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Construction of an integrated map and location of a bruchid resistance gene in mung bean

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ABSTRACT

Bruchid beetle (*Callosobruchus chinensis*) poses a serious threat to the production and storage of mung bean (*Vigna radiata*). Mapping bruchid resistance (*Br*) will provide an important basis for cloning the responsible gene(s) and elucidating its functional mechanism, and will also facilitate marker-assisted selection in mung bean breeding. Here, we report the construction of the genetic linkage groups of mung bean and mapping of the *Br1* locus using an RIL population derived from a cross between Berken, a bruchid-susceptible line, and ACC41, a bruchid-resistant line. A total of 560 markers were mapped onto 11 linkage groups, with 38.0% of the markers showing distorted segregation. The lengths of the linkage groups ranged from 45.2 to 117.0 cM with a total coverage of 732.9 cM and an average interval of 1.3 cM between loci. *Br1* was located on LG9 between BM202 (0.7 cM) and Vr2-627 (1.7 cM). Based on 270 shared SSR markers, most of the linkage groups were assigned to specific chromosomes. These results should further accelerate the genetic study of this crop.

1. Introduction

Mung bean (*Vigna radiata*) is widely grown throughout Asia, where it is a major source of protein [1]. Mung bean has been a traditional food in China for thousands of years and is the main agricultural crop in dry and semi-dry regions of the northwest of the country. Mung bean is frequently used as an intercrop and in crop rotation, because of its short growth period and nitrogen fixation. One of the most serious pests affecting the production and marketing of mung bean is the bruchid beetle (*Callosobruchus chinensis*). It attacks mung bean both in the field and in storage, resulting in heavy or even complete loss [2,3]. Searching for sources of resistance to bruchid beetles and breeding resistant varieties is the best and most effective way to protect mung bean production [1].

To date, several mung bean genotypes have been identified as resistant to bruchid beetle [4–7]. It has been reported that bruchid resistance (*Br*) is controlled by a major locus in two highly resistant wild genotypes, TC1966 and ACC41 [5]. RFLP markers have been used to map the resistance gene in both of these genotypes [8–11], but this marker system is not practical for marker-assisted selection (MAS) because of its complicated protocol. By contrast, SSR markers can be conveniently used in MAS. However, owing mainly to the limited number available in mung bean, SSR markers have not been widely used in mapping loci conferring bruchid resistance.

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Following the development and validation of a large number of SSR markers in mung bean [12], we constructed linkage maps and located the *Br1* locus using a RIL population derived from Berken and ACC41 [13]. Based on the analysis of sequences flanking mapped SSR markers [12], we also assigned linkage groups to specific chromosomes. We believe that these results will further accelerate genetic research on mung bean and related species.

2. Materials and methods

2.1. Plant materials and DNA preparation

A mapping population consisting of 201 F_{10} recombinant inbred lines (RILs) derived from a cross between Berken (cultivated type, 100% susceptible to bruchid) and ACC41 (wild type, 100% resistant to bruchid) was used in this study [14]. Fresh leaves from 5 to 6 individuals of each line and the parents were used for genomic DNA extraction using the CTAB method [15]. After assessment of quality and quantity, prepared genomic DNA was stored at −20 °C.

2.2. Measurement of bruchid resistance

Bruchid resistance was assayed in each of the RILs in a 2012 experiment, based on a method released by the National Scientific and Technological Project [16]. Owing to limited numbers of seeds, only 190 RILs were assayed again in 2013. Briefly, 30 healthy seeds, replicated three times for each line, together with Zhonglyu No. 5 (a susceptible cultivar) as a control, were placed separately into plastic dishes (diameter, 3.5 cm × 0.5 cm) without lids. All dishes were then placed in a large plastic box (diameter, 66 cm × 44 cm × 18 cm) with a cup of water to maintain humidity. Approximately 400–500 adult bruchid beetles were released into the box to ensure that each line had more than 20 adult insects to lay eggs on the seed surface. The box was covered with two layers of black cloth to maintain darkness, and placed in a room with an ambient temperature of 27 ± 2 °C. The water level was monitored regularly throughout the infestation period. Forty days later, the dishes were taken out and the damaged seeds of each line were examined and recorded. The percentage of seed damage was calculated by the formula: $SDR = \frac{\Sigma NSD}{N} \times 100\%$, where *SDR* denotes the damage percentage, *NSD* the number of damaged seeds, and *N* the total number of inoculated seeds. The percentage of damaged seeds for each RIL was used to classify each line as either resistant or susceptible as previously described [10,17].

2.3. Molecular marker analysis

Molecular markers used in this study were from several sources including SSR, EST-SSR, and STS derived from mung bean [12,18], adzuki bean [19], common bean (http://isa.ciat.cgiar.org/molphas/micros.jsp), and cowpea [20] as well as a set of RFLP markers [9,14]. PCR analysis was performed in 20-μL reactions containing 1× PCR buffer, 100 μmol L^{-1} of each dNTP, 0.4 μmol L^{-1} of each primer, 20 ng genomic DNA, and 1 U of *Taq* DNA polymerase. PCR amplification was performed using an EDC-810 thermal cycler (Dongsheng Co.) with 35 cycles of 94 °C for 30 s, 47 °C for 30 s, and 72 °C for 30 s followed by a final 5-min extension. The product was fractionated by 8% SDS-polyacrylamide gel electrophoresis (PAGE) with 0.5× TBE as buffer at 220 V. The running time was adjusted according to the expected size of products and usually ranged from 1.0 to 1.5 h. To visualize the fragments, gels were stained in 0.2% $AgNO_3$ and then developed in a solution of 1.5% NaOH plus 0.5% formaldehyde.

2.4. Linkage group construction and gene mapping

Based on profiles of all polymorphic markers in each of the RILs assessed, a linkage map was constructed using JoinMap 4.0 [21] with a minimum LOD score of 3 for grouping and a recombination frequency of 0.25 by the Kosambi mapping function [22]. Double crossovers between adjacent loci were confirmed visually. Chi-square tests were used to evaluate the segregation distortion of mapped markers. Based on the locations of SSR markers in the linkage groups and their physical positions in the mung bean genome [12,23], the linkage groups were assigned to specific chromosomes of mung bean.

3. Results

3.1. Bruchid resistance assays

Seeds of the control genotype were completely damaged in both of the two experiments conducted. Damage percentages of individual RILs ranged from 0 to 100%, with an average of 46.5% in one of the experiments and 47.7% in the other. The phenotypic values of bruchid resistance were highly correlated ($r = 0.98$) between the two experiments. Chi-square tests showed that the segregation of resistance and susceptibility in the RIL population in the 2012 and 2013 experiments fitted a 1:1 ratio.

3.2. Map construction and gene location

The 547 markers used in this study produced a total of 560 polymorphic loci. Among the RFLP markers, seven generated more than one polymorphic locus each. Among the markers used, 364 (or 66.9%) were newly developed SSRs from the mung bean genome. The 560 loci were mapped on 11 linkage groups covering a total of 732.9 cM (Fig. 1). The lengths of linkage groups ranged from 45.2 to 117.0 cM with an average of 66.6 cM. The average interval between two loci was 1.3 cM. The number of loci on each linkage group varied from 33 to 87 with an average of 50.9. Based on the resistance assay data, the bruchid resistance gene *Br1* was mapped between BM202 (a SSR marker from common bean) and Vr2-627 (a SSR marker from mung bean), covering a region of 2.4 cM on LG9 (Fig. 1).

3.3. Segregation of markers in the mapping population

There were 70 and 143 loci that showed segregation distortion at levels of $P < 0.05$ and $P < 0.01$, respectively, accounting for 12.5% and 25.5% of all markers. Among these markers showing distorted segregation, 93.9% favored alleles from the female parent Berken. The number of distorted markers

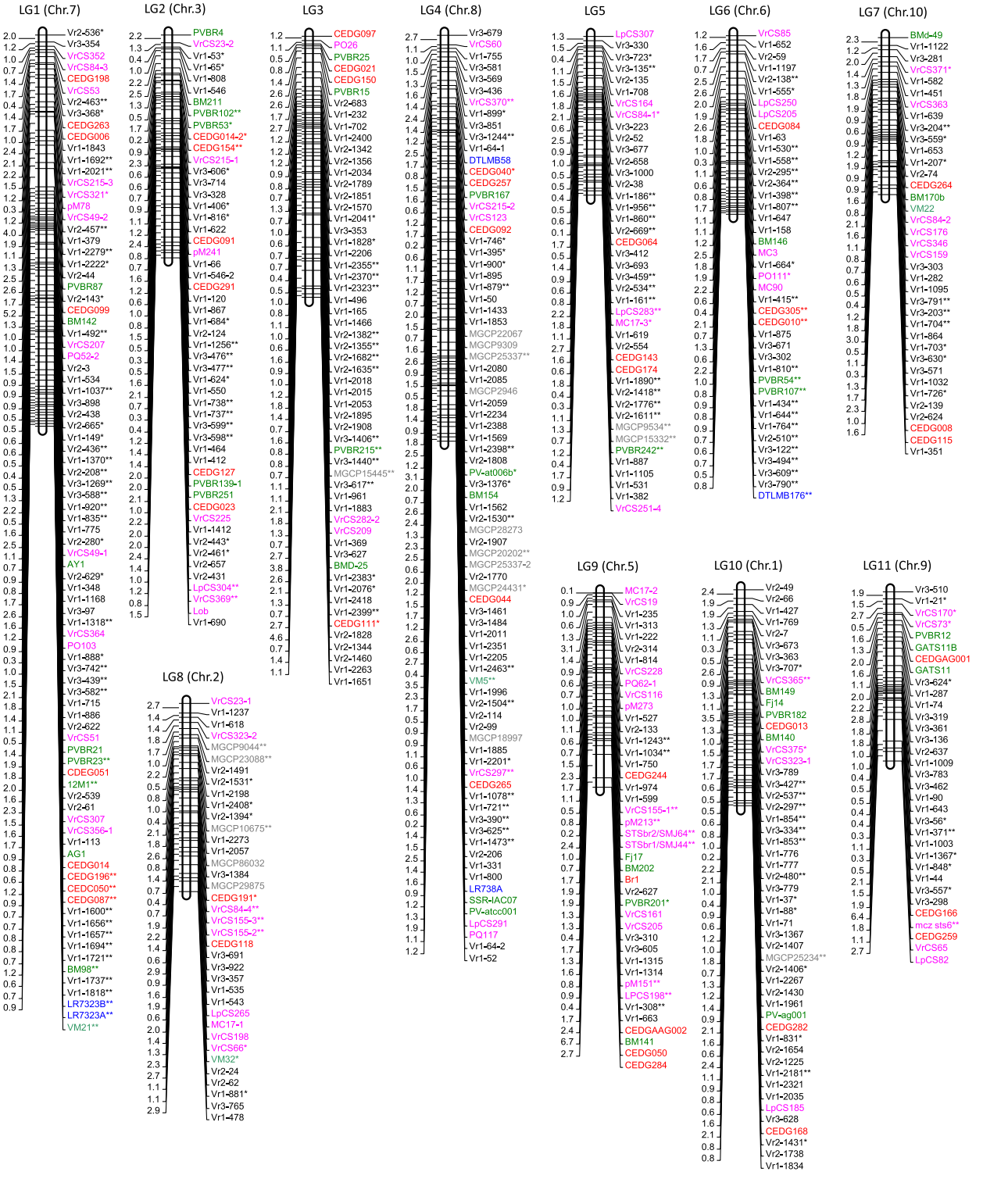

Fig. 1 – Genetic linkage map of mung bean based on DNA molecular markers from mung bean and related species. Map distances are shown on the left and marker names on the right of the linkage groups. Markers showing significant deviation from the expected segregation ratio at the 0.05 and 0.01 probability levels are indicated by * and **, respectively. For markers, black and gray text indicate SSRs and EST-SSRs derived from mung bean; green, SSRs derived from common bean; dark green, SSRs from cowpea; red, SSRs from adzuki bean; and blue, SSRs provided by Dr. Prakit Somta from Kasetsart University, Thailand. Markers in purple text indicate RFLP data provided by Dr. Chunji Liu from CSIRO Agriculture and Food, Australia. LG, linkage group; Chr., chromosome.

varied greatly between chromosomes (Fig. 2). Most of them clustered together, forming distinct segregation–distortion regions (SDRs). The longest SDR was on LG1, containing 14 markers (from CEDG196 to VM21) covering 10.6 cm. Another SDR was on LG6. It contained 12 markers (from Vr1-810 to DTLMB176) covering 9.9 cm. There were more than one SDR each on LG1, LG2, LG3, LG5, and LG6, whereas no SDR was found on either LG7 or LG11. The proportions of distorted markers on the linkage groups were, in descending order, LG6 (61.0%) > LG1 (50.6%) > LG5 (45.2%) > LG2 (42.3%) > LG8 (35.1%) > LG10 (33.3%) > LG3 (31.6%) > LG4 (30.9%) > LG11 (30.3%) > LG7 (27.0%) > LG9 (23.8%).

3.4. *The relationship between linkage groups and chromosomes*

Based on the distribution of 270 SSR markers, the relationship between chromosomes and linkage groups was analyzed. The majority of the linkage groups matched well to chromosomes, with the exception of LG3 and LG5. The 29 SSR markers on LG3 were distributed on 10 different chromosomes: seven on chromosome 11 (Chr.11), six on Chr.7, and five on Chr.4. There were no convincing data identifying the linkage group corresponding to Chr.4, whereas both LG5 and LG 9 corresponded to Chr.5 (Fig. 1).

4. Discussion

4.1. *Construction of the genetic map in mung bean*

A genetic map is a basic tool for locating and mining genes. In mung bean, marker densities of the existing genetic maps are still low and only a small number of genes have been mapped. An early map constructed with RFLP markers consisted of 14 linkage groups with an average intermarker distance of 9 cM [24]. Another two maps consisted of 12 linkage groups each [13], whereas another linkage map based on RFLP markers consisted of only nine linkage groups [25]. In none of these early studies of mung bean did the number of linkage groups coincide with the number of the haploid chromosome number of this species ($n = x = 11$). The most recent map, reported by Isemura et al. [27], consisted of 11 linkage groups. The linkage map constructed in the present study also consisted of 11 linkage groups, but with more markers and an average interval between loci of only 1.3 cM, in contrast to all earlier maps. Some RFLP markers [9,26] were incorporated into the map. SSR markers comprised the majority (66.9%) of the total markers used. Compared to RFLP and AFLP markers, the SSR markers are more applicable for MAS. The total length of the 11 genetic linkage groups obtained in this study was 732.9 cM, in close agreement with the length of 727.6 cM described in the most recent report [27]. These figures seem to suggest that the present map is almost saturated. However, there are still gaps in the linkage groups and intervals between some loci are still large. For example, the distance between BM141 and CEDG050 on LG9 is 6.7 cM and that between CEDG166 and mcz sts6 on LG11 is 6.4 cM. We are trying to reduce these gaps by developing more polymorphic markers. With the recent release of the full genome sequence of mung bean [23], the molecular genetics of this species will be greatly accelerated in the following years.

4.2. *Location of bruchid resistance gene*

Bruchid beetle resistance genes have been mapped in several species of legumes, including mung bean [9–11,28], common bean [29], rice bean [30], and wild relatives of adzuki bean [1,6].

In mung bean, work on mapping bruchid resistance has focused on two sources of resistance, TC1966 and ACC41. An early study showed that the resistance to bruchid was likely controlled by Vigna acid in the seed [31], but this hypothesis was rejected after the mapping of the *Br* gene, because an individual in the mapping F_2 population was found to produce Vigna acid but to remain susceptible to bruchid [11]. VrCRP, a cysteine-rich protein of the plant defensin family, was once considered also to confer resistance to bruchid [32]. However, this notion could not be confirmed to be directly responsible for bruchid resistance in TC1966. There were no subsequent reports about the *Br* gene until reports of mapping with SSR markers [33] and gene elucidation by genomic and transcriptomic sequencing [34]. Two STS markers, STSbr1/SMJ44 and STSbr2/SMJ64, were first identified as being linked with the *Br1* locus in the genotype ACC41 [9]. We have worked on mapping and breeding of the *Br1* locus for several years [8,35], and made some advances in genetic improvement. However, our work on gene mapping has progressed slowly, owing mainly to the lack of molecular markers. Based on

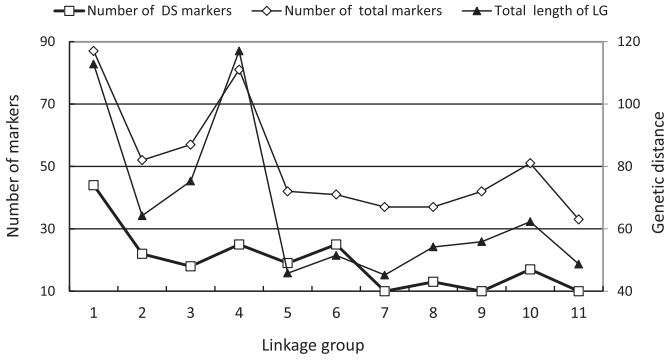

Fig. 2 – Numbers of distorted and total markers and genetic length of each linkage group. DS, distorted segregation; LG, linkage group.

previous studies and the recent development of SSR markers [8,12,35], we have managed to reduce the target region of *Br1* to 2.4 cM in the present study and identified two tightly linked markers at distances of 0.7 and 1.7 cM. In view of the genome size and the total length of genetic linkage groups in mung bean, the tightly linked markers could be effectively applied for MAS, fine mapping, and gene cloning.

4.3. Integration of linkage groups with chromosomes for mung bean

Although recent studies employing map construction have reduced the number of linkage groups to 11 and the full genome sequence has been released, there are no reported efforts to assign the various linkage groups to specific chromosomes in mung bean. In the present study, we accomplished this assignment by examining the flanking sequences of 270 SSR markers that we had mapped [12]. With the exception of LG3 and LG5, each linkage group had a best-corresponding chromosome, as labeled in Fig. 1. Both LG5 and LG9 were assigned to Chr.5, but BLAST analysis of flanking sequences of SSR markers that closely linked with *Br1* on LG9 showed that they matched Chr.5 sequences, suggesting that LG9 corresponds to Chr.5. However, the genome rearrangement and duplication that has been reported in mung bean [36] and other legume species [37] may affect the assignment of linkage groups to chromosomes. Additional markers or an improved genome assembly would be helpful for validating this assignment. Efforts are being made to clarify the relationship between the linkage groups and chromosomes in this species.

4.4. Segregation distortion

Segregation distortion is a phenomenon commonly observed in the distribution of genotypes or phenotypes within a population [38] and it is considered to be a power of genetic evolution by gametophytic selection or genome recombination [39,40]. Among legumes, segregation distortion has been reported in pea [41], soybean [42], cowpea [43], and common bean [44]. The proportion of markers showing segregation distortion usually varies between 20% and 30% in different species. The proportion observed in this study was 37.8%, much higher than that in other species. Among the distorted markers, 93.9% showed bias toward the female parent Berken, whereas only 13 showed bias toward ACC41. These results might be explained by the difference in the genetic background of the two parents, one of them a cultivated and the other a wild type.

Most of the markers with distorted segregation clustered together and formed segregation–distortion regions (SDRs) on various linkage groups [45]. A total of 17 SDRs (as a high proportion of distorted markers were observed, only a region containing more than four markers clustered together was considered an SDR) were found. LG1, LG5, and LG6 had two each, and the remainder were found on LG2, LG3, LG4, LG7, and LG10, respectively. On LG8, LG9, and LG11, the segregation-distorted markers were scattered rather than clustered together. Although segregation distortion may be caused by different factors [46–48] and does not affect the marker order on linkage groups [49], the distribution of these SDR on different regions of chromosomes would be of great interest for future study. In fact, distortion segregation of seed coat testa in mung bean has been observed [50], owing mainly to the complex genetic inheritance of this trait.

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