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*Full Length Article*

# Identification of ISSR, ISSR-RGA and SSR Markers Associated with Cercospora Leaf Spot Resistance Gene in Mungbean

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## Abstract

Cercospora leaf spot (CLS) is one of the serious diseases of mungbean in Thailand. The purpose of this study was to evaluate the inheritance and the location of a CLS resistance gene in the $F_{2:9}$ and $F_{2:10}$ recombinant inbred line (RIL) populations derived from the cross between CN72 a susceptible cultivar and V4718 a resistant line. A total of 143 RILs were planted for CLS disease response phenotyping under field conditions at Suranaree University of Technology, Nakhon Ratchasima, Thailand in the rainy seasons of 2016 and 2018. From segregation analysis, it was revealed that resistance to CLS is controlled by single dominant gene. Simple linear regression confirmed that 1 ISSR-anchored resistance gene analog (ISSR-RGA), 2 inter-simple sequence repeat (ISSR) and 3 simple sequence repeat (SSR) markers were significantly linked to CLS resistance. When quantitative trait loci (QTL) was analyzed using these markers, multiple interval mapping detected the position of the CLS resistance (*qCLSC72V18-1*) between the marker VR393 and I16274, explaining the phenotypic variability of up to 79.8%. The estimated genetic distances of VR393 and I16274 markers from the CLS resistance gene were 4 and 3 cM, respectively. These results indicate that mapping CLS resistance in mungbean can be accomplished by using ISSR, ISSR-RGA and SSR markers. In the future, the markers that are closely associated with CLS resistance gene will be beneficial for pyramiding of multiple resistance genes in mungbean. © 2020 Friends Science Publishers

**Keywords:** Mungbean; Cercospora leaf spot; ISSR-anchored resistance gene analog; Inter-simple sequence repeat; Simple sequence repeat; Recombinant inbred line

## Introduction

Mungbean (*Vigna radiata* (L.) Wilczek) is a self-pollinated crop belonging to the *Vigna* genus and has a diploid chromosome number of 2n = 2x = 22 (Kang *et al*. 2014). It is a tropical/sub-tropical crop, requiring an optimal temperature of 30–35°C. Mungbean is also known as mash bean, green gram, golden gram, green bean and green soy (Singh *et al*. 2014; Kumar and Bhat 2017). It is a main legume in South and Southeast Asia of which more than 90% of production is found in India (Chankaew *et al*. 2011). Thailand is the main exporter in international trade, mainly exporting to Japan, Taiwan, Philippines, Malaysia and Singapore at about half of its annual production of 250,000 t (Siemonsma and Lampang 2016). It provides a good source of proteins, carbohydrates and it is rich in minerals and vitamins (Kim *et al*. 2015; Hasan *et al*. 2017). Moreover, mungbean plants can be used as green manure and hay due to Their high atmospheric nitrogen fixation (Kajonphol *et al*. 2012).

Mungbean suffers from infection from several diseases including Cercospora leaf spot (CLS). The fungal pathogen *Cercospora canesens* causes CLS which occurs in rainy season (Chankaew *et al*. 2011). It was firstly reported in Delhi, India and surrounding areas (Shahzady *et al*. 2017). It attacks the crop at all stages of growth, usually at about 30–40 days after planting. Depending upon the humidity and temperature, it can spread rapidly especially in susceptible varieties (Hasan *et al*. 2017). Symptoms of CLS appear on leaves as water-soaked spots with greyish borders. Later enlarged dead area of the infected leaves can be seen. It affects petioles, stems and pods at the flowering (Shahzady *et al*. 2017), which can potentially decrease yield up to 60%. Chemicals can be used to control CLS to increase yield, however, they have a negative effect on the environment, human health, and increase the production cost. Therefore, the selection of resistant varieties is the best environmentally safe alternative in managing the disease.

Because pathogens causing CLS in legumes are genetically highly diverse, it was found that CLS obtained from different geographical locations, had different pigment production and morphological characters (Joshi *et al*. 2006). Therefore, inheritance is an important data in breeding for

CLS resistance. CLS resistance sources in mungbean can be found in many resistant genotypes in Taiwan, Pakistan, India, Bangladesh and Thailand (Iqbal *et al*. 2004; Marappa 2008; Yadav *et al*. 2014; Pandey *et al*. 2018). The inheritance of resistance to CLS in legumes has been reported in different resistance sources of several crops. In cowpea (*V. unguiculata* f. spp. *unguiculata*), a single major recessive gene controlled resistance to CLS in breeding line 'IT90K-59-120' (Duangsong *et al*. 2018). In peanut (*Arachis hypogaea* L.), it was found that tolerance to infection and resistance to CLS were primarily due to additive genetic effects (Kornegay *et al*. 1980). Previous report of Chankaew *et al*. (2011) indicated that a single dominant gene controlled resistance to CLS in mungbean. These sources of resistance to CLS are beneficial for plant breeders in breeding resistant cultivars; however, selection of resistant lines is still limited due to the seasonal occurrence of the disease (CLS mainly occurs in high humidity in the rainy season). Therefore, marker-assisted selection (MAS) is an effective method, allowing selection at the early stage of growth throughout the year.

Molecular markers have been used to tag genes in several crops and they are useful for revealing differences between individuals of different or the same species (Vidak *et al*. 2017). Most of the reports about quantitative trait loci (QTL) mapping of resistance to CLS in legumes have been conducted using single nucleotide polymorphisms (SNPs) and simple sequence repeat (SSR) markers (Duangsong *et al*. 2016; Bassi *et al*. 2017; Liang *et al*. 2017). The objectives of this work were to report the inheritance and locate the position of CLS resistance gene in 'CN72 × V4718' cross of mungbean. Using inter-simple sequence repeat (ISSR) and ISSR-anchored resistance gene analog (ISSR-RGA) markers, is highly effective for mapping resistance to powdery mildew in mungbean (Poolsawat *et al*. 2017), in the QTL mapping for mungbean resistance to CLS. We evaluated the SSR markers that have been reported to be linked to CLS resistance in legumes such as azuki bean [*V. angularis* (Willd.) Ohwi and Ohashi], mungbean and cowpea (Wang *et al*. 2004; Seehalak *et al*. 2009; Somta *et al*. 2009; Tangphatsornruang *et al*. 2009; Kongjaimun *et al*. 2012).

## Materials and Methods

### Plant materials

A 143-line $F_{2:9}$ and $F_{2:10}$ recombinant inbred line (RIL) populations obtained from the 'CN72 × V4718' cross by single-seed descent method. The female parent (Chai Nat 72; CN72) is a high yielding cultivated mungbean variety in Thailand that is susceptible to CLS while the male parent (V4718) is a line from the Asian Vegetable Research and Development Center (AVRDC) showing resistance to CLS at all stages of growth.

### Evaluation of Cercospora leaf spot resistance

Parents and the population of $F_{2:9}$ and $F_{2:10}$ RILs (143 individual plants) were screened for CLS resistance in the rainy seasons of July 2016 and 2018, respectively, under field conditions at Suranaree University of Technology, Nakhon Ratchasima, in North East Thailand. The RILs were planted in a randomized complete block design (RCBD). Seeds were planted in a 2 m long row with 50 cm inter-row spacing and 20 cm intra-row spacing. The cultivar CN72 was used as a control line to be the source of CLS inoculum distribution. After planting at 65 days, we evaluated the CLS infection using the disease rating scale (1–5) according to Chankaew *et al*. (2011). The average scores of disease severity were divided into 2 categories (resistant score of 1–2.9 and susceptible score of 3–5). The distribution of CLS resistance in the RIL population of this cross was assessed with chi-square test ($\chi^2$). We transformed scores of CLS severity in both years from the field evaluation with $(X + 1)^{1/2}$ formula to analyze the broad sense heritability ($h^2_b$) as described by Khajudparn (2009). Correlation analysis using SPSS version 14.0 (Levesque and SPSS Inc. 2006) was performed to analyze the relationship of CLS resistance between both years.

### ISSR and ISSR-RGA analysis

The genomic DNA of the parents and $F_{2:9}$ and $F_{2:10}$ RILs of this cross was extracted by using a modified CTAB extraction protocol of Lodhi *et al*. (1994) from young leaves. We used ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) to check the purity and concentration of DNA at $A_{260}$ and $A_{280}$, and diluted to be 150 ng/$\mu$L of DNA concentration for polymerase chain reaction (PCR). A set of 68 ISSR primers obtained from the University of British Columbia and 48 ISSR-RGA primer combinations that were derived from 12 ISSR primers (ISSR 807, 808, 809, 810, 827, 835, 836, 841, 856, 857, 888 and 891) and 4 RGA primers containing conserved domains including the kinase domain (Pto kin 1 and RLK for) and NBS domain (GLPLAL 1 and P-Loop) (Feuillet *et al*. 1997; Chen *et al*. 1998; Mahanil 2007) were used for the initial selection in bulk segregant analysis (BSA) (Table 1). BSA (using CN72 (S), V4718 (R), resistant bulk (RB) and susceptible bulk (SB)), and ISSR and ISSR-RGA analysis were performed as described by Poolsawat *et al*. (2017).

### SSR analysis

The genomic DNA of CN72, V4718, and their RILs was amplified by 47 SSR primer pairs from many species of legumes. Among them, 40 were from azuki bean (Wang *et*

**Table 1:** Sequences of 68 ISSR and 4 RGA primers

| Primers | Sequences (5' - 3')[a] | Primers | Sequences (5' - 3') |
|---|---|---|---|
| ISSR 807 | $(AG)_8T$ | ISSR 850 | $(GT)_8YC$ |
| ISSR 808 | $(AG)_8C$ | ISSR 851 | $(GT)_8YG$ |
| ISSR 809 | $(AG)_8G$ | ISSR 853 | $(TC)_8RT$ |
| ISSR 810 | $(GA)_8T$ | ISSR 854 | $(TC)_8RG$ |
| ISSR 811 | $(GA)_8C$ | ISSR 855 | $(AC)_8YT$ |
| ISSR 812 | $(GA)_8A$ | ISSR 856 | $(AC)_8YA$ |
| ISSR 813 | $(CT)_8T$ | ISSR 857 | $(AC)_8YG$ |
| ISSR 814 | $(CT)_8A$ | ISSR 858 | $(TG)_8RT$ |
| ISSR 815 | $(CT)_8G$ | ISSR 859 | $(TG)_8RC$ |
| ISSR 816 | $(CA)_8T$ | ISSR 860 | $(TG)_8RA$ |
| ISSR 817 | $(CA)_8A$ | ISSR 861 | $(ACC)_6$ |
| ISSR 818 | $(CA)_8G$ | ISSR 862 | $(AGC)_6$ |
| ISSR 819 | $(GT)_8A$ | ISSR 864 | $(ATG)_6$ |
| ISSR 820 | $(GT)_8C$ | ISSR 865 | $(CCG)_6$ |
| ISSR 821 | $(GT)_8T$ | ISSR 866 | $(CTC)_6$ |
| ISSR 823 | $(TC)_8C$ | ISSR 867 | $(GGC)_6$ |
| ISSR 824 | $(TC)_8G$ | ISSR 868 | $(GAA)_6$ |
| ISSR 825 | $(AC)_8T$ | ISSR 869 | $(GTT)_6$ |
| ISSR 826 | $(AC)_8C$ | ISSR 873 | $(GACA)_4$ |
| ISSR 827 | $(AC)_8G$ | ISSR 876 | $(GATA)_2(GACA)_2$ |
| ISSR 828 | $(TG)_8A$ | ISSR 878 | $(GGAT)_4$ |
| ISSR 829 | $(TG)_8C$ | ISSR 880 | $(GGAGA)_3$ |
| ISSR 830 | $(TG)_8G$ | ISSR 881 | $(GGGTG)_3$ |
| ISSR 834 | $(AG)_8YT$ | ISSR 884 | HBH $(AG)_7$ |
| ISSR 835 | $(AG)_8YC$ | ISSR 885 | BHB $(GA)_7$ |
| ISSR 836 | $(AG)_8YA$ | ISSR 886 | VDV $(CT)_7$ |
| ISSR 841 | $(GA)_8YC$ | ISSR 887 | DVD $(TC)_7$ |
| ISSR 841c | $(GA)_8CC$ | ISSR 888 | BDB $(CA)_7$ |
| ISSR 841t | $(GA)_8TC$ | ISSR 889 | DBD $(AC)_7$ |
| ISSR 842 | $(GA)_8YG$ | ISSR 890 | VHV $(GT)_7$ |
| ISSR 843 | $(CT)_8RA$ | ISSR 891 | HVH $(TG)_7$ |
| ISSR 844 | $(CT)_8RC$ | ISSR 900 | $ACTTCCC(CA)_2GGTTA(CA)_2$ |
| ISSR 846 | $(CA)_8AT$ | GLPLAL 1 | IAGIGCIAGIGGIAGICC |
| ISSR 847 | $(CA)_8RC$ | Pto kin 1 | GCATTGGAACAAGGTGAA |
| ISSR 848 | $(CA)_8RG$ | P-Loop | $(GGI)_2GTIGGIAAIACIAC$ |
| ISSR 849 | $(GT)_8YA$ | RLK for | GAYGTNAARCCIGARAA |

[a] B = C, G, T; D = A, G, T; H = A, C, T; I = inosine; N = A, G, C, T; R = A, G; V = A, C, G; Y = C, T

*al*. 2004), 6 were from mungbean (Seehalak *et al*. 2009; Somta *et al*. 2009; Tangphatsornruang *et al*. 2009) and 1 was from cowpea (Kongjaimun *et al*. 2012) (Table 2). PCR was conducted in a total volume of 20 $\mu$L containing 1× buffer [20 m*M* Tris-HCl, pH 8.4 and 50 m*M* KCl], 0.2 m*M* dNTPs, 0.5 $\mu M$ each of reverse and forward primers, 2 m*M* $MgCl_2$, 1 unit *Taq* DNA polymerase and 2 ng of DNA template. PCR amplification was performed in an Amplitronyx$^{TM}$ 6 Thermal Cycler (Nyx Technik, Inc., San Diego, CA, USA) as follows: 94°C for 2 min, 35 cycles of 94°C for 30 s, 50–65°C for 30 s and 72°C for 1 min, with a final step at 72°C for 10 min.

Denaturing polyacrylamide gel (6%) was used to separate the PCR products at 200 V and 70 min for ISSR and ISSR-RGA analysis and at 200 V and 50 min for SSR analysis. Silver nitrate method as described by Sambrook and Russell (2001) was utilized to stain the gel. The DNA bands were compared by using the molecular weights of 100 bp DNA Ladder (Invitrogen, CA, USA). Putative ISSR, ISSR-RGA and SSR loci linked to the CLS resistance gene were specified by comparing the patterns of DNA of parents (R and S) and DNA bulk (RB and SB). These putative markers were further analyzed in individual RILs to verify association with CLS resistance.

### Linkage and QTL Analysis

The linkage relationship of CLS resistance gene with markers and QTL analysis were applied as described by Poolsawat *et al*. (2017).

## Results

### Evaluation of Cercospora Leaf Spot Resistance

A population of 143 $F_{2:9}$ and $F_{2:10}$ RILs and their parents were screened for CLS resistance at 65 days after planting under field conditions in the rainy season of 2016 and 2018 (Fig. 1). In the experiment conducted in 2016, CN72 was highly susceptible to CLS with a mean score of 4.00, whereas V4718 was resistant to CLS with a mean score of 1.67. The disease severity scores of the two parents were significantly different ($P < 0.05$). In 2016, the 143-line $F_{2:9}$ RIL population was segregated into 80 resistant progenies and 63 susceptible progenies. The result from a chi-square test showed that the segregation fitted with 1:1 resistant (R) : susceptible (S) ratio ($\chi^2 = 2.02$, $P_{0.05} = 3.84$). In 2018, the level of CLS resistance was similar to that of 2016 with a mean score of 1.00 for V4718 and 4.00 for CN72. In the $F_{2:10}$ RIL population, 60 were resistant and 83 were susceptible with a segregation ratio of 1:1 (R : S) ($\chi^2 = 3.70$, $P_{0.05} = 3.84$) in 2018 (Table 3). These results indicate that in this cross the resistance to CLS was obtained from V4718 as a single dominant gene. The CLS scores of both years were significantly correlated with the correlation coefficient (r) value of 0.48 ($P < 0.001$), and the estimates of $h^2b$ for resistance to CLS were 89.74% and 82.84% in 2016 and 2018, respectively.

### Linkage and QTL Analysis

BSA was used to identify ISSR, ISSR-RGA and SSR markers potentially associated with CLS resistance gene using 68 ISSR primers, 48 ISSR-RGA primer combinations and 47 SSR primer pairs to amplify DNA of RB and SB of this cross. All 68 ISSR primers used can clearly amplify DNA of parents and DNA bulks using the annealing temperature of 50ºC. There were 1,344 total DNA bands amplified from the 68 ISSR primers, ranging from 11 bands per primer (ISSR 815, 819 and 843) to 38 bands per primer (ISSR 841) with a mean of 19.8 bands per primer. Fourteen markers amplified from 9 ISSR primers (ISSR 814, 816, 818, 827, 830, 835, 841, 884 and 888) were possibly linked to CLS resistance. When twenty RILs constituting RB and SB were individually analyzed to determine the correlation between ISSR markers and CLS resistance using simple linear regression, 7 markers (I14749, I16274, I18363,

**Table 2:** Sequences and annealing temperature of 47 SSR primers

| Primers | Forward sequences (5' - 3') | Reverse sequences (5' - 3') | Annealing temperature (°C) |
|---|---|---|---|
| CEDAAG002 | GCAGCAACGCACAGTTTCATGG | GCAAAACTTTTCACCGGTACGACC | 65 |
| CEDAAG004 | GGAGGAGAAGTCTCGGACC | GAGCGTTTTGCACAGTGTTCAC | 60 |
| CEDC008 | GGAATTAGAGATGATTGGAC | CACCACTTCATTATGTATGG | 55 |
| CEDC031 | GGGAATAAATAAACCTTTCC | TCTCAAATCACATTGCCAC | 50 |
| CEDC050 | TCCCACTTCTCCATTACCTCCAC | GAGATTATCTTCTGGGCAGCAAGG | 60 |
| CEDC055 | CAAACACTTTTGTAACTCCC | GCTTCTAACCTTGATCCTTC | 55 |
| CEDG002 | AACTGGACCTGTACCACTGG | TACAGCCTTCTTGCACCATG | 60 |
| CEDG006 | AATTGCTCTCGAACCAGCTC | GGTGTACAAGTGTGTGCAAG | 60 |
| CEDG008 | AGGCGAGGTTTCGTTTCAAG | GCCCATATTTTTACGCCCAC | 60 |
| CEDG010 | TGGGCTACCAACTTTTCCTC | TGAGCGACATCTTCAACACG | 60 |
| CEDG014 | GCTTGCATCACCCATGATTC | AAGTGATACGGTCTGGTTCC | 60 |
| CEDG021 | GCAGAATTTTAGCCACCGAG | AAAGGATGCGAGAGTGTAGC | 60 |
| CEDG024 | CATCTTCCTCACCTGCATTC | TTTGGTGAAGATGACAGCCC | 60 |
| CEDG037 | GAAGAAGAACCCTACCACAG | CACCAAAAACGTTCCCTCAG | 50 |
| CEDG040 | CGGGGTATAACTTTAGCAGC | TAACTCAGGCAAAGGTAGCC | 60 |
| CEDG043 | AGGATTGTGGTTGGTGCATG | ACTATTTCCAACCTGCTGGG | 60 |
| CEDG044 | TCAGCAACCTTGCATTGCAG | TTTCCCGTCACTCTTCTAGG | 60 |
| CEDG051 | AAACATACCCCTGGCAGTTCC | TTCTGACCTAAGAAAGAGCCTGG | 60 |
| CEDG056 | TTCCATCTATAGGGGAAGGGAG | GCTATGATGGAAGAGGGCATGG | 60 |
| CEDG059 | AGAAAAGGGTGGCCTCGTTG | GCAGGCATTTCCATCGCAG | 60 |
| CEDG063 | TTGGAAACAATTATTGGAGGTGC | GGTGCTCTTGGACGGCTGG | 55 |
| CEDG070 | CCGATCAAACTCTCCATGCTCG | TAATTTCATTGCTTTTCCCTCC | 55 |
| CEDG084 | ATCAACTGAGGAGCATCATCGA | CAACATTTCAACCTTGGGACAG | 50 |
| CEDG117 | GTACACTTCCACTAATCCAAAATT | TGGTACCTTCCTTATCTGAAATTA | 42 |
| CEDG121 | CTTTCAAAATAATGTTGAGGCATA | CAATACATAAATAACCTTTTCTGC | 50 |
| CEDG150 | GAAGGGAATGAAAATGAAACCC | GTTCAATCCATTCAGTCTCC | 50 |
| CEDG154 | GTCCTTGTTTTCCTCTCCATGG | CATCAGCTGTTCAACACCCTGTG | 55 |
| CEDG169 | CAATGCGCGTCTGCAAGTTG | CAAGGAACTTGTGGGTCC | 55 |
| CEDG176 | GGTAACACGGGTTCAGATGCC | CAAGGTGGAGGACAAGATCGG | 50 |
| CEDG186 | GGATGGGAGAGTAAGAAG | GCATGGCATGATGACTTG | 60 |
| CEDG205 | GTGGTGGTGACAGTAGCAGTAG | CAGCCACCACAAGACAACCTC | 55 |
| CEDG232 | GATGACCAAGGTAACGTG | GGACAGATCCAAAACGTG | 50 |
| CEDG259 | GATCATCGGACAGAGCTTCC | CACTCTCTGCGAACTCAATCG | 55 |
| CEDG287 | CCTTATACTAAAGATGTTGGTGG | GTGATACGCATATAGGTTCAC | 50 |
| CEDG290 | GACACTCTTTGTTTGTAGG | CAGTGATCACTCTGGTTG | 60 |
| CEDG294 | CACCTTCTTAATCTCTTCACC | GGGTTTCTCTTAATTCATTGAGTC | 55 |
| CEDG295 | CAAAGGTTAGATCCAACATCG | GGTTAGTCATCAACAACTCC | 60 |
| CEDG294 | CACCTTCTTAATCTCTTCACC | GGGTTTCTCTTAATTCATTGAGTC | 55 |
| CEDG295 | CAAAGGTTAGATCCAACATCG | GGTTAGTCATCAACAACTCC | 60 |
| CEDG304 | ACCACTTCATAATCCCTGAG | GTTGCATGCTATATTTTGGTTCAC | 50 |
| Primers | Forward sequences (5' - 3') | Reverse sequences (5' - 3') | Annealing temperature (°C) |
| CEDG305 | GCAGCTTCACATGCATAGTAC | GAACTTAACTTGGGTTGTCTGC | 55 |
| CEDGAT008 | GGATGTGAAAGACTTAACTTC | GAGGAATCTAAGTAAAACGAG | 55 |
| cp03802 | ATGTTATGAATGCACCTGCACGAT | CTGCAAGAAGCTAGTGTTGCTCCA | 55 |
| DMB-SSR 167 | TGGGACTCAAACCACACTTTC | GAACTATGAAGGTTTCACAGAAATCA | 55 |
| DMB-SSR 199 | AGAAATTAAATCCCCGTCTGCT | AGAGACAGAAGCTCTGGATGTTTT | 55 |
| DMB-SSR 59 | TGCCAGATTTGAGAAGAAAGGT | CATGCATGTGGATAAGAATTCAG | 55 |
| VR10 | GAAAGGCTATGACCAAATCCAA | CGGGAAGAGAACATAAGGGAAT | 55 |
| VR108 | GCTCCAACACTCACTCACAAAC | CAGAAATGCAGGAAAAGAGAGG | 55 |
| VR393 | TGGCACTTTCCATAACGAATAC | ATCAGCCAAAAGCTCAGAAAAC | 50 |

I41203, I88656, I88302 and I88305) were found to be significantly associated with CLS resistance ($P < 0.01$). Using a similar approach with 48 primer combinations between 12 ISSR primers and 4 RGA primers, it was found that 4 ISSR-RGA markers were significantly linked to CLS resistance (V4718) consisting of I27PL177, I35P716, I56P166 and I56P169. In addition to the ISSR and ISSR-RGA markers, 47 SSR markers that have been reported to be linked to CLS resistance were also used for the analysis. The annealing temperatures of SSR primers were 42–65°C (Table 2). Five SSR markers (CEDG008, CEDG051, CEDG084, VR108 and VR393) were found to be significantly associated with CLS resistance.

For CLS mapping, 7 ISSR, 4 ISSR-RGA and 5 SSR markers that were putatively linked to CLS resistance were further analyzed with $F_{2:9}$ and $F_{2:10}$ RIL populations to confirm the linkage with CLS resistance using linear regression, LOD analysis and recombination. The quality of the genome data was checked for the missing data of each marker, which were not more than 10%. Six markers were distributed at the ratio of 1:1 when testing with chi-square as expected, indicating that they are useful for CLS mapping. It was found that the CLS resistance gene was significantly co-segregated with all 6 markers ($P < 0.001$) with $R^2$ of 0.220

**Table 3:** Segregation in reaction to Cercospora leaf spot in $F_{2:9}$ and $F_{2:10}$ RIL populations derived from 'CN72 × V4718' cross

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

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

**Table 4:** Simple linear regression, recombination and LOD scores of SSR, ISSR and ISSR-RGA markers and resistance to Cercospora leaf spot of mungbean in 'CN72 × V4718' cross

| Year | Markers | Beta | *t* value | *P* value | $R^{2a}$ | LOD[b] |
|---|---|---|---|---|---|---|
| | VR393 | 0.766 | 10.036 | 0.000 | 0.587 | 12.969 |
| | CEDG084 | 0.678 | 7.835 | 0.000 | 0.460 | 9.549 |
| 2016 | CEDG008 | 0.469 | 4.630 | 0.000 | 0.220 | 5.720 |
| | I16274 | -0.787 | -11.038 | 0.000 | 0.619 | 15.142 |
| | I88656 | -0.545 | -5.624 | 0.000 | 0.297 | 7.324 |
| | I35P716 | -0.603 | -6.465 | 0.000 | 0.364 | 6.899 |
| | VR393 | 0.623 | 6.705 | 0.000 | 0.388 | 7.808 |
| | CEDG084 | 0.568 | 5.856 | 0.000 | 0.323 | 4.956 |
| 2018 | CEDG008 | 0.494 | 4.956 | 0.000 | 0.244 | 3.748 |
| | I16274 | -0.645 | -7.310 | 0.000 | 0.416 | 9.465 |
| | I88656 | -0.455 | -4.428 | 0.000 | 0.207 | 3.585 |
| | I35P716 | -0.520 | -5.201 | 0.000 | 0.270 | 5.694 |

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

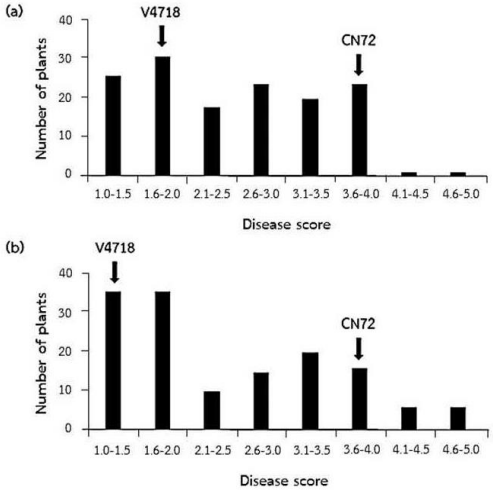


**Fig. 1:** Frequency distribution of disease severity scores for response to Cercospora leaf spot of mungbean in the $F_{2:9}$ and $F_{2:10}$ RIL populations of 'CN72 × V4718' cross, evaluated in 2016 (**a**) and 2018 (**b**), respectively

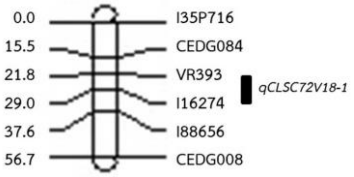


**Fig. 2:** Genetic linkage map of $F_{2:9}$ RIL population derived from 'CN72 × V4718' cross showing the position of a major QTL (*qCLSC72V18-1*) conferring Cercospora leaf spot resistance

(CEDG008) to 0.619 (I16274) in 2016 and $R^2$ of 0.207 (I88656) to 0.416 (I16274) in 2018 with LOD scores of > 3 in both years (Table 4). Two out of the 6 markers, VR393 and I16274 markers were nearest to the location of CLS resistance gene at 4 and 3 cM of distance, respectively. There would only be 0.24% recombination with the CLS resistance gene if both markers were used for the selection. For QTL analysis, multiple interval mapping detected a major QTL (*qCLSC72V18-1*) for resistance to CLS in the $F_{2:9}$ and $F_{2:10}$ RIL populations of this cross, using the WinQTLcart 2.5 program (Wang *et al*. 2007) with the permutation value of 2,000 times at $P = 0.01$. It was found that the position of the major QTL was placed between the marker VR393 and I16274, with the LOD and phenotypic variance explained (PVE) in 2016 and 2018 as being equal to 5.74, 79.8% and 3.76, 67.5%, respectively (Table 5 and Fig. 2). These markers which are located closely to the position of CLS resistance gene will be utilized for developing mungbean varieties resistant to CLS in the future.

## Discussion

From the evaluation of CLS resistance in the $F_{2:9}$ and $F_{2:10}$ RIL populations and their parents (CN72 and V4718) after planting at 65 days in 2016 and 2018, respectively, we found that V4718 was an effective source for CLS resistance in the breeding program because it showed durable CLS resistance in both years. In addition to inheritance of CLS resistance, we confirmed that a single dominant gene is inherited from CLS resistant line (V4718). Furthermore, the estimate of $h^2b$ for CLS resistance in 2016 was 89.74% and in 2018 was 82.84%. Similar results were reported by Chankaew *et al*. (2011), who studied QTL mapping of CLS in the 'KPS1 × V4718' cross. Therefore, several conventional breeding methods can be used for the selection of CLS resistance.

The success of pyramiding different resistant loci into a single variety in mungbean, depends on the development of allele-specific markers. The use of a $F_{2:9}$ RIL population together with BSA to identify a CLS resistance gene in this study identified 6 markers (2 ISSR markers, 1 ISSR-RGA marker and 3 SSR markers) that were significantly linked to CLS resistance ($P < 0.001$) (Table 4). The present results indicate that the BSA method can provide a rapid selection of markers associated with genes of interest. In a previous study, Poolsawat *et al*. (2017) detected ISSR and ISSR-RGA markers linked to resistance to powdery mildew in the 'CN72 × V4718' cross. They found one ISSR marker

**Table 5:** Quantitative trait loci (QTL) conditioning Cercospora leaf spot resistance in $F_{2:9}$ and $F_{2:10}$ RIL populations derived from 'CN72 × V4718' cross, as detected by multiple interval mapping

| Populations | Year | QTL name | Marker interval | Position[a] (cM) | LOD[b] | PVE[c] (%) | Additive effect |
|---|---|---|---|---|---|---|---|
| $F_{2:9}$ | 2016 | *qCLSC72V18-1* | VR393-I16274 | 25.78 | 5.74 | 79.8 | -0.95 |
| $F_{2:10}$ | 2018 | *qCLSC72V18-1* | VR393-I16274 | 25.78 | 3.76 | 67.5 | -1.00 |

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

(I85420) and one ISSR-RGA marker (I42PL229) flanking the major QTL, *qPMC72V18-1* in this cross. With regard to the SSR marker, Chankaew *et al*. (2011) reported that linkage group 3 was the location of a major QTL for CLS resistance, *qCLS* in mungbean. Duangsong *et al*. (2016) used the SSR markers for identification of the linkage group of CLS resistance gene in cowpea. They showed that the CEDG304 marker which was linked to CLS resistance was located on linkage group 9 of yardlong bean. Similarly, Zongo *et al.* (2017) reported that GM1911, GM1883, GM1000 and Seq13E09 markers were associated with early leaf spot resistance in groundnut. These results confirm that all 3 marker types (ISSR, ISSR-RGA and SSR) are beneficial for the identification of CLS resistance in early generations of mungbean.

The QTL analysis in our study showed a major QTL, *qCLSC72V18-1*, associated with CLS resistance with phenotypic variation up to 79.8% (Table 5). The results confirm that a major QTL controls the resistance to CLS. This is consistent with the results of Chankaew *et al.* (2011), who reported that QTL (*qCLS*) could explain up to 80.53% of the contribution to CLS resistance in the 'KPS1 × V4718' cross. The two SSR markers (VR393 and CEDG084) out of 6 markers in Table 4 were similar to those reported by Chankaew *et al.* (2011) to be associated with CLS resistance and located in the mungbean genetic map on linkage group 3. In this study, we also found that the VR393 marker was close to the major QTL (*qCLSC72V18-1*) in this cross at the distance of 4 cM. However, we also found a newly developed ISSR marker, I16274, which was closer to the CLS resistance gene (3 cM from QTL). Only 0.24% of the recombination with the CLS resistance gene of both markers will occur if both markers are used for selection, suggesting that they are highly efficient for use in marker assisted selection and pyramiding of CLS and other resistance genes in mungbean.

## Conclusion

We found that resistance to CLS in V4718 is controlled by a single dominant gene. VR393 and I16274 markers are closest to a major QTL for resistance to CLS in the $F_{2:9}$ RIL population of the 'CN72 × V4718' cross at the distance of 4 and 3 cM, respectively. The markers that were closely associated with the CLS resistance gene will be beneficial for pyramiding of the CLS resistance gene and other resistance genes in cultivated varieties of mungbean.

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