InDel Marker Development and QTL Analysis of Agronomic Traits in Mung Bean [Vigna Radiate (L.) Wilczek]

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Abstract

The stem color of young mung bean is a very useful tool in germplasm identification. Flowering time and plant height (PH) are known to be strongly correlated with crop adaption and yield. However, few studies have focused on elucidating the genetic mechanisms that regulate these five particular traits: young stem color (YSC), days to first flowering (DFF), days to maturity (DM), PH, and nodes on main stem (NMS). In this study, a genetic linkage map for the F_2 population was constructed using 129 InDel markers that were developed based on the sequence variations between parents. A total of 14 QTLs related to YSC, DFF, DM, PH, and NMS were detected. These QTLs were distributed on six chromosomes (1, 3, 4, 6, 7, and 10), which individually accounted for 1.32% to 90.07% of the total phenotypic variation. Using a short and high-density linkage map for the F_3 population, six of the seven QTLs which clustered at two intervals on chromosomes 3 and 10 were detected again. Further analysis found that four QTLs between InDel markers R3-15 and R3-19 controlled DFF, DM, PH, and NMS, and each QTL accounted for a large percent of the total phenotypic variation. Analysis of two $F_{2:3}$ lines also found that the phenotype was highly corresponded to its genotype which is between R3-15 and R3-19. Phenotype and genotype analysis for 30 mung bean accessions showed that the major effect QTL *qDFF3* was a key regulator for days to DFF. Using a map-based cloning method, the major effect QTL *qYSC4* for YSC was mapped in a 347 Kb interval on chromosome 4. Candidate gene analysis showed that sequence variations and expression level differences existed in the predicted candidate gene between the parents. These results provide a theoretical basis for cloning these QTLs and marker-assisted selection.

Introduction

Mung bean [*Vigna radiate* (L.) Wilczek] is a fast-growing warm-season leguminous species, belonging to the subgenus *Ceratotropis* (genus Vigna), the tribe Phaseoleae, and the family Fabaceae. A nutritional and health food with high-quality protein, folate and iron, mung bean is mainly cultivated in Asia, Africa, and Australia; in certain developing countries mung bean seed is regarded as a cheap source of carbohydrates [1, 2]. A benefit of mung bean is that its symbiosis with rhizobia can fix atmospheric nitrogen via biological nitrogen fixation, reducing the amount of chemical fertilizers needed while improving soil fertility and texture. Mung bean grown in rotation or intercropping with cereals can increases the cereal grain and reduce pest incidence [3, 4]. For a long time, mung bean has been regarded as a minor grain, and thus, the basic research and genomics study for mung bean still lags far behind the other major crops such as rice, wheat and corn.

InDels are an abundant form of genome variation, often more prevalent than SSRs on genomes and offering more genomic information than SNPs [5]. Compared with other markers, InDel markers have special superiority in bi-allelic, co-dominant, high-density, and low-cost genotyping. Thus, this inexpensive and convenient marker has been widely used in evaluating genetic diversity and gene mapping as well as molecular-assisted selection [6–9]. However, few studies have focused on the development of InDel markers in mung bean, not to mention using InDel markers in linkage map construction and QTL analysis, mainly because the high cost and low flux acquisition of insertion or deletion sequence

information in the past. In recent years, high-throughput sequence information has become more available along with the rapid development of next-generation sequencing (NGS) technology. This progress has greatly facilitated and accelerated the discovery process for molecular markers. In mung bean genomics studies, QTL analysis has mainly been focused on insect and disease resistance, including bruchid [10, 11], powdery mildew resistance [12, 13], yellow mosaic virus resistance [14, 15], and cercospora leaf spot resistance [16], mainly because these issues are a great threat to mung bean yield. Among most of these QTL analysis studies, genetic linkage map construction usually used RFLP, RAPD and SSR markers due to the lack of InDel markers.

The stem color of young mung bean is a very useful phenotype marker in germplasm identification. This trait is mainly determined by the kinds and content of anthocyanins. Anthocyanins are important water-soluble pigments and are widely distributed in berries and cellular fluids of leaves, stems, flowers, and fruit, attracting pollinators such as insects. Previous studies found that MYB-type transcription factor plays a regulatory role in anthocyanin biosynthesis [17, 18]. In fact, anthocyanins not only play a decisive role in the appearance color of most plants, but also protect them from various biotic and anti-biotic stresses depending on the anti-oxidation and anti-mutation ability of anthocyanins [19, 20]. Flowering time and plant height (PH) are strongly correlated with crop yield. In addition, these two agronomic traits also determine the adaptability of mung bean cultivars at different light and temperature conditions. Thus, it is necessary to explore the molecular genetic mechanism controlling these traits in mung bean. In this study, QTL analysis for young stem color (YSC), days to first flowering (DFF), days to maturity (DM), PH, and nodes on main stem (NMS) was performed using populations derived from the cross between Sulu16-10 and Weilu11. InDel marker development was based on sequence insertion or deletion between the parents. One hundred twenty-nine polymorphic markers that were uniformly distributed on 11 chromosomes were selected for genetic linkage map construction. The availability of markers linked with the major QTL controlled DFF for assist selection was verified. Fine mapping and candidate gene analysis for the major QTL associated with YSC were also performed. These newly developed InDel markers found in this study can enrich the current molecular markers resources and can be used in mung bean genomic research. Dissection of QTLs will advance our knowledge on underlying genetic mechanisms of these traits in mung bean. In addition, markers linked with major- and stable- expressed QTLs could be used in marker-assisted selection and facilitated the breeding of elite breeding varieties.

Materials And Methods

Plant materials and agronomic trait measurement

The F_2 and F_3 population consisted of 136 and 134 individuals, respectively. They were developed from a cross between Sulu16-10 and Weilu11. Sulu16-10 is a new mung bean variety with green YSC, high PH and late DFF; it was cultivated by the Jiangsu Academy of Agricultural Sciences, Jiangsu, China. Weilu11 has a purple YSC, dwarf PH and early DFF; it was bred by the Weifang Academy of Agricultural Sciences, Shandong, China. The F_2 population and the two parental lines were grown in 2019 in Hefei, China. The F_3 population, two $F_{2:3}$ lines and 30 mung bean accessions were planted in 2020 in Hefei, China. The

phenotypic data were analyzed for distribution and significance of trait variation using Excel 2007 and SPSS V22.0 software.

Anthocyanidin profiling and data analysis

Young stem samples from each of the parents were collected 10 days after germination. The samples were frozen in liquid nitrogen for further use. Each replicate was from 15–20 plants, and three biological replications were conducted. The sample preparation, extraction, anthocyanidin identification and quantification were performed following Wang [21] and Chen [22]. Sample data were collected using an LC-ESI-MS/MS system (HPLC, Shim-pack UFLC SHIMADZU CBM30A system; MS, Applied Biosystems 6500 Q TRAP). Data analysis also followed the methods described by Wang [21] and Zhuang [23]. Metabolites with significant differences in content were set with a threshold of fold change of ≥ 2 or ≤ 0.5.

Illumina sequencing and data filtering

Sulu16-10 and Weilu11 were homozygous by self-pollinating for several generations. Genomic DNA was extracted from fresh leaves of parents using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Sequencing libraries with 400-bp insertion were constructed and sequenced on Illumina HiSeq2000 sequencing system with paired-end sequencing strategy. After the production of raw sequences, all adapter sequences, short reads (length < 50 bp), and low quality reads were deleted. Duplicates (PCR duplicates and optical duplicates) produced in the sequencing process and DNA libraries amplification were removed by Picard software (http://www.psc.edu/index.php/user-resources/software/picard).

Primer design and PCR reaction

To find sequence differences between the two parents and reference genome sequence, all of the high quality sequencing data was aligned to the mung bean reference genome (https://www.ncbi.nlm.nih.gov/genome/664) using the Bwa package [24]. If the insertion/deletion sequence between the parents and the reference genome was not inconsistent at the same position, this sequence difference could regard as an InDel between parents. InDels between 5 bp and 50 bp were selected for primer design using Primer3 V2.3.6 [25]. The polymorphism of markers was validated using Sulu16-10 and Weilu11 by PCR reaction. The 10 μL PCR mixture contained 1.0 μL template DNA (50 $ng \cdot \mu L^{-1}$), 5.0 μL10×PCR MIX (TSINGKE, Beijing, China), 10 $\mu mol \cdot L^{-1}$of each forward and reverse primer 0.5 μL, and ddH_2O 3.0 μL. Thermal Cycler (Dongshenglong, Beijing, China) was used to perform PCR amplification with the following procedures: first denaturation step at 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, annealing (temperature set was based on primers, usually 57°C) for 30 s, 72°C for 30 s, final extension step at 72°C for 10 min and storage at 8°C. PCR products were separated with 6% non-denaturing polyacrylamide gel and visualized by silver staining.

Linkage map construction and QTL analysis

Polymorphic InDel markers were chosen to analyze the genotype of individuals in the F_2 population. The segregation of each marker was analyzed by a chi-square test for goodness of fit for the expected Mendelian segregation ratio (1:2:1). Markers showed segregation distortion were excluded from genetic linkage map construction. All markers used for genetic map construction are listed in Table S1. For the F_3 population, markers on chromosomes 3 and 10, additional with other seven polymorphic markers were used to construct a short but high-density genetic map. QTLs detection was performed with the QTL IciMapping software V4.1 [26, 27] using inclusive composite interval mapping of additive QTL ICIM-ADD modules. The scanning step was set at 1.0 cM. The LOD thresholds for each dataset were determined by permutation test (1000 permutations, $P < 0.05$). QTLs were declared when their LOD scores were larger than 2.0. QTLs were named according to McCouch [28].

Fine mapping for *qYSC4* and expression level analysis

New polymorphic markers used for fine mapping *qYSC4* were designed by Primer Premier 5.0 software based on sequence differences between parents. PCR-based molecular markers used to identify the genotypes of 256 F_2 individuals with green stem were listed in Table S1. Genotyping for SNP markers PCR productions were performed by the sequencing method. Young stem samples collected at 10 days after germination were used for RNA extraction with the OmniPlant RNA extraction Kit (DNase I). RNA was treated with DNase I and the first-strand cDNA was synthesized using cDNA Synthesis SuperMix (TransGen). QRT-PCR was conducted on LightCycler 96. Each 20 μL reaction system contained 2 μL of template cDNA, 10 μL 2 × SYBR Premix EX-Taq Mix, 10 $\mu mol \cdot L^{-1}$ of each forward and reverse primer 0.5 μL, and 7 μL RNase free ddH_2O. The relative expression level of each transcript was obtained by comparing with the expression of the *Vrtubulin* gene. QRT-PCR primers for candidate genes and *Vrtubulin* are listed in Table S2. The $2^{-\Delta\Delta CT}$ method [29] was used to analyze gene relative expression level.

Results

Phenotypic variation and correlations between traits

The phenotypic differences between the parents as well as the agronomic traits variation in the F_2 and F_3 populations are summarized in Table 1. For YSC, Sulu16-10 exhibited green, whereas Weilu11 showed purple. Metabolomic analysis detected 15 types of anthocyanins in the mung bean young stem. Compared with Sulu16-10, the comparative content of four types of anthocyanins (cyanidin 3-O-glucoside, delphinidin 3-O-glucoside, delphinidin 3-O-rutinoside and cyanidin 3-O-galactoside) was increased. Meanwhile, the content of other four types of anthocyanins (cyanidin 3, 5-O-diglucoside, malvidin 3, 5-diglucoside, pelargonin and cyanidin 3-p-hydroxybenzoylsophoroside-5-glucoside) in Weilu11 was decreased (Fig. S1). The stems of all of the F_1 plants showed purple, meaning purple was dominant to green. The ratio of individuals with purple to green (99 to 37) was fit to 3:1 by a chi-square test in the F_2 population ($\chi^2 = 1.3 < \chi^2_{0.05} = 3.84$), indicating the YSC was controlled by a single gene or a major effect QTL with a large contribution rate. The other four traits, DFF, DM, PH, and NMS showed

significant differences between the parents. Sulu16-10 showed higher PH and late DFF when compared with Weilu11. DFF, DM, PH, and NMS were exhibited continuous distribution in the F_2 population (Table 1). DFF, DM and PH were normally distributed, whereas the distribution of NMS was skewed and toward increased nodes. Transgressive segregation was observed for all traits. Therefore, we concluded that these traits were quantitatively inherited and controlled by multiple genes. These four traits were also observed to be continuous in the F_3 population. The mean values of DFF and DM in the F_3 population were larger than those in the F_2 population, whereas the mean values of PH and NMS were less than those in the F_2 population. Correlation analysis (Table 2) showed that all these four traits were significantly positive correlated with each other at two years. The YSC only correlated with PH in 2020.

Table 1
Phenotype analysis for the two parents and the F_2 and F_3 populations

Year	Traits	Parent lines		F_2 (2019) or F_3 (2020) population				
		Weilu11	Sulu16-10	Mean ± SD	Min	Max	Skewness	Kurtosis
2019	DFF (d)	40.5	56.2 **	47.5 ± 6.0	36.0	62.0	0.23	-0.52
	DM (d)	56.4	80.5 **	70.8 ± 8.5	55.0	88.0	-0.02	-0.70
	PH (cm)	73.4	112.5 **	105.0 ± 22.3	60.5	155.2	-0.23	-0.63
	NMS	9.0	13.0 **	12.2 ± 1.5	9.0	15.0	-0.68	-0.54
2020	DFF (d)	42.0	57.0 **	52.6 ± 8.8	38.0	65.0	-0.44	-1.50
	DM (d)	57.0	83.3 **	75.4 ± 6.2	55.0	87.0	-0.06	-1.15
	PH (cm)	53.5	108.2 **	75.4 ± 19.4	41.3	120.6	-0.13	-1.06
	NMS	8.0	12.5 **	9.9 ± 1.9	7.0	14.0	-0.11	-1.17

DFF, days to first flowering (d); DM, days to maturity (d); PH, plant height (cm); NMS, nodes on main stem. ** significant difference at the 0.01 probability level.

Table 2
Correlations between DFF, DM, PH, NMS, and YSC in the F_2 and F_3 populations

Traits	DFF	DM	PH	NMS
DM	0.873 **			
	0.902**			
PH	0.758**	0.786**		
	0.862**	0.819**		
NMS	0.722**	0.695**	0.771**	
	0.869**	0.829**	0.848**	
YSC	-0.065	0.049	0.040	0.015
	0.136	0.082	0.172*	0.120

DFF, days to first flowering; DM, days to maturity; PH, plant height; NMS, nodes on main stem; YSC, young stem color. * and ** indicate the correlation was strongly positive at the 0.05 and 0.01 significance levels, respectively. The numbers at the up or down site in the block mean correlation analysis using data collected in 2019 and 2020, respectively.

Sequencing and data filtering of Illumina paired-end reads

Whole-genome sequencing for Sulu16-10 and Weilu11 was conducted using the Illumina next-generation sequencing platform. In total, 104.0 and 106.9 million paired-end raw reads were obtained, containing 15.7 and 16.1 Gb of sequencing data with guanine and cytosine (GC) content of 34.5% and 35.6% for Sulu16-10 and Weilu11, respectively. After removal of adapter sequences and low quality reads, 88.9 and 92.9 million clean reads remained, encompassing 12.7 and 13.3 Gb for Sulu16-10 and Weilu11, respectively.

InDel marker development

All of the clean reads were mapped to the mung bean reference genome, and the mapping ratio (mapped reads/total reads) for parents were both greater than 99.0%. Finally, a total of 17, 280 InDels that ranged from 5 to 150 bp were identified between the parents based on the sequence differences. Most of these InDels were between 5 bp and 50 bp, and these InDels were chosen to design primers. Based on the flanking sequences of these InDels, PCR primer pairs were successfully designed for 14,509 loci. Two hundred nine markers that were uniformly distributed on 11 chromosomes were selected to distinguish genotype of the parents. Of these, 205 (98.1%) markers were successfully amplified clear PCR products of the expected sizes. Of these successfully amplifying markers, 180 produced polymorphic bands between the parents, accounting for 86.1% of the total tested markers.

Genetic map construction

Considering the polymorphic and position, 145 InDel markers that were uniformly distributed on the 11 chromosomes were chosen for linkage map construction. According to chi-square test and linkage analysis results, 12 markers showed serious segregation distortion and 4 markers were not mapped on chromosomes. Therefore, these 16 markers were excluded from the linkage map. Finally, 129 InDel markers could be anchored on the 11 chromosomes covering a total of 707.43 cM (Table 3, Fig. S2). The number of markers per chromosome were between 6 (Chr.10) to 20 (Chr.7) with a mean of 11.73. The length of the chromosomes ranged from 23.66 cM (Chr.10) to 158.08 (Chr.7) with an average of 64.31 cM. The average distance between the adjacent markers varied from 3.56 (Chr.2) to 10.15 cM (Chr.8) at an average marker density of 6.00 cM.

Table 3
Information of the genetic linkage map

Chr.	No. of markers	Length (cM)	Distance between two markers (cM)
1	11	51.25	5.13
2	9	28.45	3.56
3	9	31.34	3.92
4	11	54.78	5.48
5	13	67.05	5.59
6	17	86.28	5.39
7	20	158.08	8.32
8	12	111.68	10.15
9	12	51.16	4.65
10	6	23.66	4.73
11	9	43.70	5.46
Total	129	707.43	6.00

QTL analysis for agronomic traits

In total, 14 QTLs distributed on chromosomes 1, 3, 4, 6, 7, and 10 were found to be associated with YSC, DFF, DM, PH, and NMS (Table 4 and Fig. S3). LOD scores of these QTLs ranged from 2.06 to 97.45, and the phenotypic variance explained (PVE) were between 1.32% and 90.07% for each QTL. Three QTLs distributed on chromosomes 1, 4 and 6 were detected for YSC. They collectively accounted for 94.18% of the phenotypic variation. *QYSC4*, the major effect QTL for YSC could explain 90.07% of the total phenotypic variation, and the positive allele came from Weilu11. Four QTLs located on chromosomes 3, 6, and 10 were related to DFF and DM. Seven QTLs located on chromosomes 1, 3, 7, and 10 controlled PH

and NM. Among these QTLs, 7 were clustered at two intervals on chromosomes 3 (*qDFF3*, *qDM3*, *qPH3.1* and *qNMS3*) and 10 (*qDFF10*, *qPH10* and *qNMS10*).

Table 4
QTLs for DFF, DM, PH, NMS and YSC

Traits	QTL name	Chr.	Marker interval	Genetic distance (cM)	LOD	Add	PVE (%)
YSC	*qYSC1*	1	ID1-8–ID1-5	36.34–43.19	24.38	0.02	2.79
	qYSC4	4	ID4-8–ID4-9	37.25–41.38	97.45	-0.50	90.07
	qYSC6	6	ID6-16–ID6-15	72.08–86.28	4.97	0.01	1.32
DFF	*qDFF3*	3	ID3-6–ID3-7	23.40–29.12	24.31	7.04	53.67
	qDFF10	10	ID10-4–ID10-6	9.08–23.66	2.19	1.57	3.42
DM	*qDM3*	3	ID3-6–ID3-7	23.40–29.12	20.78	9.33	45.60
	qDM6	6	ID6-14–ID6-17	49.66–70.22	2.44	-0.97	3.53
PH	*qPH1*	1	ID1-8–ID1-5	36.34–43.19	3.24	7.00	6.57
	qPH3.1	3	ID3-6–ID3-7	23.40–29.12	7.42	2.83	13.60
	qPH3.2	3	ID3-8–ID3-9	30.60–31.34	15.97	20.50	31.57
	qPH7	7	ID7-19–ID7-18	9.36–14.67	2.06	-4.55	3.26
	qPH10	10	ID10-4–ID10-6	9.08–23.66	2.51	6.39	3.87
NMS	*qNMS3*	3	ID3-6–ID3-7	23.40–29.12	20.29	1.65	46.40
	qNMS10	10	ID10-4–ID10-6	9.08–23.66	2.87	0.47	5.05

LOD, logarithm of odds; Add, additive effect, positive or negative values indicate the alleles increase the effect from Su1u16-10 and Weilu11, respectively. PVE, phenotypic variation explained by the detected QTLs.

Confirmation for the two QTL hot spots

To confirm the two QTL hot spots detected in 2019. Markers on chromosomes 3 and 10 were used to construct a short linkage map. Another seven polymorphic markers on chromosome 3 were developed to narrow the distance between markers. Among these newly developed markers, two (R3-9 and R3-12) were between ID3-5 and ID3-6, and the other five (R3-5, R3-13, R3-15, R3-19, and R3-20) between ID3-6 and ID3-7. QTL analysis found 8 QTLs for DFF, DM, PH and NMS on these two chromosomes (Table 5). Six of these QTLs were detected in 2019. Based on the QTL analysis results, four QTLs between R3-15 and R3-19 controlled all of these four traits, and the PVE was ranged from 62.04–84.72%. Chromosome region between ID10-5 and ID10-6 hold QTLs for DFF, DM and NMS (Table 5). Further analysis found 49 individuals with Sulu16-10 genotype within R3-15 and R3-19 region showed late DFF and high PH (DFF = 59.0 ± 4.1, PH = 89.3 ± 13.2). The phenotype of 30 heterozygotes were similarly to these plants with

homozygous Sulu16-10 genotype (DFF = 57.9 ± 2.3, PH = 84.2 ± 7.9). Meanwhile, 49 plants with homozygous Weilu 11 genotype showed early DFF and dwarf PH (DFF = 42.0 ± 2.8, PH = 54.1 ± 8.9). Ten plants with three different genotypes from the F_2 population were selected to confirm the existence of these major QTLs between R3-15 and R3-19. All the plants with homozygous Sulu16-10 genotype in these two $F_{2:3}$ lines showed late DFF and high PH. Individuals with homozygous Weilu11 genotype in these two $F_{2:3}$ lines showed early DFF and dwarf PH. The phenotypes of descendants generated from these six heterozygotes were segregated. Two segregated $F_{2:3}$ lines were selected to analyze the consistence between genotype and phenotype. The genotypes of plants in these two lines are listed in Table S3. All the plants with Sulu16-10 or heterozygote genotype showed earlier DFF, earlier DM, dwarf PH and fewer NMS when compared with plants hold Weilu11 genotype (Table 6), and the differences reached a significant level. Consideration of combined phenotype and genotype of these two segregated $F_{2:3}$ lines, these QTLs were limited between R3-5 and ID3-7.These results indicated that QTLs with large PVE conferring DFF, DM, PH and NMS existed in this chromosome region.

Table 5
QTLs detected in 2020

Traits	QTL name	Chr.	Marker interval	Genetic distance (cM)	LOD	Add	PVE (%)
DFF	*qDFF3*$_a$	3	R3-15-R3-19	30.47–31.06	61.81	8.76	84.72
	qDFF10$_a$	10	ID10-5-R10-6	15.86–23.57	2.91	1.216	1.37
DM	*qDM3*$_a$	3	R3-15-R3-19	30.47–31.06	34.72	5.60	66.59
	qDM10$_a$	10	ID10-5-R10-6	15.86–23.57	5.37	1.69	6.09
PH	*qPH3.1*$_a$	3	ID3-6-R3-5	23.47–27.72	6.00	-0.76	6.76
	qPH3.2$_a$	3	R3-15-R3-19	30.47–31.06	35.45	17.37	62.04
NMS	*qNMS3*$_a$	3	R3-15-R3-19	30.47–31.06	43.47	1.77	73.60
	qNMS10$_a$	10	ID10-5-ID10-6	15.86–23.57	5.88	0.48	5.17

a, QTLs detected in 2020.

Table 6
Phenotype analysis of the $F_{2:3}$ lines

Type	DFF (d)	DM (d)	PH (cm)	NMS
494-I	60.8 ± 0.5 a	82.5 ± 1.0 a	73.7 ± 5.5 a	11.5 ± 1.0 a
494-II	60.1 ± 1.9 a	79.6 ± 2.2 a	71.1 ± 4.6 a	10.8 ± 1.0 a
494-	41.0 ± 1.0 b	67.3 ± 0.5 b	42.0 ± 4.8 b	7.6 ± 0.5 b
500-I	54.0 ± 1.1 a	78.0 ± 1.4 a	78.7 ± 2.5 a	10.5 ± 0.7 a
500-II	54.4 ± 0.9 a	78.2 ± 1.4 a	75.3 ± 4.9 a	10.5 ± 0.7 a
500-III	39.3 ± 1.0 b	68.0 ± 0.8 b	48.0 ± 3.0 b	7.3 ± 0.5 b
Different lowercase letters indicate significant difference at the 0.05 level in line.				

Molecular marker assistant selection

Since marker R3-15 and R3-19 were flanked with *qDFF3*, these two markers could be used in marker assistant selection. To verify the accuracy rate of marker assistant selection, 30 mung bean varieties with different DFF were divided into two groups, that is early DFF group (EF) and late DFF group (LF). Generally speaking, DFF was consistent with the genotype identified by marker R3-15 and R3-19 (Table 7, Fig. S4). Correlation analysis indicated the correlation coefficients between DFF with R3-15 and R3-19 were 0.777** and 0.857**, respectively. Among these tested mung bean varieties, 16 were showed Weilu11 genotype at *qDFF3* region, and 14 of them showed early DFF. Eleven varieties were showed Sulu16-10 genotype, and all of them showed late DFF. The accuracy rate was 92.6%. All of these results demonstrated that InDel markers R3-15 and R3-19 could be used as a very useful tool in mung bean DFF prediction and identification.

Table 7
Genotype and phenotype analysis for 30 mung bean varieties using markers R3-15 and R3-19

Code	Accession name	DFF (d)	Group	R3-15	R3-19
1	Bao942	37	EF	aa	aa
2	Baolu200212	35	EF	aa	aa
3	Ji9802-19-2	37	EF	aa	aa
4	Jilu0816	37	EF	aa	aa
5	Liaolu10L708-5	33	EF	aa	aa
6	lulu1002-3	32	EF	aa	Aa
7	Neilu2	35	EF	aa	aa
8	Pinlu08116	36	EF	aa	aa
9	Pinlu2011-06	37	EF	aa	aa
10	Suhei2	33	EF	aa	aa
11	Suheilu	34	EF	aa	aa
12	Sulu204	37	EF	aa	aa
13	Weilu7	37	EF	aa	aa
14	Weilu8	36	EF	aa	aa
15	Zhenglu8	37	EF	aa	aa
16	E1006	55	LF	AA	AA
17	E1009	48	LF	aa	aa
18	Elu5	48	LF	AA	AA
19	Lulu4-1	62	LF	aa	AA
20	Su2074	58	LF	AA	AA
21	Sukang4	51	LF	AA	AA
22	Sukang1	64	LF	AA	AA
23	Sulu11-8	64	LF	AA	AA
24	Sulu203	53	LF	AA	AA
25	Sulu4	63	LF	AA	AA

AA: Sulu16-10 genotype; aa: Weilu11 genotype; Aa: heterozygote.

Code	Accession name	DFF (d)	Group	R3-15	R3-19
26	Sulu9073	50	LF	AA	aa
27	Taiyuan52	52	LF	aa	AA
28	Taiyuanchuanfu-1	55	LF	AA	AA
29	Yinggelu	55	LF	AA	AA
30	Zhonglu3	50	LF	AA	AA
AA: Sulu16-10 genotype; aa: Weilu11 genotype; Aa: heterozygote.					

Fine mapping for the YSC gene *qYSC4*

Among these three QTLs that controlled YSC, *qYSC4* can regarded as a major gene because it could explain 90.07% of the phenotypic variation. For fine mapping *qYSC4*, four InDel and two SNP markers were used to screen the genotypes of 256 green stem individuals in the F_2 population. *QYSC4* was finally mapped within a 337 Kb region between InDel marker ID4-14.9 and SNP marker 771K (Fig. 1A). Based on the mung bean genome annotation information, there are 38 open reading frames (ORFs) with different predicted biological functions in this candidate region. Among these ORFs, *LOC106758035* and *LOC106758748* encoded MYB transcription factor were found in this target interval. Sequencing results showed there were no sequence variations in the *LOC106758035* gene between parents. The gene *LOC106758748* in Weilu11 had two synonymous SNPs in the second exon, four SNPs and an AT box (35 AT repeats) deletion in introns (Fig. 1B). QRT-PCR results showed there was no significantly different expression level for *LOC106758035* between parents. The expression level of *LOC106758748* was strongly up-regulated in Weilu11 (Fig. 1C). Based on these results, *LOC106758748* was selected candidate gene for *qYSC4*.

Discussion

Molecular marker development in mung bean had been performed in previous studies, and most of these studies were focused on SSR markers [30–32]. In this study, high-throughput development of InDel markers could be achieved by directly compare the sequence differences between parents, and the rate of polymorphic InDel markers reach 86.1%, higher than the SSR markers [30–32]. Several studies focused on genetic linkage map construction and QTL analysis for mung bean flowering time and PH have been conducted by using SSR markers [33–35]. However, the positions of these QTLs were signed with genetic distance, and the markers linked with QTLs were not anchored on mung bean chromosomes. Therefore, it is difficult to compare the positions and contribute rate among these QTLs. In this study, all the markers used in linkage map construction were anchored on mung bean chromosomes. Thus, it will facilitate fine mapping or cloning for these QTLs, and also using in marker-assistant selection in the future. A genetic linkage map consisted of 129 developed InDel markers was constructed. The map totally covered 707.43

cM (Table 3), slightly shorter than map described by Wang [11]. For the purpose of genetic analysis and QTL mapping, varieties with significant phenotypic differences were usually chosen for segregation population construction. In this study, all the agronomic traits showed significant differences between the two the parents, Sulu16-10 and Weilu11.

A total of 11 QTLs were found to be responsible for DFF, DM, PH and NMS, and 7 of them were clustered at the two chromosome regions (Table 4). It was noteworthy that QTLs located at ID3-6 and ID3-7 were responsible for these four traits and the positive alleles all from Sulu16-10. QTLs controlled correlated traits were clustered together also found in other studies [10, 33, 36]. The reasons for this phenomenon were that either a pleiotropic existed that controlled these traits or QTLs related to these traits were closely linked. In rice, *Ghd7* and *Ghd8* were pleiotropic genes, which controlled flowering time and PH at the same time [37, 38]. Thus, these four QTLs between ID3-6 and ID3-7 were considered the same loci. To distinguish chromosome regions whether contained one QTL with pleiotropy effect or closely linked QTLs, NILs need to be constructed, and then large segregation populations need to be generated to break the link relationship. QTLs detection is usually conducted at different conditions, and these stable expressed QTLs have great significance in elite cultivars breeding. However, most loci in plants of F_2 population were not homozygous, and these heterozygous loci will be segregated in the progeny. Thus, the linkage map which constructed for the F_2 population could not be used in the progeny populations. Using the F_3 population and markers on chromosomes 3 and 10 additional with other seven polymorphic InDel markers, another short and high-density linkage map was constructed. All these four QTLs located between ID3-6 and ID3-7 can be detected again, and the QTL region was narrowed at a 170 Kb region between R3-15 and R3-19 (Table 5). In addition, phenotype and genotype analysis for two segregate $F_{2:3}$ lines also verified the regulator role of *qDFF3* (Table 6). BLAST analysis for sequences of markers that closely linked with QTLs controlled flowering time [33–35, 39]; *qDFF3* was corresponding to *Dff 3 – 1*, and near *Fld5.4.1*. Comparative analyzing the candidate genomic region with soybean, a putative *phytochrome A* gene related to flowering time was found, which was an orthologue of the soybean *E3/E4* [40, 41]. Meanwhile, two minor QTLs (*qDFF10a* and *qNMS10a*) located at ID10-4 and ID10-6 could also be detected in 2020 (Table 5). Since DFF and PH were two positively correlated traits, they should share the similar change tendency in different populations or conditions. Strangely, even though the average DFF and DM in F_3 population was larger than the average value in F_2 population, the average PH and NMS in F_3 population was obviously decreased when compared with that in F_2 population (Table 1). The main reason was that the mung bean phenotype was strongly influenced by the weather since mung bean growth period in 2019 was hot and drought, but cool and rainfall in 2020. Thus, these repeatedly detected QTLs were considered stable expressed at different conditions. Genotype analysis found 14 of 15 varieties showed homozygous Weilu11 genotype in the early DFF group, and 11 of 15 varieties showed homozygous Sulu16-10 genotype in the late DFF group (Table 7, Fig. S4). This result showed *qDFF3* was the key regulator loci for DFF in mung bean germplasm. The accuracy rate of linked markers in DFF identification reached 92.6%. Thus, InDel markers R3-15 and R3-19 were very useful molecular marker in mung bean DFF prediction and identification. Future work will focused on fine mapping population construction for these major effect QTLs and depth analysis of candidate genes.

YSC is a very useful tool for varietal purity identification and assistant breeding. Previous studies showed MYB-type transcription factor were the key regulator for synthesis of anthocyanins [42, 43]. In soybean, soluble pigment in stems was considered had relationship with white mold resistance [44, 45]. Thus, it is necessary to research the relationship between disease resistance and YSC in mung bean. Metabolomic analysis showed the content of four kinds of anthocyanins was increased in Sulu16-10, demonstrating not all the content of soluble pigment were decreased in lighter colored stem when compared with darker stem in mung bean. Previous studies showed YSC was controlled by a single gene [46] or multi-genes [47]. Chi-square test found the number of plants with purple stem to green was fit to 3:1 in F_2 population, indicating there was a single gene or major QTL conferring this trait. QTL detection results showed that YSC was controlled by one major QTL *qYSC4* plus two minor QTLs: *qYSC1* and *qYSC6* (Table 4). Actually, we have identified an YSC mutant by using EMS mutagenesis strategy, and the mutation site did not allele to *qYSC4*. These results demonstrated that YSC was controlled by multi-genes. According to our phenotype investigation results, *qYSC1* and *qYSC6* could not affect the phenotype judge for YSC because alleles from Sulu16-10 could increase the effect while this parent showed green stem. Thus, the phenotype of YSC in F_2 population and parents were completely determined by *qYSC4*, which could explain 90.07% of the total phenotype variation. Map-based cloning limited the *qYSC4* within a 347 Kb region which contained two predicted MYB-type transcription factor, *LOC106758035* and *LOC106758748* (Fig. 1). Homology analysis showed LOC106758748 was homologs to soybean *R* gene, which controlled seed coat color [48]. Sequence variations were existed in *LOC106758748* and may affect the expression level between parents. In summary, sequence variation and expression analysis revealed *LOC106758748* was the preferred candidate gene for *qYSC4*.

Conclusions

High-throughput development of InDel marker could be performed based on the sequence variation between parents' genome sequence obtained from NGS. The ratio of polymorphic InDel marker could reach 86.1%. A genetic linkage map which covered 707.43 cM for a F_2 population developed by the cross between Sulu16-10 and Weilu11 was constructed. QTL analysis detected 14 QTLs for YSC, DFF, DM, PH and NMS. The phenotypic variance explained by each QTL was between 1.32% and 90.07%. Seven of these QTLs were clustered at two intervals on chromosomes 3 and 10. Six of these co-located QTLs were detected again in the F_3 population by using a short and high-density linkage map. Phenotype and genotype analysis for 30 mung bean accessions found that the *qDFF3* was the key regulation factor for DFF and the linked markers could be used in marker assisted selection. Map-based cloning limited the *qYSC4*, the major gene for YSC within a 347 Kb region. Sequence variation and expression level analyses confirm that the MYB transcription factor *LOC106758748* was the prefer candidate gene for *qYSC4*. These results provide a foundation for cloning these QTLs and clarifying the molecular regulation mechanisms for these traits.

Declarations

Declaration of Competing Interest

The authors declared that they have no competing interests.

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Ethics approval Not applicable

Consent to participate Not applicable

Consent for publication Not applicable

Availability of data and material

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable

Author's contributions

All authors contributed to the study conception and design. The experiments were designed by Weijun Ye, Liya Zhang, Lei Zhang, and Bin Zhou. Material preparation, data collection and analysis were performed by Weijun Ye, Yong Yang, Peiran Wang, Yin Zhang, Dongfeng Tian, Lingling Zhang and Bin Zhou. The first draft of the manuscript was written by Weijun Ye and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Supplemental Data

Supplemental figures and tables not available with this version

Figures

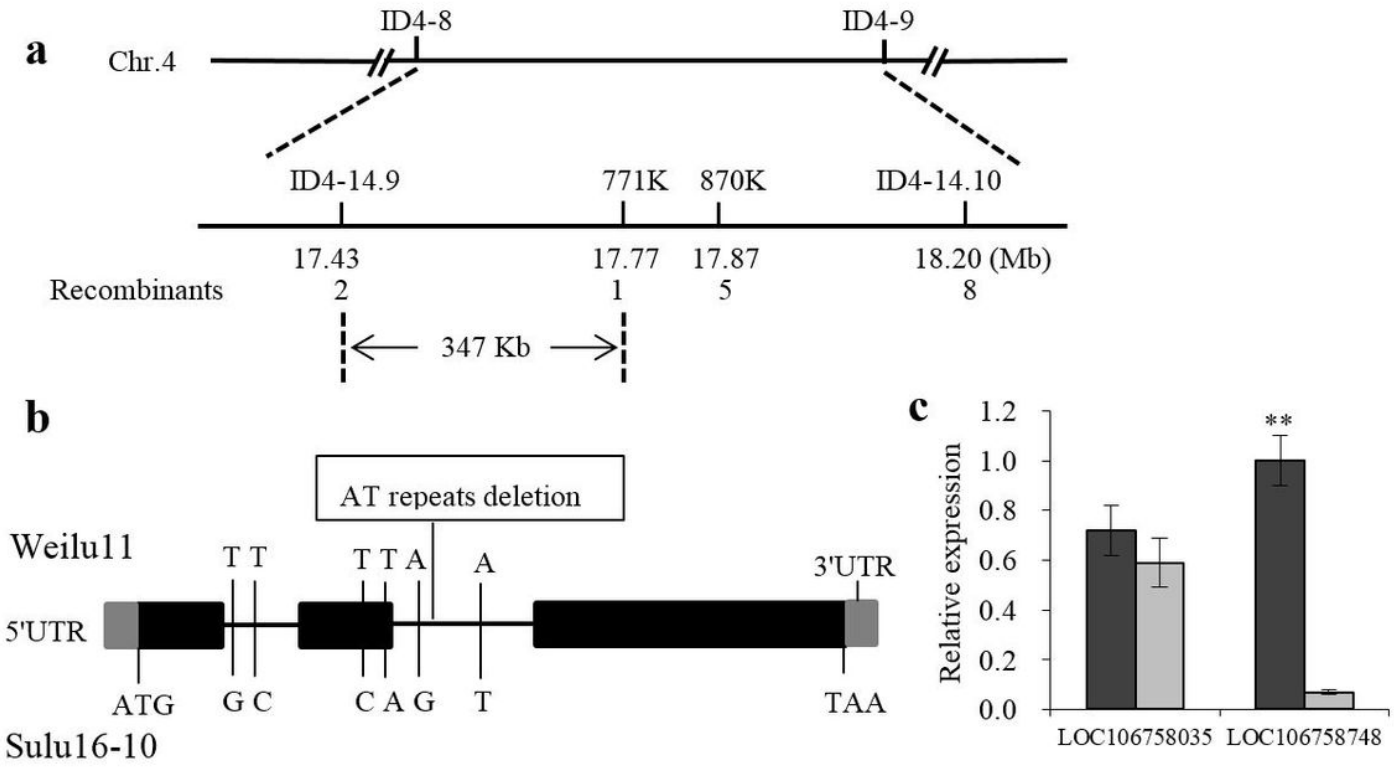

Figure 1

Fine mapping and candidate gene analysis for qYSC4. a. Fine mapping for qYSC4. b. Sequence differences of LOC106758748 between Sulu16-10 and Weilu11. c. Expression analysis of LOC106758035 and LOC106758748 between the parents. The black and grey columns represent Weilu11 and Sulu16-10, respectively. ** represents significant difference at 0.01 level.