QTL conferring resistance to PM in the cross between CN72 and V4718, and one was a dominant ISSR-RGA marker (I27R565) associated with PM resistance in the cross between CN72 and V4785 (Arsakit et al., 2017; Poolsawat et al., 2017; Tantasawat et al., 2021).

For background selection, we screened a total of 37 SSR and EST-SSR markers but found that only 15 of them were polymorphic between KING and D2 (40.5%). Among these, 6 polymorphic SSR markers (Set A) were reported to be linked to domestication related traits i.e., seed length, seed width, pod length, pod width, 100-seed weight or seeds per pod and located on linkage groups (LG) 1, 4, 6, 7, 8, and 10 (Isemura et al., 2012). Meanwhile, 6 polymorphic EST-SSR markers reported to be related to other putative protein functions and three polymorphic EST-SSR and SSR markers with unknown functions (Set B) were located on LG 1, 2, 4, and 5 (Isemura et al., 2012; Chen et al., 2015). The number of polymorphic SSR and EST-SSR markers obtained from this study may be lower than those in other studies, which have reported using 26 to 160 polymorphic loci for background selection (Divya et al., 2014; Miah et al., 2015; Pradhan et al., 2015; Ahmed et al., 2016; Arunakumari et al., 2016; Xiao et al., 2016; Krishna et al., 2017; Yadawad et al., 2017; Baliyan et al.,

2018; Jamaloddin et al., 2020). This may be due to the number of markers and genetic variation of the parents used. Therefore, an additional 34 polymorphic fragments amplified by 11 ISSR primers (set C), which were randomly distributed throughout the genome, were also used to cover more chromosomal regions of the genome. Using all of these 49 polymorphic fragments/loci, 94.4 to 100.0% RPG was recovered in the two pyramided BC lines. Interestingly, one of the two pyramided BC lines (H3) displayed RPG recovery of 100.0, 100.0, and 98.2% for Sets A, B, and C, respectively. This is in agreement with Sundaram et al. (2008), who suggested that background selection with approximately 50 polymorphic SSR markers in conjunction with four generations of backcrossing is sufficient for the recovery of grain yield and other characteristics of the recurrent parent while transferring a resistance gene or a trait of interest. These results demonstrate that background selection with these polymorphic marker loci facilitated the recovery of the recurrent parent genome from BC_4 generation, accelerating the backcrossing, which can reduce at least 2 generations of backcrossing in breeding programs, thereby leading to time and cost savings.

The combinations between phenotypic selection and marker-assisted background selection have long been reported to be effective for breeding programs (Gopalakrishnan et al., 2008). Therefore, we also evaluated the yield performance and agronomic traits of these two pyramided BC lines, their parents and check varieties under field conditions in several seasons, years, and locations. Both pyramided BC lines displayed some levels of resistance to both CLS and PM in comparison to the recurrent parent KING and they exhibited most agronomic traits similar or superior to those of KING. A tendency for higher clusters per plant and pods per plant in BC

lines may be inherited from the donor parents. However, pod length and 100-seed weight of both pyramided BC lines were lower than those of KING in 1-2 environments. These may stem from the low number of selected BC lines used for evaluating field performances, resulting in slightly lower levels of a few traits in our pyramided BC lines in some environments, especially 100-seed weight. In addition, in each generation of backcrossing, using the stringent phenotypic selections together with background selection based on markers may allow more efficient RPG recovery than using only markers, especially in case of limited number of polymorphic markers available for background selection. This is in agreement with Miah et al. (2015), who recommended that using background selection coupled with visual selection led to increasing the recurrent parent genome recovery. In a growing season without any disease outbreaks, yield performance of both pyramided BC lines was not significantly different from that of KING, whereas under conditions of CLS and PM outbreaks, one of these BC lines (H3) not only had a higher yield performance than KING (18-32%) but also tended to have higher 100-seed weight than check varieties KPS1 and SUT1, possessing a larger seed size than other varieties commonly grown in Thailand, in all environments. The weather data during CLS evaluation showed that the relative humidity (RH %) levels at SUT Farm and Chai Nat Field Crops Research Center was 71.1% RH and 76.2% RH, respectively (a 5.1% RH difference). The average temperature at both locations was not different (25.4-35.2°C). By contrast, the average amount of rain at SUT Farm (203.8 mm) was higher than Chai Nat Field Crops Research Center (130.0 mm) (Table A.3). However, the highest reduction of grain yield (30-54%) was observed at Chai Nat Field Crops Research Center. While, grain yield at SUT Farm was decreased 15-55%. This may be due to the CLS outbreak

at Chai Nat Field Crops Research Center was more severe than at SUT Farm possibly due to higher RH than at SUT Farm. The results indicated that high humidity may increase the CLS outbreak. Similarly, Kumar et al. (2011) demonstrated that higher RH and temperature promoted the germination of conidia of *Cercospora canescens.* In addition, this location was also affected by virus infection, possibly from severe uncontrollable insect infestation. During PM evaluation at SUT Farm, we found that grain yield of most mungbean genotypes was decreased up to 15-51% when compared to the condition without disease outbreak. The average temperatures in this season ranged from 18.3-31.0°C, which were slightly lower than at SUT Farm without disease outbreak (19.4-33.2°C) (Table A.2, A.4). Differences in temperatures may affect PM outbreak. These results indicated that CLS and PM outbreaks affected mungbean production. However, other environmental factors may also contribute to the yield reduction. Our results confirmed that the use of foreground and background selection in MAS is efficient for improving mungbean variety. The pyramided BC line H3 not only exhibited higher levels of resistance to CLS and PM than the recurrent parent KING but also had most agronomic traits similar or superior to those of the recurrent parent KING.

4.6 Conclusion

We successfully pyramided CLS and PM resistance genes from the donor parent into a high yielding mungbean variety KING using MABC technique by performing selection with five markers linked to CLS and PM resistance genes for foreground selection and 15 polymorphic SSR and EST-SSR and 34 polymorphic ISSR loci for background selection. Our pyramided BC_4F_2 progenies had a high RPG recovery up

to 98.0-100.0%, depending on the marker set, indicating the effectiveness of background selection in accelerated backcrossing. The pyramided BC lines, namely H3 and H4, displayed moderate resistance to PM, one of which (H3) was also resistant to CLS. We found that the H3 line had most agronomic traits similar or superior to the recurrent parent KING. Interestingly, H3 line tended to have higher pods per plant, clusters per plant, seeds per pod, and yield per plant than KING. Therefore, this line can be potentially developed into a new resistant variety in the future.

4.7 References

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CHAPTER V

PYRAMIDING OF CERCOSPORA LEAF SPOT AND POWDERY MILDEW RESISTANCE GENES IN MUNGBEAN VARIETY SUT1 THROUGH MARKER-ASSISTED BACKCROSSING

5.1 Abstract

The utilization of markers linked to R genes for developing new resistant varieties is essential for overcoming mungbean diseases. In this study, a Cercospora leaf spot (CLS) resistance gene and 2 powdery mildew (PM) resistance genes from the donor parent A2 (a double cross of RILs from the crosses between CN72 and V4718, V4758, and V4785) were transferred into a recommended mungbean variety SUT1 using marker-assisted backcrossing (MABC). Five markers linked to CLS and PM resistance genes and three marker sets with 72 polymorphic loci were used for foreground and background selection, respectively. As a result, six pyramided BC_4 lines (A1, B1, B2, D2, D5, and G1) carrying all marker loci in homozygosity, except for marker CEDG084, were developed. The recurrent parent genome (RPG) recovery of these pyramided BC lines was 100.0, 100.0, and 98.8% for Set A, B, and C, respectively. During field evaluation, we found that a moderate to high level of PM resistance was observed in these BC lines, and one of these lines (B2) was also moderately resistant to CLS. Our pyramided BC lines had most agronomic traits similar or superior to the re-

current parent SUT1. In addition, we found that yield performance of B2 line with resistance to both CLS and PM was slightly higher than SUT1 (3.5-5.3%) during CLS and PM outbreaks. Interestingly, other selected BC lines (A1, B1, and D5) with moderate resistance to PM had higher yield up to 3.7-31.0% than SUT1 under CLS and PM outbreaks. Thus, these pyramided BC lines can be further used to develop a new resistant mungbean variety in the future.

5.2 Introduction

In Thailand, mungbean [*Vigna radiata* (L.) Wilczek] cultivated area was approximately 1.3 hundred thousand hectares (Office of Agricultural Economics, 2019). Mungbean plays an important role in crop rotation with rice and corn or used in intercropping system. Moreover, they are a rich source of proteins, minerals, and vitamins. Thus, it is a supplement of cereal-based human diet. Thai certified mungbean cultivars such as CN72, CN84-1, CN36, SUT1, KPS1, and KPS2, are commonly grown by Thai farmers in wet and dry seasons. The yield potential of these cultivars is in the range of 165 to 226 kg/rai (Ngampongsai et al., 2011; Chai Nat Field Crops Research Center, 2018). The low productivity is constrained by biotic and abiotic stresses such as susceptibility to pests and diseases or weakness to environments.

Cercospora leaf spot (CLS), powdery mildew (PM), and yellow mosaic virus are the three most severe diseases in mungbean. The infection of these diseases can cause 10-100% yield loss depending on their growth stage (Rana et al., 2016). CLS is caused by the hemibiotrophic fungus *Cercospora canescens* Ellis & Martin (Chand et al., 2015). The first visible symptom of CLS infection is spotting on older leaves at the bottom of the plants. The severest effect of CLS occurs during flowering until the pod-filling stage in the

warm wet growing season, resulting in a reduction in the size of pods and seeds (Grewal et al., 1980). On the other hand, PM is caused by the biotrophic fungus *Sphaerotheca phaseoli.* Symptoms of PM include white spotting on both sides of the leaf surface and stems. This disease is widely spread in cool-dry seasons and can reduce mungbean seed yield, up to 50% (Khajudparn et al., 2010). Therefore, the improvement of new resistant mungbean cultivars is essential for increasing yield potential. A few resistant sources of CLS and PM have been identified, such as the V4718 line for CLS and PM resistance (Chankaew et al., 2011; Tantasawat et al., 2020) and V4758 and V4785 for PM resistance (Khajudparn et al., 2010). The inheritance of CLS resistance gene in the V4718 line is controlled by a single dominant gene, while PM resistance gene in V4718, V4758, and V4785 is also controlled by a single dominant gene which is non-allelic (Khajudparn et al., 2010; Chankaew et al., 2011; Tantasawat et al., 2020). This knowledge is helpful for plant breeders in developing new resistant varieties.

Recently, the identification of CLS and PM resistance genes has been reported using different molecular marker systems. Yundaeng et al. (2020) identified closer flanking markers (Vr6gCLS085 and VrTAF5_indel) located nearer the QTL (*qCLS*) controlling CLS resistance in KPS1 × V4718 cross than those reported by of Chankaew et al. (2011). These markers were only 12-13 Kb from a candidate gene (*VrTAF5*) for CLS resistance in resistant line V4718. Tantasawat et al. (2020) found a major QTL associated with CLS resistance in the CN72 × V4718 cross that was located between an inter-simple sequence repeat (ISSR) marker I16274 and SSR marker VR393. In addition, the closest markers, I85420 and I42PL229, linked to the PM resistance gene were also found in a cross between CN72 and this resistant line V4718 (Poolsawat et al., 2017). Two ISSR-anchored resistance gene analog (ISSR-RGA) markers, I41tP379

and I27R565, were identified to be linked to PM resistance genes obtained from crosses of CN72 × V4758 and CN72 × V4785, respectively (Tantasawat et al., 2021; Tantasawat et al., unpublished data). These markers linked to CLS and PM resistance genes are helpful in marker-assisted selection (MAS) for improving mungbean varieties with resistance to CLS and PM.

The use of MAS is becoming an alternative tool for selecting putatively desirable plants at DNA level. This technique can select plants at all growth stages and all environments, which allows year-round selection. Besides, it enables differentiation among plants with 1, 2, or multiple resistance genes, which is useful for resistance gene pyramiding. Thus, it can resolve the limitation of conventional breeding. In addition, marker-assisted gene pyramiding (MAGP) is widely used through marker-assisted backcrossing (MABC), which allows multiple genes to be combined simultaneously into an elite variety, while the improved lines still having a similar genetic background to the elite variety. Their phenotypic traits are similar to the recurrent parent but have broad-spectrum resistance to pests or diseases (Collard and Mackill, 2008). Therefore, the pyramiding of CLS and PM resistance genes in mungbean may enhance resistance to CLS and PM. In this study, a CLS resistance gene and 2 PM resistance genes were introgressed into a recommended mungbean variety SUT1 through MABB to develop a new variety with resistance to CLS and PM. We also aimed to evaluate their agronomic traits for potential commercialization in the future.

5.3 Materials and Methods

5.3.1 Plant materials and breeding scheme

The plant materials consisted of Suranaree University of Technology 1

(SUT1) variety as a recurrent parent, and A2 [(14B × 19C) × (67A × 5B)-2], as a donor parent having a CLS resistance gene and 2 PM resistance genes. SUT1 variety is a recommended variety with high yield and large seeds and has been reported to have moderate resistance to CLS and PM. It was developed at Suranaree University of Technology, Thailand. Meanwhile, A2 was a double cross of recombinant inbred lines (RILs) from three populations, including CN72 × V4758 (A), CN72 × V4718 (B), and CN72 × V4785 (C) (Poolsawat et al., unpublished data). These three resistant lines (V4718, V4758, and V4785) are sources of CLS and PM resistance, which originated in India. They were found to have high yield and seeds per pod, but seed size is tiny.

For MABC scheme, SUT1 was hybridized with A2 to generate F_1 seeds, and the F_1 plants were further backcrossed to recurrent parent (SUT1). MABC was carried out up to BC_4F_1 generation. In each generation, only desirable plants possessing all the resistance loci were advanced to the next generation. Then, selected plants were selfed to produce BC_4F_2 till BC_4F_7 generations (Figure 5.1). Marker-assisted foreground and background selection was performed in each generation. In brief, foreground selection was used to select F_1 until BC_4F_5 to identify heterozygous-/homozygous plants for all three target resistance genes. While, background selection was used in BC_1F_1 to BC_4F_2 generations to achieve high recurrent parent genome recovery. F_1 to BC_3F_1 generations were implemented by Chueakhunthod (2019). In continuation of this work, selected BC_3F_1 plants were further backcrossed to SUT1 to produce BC_4F_1 generation and followed by selfing to identify homozygous plants. In addition, the non segregation of markers linked to resistance genes in BC_4F_4 and BC_4F_5 was also confirmed with foreground selection to ensure homozygosity.

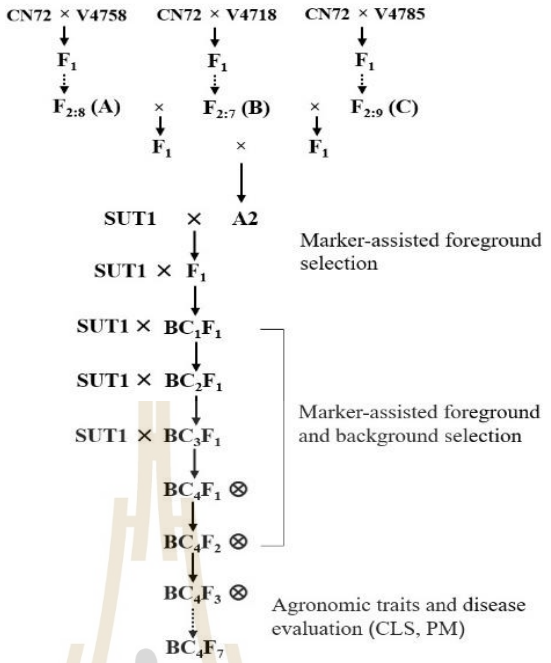

Figure 5.1 Schematic workflow for pyramiding CLS and PM resistance genes into mungbean variety SUT1 through MABC.

5.3.2 DNA extraction and PCR amplification

DNA isolation for PCR analysis was carried out using a modified CTAB extraction protocol described by Lodhi et al. (1994). Details of the primer sequences of markers used for marker-assisted foreground and background selection are presented in Tables 5.1 to 5.3. The PCR reaction mixture of ISSR and ISSR-RGA markers contained 150 ng of genomic DNA template, 1× buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.1, 0.01% TritonTM X-100), 3.5 mM $MgCl_2$, 250 µM of each dNTPs, 0.4 µM of ISSR primer, 1 µM of RGA primer (only ISSR-RGA marker), and 1 unit of Taq DNA polymerase in a volume of 20 µl and PCR amplification of these markers was described by Poolsawat et al. (2017). While each 20 µl of PCR reaction mix of SSR and EST-SSR markers consisted of 1× buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.1, 0.01% Triton™

X-100), 2 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.5 µM each of forward and reverse primers, 1 unit of *Taq* DNA polymerase, and 150 ng of genomic DNA template. PCR amplification of SSR and EST-SSR markers was described by Isemura et al. (2012), Chen et al. (2015) and Arsakit et al. (2017).

5.3.3 Foreground and background selection

A total of 5 markers linked to target resistance genes were used for foreground selection in F_1 to BC_4F_5 generations (Table 5.1). Among these, two SSR markers (VR393 and CEDG084) flanked a CLS resistance gene in V4718 (Arsakit et al., 2017) at 4 and 6 cM. Two dominant ISSR and ISSR-RGA markers consisting of I85420 and I42PL222 were linked to PM resistance genes in V4718 at 9 and 13 cM. While the dominant ISSR-RGA marker I27R656 was linked to PM resistance genes in V4785 (Poolsawat et al., 2017; Tantasawat et al., 2021). These five markers were polymorphic between SUT1 and A2, and were used for foreground selection (Chueakhunthod, 2019). For background selection, a set of 37 SSR and EST-SSR markers were used to survey polymorphisms between SUT1 and A2. These markers were found to be linked to domestication related traits or designed from other putative protein functions in mungbean (Isemura et al., 2012; Chen et al., 2015). In addition, a total of 12 ISSR primers developed from the University of British Columbia were also used to detect DNA polymorphisms which are possibly distributed well throughout the genome among parents. Only the polymorphic markers/loci were used to identify desirable plants with a high recurrent parent genome (RPG) recovery. Of these, 20 SSR and EST-SSR were found to be polymorphic (Table 5.2). Among these, 10 polymorphic SSR and EST-SSR markers gave similar allele size to those reported by Isemura et al. (2012) and were assigned as Set A. Other SSR and EST-SSR were assigned as Set B.

While, 52 polymorphic fragments amplified by 12 ISSR primers were classified as Set C (Table 5.3). For data analysis, all clearly amplified DNA bands were scored as allele sizes at each locus for SSR and EST-SSR markers, while polymorphic ISSR markers were scored as presence/absence of DNA bands. Similarity coefficients between BC lines and their parents were performed following the procedure of Chueakhunthod (2019).

5.3.4 Evaluation of CLS and PM resistance and agronomic traits under field conditions

For field evaluation to access resistance against CLS and PM, and agronomic traits, the pyramided BC_4F_4 to BC_4F_7 lines, parental lines (SUT1, V4718, V4758, and V4785), and check cv. KPS1 were grown in several seasons, years, and locations during 2019-2021. They were grown in a single row of 2 m long or double rows of 6 m long with 10 or 30 hills/row/entry at 0.2 × 0.5 m spacing, and three plants per hill (ca. 30 or 90 plants/row) were kept under a randomized incomplete block design with two to four replications depending on seasons, years and locations. Briefly, in a growing season without disease outbreak, they were gown in December 2019-February 2020 at Suranaree University of Technology (SUT) Farm, Muang district, Nakhon Ratchasima province and in December 2020-March 2021 at Pak Thong Chai District, Nakhon Ratchasima province. While for CLS outbreak, they were grown during the rainy season in July 2020-September 2020 at SUT Farm, Nakhon Ratchasima province, and Chai Nat Field Crops Research Center, Chai Nat province. In this season, CLS evaluation was performed at 65 days after planting (DAP) using a scoring system described by Chankaew et al. (2011). The scale of CLS severity was divided into three categories (resistance= 1.0-2.5, moderate resistance = 2.6-3.4 and susceptibility = 3.5-5.0). In a growing season with PM outbreak, they were grown during cool-dry season

in November 2020-February 2021 at SUT farm. The observation of PM severity was scored at 65 DAP using a scoring system described by Khajudparn et al. (2010). The observation of resistance level was divided into four categories (resistance = 1.0-3.0, moderate resistance = 3.1-4.5, moderate susceptibility = 4.6-6.0 and susceptibility = 6.1-9.0). Samples of CLS and PM responses were presented in Appendix (Figures A.1, A.2). In addition, the susceptible variety CN72 was sown around the experimental blocks as a source of CLS and PM inoculums.

For agronomic traits, days to flowering, days to maturity, plant height, clusters per plant, pods per plant, pod length, seeds per pod, 100-seed weight, and yield per plant, were recorded in pyramided BC lines along with their parents and check variety (KPS1). Data were measured from10 randomly selected plants in the middle row of each replication. The techniques for measuring agronomic traits were described by Chai Nat Field Crops Research Center (2018) and Chueakhunthod et al. (2020) which were presented in Appendix (Table A.1). Data analysis was carried out using SPSS version 14.0 (Levesque and SPSS Inc., 2006). The significant difference of means was performed using Duncan's Multiple Range Test (DMRT).

Table 5.1 Markers used for foreground selection of a CLS resistance gene and 2 PM resistance genes in MABC.

Resistance genes	QTL/genes	Marker types	Markers	Primer sequences (5'-3')[c]	Expected size (bp)	Reference
CLS (from V4718)	*qCLSC72V18-1*	SSR	CEDG084	F: ATCAACTGAGGAGCATCATCGA R: CAACATTTCAACCTTGGGACAG	168/170 (S/R)	Arsakit et al. (2017)
		SSR	VR393	F: TGGCACTTTCCATAACGAATAC R: ATCAGCCAAAAGCTCAGAAAAC	158/160 (S/R)	
PM (from V4718)	*qPMC72V18-1*	ISSR	I85420	BHB $(GA)_7$	420 (R)	Poolsawat et al. (2017)
		ISSR-RGA	I42PL222[a]	$(GA)_8$YG and $(GGI)_2$GTIGGIAAIACIAC	222 (R)	
PM (from V4785)	*qPMC72V85*	ISSR-RGA	I27R565[b]	$(AC)_8$G and GAYGTNAARCCIGARAA	565 (R)	Tantasawat et al. (2021)

[a] I42P222 was derived from the resistant line (V4718) and was used instead of I42P229, which linked to a susceptible allele of a susceptible variety (CN72).
[b] I27R565 which linked to PM resistance 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).

Table 5.2 Polymorphic SSR and EST-SSR markers were used for background selection.

Marker sets	Primer name	Primer sequences (5'-3')	LG[c]	Traits/ putative functions[d]
Set A	CEDG132[a]	F: GGGTGTAATCCGTCAGAGGC R: CTTCCCCCTCTTCCGTTCTC	5	BRP
	CEDG150[a]	F: GAAGGGAATGAAAATGAAACCC R: GTTCAATCCATTCAGTCTCC	10	HECL, ITL, STL
	CEDG174[a]	F: GAGGGATCTCCAAAGTTCAACGG R: GAAGGCTCCGAAGTTGAAGGTTG	7	SDL, SDW, PDL
	CEDG220[a]	F: GGTATTGAAGTCACATGGTCC R: GGTTGTTATCTTTGTGCACTCC	1	SDL, SDNPPD
	CEDG245[a]	F: GATAGAGCTTAAACCCTC R: CTTTTGATGACAAATGCCC	6	RSP
	MBSSR015[a]	F: ATCATCATGACTCCGACACTC R: GTCGCGTAGCATGTTGGAG	4	FLD, PDDM
	VM37[a]	F: TGTCCGCGTTCTATAAATCAGC R: CGAGGATGAAGTAACAGATGATC	8	SD100WT, SDW, SDL, SDT, PDW
	GATS11[a]	F: CACATTGGTGCTAGTGTCGG R: GAACCTGCAAAGCAAAGAGC	9	SDNPPD, PDL, ITL, PDDM
	GMES0477[b]	F: ATTCCGACCTCGAAGATTCC R: CGTCTCTCGAAGAAGGGTTG	2	SD100WT, SDL, SDW, SDT, STT, ITL, STL, BRN, FLD
	GMES3893[b]	F: TTACCGGCTGAGGGTTATTG R: GCAAGAAGGAGAATGAACAGTG	11	SD100WT, PDL, SDT
Set B	MB14180[b]	F: CAGATTCCAACCCGAAGCCA R: GCGAAAGAAGCTCGTCCTCT	-	Protein FRIGIDA-like
	MB15686[b]	F: CCCAACCTCTCCGCAAAGAT R: ACAGCCAATCCACGTACCTC	-	Glucose-6-phosphate isomerase 1, chloroplastic-like
	MB21347[b]	F: GCCATCACCAACTACCCCTC R: AGGGGAGGGCGTAGATGTAG	-	Xyloglucan galactosyltransferase KATAMARI1 homolog
	MB27164[b]	F: CTCAACAAGTTCCTCAGCGC R: CCAGAACCGGTGGAAGTCTC	-	Mitochondrial import inner membrane trans- locase subunit TIM17-2-like
	MB33094[b]	F-ATTGCCACCCCCATTTCCAT R-AGCAGTCCACCACTCTCTCT	-	Leucine-rich repeat extensin-like protein 4-like
	MB64504[b]	F: CTCCTGAGGGCACTGAACTG R: GCTTCTGCAACGAGTTTCAACT	-	Dof zinc finger protein DOF4.6-like
	GMES0216[b]	F: CCGGGACAGGGTTTCTAACT R: CCGAAGAAGACGACGAAATC	2,4	-
	GMES5572[b]	F: GCAGCAGCACTACATGGGTA R: AGATGGCATAGGAGGTGGTG	5	-
	BM149[a]	F: CGATGGATGGATGGTTGCAG R: GGGCCGACAAGTTACATCAAATTC	11	
	cp05137[a]	F: CCGATTGTAGATGATCCCGATTGT R: TGATGATTGCTGTGGGGAAATATG	1	-

Note: Set A = polymorphic SSR and EST-SSR markers linked to domestication related traits; Set B = polymorphic SSR and EST-SSR markers related to putative protein functions and other fragments with unknown functions.

[a] SSR markers

[b] EST-SSR markers

[c] Linkage group

[d] BRP = Position of first branch, BRN = Branch number, FLD = Days to first flower, HECL = Hypocotyl plus epicotyl length, ITL = Internode length, PDL = Pod length, PDDM = Days to maturity of first pod, PDW = Pod width, RSP = Rate of shattered pods, SDL = Seed length, SDW = Seed width, SDNPPD = Number of seed per pod, SD100WT = 100-seed weight, SDT = Seed thickness, STL = Stem length, STT = Stem thickness.

Table 5.3 ISSR markers were used for background selection.

Primers	Primer sequences (5'-3')[a]	Number of scorable DNA bands	Number of polymorphic bands (male-female parents)
809	$(AG)_8G$	21	3
811	$(GA)_8C$	19	5
830	$(TG)_8G$	20	2
834	$(AG)_8YT$	30	6
835	$(AG)_8YC$	23	5
841c	$(GA)_8CC$	16	6
850	$(GT)_8YC$	17	3
857	$(AC)_8YG$	18	4
864	$(ATG)_6$	14	4
884	HBH $(AG)_7$	32	4
887	DVD $(TC)_7$	20	5
890	VHV $(GT)_7$	23	5
Total		253	52
Average		23	4.3

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

5.4 Results

5.4.1 Foreground and background selection

Parental polymorphisms were detected between the donor parent (A2) and the recurrent parent (SUT1) with markers VR393 and CEDG084 for CLS resistance gene, I85420, I42PL222, and I27R565 for PM resistance genes (Table 5.1). These five markers were polymorphic between parents and were used for foreground selection in F_1 to BC_4F_5 generations (Figure 5.2). In addition, they were screened with 37 EST-SSR primer pairs, of which 20 EST-SSR markers were polymorphic and divided into 2 sets (Set A and Set B) (Table 5.2). Meanwhile, a total of 52 polymorphic fragments were amplified by 12 ISSR primers and assigned as Set C (Table 5.3). All of these 72 marker loci were used for background selection in generations BC_1F_1 to BC_4F_2. Out of 29 F_1 progenies screened, 4 progenies were identified as heterozygous for all five markers associated with CLS and PM resistance gene loci and were further used as male parent for backcrossing to SUT1 to generate BC_1F_1 generation. The results are presented in Table 5.4.

Of the total 149 BC_1F_1 progenies generated, 3 BC_1F_1 progenies (2.0%) were heterozygous for all marker loci, except for CEDG084. Therefore, only four markers were used for foreground selection in the next generation. These progenies were then subjected to background selection using 72 marker loci, and they displayed 81.8 to 95.0% RPG recovery with an average of 91.7, 86.4, and 87.5% for Sets A, B, and C, respectively. These three BC_1F_1 progenies had higher RPG recovery than expected (75.0%). Two of the three BC_1F_1 progenies with RPG recovery up to 90.0-91.0% were backcrossed to the recurrent parent to produce BC_2F_1 generation.

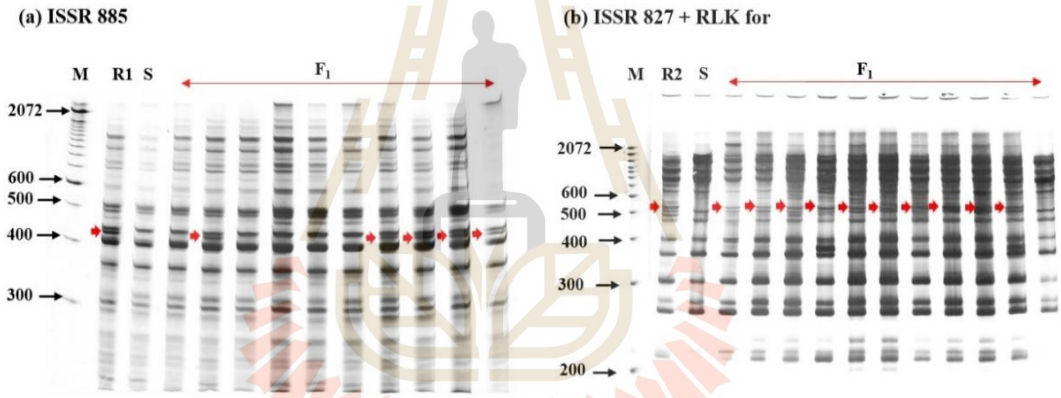

Figure 5.2 Detection of PM resistance genes in F_1 generation using ISSR 885 primer (I85420 marker) (a) and ISSR 827 + RLK for primers (I27R565 marker) (b). M = 100 bp DNA ladder, R1 = donor resistant parent (V4718), R2 = donor resistant parent (V4785), S = susceptible recurrent parent (SUT1). Arrow shows markers putatively linked to target resistance genes.

A total of 280 BC_2F_1 progenies were screened, and 13 BC_2F_1 progenies (4.6%) were heterozygous for all marker loci. These selected BC_2F_1 plants showed RPG recovery ranging from 87.2 to 97.6% with an average of 92.3, 90.9, and 91.8% for Sets

A, B, and C, respectively, which was higher than expected (87.5%). Three out of thirteen progenies with RPG recovery up to 91.0-95.0% of all marker sets were backcrossed to the recurrent parent to produce BC_3F_1 generation.

Of the total 58 BC_3F_1 progenies produced, 6 BC_3F_1 progenies (10.3%) were heterozygous for all marker loci. These progenies were further selected with background selection. RPG recovery ranged from 90.0 to 100.0 % with an average of 96.7, 95.8, and 96.9 % for Sets A, B, and C, respectively. Five out of six progenies showed RPG recovery up to 96.2-100.0%, which was higher than expected (93.7%). However, only three progenies with a high number of pyramided BC_3F_1 seeds were further backcrossed to the recurrent parent to produce BC_4F_1 generation.

Of 136 BC_4F_1 progenies examined, 26 progenies (19.1%) were identified as heterozygous for all marker loci. The RPG recovery of these progenies based on all marker sets ranged from 95.0 to 100.0% with an average of 99.2, 99.2, and 98.6% for Sets A, B, and C, respectively. Among them, nineteen BC_4F_1 progenies had higher RPG recovery (up to 98.8-100.0%) than expected (96.9%), and they were selfed to produce BC_4F_2 generation.

A total of 309 BC_4F_2 progenies were obtained from six BC_4F_1 progenies. Among them, 44 BC_4F_2 progenies (14.2%) were homozygous for the SSR marker (VR393) and heterozygous/homozygous for ISSR and ISSR-RGA markers. When background selection was performed, the percentages of RPG recovery of the foreground marker positive plants based on all sets of markers ranged from 95.0 to 100.0% with an average of 99.2, 100.0, and 99.0% for Sets A, B, and C, respectively, which was higher than expected (96.9%). All promising plants were selfed to produce BC_4F_3 generation.

Table 5.4 Number of triple resistant gene heterozygotes or homozygotes identified and estimation of recurrent parent genome (RPG) contribution.

Generation	# of plants	# of plants that are triple resistant gene heterozygotes/homozygotes	Estimated contribution of RPG (%) to selected backcross plant						Expected % contribution of RPG to selected backcross plant
			Set A[b]		Set B[c]		Set C[d]		
			Range	Average	Range	Average	Range	Average	
F_1[a]	29	4 (13.8%)	-	-	-	-	-	-	50
BC_1F_1[a]	149	3 (2.0%)	90.0-95.0	91.7	81.8-90.9	86.4	84.7-90.0	87.5	75
BC_2F_1[a]	280	13 (4.6%)	90.0-95.0	92.3	90.0	90.9	87.2-97.6	91.8	87.5
BC_3F_1	58	6 (10.3%)	90.0-100.0	96.7	95.0-100.0	95.8	96.2-97.5	96.9	93.7
BC_4F_1	136	26 (19.1%)	95.0-100.0	99.2	95.0-100.0	99.2	97.5-100.0	98.6	96.9
BC_4F_2	309	44 (14.2%)	95.0-100.0	99.2	100.0	100.0	98.8-100.0	99.0	96.9

[a] F_1 to BC_2F_1 generations were reported by Chueakhunthod (2019).
[b] SSR and EST-SSR markers linked to domestication related traits derived from Isemura et al. 2012 (10 polymorphic markers).
[c] EST-SSR markers related to other putative protein functions derived from Chen et al. (2015) and other SSR and EST-SSR markers with unknown functions derived from Isemura et al. (2012) (10 polymorphic markers).
[d] ISSR markers developed from the University of British Columbia (52 polymorphic markers).

5.4.2 Confirmation of homozygosity for all marker loci linked to the three target resistance genes

In BC_4F_3 generation, a total of 24 BC_4F_3 lines were grown in plant to row for seed multiplication. In each line, seeds were separately harvested from 10-20 individuals. In total, 110 BC_4F_4 lines were obtained and used for confirmation of resistance gene homozygosity at all linked marker loci. Among these, 15 BC_4F_4 lines that had good performance in the field condition were confirmed for marker homozygosity by randomly selected 20 plants per BC line to perform marker analysis. Seven out of 15 lines, namely A1, B1, B2, D2, D5, D6 and G1 possessed all marker loci in all 20 plants (not segregating). They were again confirmed for the presence of 4 marker loci in BC_4F_5 generation. Six of these BC lines possessed all marker loci in all 20 plants (not segregating), confirming that these BC lines had homozygous alleles for all marker loci. In contrast, D6 was found segregating at some marker loci so this line was not evaluated further. Therefore, these six BC lines were used to evaluate for CLS and PM resistance and agronomic traits in the field conditions together with their parents and check variety (KPS1) in different locations, seasons, and years.

5.4.3 Comparison of agronomic traits and disease resistance under field condition

5.4.3.1 Under no disease outbreak

Six BC lines, their parents, and check variety were evaluated for yield performance and agronomic traits during no disease outbreak in December 2019-February 2020 and December 2020-March 2021 at SUT Farm and Pak Thong Chai, Nakhon Ratchasima, respectively. When the pyramided BC_4F_4 lines were evaluated in December 2019-February 2020 at SUT Farm, yield performance was found not

significantly different ($P > 0.05$) among BC lines, their parents, and check variety. We found that yield per plant of BC lines ranged from 6.59 (G1) to 8.49 (B1) g, whereas yield per plant of the recurrent parent SUT1 was 8.01 g and donor parents were 5.71-8.18 g. In addition, yield of check cv. KPS1 was 8.01 g (Figure 5.3). However, data of A1 line could not be analyzed because only one replication was recorded. Although yields were not significantly different among BC lines, parents, and check variety, B1 line tended to have slightly higher yield than others at this environment. Conversely, at Pak Thong Chai in December 2020-March 2021, yield performances of the pyramided BC_4F_7 lines were highly significantly different from parents and check variety ($P < 0.01$). At this location and year, yield per plant of pyramided BC_4F_7 lines varied from 6.94 (G1) to 9.03 (A1) g and most of which were not different from the recurrent parent SUT1 (9.18 g), but they tended to be slightly lower than that of the recurrent parent SUT1. Yield per plant of three donor parents were 6.41-9.15 g and that of check cv. KPS1 was 9.05 g (Figure 5.3).

In addition, the other eight agronomic traits were also evaluated, which are presented in Table 5.5. A significant variation was observed among pyramided BC lines, their parents, and check cv. KPS1 for days to maturity, plant height, clusters per plant, pods per plant, pod length, seeds per pod, and 100-seed weight in both environments ($P < 0.05$ or $P < 0.01$). While days to flowering was found not significantly different among pyramided BC lines, their parents and check cv. KPS1 in both environments ($P > 0.05$). At SUT Farm, most pyramided BC lines (B2, D2, D5 and G1) exhibited all agronomic traits similar to the recurrent parent SUT1 while B1 had greater plant height and seeds per pod than SUT1. Meanwhile, at Pak Thong Chai, only D5 line exhibited all agronomic traits similar to the recurrent parent SUT1. Some

pyramided BC lines had higher or lower levels of a few agronomic traits than SUT1, such as A1, B1, B2, and D2 had higher number of seeds per pod than SUT1, B2 and D2 had higher pod length than SUT1, A1 and B1 had lower 100-seed weight than SUT1, B2 had lower clusters per plant and pods per plant than SUT1, and G1 had lower pods per plant and pod length than SUT1. Moreover, the recurrent parent SUT1 and all BC lines had higher pod length and 100-seed weight than three donor parents in both environments. They also tended to have lower number of pods per plant than those of donor parents in both environments. When BC lines were compared with check cv. KPS1, it was found that B1 had higher number of seeds per pod than KPS1 in December 2019-February 2020 at SUT Farm. Meanwhile, KPS1 had higher pod length than some BC lines in December 2020-March 2021 at Pak Thong Chai (Table 5.5).

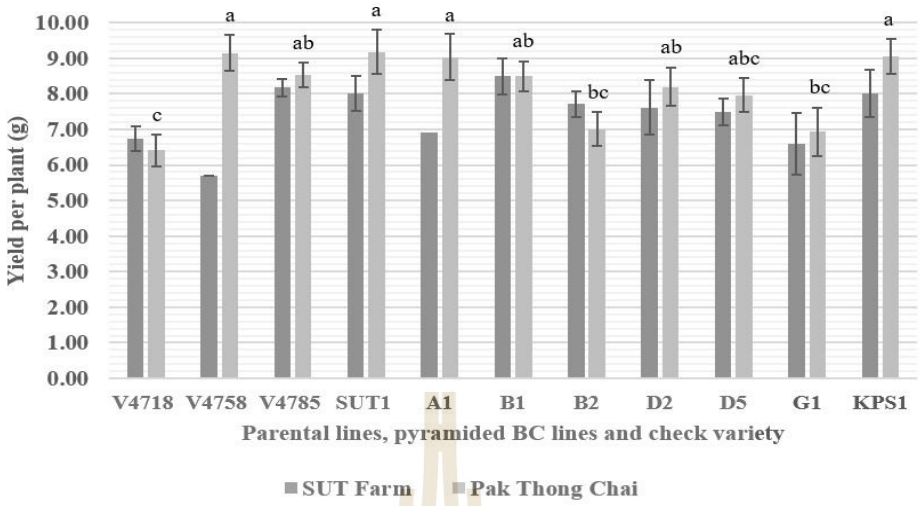

Figure 5.3 Yield performance of parental lines, check variety and pyramided BC lines when growing without disease outbreak. They were grown in December 2019-February 2020 at SUT Farm and in December 2020-March 2021 at Pak Thong Chai, Nakhon Ratchasima. Different letters are significantly different ($P < 0.05$) while no letter is not significantly different ($P > 0.05$) based on DMRT. V4785, V4758, V4718 and SUT1 are parental lines; KPS1 is check variety; A1, B1, B2, D2, D5 and G1 are pyramided BC lines, respectively. A1 at SUT Farm had only one replication.

Table 5.5 Comparison of eight agronomic traits between parental lines and pyramided BC lines as well as check variety under field condition without disease outbreak.

Locations	Lines/varieties /BC lines	Days to flowering	Days to maturity	Plant height (cm)	Clusters/plant	Pods/plant	Pod length (cm)	Seeds/pod	100 seed weight (g)
SUT	**V4718**	46.50 ± 1.80	62.83 ± 1.17 cd[c]	40.85 ± 4.54 bc	6.74 ± 0.84 a	20.85 ± 3.13 a	6.85 ± 0.18 c	10.83 ± 0.13 ab	3.68 ± 0.23 b
Farm[a]	**V4758**	47.50 ± 3.50	62.00 ± 2.00 d	32.49 ± 2.79 c	6.05 ± 1.45 ab	17.49 ± 1.89 ab	6.95 ± 0.12 c	11.32 ± 0.14 a	3.55 ± 0.05 b
	V4785	47.00 ± 1.26	65.17 ± 0.60 abc	38.15 ± 1.81 bc	7.86 ± 0.22 a	21.13 ± 0.50 a	7.12 ± 0.17 c	11.34 ± 0.04 a	3.86 ± 0.08 b
	SUT1	46.31 ± 0.60	65.25 ± 0.90 abc	38.05 ± 0.72 bc	5.54 ± 0.33 ab	16.4 ± 0.72 ab	9.06 ± 0.12 ab	9.63 ± 0.23 c	6.1 ± 0.70 a
	A1[d]	45.00	64.00	44.27	4.57	10.71	8.6	9.56	6.49
	B1	44.50 ± 0.50	63.00 ± 1.00 bcd	49.44 ± 0.52 a	6.07 ± 1.36 ab	15.36 ± 2.93 ab	8.99 ± 0.08 ab	10.86 ± 0.01 ab	5.94 ± 0.24 a
	B2	47.33 ± 0.67	66.00 ± 1.15 a	39.02 ± 2.39 bc	5.81 ± 0.54 ab	14.50 ± 3.04 ab	9.31 ± 0.01 a	10.30 ± 0.25 abc	6.35 ± 0.07 a
	D2	46.50 ± 1.50	65.00 ± 1.00 abc	37.71 ± 1.59 bc	5.91 ± 1.49 ab	15.44 ± 1.16 ab	9.31 ± 0.38 a	10.44 ± 0.78 abc	6.38 ± 0.16 a
	D5	48.33 ± 1.33	66.67 ± 0.67 a	32.88 ± 1.07 c	4.21 ± 0.23 b	11.40 ± 0.61 b	8.63 ± 0.23 b	10.15 ± 0.56 bc	6.48 ± 0.13 a
	G1	47.67 ± 0.67	65.67 ± 0.33 ab	35.70 ± 0.60 bc	4.09 ± 0.49 b	11.68 ± 1.00 b	8.43 ± 0.20 b	9.98 ± 0.26 bc	6.02 ± 0.10 a
	KPS1	45.89 ± 0.93	64.22 ± 0.89 a-d	44.12 ± 3.45 ab	5.51 ± 0.19 ab	15.08 ± 1.12 ab	8.89 ± 0.19 ab	9.68 ± 0.03 c	6.41 ± 0.22 a
	F-test	ns	*	*	*	*	**	*	**
Pak	**V4718**	45.00 ± 0.00	64.67 ± 0.33 c	38.94 ± 3.25 b	7.78 ± 0.98 ab	27.23 ± 2.54 a	6.04 ± 0.10 f	11.78 ± 0.22 ab	2.31 ± 0.05 f
Thong	**V4758**	45.00 ± 0.00	66.00 ± 0.58 bc	51.73 ± 1.33 a	8.54 ± 0.83 a	24.51 ± 1.40 a	7.84 ± 0.15 d	12.44 ± 0.29 a	3.36 ± 0.01 e
Chai[b]	**V4785**	45.00 ± 0.00	67.50 ± 1.50 ab	48.84 ± 0.41 a	7.71 ± 1.29 abc	27.13 ± 3.13 a	7.16 ± 0.26 e	12.22 ± 0.09 a	3.11 ± 0.19 e
	SUT1	45.67 ± 1.76	68.67 ± 0.88 a	53.82 ± 3.69 a	7.11 ± 0.12 abc	19.03 ± 1.01 b	8.73 ± 0.22 c	9.43 ± 0.18 e	5.67 ± 0.06 ab
	A1	48.50 ± 0.50	69.00 ± 0.00 a	53.87 ± 3.73 a	6.59 ± 0.01 bcd	19.33 ± 1.47 b	8.93 ± 0.10 bc	11.14 ± 0.12 bc	5.04 ± 0.25 d
	B1	46.67 ± 1.67	68.67 ± 0.33 a	54.48 ± 4.96 a	6.52 ± 0.26 bcd	17.04 ± 1.31 bcd	8.65 ± 0.07 c	10.76 ± 0.24 c	5.30 ± 0.13 cd
	B2	46.67 ± 1.67	68.33 ± 0.33 ab	51.44 ± 3.45 a	5.21 ± 0.40 d	13.33 ± 0.91 d	9.27 ± 0.10 ab	10.49 ± 0.35 cd	5.78 ± 0.10 a
	D2	48.33 ± 1.67	69.00 ± 1.00 a	54.58 ± 4.79 a	6.45 ± 0.19 bcd	16.45 ± 0.28 bcd	9.57 ± 0.10 a	10.45 ± 0.23 cd	5.35 ± 0.16 bcd
	D5	49.00 ± 1.53	69.67 ± 1.20 a	53.50 ± 3.64 a	6.15 ± 0.05 bcd	17.15 ± 1.33 bcd	8.74 ± 0.12 c	9.64 ± 0.36 de	5.53 ± 0.02 abc
	G1	45.50 ± 0.50	69.50 ± 0.50 a	53.17 ± 2.27 a	5.85 ± 0.15 cd	14.92 ± 0.42 cd	8.24 ± 0.09 d	9.63 ± 0.02 de	5.43 ± 0.24 abc
	KPS1	48.33 ± 1.76	70.00 ± 0.58 a	58.31 ± 1.32 a	7.69 ± 0.48 abc	18.84 ± 0.77 bc	9.55 ± 0.21 a	10.45 ± 0.45 cd	5.64 ± 0.07 abc
	F-test	ns	**	*	*	**	**	**	**

[a] SUT Farm, BC_4F_4 lines were grown and evaluated in December 2019 - February 2020.
[b] Pak thong Chai, BC_4F_7 lines were grown and evaluated in December 2020 - March 2021.
[c] Means ± SE in the same column with different letters are significantly different ($P < 0.05$) based on DMRT.
[d] BC line that had only one replication.

5.4.3.2 Under warm-wet growing season for CLS evaluation

Six BC lines along with the recurrent parent and donor parents as well as check cv. KPS1 were evaluated for CLS resistance and their agronomic traits during wet season, 2020 at SUT Farm, Nakhon Ratchasima, and Chai Nat Field Crops Research Center, Chai Nat. Highly significant differences were observed on CLS responses among BC lines, their parents, and check cv. KPS1 at both locations ($P < 0.01$) (Figure 5.4a). Among BC_4F_5 lines, only B2 was observed to be moderately resistant to CLS with a severity score of 2.75 when growing at SUT Farm. The disease severity score of this BC line was comparable to that of the donor parent V4718 (2.50) and tended to be lower than that of SUT1 (3.50). Other BC lines were found to have similar or more disease severity than the recurrent parent SUT1 with severity scores of 3.50-4.75, which were identified as susceptible to CLS (Figure 5.5). In addition, V4785 was found to be resistant to CLS with a severity score of 2.50 while V4758 and check variety KPS1 were moderately resistant to CLS with severity scores of 3.33 and 3.25, respectively. However, under severe disease pressure at Chai Nat Field Crops Research Center, all BC_4F_6 lines, check variety, and most parental lines including V4718 and V4785 were susceptible to CLS. Only V4785 was moderately resistant to CLS with a severity score of 3.00 at this location.

A significant variation of yield performance was found among BC lines, their parents, and check cv. KPS1 at SUT Farm ($P < 0.01$) while no significant difference was observed at Chai Nat Field Crops Research Center ($P > 0.05$). At SUT Farm, yield performance of six pyramided BC lines ranged from 5.04 (D2) to 7.68 (D5) g, but most of these were not significantly different from that of the recurrent parent SUT1 (5.32 g). While those of donor parents were 4.59, 4.80, and 5.05 g for V4718,

V4758, and V4785, respectively. In addition, yield performance of B2 line with moderate resistance to CLS was slightly higher than SUT1 (5.3%). In addition, yields of some BC lines including A1, B1, D5, and G1 were 14.1-31.0% higher than SUT1 although they were susceptible to CLS (Figure 5.4). The highest yielding D5 line had significantly higher yield than all parental lines and KPS1. The yields of all BC lines, the recurrent parent SUT1, and donor parent V4718 at Chai Nat Field Crops Research Center were lower than at SUT Farm (9-49%) because the CLS outbreak was much more severe than at SUT Farm and they were affected by insect infestation and virus, resulting in lower yield in most lines (Figure 5.4). Moreover, when we compared to the condition without disease outbreak, the reduction of yield ranging from 1% to 55% was found at SUT Farm while at Chai Nat Field Crops Research Center yield was found to be reduced up to 30-54%. In addition, the analysis of variance displayed significant differences ($P < 0.05$ or $P < 0.01$) for days to flowering, days to maturity, plant height, clusters per plant, pods per plant, pod length, seeds per pod, and 100-seed weight at both locations (Table 5.6). All pyramided BC lines had most agronomic traits similar to the recurrent parent SUT1, especially B2 and D2, which had all agronomic traits similar to SUT1 at both locations. While those of A1 and G1, as well as D5, were either similar to SUT1 at SUT Farm or Chai Nat Field Crops Research Center. At SUT Farm, D5 not only had higher yield than SUT1 but also had higher pods per plant (16.02 pods) and seeds per pod (10.79 seeds) than SUT1 (11.54 pods and 9.46 seeds, respectively). Moreover, its days to maturity (52.67 days) was earlier than SUT1 (57.25 days). B1 had lower 100-seed weight (5.50 g) than SUT1 (5.97 g). Meanwhile, A1 and B1 were taller (100.70-101.05 cm) than SUT1 (90.44 cm), and days to flowering of G1 (44.00 days) was later than SUT1 (39.83 days) when observed at Chai Nat Field Crops Research

Center. These BC lines and SUT1 not only had higher pod length and 100-seed weight but also tended to have lower pods per plant and seeds per pod than those of donor parents at both locations. Interestingly, D5 had higher pods per plant, seeds per pod, and 100-seed weight than check cv. KPS1 at some locations.

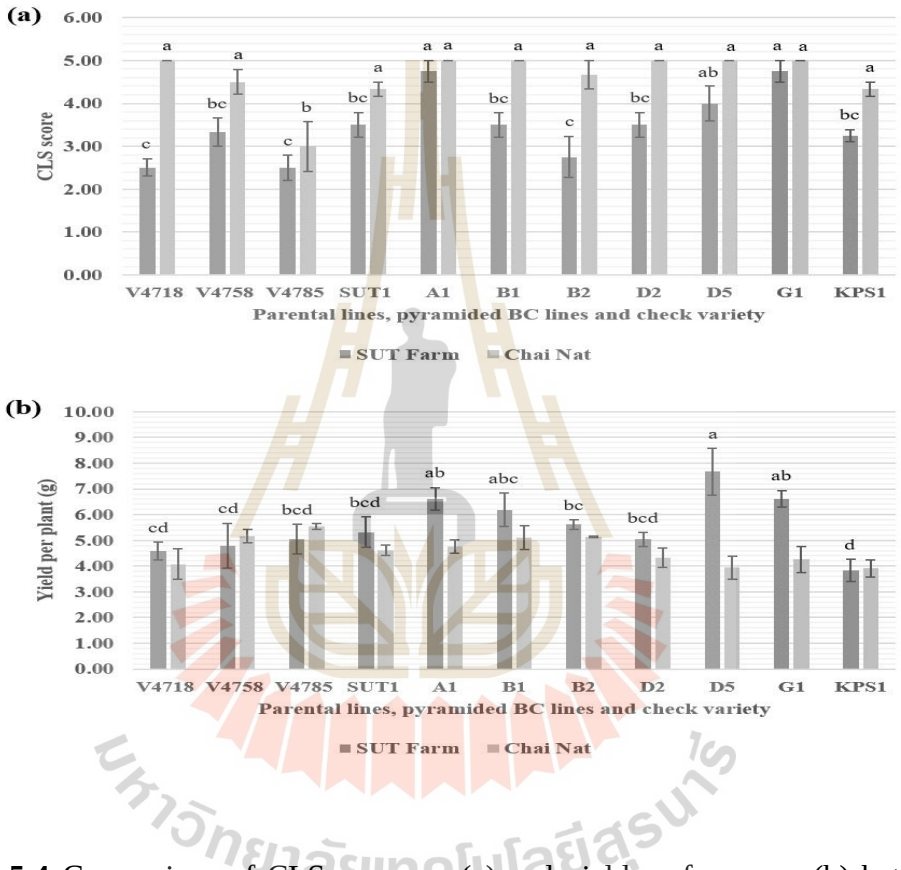

Figure 5.4 Comparison of CLS response (a) and yield performance (b) between the parental lines, check variety and pyramided BC lines. They were grown in July 2020-September 2020 at SUT Farm, Nakhon Ratchasima and Chai Nat Field Crops Research Center, Chai Nat. Different letters are significantly different ($P < 0.05$) while no letter is not significantly different ($P > 0.05$) based on DMRT. V4785, V4758, V4718 and SUT1 are parental lines; KPS1 is check variety; A1, B1, B2, D2, D5 and G1 are pyramided BC lines, respectively.

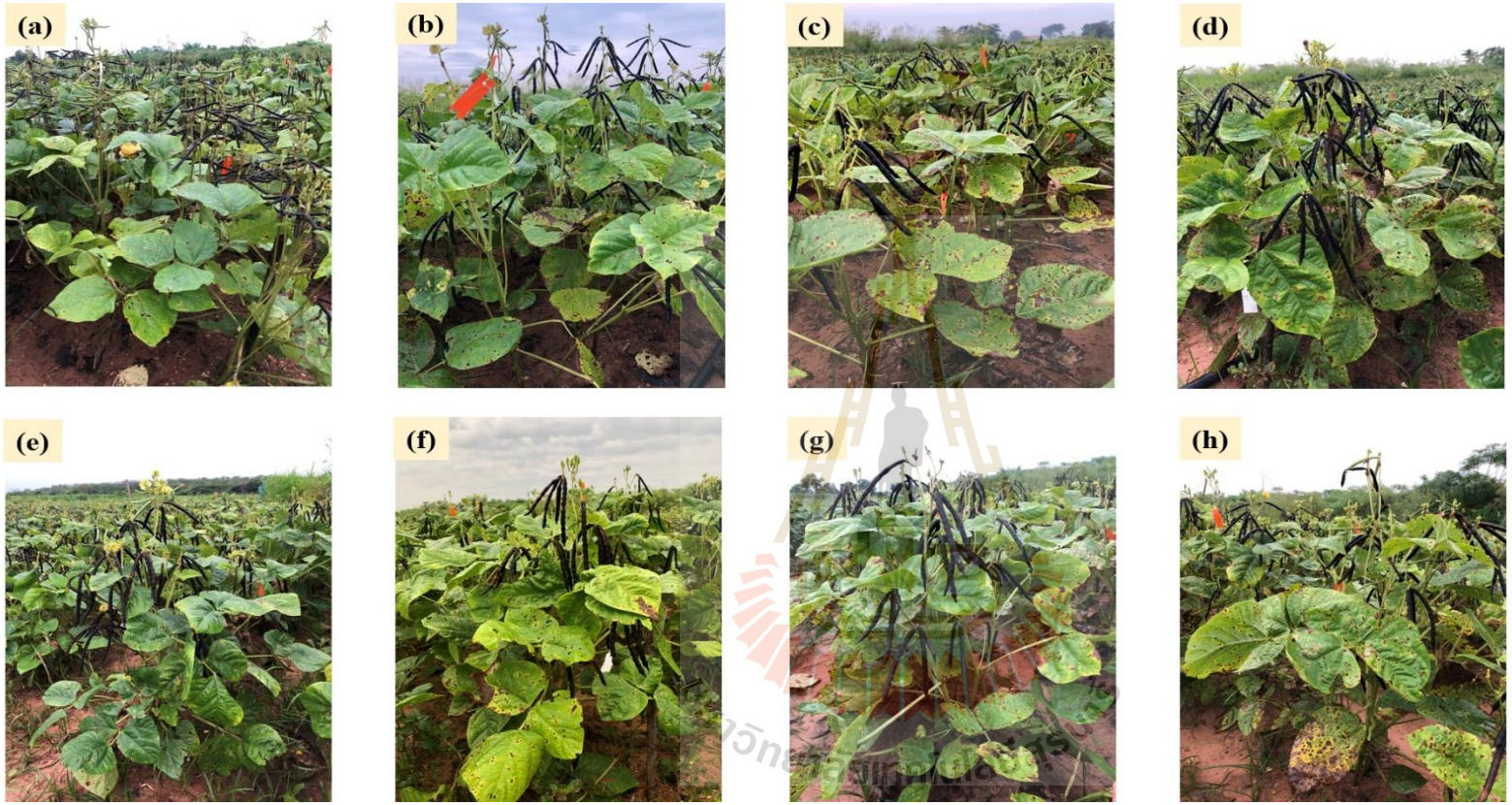

Figure 5.5 Evaluation of pyramided BC_4F_5 lines and parental lines against CLS disease under field condition at SUT Farm. a: donor resistant parent V4718; b: susceptible recurrent parent SUT1; c-h: pyramided BC lines (A1, B1, B2, D2, D5, and G1, respectively).

Table 5.6 Comparison of eight agronomic traits between parental lines and pyramided BC lines as well as check variety under field condition with CLS outbreak.

Locations	Lines/varieties /BC lines	Days to flowering	Days to maturity	Plant height (cm)	Clusters/plant	Pods/plant	Pod length (cm)	Seeds/pod	100 seed weight (g)
SUT Farm[a]	**V4718**	36.88 ± 0.66 f[c]	50.50 ± 0.29 e	48.70 ± 4.03 c	5.26 ± 0.12 b	17.05 ± 1.27 ab	6.15 ± 0.10 d	10.82 ± 0.39 ab	3.01 ± 0.04 c
	V4758	43.83 ± 0.60 ab	61.50 ± 0.76 ab	68.40 ± 2.79 a	7.12 ± 0.67 a	17.90 ± 2.96 a	7.17 ± 0.04 c	11.18 ± 0.55 ab	3.29 ± 0.07 c
	V4785	44.50 ± 0.76 a	63.17 ± 2.32 a	65.81 ± 8.54 ab	8.04 ± 0.68 a	18.56 ± 1.99 a	6.98 ± 0.10 c	11.46 ± 0.70 a	3.35 ± 0.06 c
	SUT1	40.25 ± 0.95 cde	57.25 ± 1.64 c	48.63 ± 2.63 c	4.28 ± 0.27 b	11.54 ± 1.87 d	8.57 ± 0.11 ab	9.46 ± 0.18 cd	5.97 ± 0.07 a
	A1	40.00 ± 0.91 cde	55.75 ± 1.38 cd	55.61 ± 3.34 bc	5.14 ± 0.55 b	12.95 ± 0.73 bcd	8.50 ± 0.11 ab	10.35 ± 0.16 bc	5.62 ± 0.05 ab
	B1	41.75 ± 1.49 a-d	57.00 ± 1.87 c	51.97 ± 3.70 c	4.51 ± 0.56 b	12.59 ± 1.48 cd	8.29 ± 0.13 b	10.15 ± 0.19 bc	5.50 ± 0.16 b
	B2	42.67 ± 1.20 abc	59.00 ± 1.53 bc	52.19 ± 3.73 c	4.64 ± 0.31 b	11.84 ± 0.87 cd	8.8 ± 0.12 ab	9.07 ± 0.26 cd	5.75 ± 0.09 ab
	D2	40.75 ± 0.85 cde	57.00 ± 1.08 c	49.24 ± 2.89 c	4.41 ± 0.17 b	10.36 ± 0.23 d	8.99 ± 0.14 a	9.59 ± 0.30 cd	5.73 ± 0.05 ab
	D5	38.00 ± 0.58 ef	52.67 ± 0.88 de	54.14 ± 4.78 bc	5.29 ± 0.73 b	16.02 ± 2.79 abc	8.88 ± 0.25 a	10.79 ± 0.46 ab	5.80 ± 0.16 ab
	G1	41.25 ± 1.44 bcd	57.00 ± 1.00 c	56.60 ± 1.72 abc	5.21 ± 0.35 b	13.26 ± 0.3 bcd	8.50 ± 0.18 ab	10.21 ± 0.30 bc	5.70 ± 0.06 ab
	KPS1	39.50 ± 0.65 def	56.38 ± 1.14 c	60.37 ± 4.67 abc	4.57 ± 0.36 b	9.09 ± 0.81 d	8.89 ± 0.41 a	8.73 ± 0.21 d	5.96 ± 0.19 a
	F-test	**	**	*	**	**	**	**	**
Chai Nat[b]	**V4718**	38.17 ± 0.17 d[1]	55.33 ± 0.17 d	76.91 ± 1.84 e	7.06 ± 0.75 ab	13.91 ± 1.32 b	6.40 ± 0.04 e	11.41 ± 0.03 bc	3.01 ± 0.06 e
	V4758	48.17 ± 0.17 a	64.00 ± 2.50 ab	104.23 ± 1.51 ab	6.76 ± 0.42 ab	19.07 ± 0.93 a	7.18 ± 0.10 d	12.28 ± 0.63 a	2.68 ± 0.05 e
	V4785	46.00 ± 0.76 b	65.50 ± 2.57 a	104.04 ± 2.53 ab	7.55 ± 0.94 a	17.96 ± 0.79 a	7.15 ± 0.19 d	12.12 ± 0.20 ab	3.08 ± 0.08 e
	SUT1	39.83 ± 0.33 d	58.50 ± 1.00 cd	90.44 ± 2.86 cd	5.38 ± 0.02 bcd	9.04 ± 0.55 c	8.69 ± 0.05 abc	10.07 ± 0.15 de	5.64 ± 0.09 abc
	A1	39.67 ± 0.33 d	60.00 ± 0.58 bcd	100.70 ± 1.45 ab	5.96 ± 0.38 a-d	8.47 ± 0.53 c	8.69 ± 0.15 abc	10.79 ± 0.38 cd	5.65 ± 0.09 abc
	B1	39.33 ± 0.33 d	59.00 ± 1.53 bcd	101.05 ± 2.26 ab	5.45 ± 0.53 bcd	10.26 ± 1.22 c	8.32 ± 0.14 bc	10.16 ± 0.36 de	5.56 ± 0.06 bc
	B2	39.67 ± 0.33 d	59.00 ± 0.58 bcd	97.79 ± 1.65 abc	5.34 ± 0.34 bcd	9.61 ± 0.48 c	8.98 ± 0.08 a	10.36 ± 0.14 de	5.71 ± 0.05 ab
	D2	40.00 ± 0.58 d	60.33 ± 1.20 bcd	87.29 ± 3.12 d	4.28 ± 0.38 d	9.02 ± 1.01 c	9.11 ± 0.30 a	9.76 ± 0.19 e	5.21 ± 0.09 cd
	D5	39.67 ± 1.20 d	62.33 ± 2.40 abc	84.13 ± 1.74 de	4.71 ± 0.59 cd	7.09 ± 0.65 c	8.87 ± 0.19 ab	10.46 ± 0.16 de	6.09 ± 0.24 a
	G1	44.00 ± 0.00 c	61.67 ± 0.33 abc	96.60 ± 2.91 bc	5.36 ± 0.52 bcd	8.94 ± 1.65 c	8.08 ± 0.38 c	9.70 ± 0.51 e	5.49 ± 0.18 bc
	KPS1	42.33 ± 0.88 c	63.17 ± 1.96 abc	105.83 ± 4.70 a	6.51 ± 0.77 abc	9.06 ± 1.00 c	8.58 ± 0.08 abc	10.06 ± 0.04 de	4.94 ± 0.30 d
	F-test	**	**	**	*	**	**	**	**

[a] SUT Farm, BC_4F_5 lines were grown and evaluated in July 2020 - September 2020.
[b] Chai Nat Field Crops Research Center, BC_4F_6 lines were grown and evaluated in July 2020 - September 2020.
[c] Means ± SE in the same column with different letters are significantly different ($P < 0.05$) based on DMRT.

5.4.3.3 Under cool-dry growing season for PM evaluation

When six BC_4F_6 lines along with the recurrent parent and donor parents were evaluated for PM resistance and their agronomic traits in November 2020-February 2021 at SUT Farm. Analysis of the PM responses revealed highly significant difference among BC lines and their parents ($P < 0.01$). In pyramided BC lines, B2 and G1 were resistant (2.50-3.00), and other lines were moderately resistant to PM (4.00-4.50), while the recurrent parent SUT1 was moderately susceptible to PM with a severity score of 5.67. By contrast, two donor resistant parents were resistant and moderately resistant to PM with a score of 2.00 and 4.00 for V4718 and V4785, respectively (Figure 5.6a). Yield performance was also highly significantly different between BC lines and their parents ($P < 0.01$). The highest yield per plant (6.77 g) was found in the donor parent V4718 but was not significantly different from the other donor parents (V4758 and V4785) and D5 (5.58-6.35 g). However, it was lowest in some BC lines, D2 and G1, which were moderately resistant and resistant to PM, respectively. Interestingly, D5 line tended to have higher yield than SUT1 (6.3%). While other lines including A1, B1, and B2 had slightly higher yields (3.5-4.0%), but they were not significantly different from SUT1 (Figure 5.6b). When compared to under no disease outbreak, the yield reduction of most mungbean genotypes was up to 15-53%.

In addition, analysis of other agronomic traits revealed a highly significant difference ($P < 0.01$) among BC lines and their parents for pods per plant, pod length, seeds per pod, and 100-seed weight. Clusters per plant also displayed significant difference among various lines/varieties ($P < 0.05$). No significant difference ($P > 0.05$) was observed in days to flowering, days to maturity, and plant height (Table 5.7). In this season, five out of six BC lines (A1, B1, B2, D2, and D5) were similar to

the recurrent parent SUT1 for all agronomic traits, while G1 had lower pod length than SUT1. We also found that all BC lines and the recurrent parent SUT1 had higher pod length and 100-seed weight than three donor parents. Meanwhile, these three donor parents had higher pods per plant and seeds per pod than SUT1 and some BC lines.

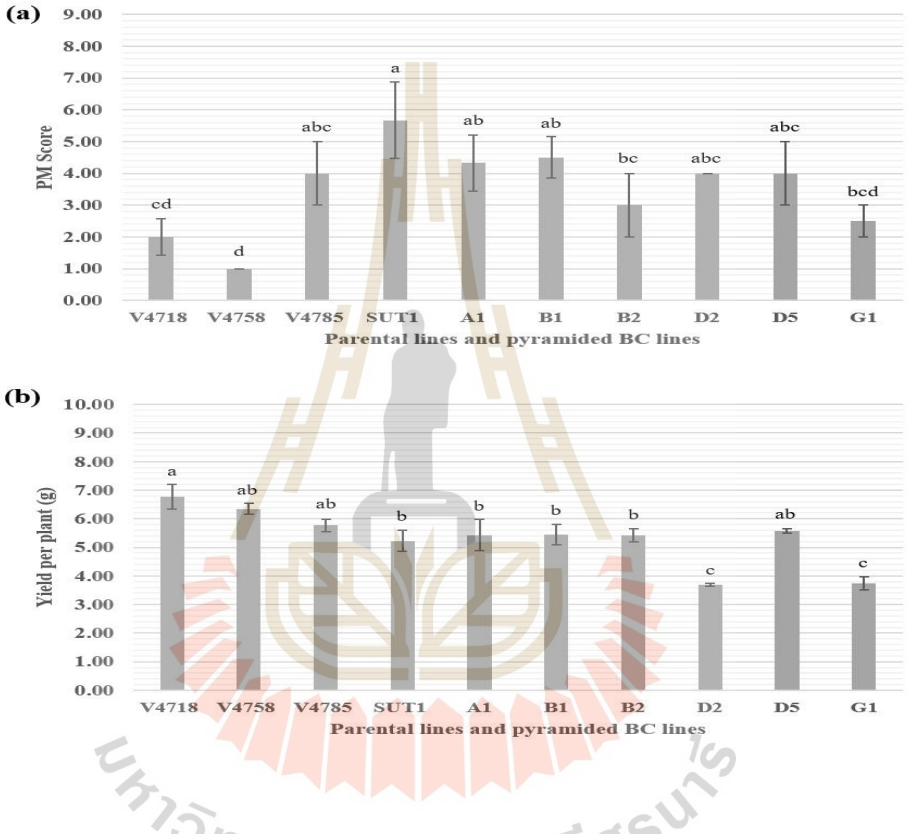

Figure 5.6 Comparison of PM response (a) and yield performance (b) between the parental lines and pyramided BC lines. They were grown in November 2020-February 2021 at SUT Farm, Nakhon Ratchasima. Different letters are significantly different ($P < 0.05$) based on DMRT. V4785, V4758, V4718 and SUT1 are parental lines; A1, B1, B2, D2, D5 and G1 are pyramided BC lines, respectively.

Table 5.7 Comparison of eight agronomic traits between parental lines and pyramided BC_4F_6 lines under field condition with PM outbreak.

Lines/varieties /BC lines	Days to flowering	Days to maturity	Plant height (cm)	Clusters/plant	Pods/plant	Pod length (cm)	Seeds/pod	100 seed weight (g)
V4718	43.00 ± 2.08	59.00 ± 2.08	38.28 ± 8.32	7.40 ± 0.48 ab[a]	23.19 ± 1.95 a	6.28 ± 0.20 f	10.68 ± 0.45 a	3.18 ± 0.14 b
V4758	44.67 ± 3.28	62.67 ± 3.18	43.97 ± 5.55	6.40 ± 0.79 abc	18.50 ± 1.19 b	7.24 ± 0.13 de	10.42 ± 0.18 a	3.77 ± 0.03 b
V4785	46.33 ± 2.19	65.67 ± 0.88	46.31 ± 6.56	8.15 ± 1.20 a	20.14 ± 0.79 ab	6.75 ± 0.14 ef	9.88 ± 0.31 ab	3.60 ± 0.13 b
SUT1	47.00 ± 1.53	64.00 ± 1.15	44.10 ± 5.04	4.71 ± 0.22 cd	10.63 ± 0.66 c	8.23 ± 0.21 ab	8.66 ± 0.27 cd	6.05 ± 0.01 a
A1	45.33 ± 0.88	63.67 ± 0.33	36.31 ± 1.75	4.98 ± 0.09 bcd	10.91 ± 0.89 c	7.86 ± 0.21 bc	8.56 ± 0.20 cd	6.27 ± 0.17 a
B1	44.00 ± 0.41	62.00 ± 0.41	40.38 ± 0.76	4.72 ± 0.77 cd	10.49 ± 0.73 c	8.17 ± 0.12 ab	9.08 ± 0.21 bc	6.02 ± 0.09 a
B2	47.00 ± 3.00	64.50 ± 1.50	37.30 ± 3.30	4.37 ± 1.03 cd	9.82 ± 0.98 c	8.56 ± 0.02 a	9.23 ± 0.10 bc	6.42 ± 0.06 a
D2	45.50 ± 1.50	63.50 ± 1.50	33.19 ± 2.19	2.92 ± 0.08 d	7.60 ± 0.23 c	8.54 ± 0.17 a	8.59 ± 0.55 cd	6.12 ± 0.58 a
D5	44.00 ± 0.00	63.00 ± 0.00	35.38 ± 8.24	3.81 ± 0.19 cd	10.16 ± 0.41 c	7.88 ± 0.28 bc	9.21 ± 0.01 bc	6.26 ± 0.06 a
G1	48.00 ± 1.00	65.50 ± 0.50	31.85 ± 1.99	4.02 ± 0.31 cd	7.95 ± 1.38 c	7.47 ± 0.20 cd	7.79 ± 0.26 d	6.22 ± 0.42 a
F-test	ns	ns	ns	*	**	**	**	**

Note: They were grown in November 2020 - February 2021 at SUT Farm, Nakhon Ratchasima.
[a] Means ± SE in the same column with different letters are significantly different ($P < 0.05$) based on DMRT.

5.5 Discussion

Three PM resistance genes in each mungbean accession V4718, V4758, and V4785 and a CLS resistance gene in V4718 have been reported to provide resistance against both diseases in Thailand and can be used as resistance sources to develop resistant mungbean varieties (Khaiudparn et al., 2010; Chankaew et al., 2011; Arsakit et al., 2017; Poolsawat et al., 2017; Chueakhunthod et al., 2020; Tantasawat et al., 2020; Yundaeng et al., 2020). However, development of mungbean varieties with PM and CLS resistance through conventional breeding is difficult. This is because the presence of 1, 2, or multiple resistance genes cannot be differentiated and both diseases only occur substantially in one season (the cool dry season for PM and the raining season for CLS). Therefore, phenotypic selection under field condition can be performed only once per year for each disease although 3-4 crops can be grown annually. Several molecular markers linked to these resistance genes i.e., I85420 and I42PL222 (flanked to PM resistance gene in V4718 (Poolsawat et al., 2017)), VR393 and CEDG084 (flanked CLS resistance gene in V4718 (Arsakit et al., 2017)), and I27R565 (linked PM resistance gene in V4785 (Tantasawat et al., 2021)) have been developed using the CN72 × V4718 and CN72 × V4785 crosses. In this cross, the marker I42PL222 derived from the resistant line V4718 which was located further to the *qPMC72V18-1* (13 cM) than the marker I42PL229 linked to susceptible allele of CN72 (4.0 cM) was used instead because I42PL229 was monomorphic between parents. When using both I85420 and I42PL222 markers for PM selection, there will be 2.34% recombination between both markers and the PM resistance gene in the CN72 × V4718 cross. However, the distance and recombination frequency may differ in the SUT1 × A2 cross used in this study. Nevertheless, these linked markers can be applied to combine these resistance genes

into a single variety through MAS which can overcome the limitation of season-dependent selection for PM and CLS resistance encountered by conventional selection approaches. In the beginning of our backcross breeding, 5 marker loci linked to 2 PM resistance genes from V4718 and V4785 and a CLS resistance gene from V4718 were found to be polymorphic between SUT1 (recurrent parent) and the donor A2, and were used for foreground selection in marker-assisted backcrossing (MABC). Although these marker loci were previously developed in other crosses (CN72 × V4718 or CN72 × V4785), they can also be used in the SUT1 × A2 cross because the donor A2 was double cross of RILs from the crosses between CN72 and V4718, V4758, and V4785, thus the PM and CLS resistance genes were the same. However, the marker CEDG084 linked to CLS resistance gene disappeared since BC_1F_1 generation although we selected 149 BC_1F_1 progenies. This may stem from segregation of marker and gene in this generation since a double cross was used as donor parent. Therefore, the selection of CLS resistance gene was only performed with the marker VR393 linking to the resistance gene at 4.0 cM from then on. In addition, the use of molecular markers for background selection allows estimation of the extent of recurrent parent genome (RPG) recovered in the pyramided backcross progenies carrying 2 PM and CLS resistance genes so that the progenies with maximum RPG recovery can be selected and advanced to the next generation. Therefore, only 2-4 generations of backcrossing are required to recover the RPG (Ragot et al., 1995; Pradhan et al., 2015; Krishna et al. 2017; Baliyan et al., 2018; Sagare et al., 2019), while conventional breeding takes 6-7 generations (Ahmed et al., 2016). Servin and Hospital (2002) recommended that two to four markers on each chromosome are sufficient for background selection. In this study, we used 10 polymorphic SSR and EST-SSR markers (Set A) which were reported to be linked to

domestication related traits i.e., 100-seed weight, pod length, pod width, seed length, seed width or seeds per pod on all 11 linkage groups (LG) of mungbean except LG 3 (Isemura et al., 2012). Moreover, six EST-SSR markers related to other putative protein functions (Chen et al., 2015) and four other polymorphic EST-SSR and SSR markers with unknown functions (Isemura et al., 2012) (Set B), which were located on LG 1, 2, 4, 5, and 11 were also included. To cover more chromosomal regions of the genome, we also included 52 polymorphic ISSR fragments from 12 ISSR primers (Set C) which may be randomly distributed throughout the genome. Using all of these 72 polymorphic loci/ fragments, 100, 100, and 98.8% RPG recovery for Sets A, B, and C, respectively were observed in six pyramided selected BC lines (A1, B1, B2, D2, D5, and G1). Recovery of the RPG depends on several factors such as plant breeder's preference, the required levels of line conversion and the genetic background between both parents. It should be noticed that genetic background of the donor parent used in this study may be partly shared in common with SUT1 because the donor parent A2 [[(14B × 19C) × (67A × 5B)-2]; A = RIL from CN72 × V4758, B = RIL from CN72 × V4718, and C = RIL from CN72 × V4785] is largely derived from V4718. V4718 was reported to be more genetically related to SUT1 (84.8% similarity) than V4758 and V4785 (79.0% similarity) by means of EST-SSR analysis. However, there were substantial genetic distances between SUT1 and A2 as SUT1 and V4718, V4758, and V4785 located in different clusters of dendrogram based on 54 polymorphic EST-SSR marker loci (Chueakhunthod, 2019). These differences were clearly observed on seed size, pod length, seeds per pod, and pods per plant. Thus, these portions may be introgressed along with the target genes into the recurrent parent, resulting in increased/decreased performance of other traits.

Field performance based on agronomic characters and disease responses in several seasons, years, and locations during 2019-2021 among the six pyramided BC_4 lines with similar genetic background to SUT1 identified by background markers revealed that most pyramided BC_4 lines were similar or superior to SUT1 for yield performance and their agronomic traits in several environments. This may be due to the inheritance of high yielding potential from SUT1. The yield related traits are normally controlled by polygenes, and they are distributed throughout the genome. Therefore, the use of markers distributed throughout the genome for background selection help ensure the recovery of genes controlling yield related traits in BC progenies. During CLS outbreak at SUT Farm, most pyramided BC_4 lines (A1, B1, B2, D5, and G1) tended to have higher yield than SUT1 (5.3-31.0%) and check cv. KPS1 (32-50%), although most of these lines (A1, B1, D5, and G1) were susceptible to CLS and only B2 was moderately resistant. These BC_4 lines tended to have higher number of seeds per pod and pods per plant than SUT1 which were inherited from the donor parent. This study confirmed the results of Chueakhunthod et al. (2020) and Papan et al. (2021), who reported that grain yield of mungbean was significantly and positively correlated with pods per plant and seeds per pod. Therefore, increasing number of pods per plant and seeds per pod can increase mungbean yield. Moreover, the reason why we loss CLS resistance in most BC lines may stem from the fact that the selection of CLS resistance gene was only performed using one side of flanking markers (VR393) because another side (CEDG084) disappeared since the first generation of backcrossing, making it possible for the crossing over between VR393 and CLS resistance gene (Chueakunthod, 2019). These results implied that the use of single marker for selection were less efficient than using both flanking markers as suggested by Collard and Mackill (2008).

Therefore, confirmation of resistance should be performed using detached leaf assay in each generation. While evaluation at Chai Nat Field Crops Research Center revealed that although the yields of pyramided BC lines were not significantly different from parents and KPS1, some of them i.e., A1, B1, and B2 tended to have slightly higher yield than SUT1 and KPS1. Note that most of the parents, KPS1 and BC lines were susceptible to CLS in this location, possibly due to heavy rain during flowering stage and pod setting and they were also affected by virus and insect infestation. This is in agreement with the weather data, which showed that the relative humidity (RH) in this location was found to be higher than that at SUT Farm (5.1% RH) though the amount of accumulated rain was lower than that of SUT Farm (Table A.3). A high RH level promoted the germination of conidia of *Cercospora canescens* (Kumar et al., 2011). Hence, the highest CLS severity was observed in this location, resulting in yield reduction of up to 9-49% when compared to SUT Farm location. During PM outbreak at SUT Farm, all of the pyramided BC lines showed moderate (A1, B1, D2, and D5) to high (B2 and G1) resistance to PM. The average temperature in this season was favorable for disease development and appeared to be slightly lower than the condition without disease outbreak (Tables A.2, A.4). In addition, we found that A1, B1, B2, and D5 tended to have higher yield than SUT1 (3.5-6.3%) while D2 and G1 had lower yield than SUT1. In this season, the yield of most mungbean genotypes was reduced of 15-53% comparing to the condition without disease outbreak. However, other environmental effects may also contribute to yield reduction. Meanwhile, under no disease outbreak, yield performances of the pyramided BC lines were not significantly different from those of parents and KPS1 at SUT Farm while differences were observed at Pak Thong Chai. We found that most pyramided BC lines also had higher number of

seeds per pod than the recurrent parent SUT1. Besides, yield potential and important yield components such as seeds per pod and pods per plant tended to be improved in some pyramided BC lines, A1, B1, B2, and D5. One of which (B2) was also resistant to both CLS and PM. This study demonstrates the application of MABC in pyramiding PM and CLS resistance genes to provide multiple resistance gene barrier against PM and CLS diseases.

5.6 Conclusion

MABC approach was successfully used to transfer a CLS resistance gene and 2 PM resistance gene from the donor parents into a recommended variety SUT1. Four markers linked to the target resistance genes were used for foreground selection, while three marker sets (72 polymorphic loci) were used for background selection. The RPG recovery of six pyramided BC lines was 100.0, 100.0, and 98.8% for Sets A, B, and C, respectively after only 4 generations of backcrossing. All pyramided BC_4 lines were moderately resistant or resistant to PM, and one of which (B2) was also moderately resistant to CLS. Most of pyramided BC lines were found to have all agronomic traits similar or superior to the recurrent parent SUT1. Some of them also had a tendency to produce higher yields than SUT1 under CLS and PM outbreaks. These BC lines are currently being evaluated in yield trial at multiple locations and seasons to select the ones that can be potentially developed into new resistant varieties.

5.7 References

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CHAPTER VI

EVALUATION OF GENETIC RELATIONSHIP BETWEEN PARENTAL LINES AND THEIR BACKCROSS PROGENIES

6.1 Abstract

Genetic relationship is a key factor for selecting and identifying parental lines and desirable progenies in plant breeding programs. In this study, we evaluated genetic relationship among mungbean genotypes based on agronomic and photosynthetic characters. Nine agronomic and four photosynthetic characters as well as CLS response were measured in 11 mungbean genotypes consisting of 6 BC_4F_5 progenies, 4 parental lines (V4718, V4758, V4785; powdery mildew (PM) and Cercospora leaf spot (CLS) resistant donor parents and SUT1; recurrent parent) and a check variety, KPS1. The results showed that all agronomic traits and CLS response were significantly different among mungbean genotypes ($P < 0.05$ or $P < 0.01$) but only pods per plant, pod length and seeds per pod were correlated with yield/plant. However, their photosynthetic traits were neither significantly different ($P > 0.05$) nor correlated with yield/plant. Pair-wise Euclidean distance coefficient ranged from 1.399 to 7.688 with a mean of 4.732. Using UPGMA analysis with photosynthetic and agronomic data, two clusters and two individual genotypes (V4718 and KPS1) were classified. We found that two Indian mungbean genotypes which are PM resistant lines were grouped into cluster I. While, SUT1 and all BC_4F_5 progenies were grouped into cluster II with B2 and D2 having the

closest genetic relationship with SUT1, the recurrent parent. The close relationship among all backcross progenies and SUT1 was found and some of these such as B2 was also resistant and moderately resistant to PM and CLS, respectively. These results confirmed the usefulness of genetic relationship evaluation based on phenotypic data.

6.2 Introduction

Mungbean [*Vigna radiata* (L.) Wilczek] is one of the most important grain legume crops in Asian. It is mainly grown in India followed by China, Myanmar, Indonesia, Pakistan, Thailand and Bangladesh, providing 90% of the world's production (Chankaew et al., 2011; Nair et al., 2014). It is cultivated for human consumption, feeds as well as green manure. Mungbean seeds contain essential nutrients such as minerals, vitamins, and digestible proteins. However, in Thailand, mungbean planting area and production has decreased due to high cost of production, low compensation and low yield (Tantasawat et al., 2010; Office of Agricultural Economics, 2019). The major constraints of mungbean production are lack of the varieties having high yield, susceptibility to pests and diseases or weakness to climate changes. Therefore, the improvement of mungbean with high yield and resistance to pests or diseases or more adaptability is needed for solving these constraints.

Genetic relationship is a basic knowledge for any breeding program and can be used for finding suitable parental lines or selecting desirable progenies. Phenotypic evaluation and photosynthesis performances had been used to determine genetic diversity and relationship in *Vigna* species (Ehlers and Hall, 1997; Bisht et al., 2005; Tantasawat et al., 2010; Islam and Razzaque, 2010; Chueakhunthod et al., 2020). The measurement of these phenotypic and photosynthetic characters provides criteria for

helping breeders select parental lines i.e., days to first flowering, days to the pod maturity, plant height, clusters per plant, branches per plant, pods per plant, pod length, pod width, seeds per pod, 100-seed weight, yield per plant, terminal leaf length, terminal leaf width, seed shape, seed color, photosynthetic rate, transpiration rate, stomatal conductance, and water use efficiency etc. In addition, several agronomic and photosynthetic traits consisting of plant height, pods per plant, pod length, seeds per pod, photosynthetic rate and stomatal conductance had been found to be positively correlated to yield of mungbean (Chueakhunthod et al., 2020). Therefore, the objective of this study was to estimate genetic relationship among mungbean genotypes based on agronomic and photosynthetic performances.

6.3 Materials and methods

6.3.1 Plant materials

A total of 11 mungbean genotypes (6 BC_4F_5 progenies, 4 parental lines and 1 check variety) were studied for their agronomic and photosynthetic performances. Six BC_4F_5 progenies consisting of A1, B1, B2, D2, D5 and G1 were obtained from the SUT1 × A2 [(14B × 19C) × (67A × 5B)-2] cross. A2 is a donor parent containing a CLS resistance gene and 2 PM resistance genes which was developed by double crosses of recombinant inbred lines (RILs) from three populations including CN72 × V4758 (A), CN72 × V4718 (B) and CN72 × V4785 (C). Four parental lines included SUT1 (recurrent parent), and V4718, V4758 and V4785, resistant lines which were derived from the World Vegetable Center (WORLDVEG) in Taiwan. SUT1 is a recommended variety with high yield and moderately resistant to CLS and PM. It was developed at Suranaree University of Technology, Thailand. Meanwhile, KPS1which is a Thai certified

variety was used as check variety.

6.3.2 Site description

A field experiment was carried out during July to September 2020 at Suranaree University of Technology Farm, Nakhon Ratchasima, Thailand (latitude: 14°52'39"N, longitude: 102°00'15"E, altitude: 227 m). This experiment was conducted in a randomized incomplete block design with 3-4 replications. Each genotype was grown in two rows of 6 m long with spacing of 20 and 50 cm intra-row and inter-row, respectively. Three plants per hill were kept (ca. 90 plants per row).

6.3.3 Agronomic and photosynthetic evaluation

Nine agronomic traits were evaluated including days to first flowering, days to the pod maturity, plant height, clusters per plant, pods per plant, pod length, seeds per pod, 100-seed weight and yield per plant. Days to first flowering and pod maturity were measured from sowing to 50% of plants in the plot showing the first flower opening and first pod ripening, respectively. Other traits were counted from 10 randomly selected plants from the middle of each block. Agronomic measurements were carried out using the techniques described by Chueakhunthod et al. (2020) and Chai Nat Field Crops Research Center (2018) (Table A.1). In addition, CLS response was evaluated at 65 days after planting using scoring system described by Chankaew et al. (2011) (Figure A.1).

Four photosynthetic parameters consisting of net photosynthetic rate (Pn), transpiration rate (Tr), stomatal conductance (Gs) and water use efficiency (WUE) were evaluated at 40 days after planting from three representative plants that were also used for agronomic traits evaluation. Three upper fully-expanded terminal leaves (fully bloom stage) of each plant were measured by a portable photosynthesis system (model

LCA 4) (ADC Ltd., Hoddesdon, England) during 9.30 am. to 2.30 pm. according to Chueakhunthod et al. (2020).

6.3.4 Data analysis

The analysis of variance (ANOVA) of all agronomic and photosynthetic data as well as CLS score was carried out using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). The significant difference of sample means was performed by Duncan's Multiple Range Test (DMRT). In addition, the correlation coefficients among four photosynthetic and nine agronomic traits were also calculated by using SPSS 16.0 software. Using photosynthetic and agronomic data, the Unweighted pair-group method arithmetic average (UPGMA) and Euclidean distance were applied to construct the clustering using the XLSTAT 2015 software (Addinsoft, Inc., Paris, France). The genetic dissimilarity coefficients in pair-wise comparison across all mungbean genotypes were computed using Euclidean distance function.

6.4 Results

6.4.1 Agronomic and photosynthetic evaluation

The analysis of variance (ANOVA) showed highly significant differences ($P < 0.01$) for eight of the nine agronomic traits including days to first flowering, days to maturity, clusters per plant, pods per plant, pod length, seeds per pod, 100-seed weight, and yield per plant, and for CLS resistance response. While plant height showed significant differences among mungbean genotypes ($P < 0.05$) (Table 6.1). In addition, we found that all backcross progenies had all agronomic traits similar to their recurrent parent (SUT1), except D5 and B1. D5 had significantly higher number of pods per plant (16.02 pods), seeds per pod (10.79 seeds) and yield per plant (7.68 g) than SUT1 (11.54

pods, 9.46 seeds and 5.32 g, respectively), and its days to maturity (52.67 days) was earlier than that of SUT1 (57.25 days). Whereas, B1 had less 100-seed weight (5.50 g) than SUT1 (5.97 g). When comparing other BC progenies and SUT1, we found that A1, B1, and G1 also tended to have higher pods per plant, seeds per pod, and yield per plant than SUT1 although all of which were not significantly different from SUT1. Furthermore, SUT1 and all BC progenies had significantly higher 100-seed weight and pod length than three donor parents. While three donor parents had higher pods per plant and seeds per pod than SUT1 and some BC progenies. Moreover, some BC progenies (A1, B1, D5, and G1) had significantly higher seeds per pod and yield per plant than check cv. KPS1. Only B2 was identified as moderately resistant to CLS with disease severity score of 2.75, while other BC progenies were susceptible to CLS with disease severity scores of 3.50-4.75. The disease severity score of B2 was comparable to that of V4718 which was the donor of CLS resistance gene (2.50). In contrast, SUT1 was identified as susceptible to CLS with disease severity score of 3.50. Check variety KPS1 was found to be moderate resistance to CLS (Table 6.1). In contrast, all photosynthetic traits were found not significantly different ($P > 0.05$) among mungbean genotypes (Table 6.2). We found that three resistant lines (V4718, V4758 and V4785) and check variety (KPS1) tended to have higher photosynthetic rate (Pn), transpiration rate (Tr), and stomatal conductance (Gs) than SUT1 and BC progenies (Table 6.2).

Table 6.1 Agronomic characters of selected BC_4F_5 progenies obtained from the SUT1 × A2 [(14B × 19C) × (67A × 5B)-2] cross, parental lines and check variety.

Lines/varieties /BC	Days to flowering	Days to maturity	Plant height (cm)	Clusters/plant	Pods/plant	Pod length (cm)	Seeds/pod	100 seed weight (g)	Yield/plant (g)	CLS score [b]	
V4718	36.88 ± 0.66 f [a]	50.50 ± 0.29 e	48.70 ± 4.03 c	5.26 ± 0.12 b	17.05 ± 1.27 ab	6.15 ± 0.10 d	10.82 ± 0.39 ab	3.01 ± 0.04 c	4.59 ± 0.35 cd	2.50 ± 0.20 c	R
V4758	43.83 ± 0.60 ab	61.50 ± 0.76 ab	68.40 ± 2.79 a	7.12 ± 0.67 a	17.90 ± 2.96 a	7.17 ± 0.04 c	11.18 ± 0.55 ab	3.29 ± 0.07 c	4.80 ± 0.87 cd	3.33 ± 0.33 bc	MR
V4785	44.50 ± 0.76 a	63.17 ± 2.32 a	65.81 ± 8.54 ab	8.04 ± 0.68 a	18.56 ± 1.99 a	6.98 ± 0.10 c	11.46 ± 0.70 a	3.35 ± 0.06 c	5.05 ± 0.57 bcd	2.50 ± 0.29 c	R
SUT1	40.25 ± 0.95 cde	57.25 ± 1.64 c	48.63 ± 2.63 c	4.28 ± 0.27 b	11.54 ± 1.87 d	8.57 ± 0.11 ab	9.46 ± 0.18 cd	5.97 ± 0.07 a	5.32 ± 0.59 bcd	3.50 ± 0.29 bc	S
A1	40.00 ± 0.91 cde	55.75 ± 1.38 cd	55.61 ± 3.34 bc	5.14 ± 0.55 b	12.95 ± 0.73 bcd	8.50 ± 0.11 ab	10.35 ± 0.16 bc	5.62 ± 0.05 ab	6.61 ± 0.43 ab	4.75 ± 0.25 a	S
B1	41.75 ± 1.49 a-d	57.00 ± 1.87 c	51.97 ± 3.70 c	4.51 ± 0.56 b	12.59 ± 1.48 cd	8.29 ± 0.13 b	10.15 ± 0.19 bc	5.50 ± 0.16 b	6.19 ± 0.65 abc	3.50 ± 0.29 bc	S
B2	42.67 ± 1.20 abc	59.00 ± 1.53 bc	52.19 ± 3.73 c	4.64 ± 0.31 b	11.84 ± 0.87 cd	8.8 ± 0.12 ab	9.07 ± 0.26 cd	5.75 ± 0.09 ab	5.62 ± 0.19 bc	2.75 ± 0.48 c	MR
D2	40.75 ± 0.85 cde	57.00 ± 1.08 c	49.24 ± 2.89 c	4.41 ± 0.17 b	10.36 ± 0.23 d	8.99 ± 0.14 a	9.59 ± 0.30 cd	5.73 ± 0.05 ab	5.04 ± 0.28 bcd	3.50 ± 0.29 bc	S
D5	38.00 ± 0.58 ef	52.67 ± 0.88 de	54.14 ± 4.78 bc	5.29 ± 0.73 b	16.02 ± 2.79 abc	8.88 ± 0.25 a	10.79 ± 0.46 ab	5.80 ± 0.16 ab	7.68 ± 0.91 a	4.00 ± 0.41 ab	S
G1	41.25 ± 1.44 bcd	57.00 ± 1.00 c	56.60 ± 1.72 abc	5.21 ± 0.35 b	13.26 ± 0.3 bcd	8.50 ± 0.18 ab	10.21 ± 0.30 bc	5.70 ± 0.06 ab	6.61 ± 0.33 ab	4.75 ± 0.25 a	S
KPS1	39.50 ± 0.65 def	56.38 ± 1.14 c	60.37 ± 4.67 abc	4.57 ± 0.36 b	9.09 ± 0.81 d	8.89 ± 0.41 a	8.73 ± 0.21 d	5.96 ± 0.19 a	3.84 ± 0.44 d	3.25 ± 0.14 bc	MR
F-test	**	**	*	**	**	**	**	**	**	**	

[a] Means in the same column with different letters are significantly different ($P < 0.05$) based on Duncan's multiple range test (DMRT).
[b] CLS response; 1.0-2.5 = resistant (R), 2.6-3.4 = moderately resistant (MR) and 3.5-5.0 = susceptible (S)

Table 6.2 Photosynthetic characters of parental lines, selected BC_4F_5 progenies and check variety.

Genotypes	Pn (μmol m^{-2} s^{-1})	Tr (mmol m^{-2} s^{-1})	Gs (mol m^{-2} s^{-1})	WUE (mmol mol^{-1})
V4718	11.08	1.40	1.09	11.21
V4758	12.68	1.45	0.49	12.06
V4785	14.78	1.36	0.49	11.56
SUT1	6.82	0.59	0.15	11.81
A1	8.94	0.55	0.18	15.31
B1	9.40	0.52	0.19	18.46
B2	7.33	0.58	0.25	21.37
D2	5.99	0.50	0.12	17.38
D5	8.44	0.94	0.23	9.13
G1	9.73	0.90	0.33	11.14
KPS1	12.02	1.62	0.46	8.36
F-test	ns	ns	ns	ns

Note: Pn = net photosynthetic rate; Tr = transpiration rate; Gs = stomatal conductance; WUE = water use efficiency
ns = not significantly different at $p > 0.05$.

6.4.2 Correlation coefficient analysis

To analyze the correlation coefficient of photosynthetic traits together with agronomic traits. The results are showed in Table 6.3. Grain yield per plant was significantly and positively correlated with pods per plant ($r = 0.442^{**}$), pod length ($r = 0.314^{*}$), and seeds per pod (0.415^{**}), whereas it was not correlated with days to first flowering, days to maturity, clusters per plant, plant height, 100-seed weight as well as net photosynthetic rate (Pn), transpiration rate (Tr), stomatal conductance (Gs) and water use efficiency (WUE). In addition, the correlations of other characters were also evaluated. 100-seeds weight was strongly and positively correlated with pod length ($r = 0.883^{**}$), whereas it was negatively related to clusters per plant ($r = -0.614^{**}$), and pods per plant ($r = -0.654^{**}$), indicating that selection of large seed mungbean genotypes may reduce number of clusters per plant and pods per plant or increase pod length. We

also found that number of seeds per pod was significantly correlated with clusters per plant ($r = 0.709^{**}$), plant height ($r = 0.482^{**}$), pods per plant ($r = 0.825^{**}$), pod length ($r = -0.451^{**}$) and 100-seeds weight ($r = -0.632^{**}$) (Table 6.3), suggesting that the improvement of number of seeds per pod can be selected through increasing clusters per plant, plant height and pods per plant. Furthermore, Tr was positively correlated with plant height ($r = 0.450^{*}$), pods per plant ($r = 0.379^{*}$) and Pn ($r = 0.665^{**}$) but it was negatively correlated with 100-seed weight ($r = -0.466^{*}$). Gs was significantly related to pod length ($r = -0.548^{**}$), 100-seed weight (-0.568^{**}), Pn ($r = 0.459^{**}$) and Tr ($r = 0.746^{**}$). While WUE was correlated with Tr ($r = -0.355^{*}$).

6.4.3 Genetic relationships

The estimation of genetic dissimilarity coefficient based on photosynthetic and agronomic traits for all pair-wise combinations of the eleven mungbean genotypes ranged from 1.399 (V4758 vs. V4785) to 7.688 (V4785 vs. D2) with an average of 4.732. For cluster analysis, two different clusters and two individual genotypes (V4718 and KPS1) were identified (Figure 6.1). Cluster I contained two donor parents, V4758 and V4785 which were originated in India. It was distantly related to SUT1 and all BC_4F_5 progenies. On the other hand, cluster II contained recurrent parent (SUT1) and their BC_4F_5 progenies, indicating that these BC progenies had the genetic relationship similar to SUT1, especially D2 and B2. The results confirmed that BC_4F_5 progenies which were obtained from 4 times backcrossing to recurrent parent had sufficiently similar characters to recurrent parent. In addition, check cv. KPS1 was found to be different from BC_4F_5 progenies and their parental lines.

Table 6.3 Pearson correlation coefficients of agronomic and photosynthetic characters of selected BC_4F_5 progenies obtained from the SUT1 × A2 [(14B × 19C) × (67A × 5B)-2] cross, parental lines and check variety.

Characters	YPP	DTF	DTM	CPP	PH	PPP	PL	HSW	SPP	Pn	Tr	Gs	WUE
YPP		-0.214^{ns}	-0.26^{ns}	0.184^{ns}	0.125^{ns}	0.442**	0.314*	0.291^{ns}	0.415**	-0.272^{ns}	-0.212^{ns}	-0.209^{ns}	-0.008^{ns}
DTF			0.913**	0.106^{ns}	0.121^{ns}	-0.091^{ns}	-0.008^{ns}	-0.112^{ns}	0.033^{ns}	0.265^{ns}	0.048^{ns}	-0.190^{ns}	0.089^{ns}
DTM				0.160^{ns}	0.125^{ns}	-0.113^{ns}	0.010^{ns}	-0.077^{ns}	-0.050^{ns}	0.166^{ns}	0.092^{ns}	-0.216^{ns}	0.065^{ns}
CPP					0.725**	0.747**	-0.400*	-0.614**	0.709**	0.013^{ns}	0.309^{ns}	0.099^{ns}	-0.264^{ns}
PH						0.452**	0.003^{ns}	-0.266^{ns}	0.482**	0.170^{ns}	0.450*	0.181^{ns}	-0.358^{ns}
PPP							-0.540**	-0.654**	0.825**	0.147^{ns}	0.379*	0.364^{ns}	-0.194^{ns}
PL								0.883**	-0.451**	-0.296^{ns}	-0.292^{ns}	-0.548**	0.046^{ns}
HSW									-0.632**	-0.348^{ns}	-0.466*	-0.568**	0.132^{ns}
SPP										0.222^{ns}	0.303^{ns}	0.335^{ns}	-0.161^{ns}
Pn											0.665**	0.459**	0.092^{ns}
Tr												0.746**	-0.355*
Gs													-0.154^{ns}

YPP = Yield/plant; DTF = Days to flowering; DTM = Days to maturity; CPP = Clusters/plant; PH = Plant height; PPP = Pods/plant; PL = Pod length; HSW = 100 seed-weight; SPP = Seeds/pod; Pn = net photosynthetic rate; Tr = transpiration rate; Gs = stomatal conductance; WUE = water use efficiency.
** significant correlation at $P < 0.01$; * significant correlation at $P < 0.05$; ^{ns} not significant correlation at $P > 0.05$.

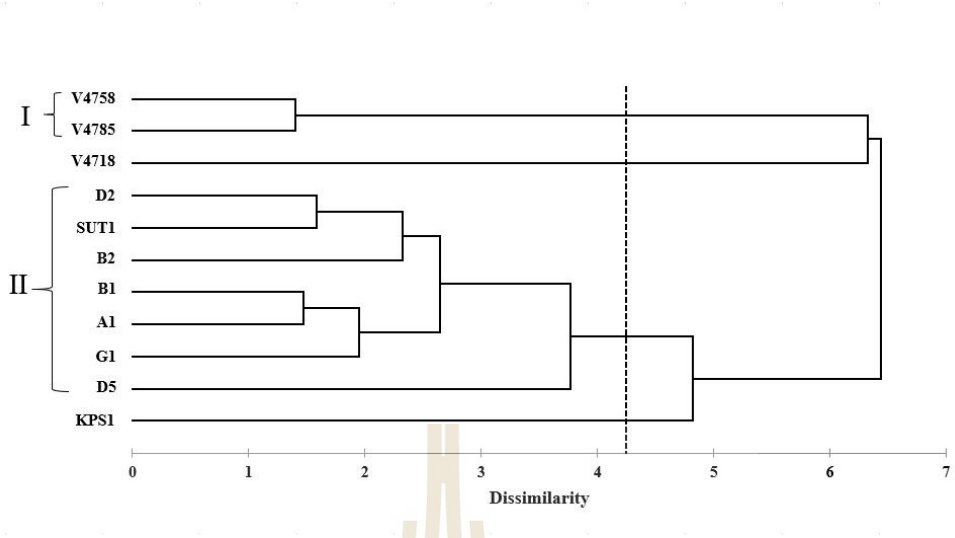

Figure 6.1 Agronomic and photosynthetic characters derived dendrogram of 11 mungbean genotypes constructed by an unweighted pair group method average (UPGMA) using XLSTAT 2015 software.

6.5 Discussion

Previously, the genetic resources of mungbean have been evaluated based on morpho-agronomic traits, photosynthetic performances, protein banding, and molecular markers (Tomooka et al., 1992; Lawn and Rebetzke, 2006; Sangiri et al., 2007; Tantasawat et al., 2010; Chueakhunthod et al., 2020). Knowledge of the genetic relationship enables plant breeders to select germplasm more effectively and create efficient strategies in their breeding programs (Tantasawat et al., 2010). Therefore, we aimed to evaluate the genetic relationship of BC_4F_5 progenies along with their parents and check variety based on agronomic and photosynthetic performances. All agronomic traits and CLS response were found significantly different among mungbean genotypes ($P < 0.05$ or $P < 0.01$) but no significant difference was found on their photosynthetic traits ($P > 0.05$) in July- September 2020 at SUT Farm. We found that most BC

progenies had all characteristics similar or superior to the recurrent parent SUT1. Moreover, B2 progeny was moderately resistant to CLS while other progenies were susceptible to CLS. These backcross progenies were also found to be resistant and moderately resistant to PM under field conditions in cool-dry season (Papan et al., unpublished data). A high photosynthetic rate (Pn), transpiration rate (Tr), and stomatal conductance (Gs) were observed in three resistant lines (V4718, V4758 and V4785) and check variety (KPS1) in contrary to the results reported by Chueakhunthod et al. (2020), who revealed that mungbean cv. SUT1 had higher Pn than KPS1, V4718, V4758 and V4785 when evaluated during March-June 2020. They also found no significant difference of Pn, Tr, Gs and water use efficiency (WUE) among 23 mungbean and 4 blackgram genotypes. These results revealed that the differences of growing season may affect Pn of mungbean genotypes. In addition, all mungbean genotypes in this study were grown during wet season (July-September 2020), most of which had lower Pn than those observed by Chueakhunthod et al. (2020). These results are consistent with those of Hamid et al. (1990) who found that Pn was lowest at flowering and pod development stages when growing under high soil moisture. However, not only growing seasons, but growth stages, plant materials, locations, and nitrogen content in the soil also affected Pn (Hamid et al., 1990; Chowdhury et al., 2005; Hossain et al., 2009; Chueakhunthod et al., 2020).

When the correlation coefficient of photosynthetic traits together with agronomic traits was calculated to identify their relative significance. The significant correlation between grain yield and number of pods per plant, pod length and seeds per pod in this study confirmed the results of Makeen et al. (2007), Canci and Toker (2014) and Chueakhunthod et al. (2020). These results revealed that increasing number of pods per

plant, pod length and seeds per pod can improve mungbean yield. However, in previous reports, days to first flowering, 100-seed weight, and plant height were also found to be positively correlated to yield (Khattak et al., 1995; Canci and Toker, 2014, Chueakhunthod et al., 2020). A negative correlation between seeds per pod and 100-seed weight in the present study was in agreement with the results of Khattak et al. (1995) and Makeen et al. (2007). In contrast, a negative correlation between seeds per pod and pod length was different from those reported by Makeen et al. (2007) and Chueakhunthod et al. (2020). Moreover, all photosynthetic characters were not correlated with yield, consistent with the results of Islam and Razzaque (2010), who also found that these photosynthetic traits were not associated with mungbean yield. In contrast, Chueakhunthod et al. (2020) found that all photosynthetic characters were significantly and positively correlated with seed yield when evaluated in 23 mungbean and 4 blackgram genotypes from nine countries. In our study, a significantly positive correlation between Pn and Gs, and negative correlation between Tr and WUE agreed with the previous results reported by Chueakhunthod et al. (2020), while a significantly positive correlation between Pn and Tr as well as Tr and Gs in this study was not observed in their results. These inconsistencies may stem from the differences in plant materials, seasons and locations.

The cluster analysis divided these 11 mungbean genotypes into two clusters and two out group genotypes (V4718 and KPS1). We found that two donor parents, V4758 and V4785 were classified into cluster I, and all BC_4F_5 progenies and the recurrent parent SUT1 were classified into cluster II. These donor parents originated in India and their genetics are associated with PM resistance (Khajudparn et al., 2010; Chathiranrat et al., 2018; Chueakhunthod et al., 2020). Similarly, V4718 which was also originated

in India was found to be resistant to PM and CLS (Hartman et al., 1993; Poolsawat et al., 2017; Arsakit et al.,2017).

6.6 Conclusion

We successfully identified the genetic relationship among 11 mungbean genotypes based on photosynthetic and agronomic traits. The results demonstrate that close genetic relationship was found between recurrent parent (SUT1) and their BC_4F_5 progenies which were classified into the same cluster. We found that B2 and D2 had the highest genetic similarity to SUT1. One of these (B2) was resistant and moderately resistant to PM and CLS. In addition, D5 had higher yield per plant than other BC lines. These BC lines can be potentially developed into a new resistant variety in the future.

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CHAPTER VII

CONCLUSION

In mungbean breeding programs, selection for desirable traits through conventional breeding is typically complicated by ambiguous phenotypes and the requirement for specific environmental conditions, especially gene pyramiding for disease resistance, of which the presence of 1, 2, or multiple resistance genes cannot be differentiated and monitored. Recently, the use of marker-assisted selection (MAS) can overcome these limitations which is useful for monitoring the presence of multiple genes. It can also select at all developmental stages without environmental effects, allowing year-round selection. As a result, the development of markers tightly linked to the traits of interest is essential. In this chapter, we summarized the answers to research objectives mentioned in chapter I as follows:

We found five out of 28 markers (ISSR; I16274, I88656, ISSR-RGA; I35P716, SSR; CEDG008, and InDel; VrTAF5_Indel) that were associated with the CLS resistance gene with a LOD score of more than 3.0 in two years, indicating their usefulness for CLS mapping. Later, we used these five markers together with markers VR393 and CEDG084 to refine QTL mapping of a gene conferring CLS resistance in the CN72 × V4718 cross. Inclusive composite interval mapping (ICIM) identified a major QTL (*qCLS72V18-1*) conferring CLS resistance. This QTL was flanked between the I16274 and VrTAF5_Indel markers, accounting for 32.86% to 41.56% of the phenotypic variation depending on years. Moreover, I16274 and VrTAF5_Indel markers were closest to the location of CLS resistance gene at distances of 4 and 5 cM,

respectively. If both markers are used in MAS, only 0.40% recombination between both markers and the CLS resistance gene will be observed. Interestingly, when we evaluated these closest markers in an additional 21 mungbean varieties/lines with known CLS reactions, 14 out of 21 genotypes/lines, including V4758, CN36, CN72, CN84-1, SUT1, SUT4, PUSA-105, NM92, NM94, EG-MD-6D, CES55, MG50-10A (Y), BPI GLABROUS #3, and KING did not have either of the resistance associated amplicons (aabb). These 14 genotypes/lines were found to be susceptible to CLS and polymorphic at these loci. Therefore, we can transfer the CLS resistance gene into these genotypes through MAS using these two tightly linked markers.

Furthermore, using the marker-assisted backcrossing (MABC) technique, this work effectively transferred a CLS resistance gene and two powdery mildew (PM) resistance genes from donor parents (D2 and A2) into high yielding mungbean varieties KING and SUT1. Two pyramided BC lines (H3 and H4) for KING and six pyramided BC lines (A1, B1, B2, D2, D5, and G1) for SUT1 were developed by using marker-assisted foreground and background selection. In addition, when we used 49 and 72 polymorphic marker loci for KING and SUT1, respectively to estimate recurrent parent genome (RPG) recovery of pyramided BC progenies. Depending on marker sets, RPG recovery of these pyramided BC lines ranged from 94.4 to 100.0% within BC_4 generation. Using this technique, we can reduce the number of generations of backcrossing, thereby saving more time and cost than conventional breeding. However, both visual phenotypic selection together with marker-assisted selection should be used to select pyramided BC lines in each generation of backcrossing be to enhance RPG recovery. In addition, after marker-assisted foreground selection, selected BC progenies should be confirmed with detached leaf assay.

We discovered that H3 line was resistant to CLS and moderately resistant to PM in the KING × D2 cross. H3 line had most agronomic traits similar or superior to the recurrent parent KING, and had 18.0-32.0% greater yields than KING during CLS and PM outbreaks. In addition, we observed that most pyramided BC lines in the SUT1 × A2 cross exhibited most agronomic traits similar or superior to SUT1. They were resistant or moderately resistant to PM, and one of them (B2) was also moderately resistant to CLS. Under CLS and PM outbreaks, B2 line provided a slightly higher yield than SUT1 (3.5-5.3%), whereas other lines (A1, B1, and D5) gave 3.7-3.10% higher yields than SUT1 in some environments.

The final experiment was carried out to confirm the genetic relationship between six BC_4F_5 lines, their parents and check cv. KPS1, using agronomic and photosynthetic traits in the field during the wet season. In this season, we found that all six BC_4F_5 lines had most agronomic and photosynthetic performances similar or superior to recurrent parent SUT1. In addition, yield per plant was significantly related to pods per plant and seeds per pod. When using UPGMA analysis with photosynthetic and agronomic data, the closest genetic relationship was found between recurrent parent SUT1 and their BC_4F_5 lines which were classified into the same cluster, especially B2 and D2. These results confirmed the effectiveness of background selection for accelerated backcrossing.

Finally, these promising pyramided BC lines such as H3, H4, B1, B2, D5, and G1 are currently under investigation in multi-location to evaluate their potential. Some of these pyramided BC lines can be expected to replace KING and SUT1 in the future.

APPENDIX

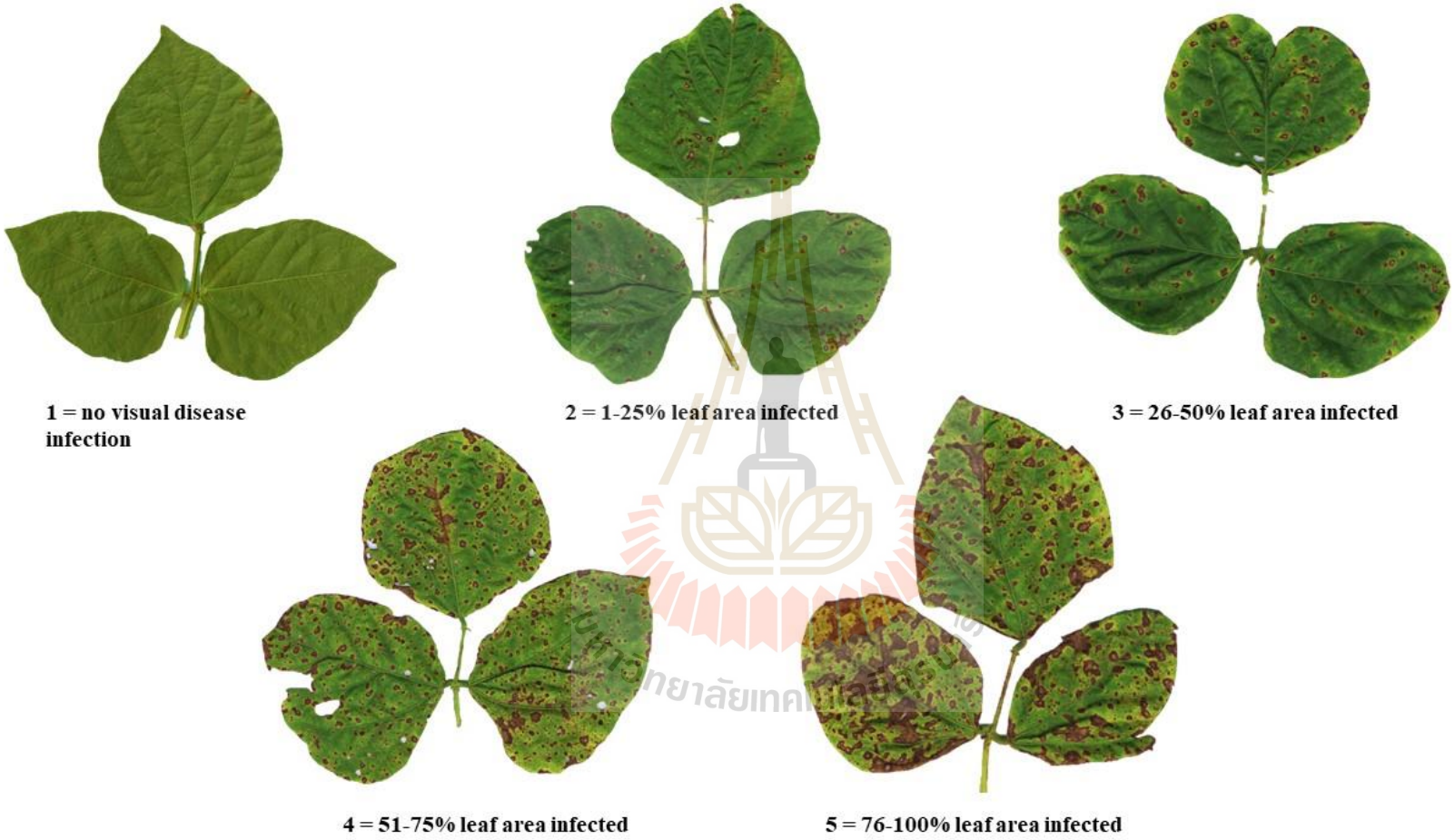

Figure A.1 Samples of CLS symptoms in mungbean leaves.

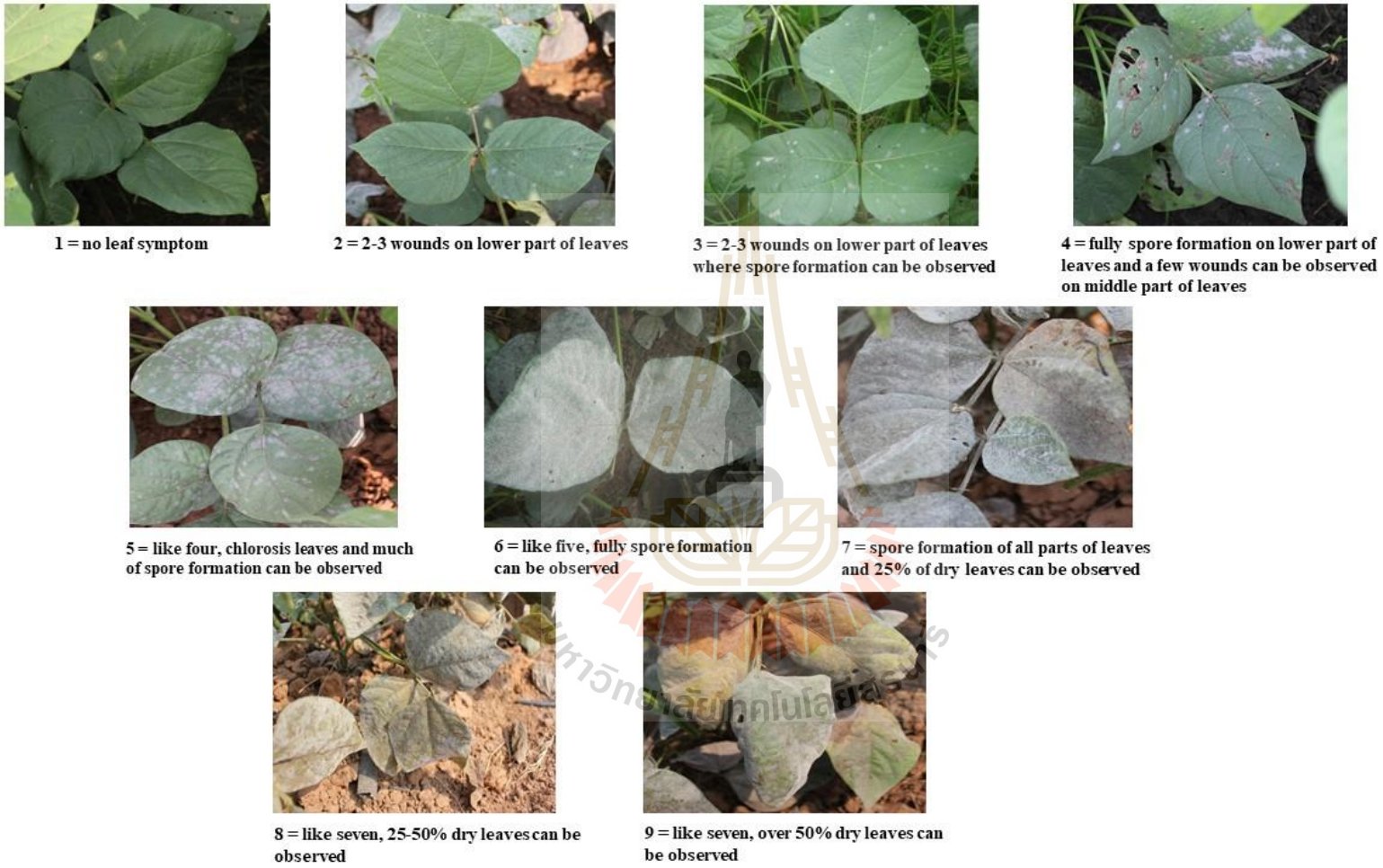

Figure A.2 Samples of PM symptoms in mungbean leaves.

Table A.1 Evaluation methods of agronomic and photosynthetic characters.

No.	Characters	Evaluation methods
1	Days to flowering	Measured from sowing to 50% of plants in the plot showing the first flower opening.
2	Days to maturity	Measured from sowing to 50% of plants in the plot showing the first pod ripening.
3	Clusters/plant	Counted from clusters having at least one fully grown pod at first harvest including both main stem and branches. Average of ten plants/block.
4	Plant height (cm)	Measured from soil level to the highest point after the first harvest. Average of ten plants/ block.
5	Pods/plant	Counted number of pods from all harvests. Average of ten plants/ block.
6	Pod length (cm)	Measured from maximum length of ten pods per plant. Average of ten plants/ block.
7	100-seed weight (g)	Measured from 100 randomly selected seeds. Average of ten plants/block (50 seeds from two plants were combined if necessary).
8	Seeds/pod	Counted number of seeds per pod from ten pods. Average of ten plants/ block.
9	Yield/plant (g)	Measured from total seed yield of each plant. Average of ten plants/ block.
10	Photosynthetic rate (Pn) (μmol m^{-2} s^{-1})	Three upper most fully-expanded terminal leaves of 40 days-old plant (full bloom stage, R2 showing the greatest photosynthetic potential in mungbean were measured by a portable photosynthesis system (model LCA 4) at 9:30 am to 2:30 pm. Average of three plants/block.
11	Transpiration rate (Tr) (mmol m^{-2} s^{-1})	
12	Stomatal conductance (Gs) (mol m^{-2} s^{-1})	
13	Water use efficiency (WUE) (mmol mol^{-1})	

Table A.2 Weather data of two locations during cool-dry season without disease outbreak in November 2019-March 2020.

Month	Maximum temperature (°C)		Minimum temperature (°C)		Relative humidity (% RH)		Amount of rain (mm)	
	SUT Farm	Pak Thong Chai	SUT Farm	Pak Thong Chai	SUT Farm	Pak Thong Chai	SUT Farm	Pak Thong Chai
Nov	31.6	-[a]	20.1	-	54.2	-	9.1	-
Dec	30.2	-	15.9	-	62.0	-	0.0	-
Jan	33.4	-	18.4	-	74.0	-	0.0	-
Feb	33.9	-	18.6	-	61.8	-	0.0	-
Mar	37.0	-	23.8	-	67.3	-	101.4	-
Mean	33.2	-	19.4	-	63.9	-	22.1	-

Note: Data were obtained from Huybanyang Meteorological, Irrigation Water Management Research Station 3, Nakhon Ratchasima province.
[a] Not available.

Table A.3 Weather data of two locations during rainy season in June-August 2020 for CLS evaluation.

Month	Maximum temperature (°C)		Minimum temperature (°C)		Relative humidity (% RH)		Amount of rain (mm)	
	Chai Nat	SUT Farm	Chai Nat	SUT Farm	Chai Nat	SUT Farm	Chai Nat	SUT Farm
Jun	36.3	34.8	25.5	26.1	73.7	73.4	79.3	155.8
Jul	35.9	34.9	25.5	26.1	73.6	70.3	160.7	172.0
Aug	34.6	33.1	25.3	25.8	75.9	66.3	112.1	208.7
Sep	33.9	33.1	25.4	25.4	81.6	74.6	167.7	278.7
Mean	35.2	34.0	25.4	25.8	76.2	71.1	130.0	203.8

Note: Data were obtained from Bang Luang Meteorological, Chai Nat Province and Huybanyang Meteorological, Irrigation Water Management Research Station 3, Nakhon Ratchasima province.

Table A.4 Weather data at SUT Farm during cool-dry season in November 2020-February 2021 for PM evaluation.

Month	Temperature (°C)		Relative humidity (% RH)	Amount of rain (mm)
	Maximum	Minimum		
Nov	31.1	21.3	70.3	2.6
Dec	29.9	18.1	66.8	0.0
Jan	29.3	15.6	62.8	0.0
Feb	33.4	18.4	59.9	21.0
Mean	31.0	18.3	64.9	5.9

Note: Data were obtained from Huybanyang Meteorological, Irrigation Water Management Research Station 3, Nakhon Ratchasima province.

BIOGRAPHY

Mr. Pitchakon Papan was born on April 21, 1994 at Buriram, Thailand. He graduated from Bualuangwitthayakhom Senior High School, Buriram and attended a Bachelor of Science Degree Program in Crop Production Technology at Suranaree University of Technology in 2012. He graduated in March 2016. Then, in the same year, he pursued in a Ph. D. program in School of Crop Production Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. He received a *Kittibandit* scholarship from Suranaree University of Technology which supported his tuition, fee, and salary. During his study, he had a chance to present his work in the ISER-333rd International Conference on Agricultural and Biological Science (ICABS) during March 26-27, 2018 in Macau, China (Poster presentation on "Selection of mungbean resistant to powdery mildew in BC_1F_1 progenies based on ISSR and ISSR-RGA markers"). This work was published in the proceeding of ISER 117th international conference. Recently, he had a chance to present his work in the 2nd SUT International Virtual Conference on Science and Technology during August 6, 2021 in Nakhon Ratchasima, Thailand (Oral Presentation on "Evaluation of genetic relationship between parental lines and their backcross progenies"), and received the best presentation award.