QTL mapping and identification of genes associated with the resistance to *Acanthoscelides obtectus* (Say) in cultivated common bean using a high-density genetic linkage map

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Abstract

Background: Common bean (*Phaseolus vulgaris* L.) is an important agricultural product with large nutritional value, and the insect pest *Acanthoscelides obtectus* (Say) seriously affects its product quality and commodity quality during storage. Few researches on genes of bruchid resistance have investigated in common bean cultivars.

Results: In this study, a bruchid-resistant cultivar black kidney bean and a highly susceptible accession Longyundou3 from different gene banks were crossed to construct a segregated population. The genetic analysis indicated a quantitative inheritance of the bruchid resistance trait controlled by recessive polygenes. A high-density genetic map of a total map distance of 1,283.68 cM with an average interval of 0.61 cM between each marker was constructed using an F_6 population of 157 recombinant inbred lines containing 2234769 SNP markers. Using the high-density genetic map, a new quantitative trait locus (QTL) for both the percentage of seeds damaged by *Acanthoscelides obtectus* and the number of perforations was identified on chromosome 6. This locus was further delimited to an interval of 122.3 kb between SSR marker I6-4 and I6-16 using an F_2 population. This region comprised five genes.

Phvul.006G003700, which encodes a bifunctional inhibitor, may be a potential candidate gene for bruchid resistance. Sequencing analysis of candidate gene identified an 5bp insertion-deletion site in promoter of gene *Phvul.006G003700* between two parents. Expression analysis of candidate genes revealed that the expression level of *Phvul.006G003700* in bruchid-resistant parent was markedly higher than that in bruchid-susceptible parent both in seeds and leaves.

Conclusions: A high-density genetic map was constructed utilizing whole-genome resequencing and one new QTL for bruchid resistance was identified on chromosome 6 in common bean cultivar.

Phvul.006G003700 (encoding a bifunctional inhibitor) may be a potential candidate gene. These results may form the basis for further research to reveal the bruchid resistance molecular mechanism of common bean.

Background

Common bean (*Phaseolus vulgaris* L.) is one of the most widely adapted legumes in the developing world and the most important food legume for direct human consumption in the world [74, 75]. This autodiploid ($2n = 2x = 22$) species has 11 chromosomes and a genome size of 600 Mb [74, 11, 45]. Common bean is an annual and self-pollinating plant, and its seeds are rich in protein, amino acids, flavonoids, alkaloids and terpenoids [18, 52], which are necessary for human life. Common bean originated from Mesoamerica, and China is considered a secondary centre of common bean diversity and has a long history of cultivation [41, 77]. Common bean is an important source of plant protein in the human dietary structure and is one of the main export products of China [18, 68].

During their storage, legume seeds are often damaged by legume weevils, which leads to a serious decrease in yield. Bean weevils [*Acanthoscelides obtectus* (Say)] and Mexican bean weevils [*Zabrotes*

subfasciatus (Boheman)] are the main insect pests of common bean during storage, and the latter mainly infects wild common bean and has little influence on cultivated species [46, 72]. *Acanthoscelides obtectus* (Say) originated from South America and Central America and has been introduced into dozens of countries and regions, such as Afghanistan, Japan, Myanmar and China, through international trade and introduction channels [3]. Common bean is mainly damaged by young insects of bean weevils into seeds, and adults can breed in fields and warehouses, which causes great losses [25, 61]. The damage and weight loss rates of common bean in the field are 30% ~ 38% and 6% ~ 8%, respectively, and storage increases these values to 74% and 64%, respectively, which indicates that the damage created by bruchids is more serious during the storage period [20, 44, 48]. At present, the commonly used physical methods, including boiling and seed dressing treatment as well as fumigation with chemical agents such as phosphine, aluminium phosphide, methyl bromide and ethyl formate, and biological control are applied to control seed damage by bruchids [32, 43, 47, 58], but the negative impacts on the seeds themselves and the environment are substantial.

Because the growth and development of insects are affected by the chemical components in plants [9], which can kill, resist, avoid insects or inhibit their growth, the direct utilization of plants themselves to prevent and control pests has become a research hotspot. Many common bean varieties are resistant to azuki bean weevil (*Callosobruchus chinensis*) and cowpea weevil (*Callosobruchus maculatus*) because they contain special seed proteins [28]. This protein is a component of a polygene family encoded by APA loci [arcelin (Arc), phytohemagglutinin (PHA), and α -amylase inhibitor (α AI)] [34] and is most abundant in PHA and α AI [31, 39, 51, 56, 71]. PHA and α AI are the most representative members of the lectin family and normally occur in wild and cultivated accessions of bean. Arcelin is found only in some wild Mexican bean varieties but not in cultivated bean varieties [35, 50, 64]. The wild common bean germplasms G02771 (containing Arc-5 protein) [26], G12882 (including ARC-1) [71], RAZ106 (containing the arcelin 1 allele), G12866 (including ARC-2) [13], G24582 (including ARC-7), G24582A (including ARC-7) and G24584 (including ARC-7) [2] are effective against the Mexican bean weevil. The wild bean germplasms G12952 (ARC-4), G12949 (ARC-4) ([28]Santino et al. 1991) and QUES (ARC-8) [76] are resistant to both *A. obtectus* and *Z. subfasciatus*. Baldin et al. [8] screened 18 common bean genotypes of Andean American and Mesoamerican origin, and the common bean genotypes Arc.1, Arc.2, Arc.1S, Arc.3S, and Arc.5S were identified as resistant to *A. obtectus*. Some Bulgarian common bean genotypes and commonly grown Turkish bean genotypes were resistant against *A. obtectus* [4, 5]. Although an increasing number of common bean germplasms resistant to weevils have been discovered, most of these are concentrated in wild beans, and few are concentrated in cultivated beans.

At present, research on bruchid resistance gene cloning is lagging behind both at home and abroad, and in particular, few studies have investigated resistance genes in common bean cultivars using molecular markers. Blair et al. [15] used SSR molecular markers to identify a locus linked to the Brazilian weevil resistance gene (*arcelin* 1 allele) from the wild common bean accession RAZ106 and found that this locus is located near the APA locus in the B04 linkage group. Kamfwa et al. [30] mapped two QTLs on chromosome 4 (one associated with the APA locus) and one QTL on chromosome 6 using an exhibited *A. obtectus* resistant breeding line introgressed from the wild tepary bean (contained Arclin gene). Lectin

can be encoded by a single locus or by several loci; for example, APA is located on chromosome 4, *Lec-2* and *Lec-3*, and the *lec* genes mapping B7 [21, 29].

In this study, we found that the black kidney bean, a cultivar of common bean, has high resistance to *Acanthoscelides obtectus* (Say). We then constructed a high-density genetic linkage map using a recombinant inbred line population obtained from a cross between the bruchid-susceptible landrace Longyundou3 and black kidney bean and performed gene mapping of bruchid resistance. This study comprises the investigation of resistance genes for bean weevils in cultivated common bean, and as a result, this study lays a foundation for further study of the mechanism of bruchid resistance in common bean and provides a possibility for improved seed preservation.

Results

Bruchid resistance segregation and genetic analysis

BKB exhibited resistance to *Acanthoscelides obtectus* (Say), with a mean percentage of damaged seeds of 28.3%. In contrast, LYD3 showed high susceptibility, and all the seeds were damaged by bruchids (percentage of damaged seeds equal to 100%) (Fig. 1a, b).

The resistance of the F_1 and $F_{2:3}$ populations and RILs was identified. Two traits, namely, the percentage of seeds damaged by bruchids (PDB) and the number of perforations (NP), were investigated. The percentage of F_1 seeds damaged by bruchids was 100%, which was the same as that of the susceptible parents, and this finding indicated that the trait of resistance to *A. obtectus* was a recessive trait. The PDS among the $F_{2:3}$ individuals ranged from 0 to 100%, and the NP ranged from 0 to 360, showing significant resistance segregation (Fig. 1c). The PDS in the RILs ranged from 0 to 100%, and the NP ranged from 0 to 420. However, the minimum damage rate of seeds was lower than that of resistant parents, which indicated that resistant traits had a positive superparent advantage. The distribution of bruchid resistance was continuous in the $F_{2:3}$ generation, and the ratio of susceptibility to resistance was not consistent with Mendelian inheritance. The kurtosis and skewness values were 0.35 and -0.84, respectively, with an absolute value lower than one, which was approximately normal, and this finding indicates the quantitative inheritance of the resistance trait controlled by recessive polygenes.

High-density genetic linkage map construction

Based on the technical characteristics of whole genome resequencing, the 157 RILs and two parents of common bean were resequenced. Raw reads were obtained by CASAVA base calling and conversion from the raw image data acquired by high-throughput sequencing. After filtering, 80029096 (17.12X coverage) and 71913622 (16.95X coverage) clean reads were generated from BKB (resistant parent) and LYD3 (susceptible parent), respectively, the clean reads from 157 RILs ranged from 39686552 to 105924272, with an average sequencing depth of 14.38 X coverage. By comparing the sequencing data of BKB, LYD3

and 157 RILs with the reference genome, a total of 300,003 SNPs among the parents were screened out and found to be distributed on 11 chromosomes (Table 1) using GATK 3.8 software [19].

The sliding window approach, with 15 SNPs in each sliding window, was used in this study. According to the proportion of SNP markers in the sliding window from the male parent to the female parent, the genotype of the segment where the sliding window was located was determined, and the location of the recombination breakpoint of the chromosome segment was determined. When the sliding window hit a homozygous/homozygous breakpoint, the genotype transiently changed from homozygous to heterozygous and then changed to the other homozygous genotype. A genotype remained unchanged until it hit a recombination breakpoint [27]. After all genotypes were called and the recombination breakpoints were determined, we identified a total of 48,689 recombinant breakpoints, with an average of 310 breakpoints per RIL (Fig. S1a).

By consolidating continuous non-recombination markers on the genome into a bin, a total of 4214 recombination bins were obtained for the 157 RILs. The average physical length of the recombination bins was 135.2 kb, including 712 SNPs. Each bin was used as a marker for genetic marker screening and genetic linkage map construction. Because segregation distortion could affect the results from map construction and QTL positioning, a ratio of 1:1 was used to perform segregation filtration with 4,214 candidate markers, and 1088 biased markers were filtered out to yield 3126 valid markers, which accounted for 74.18% of all bin markers. According to the genome information [59], the linkage groups were constructed using JoinMap 4.0 software [70] by controlling the LOD value between 2.0 and 10.0. A total of 11 linkage groups were generated with 3106 bin markers, containing 2234769 SNP markers. Taking the linkage group as the unit, the genetic distance between the markers was calculated using the Kosambi algorithm. A high-density genetic map of a total map distance of 1,283.68 cM, with an average interval of 0.61 cM between the bin markers, was constructed (Table 1, Fig. 2). The number of effective bin markers in each chromosome ranged from 134 to 417. The length of each chromosome ranged from 79.23 cM to 166.73 cM, and the average distance between adjacent markers ranged from 0.43 cM to 0.80 cM. With the exception of two >10-cM gaps between adjacent markers on LG05 and LG08, all other gaps were < 8 cM. The conversion ratio between genetic distance and physical distance ranged from 1.67 to 2.79. LG08 was the longest with a map length of 166.73 cM, containing 417 bin markers with 297249 SNPs, and had an average genetic distance of 0.71 cM. LG03 was the second with a map length of 159.84 cM containing 289659 SNPs. LG09 and LG01 were the two shortest with map lengths of 79.23 cM and 95.36 cM, including 134 bin markers with 110344 SNPs and 364 bin markers of 246412 SNPs, respectively, and the average genetic distances of LG09 and LG01 were 0.43 cM and 0.63 cM.

Collinearity analysis was performed based on the positions of markers on the genome and the genetic map, and the results showed that the order of most marker positions on each linkage group was consistent with the chromosome (Fig. S1b), indicating good collinearity, high accuracy of the genetic recombination rate and high accuracy of the map construction.

Quantitative trait loci (QTL) analysis

The genetic map was used to identify QTLs controlling bruchid resistance in common bean. After the identification of bruchid resistance, the PDS of the RILs ranged from 0 to 100%, and the NP ranged from 0 to 420. The ICIM-ADD method in QTL ICIMapping4.2 software was used to analyse PDS and NP. The analysis of the 157 RILs detected one QTL associated with PDS was also located on chromosome 6 between markers Bin1565 and Bin1566. The LOD values were 5.49, explaining 16.10% of the variation, and the additive effects were -0.11. One QTL related to NP, which was located on chromosome 6 between markers Bin1565 and Bin1566, had an LOD value of 4.31 and additive effects of -27.48, and explained 16.37% of the variation (Table 2; Fig. 3a; Fig. S2). This QTL for both PDS and NP was located at the physical positions between 1,250,000 and 1,500,000bp on chromosome 6 (Fig. 3b). We named the QTL qCBr6, and the beneficial allele at this locus came from BKB, the resistant parent. The qCBr6 might be a major locus for resistance to *Acanthoscelides obtectus* (Say).

For narrowing down the regions identified by QTL-seq, we developed 44 SSR markers and 5 InDel markers (Table S1) on chromosome 6 in candidate regions to detect polymorphisms between BKB and LYD3. A total 11 SSR markers and 3 InDel markers on chromosome 6 were polymorphic between the two parents. The regional linkage maps for chromosome 6 and chromosome 9 were constructed with polymorphic DNA markers using F₂ mapping population consisted of 188 individual plants. We identified ten recombinants from the fine-mapping population using seven molecular markers. The qCBr6 locus was delimited to an interval of approximately 122.3 kb between SSR marker I6-4 and I6-16 (Fig.3c).

To further verify the correlation between this locus and bruchid resistance, an association study in candidate region was conducted using SNP markers and the phenotypic data of two traits, PDS and NP, of one natural population (contained 625 common bean accessions) (Table S2). A total 437 SNPs were generated from the whole genome resequencing project between I6-4 (Chr06,1367622bp) and I6-16 (Chr06,1489891bp) on chromosome 6. The results showed that 70 SNPs (P -value $< 1 \times 10^{-6}$) associated with PDS and 7 SNPs (P -value $< 1 \times 10^{-6}$) associated with NP were detected (Table S3). Seven SNPs (Chr06_1413364, Chr06_1429130, Chr06_1429225, Chr06_1429229, Chr06_1429376, Chr06_1432127, Chr06_1432316) were associated with both two traits. The peak SNP (SNP with the lowest P value) was located in 1432316bp on chromosome 6 (PDS-associated Chr06_1432316, $P = 8.38 \times 10^{-28}$; NP-associated Chr06_1432316, $P = 3.44 \times 10^{-12}$) (Fig S3). Four SNPs associated with PDS (Chr06_1376148, Chr06_1376347, Chr06_1376967 and Chr06_1407036) were located within genes, others lied between genes. These results suggested that this locus might be strongly associated with bruchid resistance.

Annotation and prediction of candidate regions for bruchid resistance

According to the common bean reference genome sequence, the region between marker I6-4 and I6-16 contained five genes, *Phvul.006G003700* (chr06,1376123–1378343bp), *Phvul.006G003800*

(chr06,1406616–1408098bp), *Phvul.006G003900* (chr06,1417873–1419245bp), *Phvul.006G004000* (chr06,1454577–1457697bp) and *Phvul.006G004100* (chr06,1486729–1491368bp) (Fig. 3d; Table 3). *Phvul.006G003700* was found to be a homologous gene in *A. thaliana* according to the NCBI database and encoded a protease inhibitor/seed storage/LTP family protein, belonging to the AAI-LTSS [alpha-amylase inhibitor (AAI), lipid transfer (LT) and seed storage (SS)] protein superfamily. The AAI-LTSS family is known to play important roles in defending plants from insects and pathogens, in lipid transport between intracellular membranes, and in nutrient storage. The BLAST searches against the NCBI and UniProt databases revealed that *Phvul.006G003700* showed the strongest similarity to an AAI domain-containing protein in *Phaseolus vulgaris* (kidney bean) (identity = 91.29%) and an AAI domain-containing protein in *Glycine max* (Soybean) (E-value = 0.0, score = 1,614, identity = 77.9%), which speculated that the protein encoded by *Phvul.006G003700* might have a similar function of inhibiting α -amylase activity. *Phvul.006G003800* encodes a carbohydrate-binding X8 domain superfamily protein. *Phvul.006G004000* and *Phvul.006G004100* encode homologues of yeast ADA2 2B and ADA2 2A, respectively. *Phvul.006G003900* has unknown function.

Sequence analysis and expression of candidate genes

The coding DNA sequences (CDS) of *Phvul.006G003700* in BKB and LYD3 were determined. The amplification of the candidate gene indicated that *Phvul.006G003700* had a length of 999 bp in cDNA in the resistant genotype BKB and encoded 332 amino acid residues. No difference was found between BKB and LYD3 (Fig. S4). The results from the search of the SMART Database showed that the *Phvul.006G003700* protein contained an AAI domain within the region from amino acids 55 to 329 (Fig. S5).

To further examine the transcriptional regulation of this gene, we analysed the upstream promoter sequences of the genes using the PlantCARE database. The BLAST searches revealed that the amplified sequence included essential cis-regulatory elements of the promoters such as the core promoter element TATA and the common element CAAT that were highly conserved across the investigated species. We obtained the sequence of promoter region for *Phvul.006G003700* gene. A 5-bp difference on 800 bp upstream of the start codon in the promoter region of the *Phvul.006G003700* gene was found between LYD3 and BKB, which resulted in the absence of essential cis-regulatory element TATA box in the susceptible material (Fig. 4). TATA box was known to be an essential transcription regulator. The absence of the TATA box may reduce the efficiency of transcription and thus affect the gene expression of *Phvul.006G003700* in the susceptible parent LYD3.

The qRT-PCR results showed that the expression level of *Phvul.006G003700* in BKB was markedly higher than LYD3 both in seeds and leaves (Fig. 5), which revealed that the gene expression level was higher in the bruchid-resistant accession than in the bruchid-susceptible accession. In seeds, gene *Phvul.006G003700* in BKB was about 40 times more expressed than it in LYD3, and about 3.5 times more expressed in leaves.

Through the results of association study between SNP markers and two traits of PDS and NP in the natural population, we found that three significant SNPs associated with PDS (Chr06_1376148, $p = 5.85 \times 10^{-10}$; Chr06_1376347, $p = 9.97 \times 10^{-10}$; Chr06_1376967, $p = 1.11 \times 10^{-9}$) were localized in the 3'-UTR region of the candidate gene *Phvul.006G003700* (Fig S6). These findings further support the likelihood that *Phvul.006G003700* was the candidate gene for bruchid resistance.

Identification and homology sequence analysis of candidate genes

Homologous sequences were accessed of candidate gene *Phvul.006G003700* within the G19833 reference genome and other crops. We searched for homologous sequences of the candidate genes within the common bean G19833v1.0 reference genome and identified 14 additional specific genes located on chromosomes 1, 3, 6, 8 and 9. Seven homologous genes, *Phvul.003G169100*, *Phvul.003G218600*, *Phvul.003G218700*, *Phvul.003G218800*, *Phvul.003G218900*, *Phvul.003G219000* and *Phvul.003G219100*, were located on chromosome 3. Four homologous genes, *Phvul.006G138600*, *Phvul.006G138800*, *Phvul.006G138900* and *Phvul.006G139100*, were located on chromosome 6. *Phvul.001G050300*, *Phvul.008G142500* and *Phvul.009G092000* were located on chromosomes 1, 8 and 9, respectively. These homologous genes all had a similar protein domain as the candidate gene and encoded a bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin protein belonging to the AAI-LTSS protein superfamily. The analysis of conserved motifs revealed two motifs with an e-value >E-60 within the AAI domain region of *Phvul.006G003700* and homologous proteins in the *P. vulgaris* genome (Fig. S7).

To further clarify the relationship between the *Phvul.006G003700* gene and homologous genes from other crops, a BLAST search against the NCBI and UniProt databases was performed to find the homologous protein of *Phvul.006G003700* in soybean (*Glycine max*), cowpea (*Vigna angularis var. angularis*), mung bean (*Vigna radiata var. radiata*), lupine (*Lupinus albus*), wheat (*Triticum aestivum*), maize (*Zea mays*), rice (*Oryza glumipatula*), barley (*Hordeum vulgare subsp. vulgare*), *Medicago truncatula*, *Arabidopsis thaliana* and other crops. The protein sequence encoded by *Phvul.006G003700* was compared with the homologous protein sequence in other crops by phylogenetic tree analysis (Fig. S8). The results showed that the *Phvul.006G003700* protein was closely related to the AAI protein in soybean, which suggested that it might have a similar function to AAI. The analysis of conserved motifs revealed three motifs with an e-value >E-60 among *Phvul.006G003700* and homologous proteins in other crops, and the first two conserved motifs were located in the AAI domain region (Fig. S9).

Discussion

During the storage of common bean, the damage caused by the common bean weevil leads to a decrease in stored grain [3, 5]. Although some physical and chemical methods [6, 43, 47] can be used to prevent and control the damage, the treated seeds are difficult to eat or sell again, resulting in relatively large

economic losses [44]. The screening of resistant materials and the identification of resistance genes of common bean are needed to provide a green and environmental protection approach. Previous studies have found some common bean germplasms that are resistant to bean weevils. Most resistant germplasms were concentrated in wild bean, and few were concentrated in cultivars/landraces. Based on the preliminary screening and identification of resistance to *A. obtectus* among several hundred common bean germplasm resources, we identified one cultivated common bean variety with good resistance to weevils in China. It was named BKB, and presented an average seed infection rate of less than 50%. Using the cross between BKB and LYD3, a landrace completely susceptible to *A. obtectus*, we constructed a segregated offspring population. Through the identification of resistance to *A. obtectus* among the F₁ and F_{2:3} populations, we demonstrated that the trait of bruchid resistance was a quantitative trait and the inheritance of resistance to *A. obtectus* was recessive. This result was consistent with Minney [42].

Genetic linkage maps are a powerful tool for gene mapping and cloning and are generally constructed using various molecular markers, such as SSRs and SNPs. In our study, a high-throughput method for genotyping recombinant populations utilizing whole-genome resequencing data [53, 54, 73, 78] was implemented, and a sliding window approach was designed to collectively examine genome-wide SNPs for genotype calling and recombination breakpoint determination. This method is efficient and fast, and widely used in other crops, such as wheat and cucumber [27, 79]. We constructed a high-density genetic linkage map included 2,234,769 SNP markers for 157 common bean RILs derived from a cross between BKB and LYD3. The number of markers were many more than those in previous studies [12, 14, 24, 59]. It can effectively reduce the influence of the dominant effect and reveal the additive effect of QTLs using an RIL as the mapping population compared to using F₂ mapping population[36].

The level of research on bruchid resistance genes in common bean is relatively low at present, and most of the genes associated with bruchid resistance exist in wild species, such as *Arclin1* to *Arclin8* loci. In this study, we detected one QTL associated with both two traits, PDS and NP, and this QTL mapped to the same region on chromosome 6 between markers Bin1565 and Bin1566 and had additive effects of -27.48 and -0.11, explaining 16.37 % and 16.10 % of the variation, respectively. The additive effects were negative, indicating the beneficial alleles at this locus came from BKB, the resistant parent. We delimited this QTL to an interval of approximately 122.3 kb between SSR marker I6-4 and I6-16, and it may be a major locus for resistance to *Acanthoscelides obtectus* (Say). This QTL was same located on chromosome 6, but approximately a distance of 22.9 Mb upstream of the QTL reported by Kamfwa [30], indicating that the qCBr6 identified in this study was a new bruchid resistance related locus.

The *Phvul.006G003700* encodes a bifunctional inhibitor/seed storage/LTP family protein that belongs to the AAI-LTSS protein superfamily, which is known to play important roles in defending plants from insects and pathogens, in lipid transport between intracellular membranes and in nutrient storage. The results from the search of the SMART Database showed that the *Phvul.006G003700* protein contained an AAI domain. Previous studies have been reported that a double-headed inhibitor (subtilis protease/α-amylase inhibitor and a trypsin/α-amylase inhibitor) in Ragi (finger millet, *E. coracana* Gaertneri) could simultaneously bind a molecular protease (subtilis protease and trypsin) with a molecular α-amylase [57,

60, 65]. The α -amylase was a kind of important digestive enzymes in the saliva and gut of insects [16, 67], and proteinase inhibitors can combine with digestive enzymes to prevent insects from eating [48]. For example, proteinaceous inhibitors from finger millet could inhibit α -amylases of insect pests, and the highest inhibition percentage was found against α -amylase of *Callosobruchus chinensis* [62, 63]. The α -amylases inhibitor is an important biochemical substance in common bean, and plays an important role in insect resistance. The previous studies demonstrated that the seeds of adzuki bean with a transferred α -amylase inhibitor gene could accumulate a high amount of amylase inhibitor and had a high resistance to bruchid [28]. Similarly, one transgenic chickpea and four cowpea lines expressing α AI-1 (an α -amylase inhibitor from common bean) were highly resistant to both bruchid pest species *Callosobruchus chinensis* L. and *Callosobruchus maculatus* F. [38]. Franco et al. [22, 23] confirmed plant α -amylase inhibitors can inhibit the enzymes from the bean weevil *Acanthoscelides obtectus*. According to the analysis of candidate gene sequences, we found that there was a 5bp difference in the promoter region of *Phvul.006G003700* between the two parents, which was just located in the TATA box region. TATA box is an essential transcription regulator and may affect gene expression level. The qRT-PCR analysis of gene *Phvul.006G003700* showed that the expression level of *Phvul.006G003700* in bruchid-resistant parent was much higher than that in bruchid-susceptible parent, which corresponded to the presence of TATA box in resistant parents and the absence of TATA box in susceptible parent due to 5bp insertion. Basí's studies [10] showed that the stepwise deletions of the TATA box of nmt1 gene highly expressed in *Schizosaccharomyces pombe* resulted in a decrease in transcription efficiency and level of expression down-regulated. Therefore, we speculated that the high level of *Phvul.006G003700* gene expression led to the high content of α -amylase inhibitor, thus enhancing the inhibition to α -amylases of *Acanthoscelides obtectus*, and producing anti-insect effect. Further studies for the functional validation of this candidate gene are required, for example using complementation or gene knockout analyses, and physiological and biochemical researches.

Conclusions

we constructed a high-density genetic map by whole-genome resequencing using a RIL population, containing 2,234,769 SNPs. A new major QTL for resistance to *A. obtectus* on chromosome 6 was identified from a common bean cultivar based on the constructed genetic map. *Phvul.006G003700* encodes a bifunctional amylase/protease-inhibited protein and is likely to be a candidate gene responsible for bruchid resistance. This research was very useful for screening and identifying bruchid resistance resources in common bean, and further research on the excavation of bruchid-resistant genes.

Methods

Plant materials

The black kidney bean (BKB), a Chinese cultivar in the Mesoamerican gene pool of common bean that has a determinate bush plant and small black seeds (100-seed weight of $17.053 \text{ g} \pm 1.379$), displays bruchid resistance. Longyundou3 (LYD3), a Chinese landrace originating from the Andean gene pool, is

an indeterminate viny plant with large yellow seeds (100-seed weight of $59.228 \text{ g} \pm 4.004$) with susceptibility to *Acanthoscelides obtectus* (Say). A genetic mapping population was created from a cross between LYD3 and BKB, and the F_6 population, which included 157 recombinant inbred lines (RILs), was obtained after continuous self-crossing. All seeds production was carried out at the experimental field of Heilongjiang Academy of Agricultural Sciences at the Harbin and Changping experimental sites of the Chinese Academy of Agricultural Sciences in Beijing, China.

Evaluation of bruchid resistance

The improved indoor artificial pest grafting method was used to identify the resistance to *Acanthoscelides obtectus* (Say). *Acanthoscelides obtectus* (Say) was collected from local red kidney beans infected with partially feathered bean weevils located in several cities and counties with severe bruchid damage in Yunnan Province. Thirty healthy mature seeds of each single $F_{2,3}$ line and F_6 line were randomly selected, and 50 healthy F_1 seeds and parents were used as controls. Each material was placed into a 5.5-cm-diameter petri dish and randomly placed on the insect rack. The collected feathered adults were placed into the breeding rack. The room temperature was maintained at $18\sim 25^\circ\text{C}$, the humidity was maintained at approximately 70%, and timely ventilation was performed. After 100 days, the damage degree of each material was investigated. The percentage of seeds damaged by bruchids (PDB) and the number of perforations (NP) of the seeds were calculated. The percentage of seeds damaged by bruchids was the percentage of infected seeds in the total number of identified seeds, and the number of perforations was the total number of infected holes of thirty identified seeds in each material.

Genetic map of the RIL population

BKB, LYD3 and 157 RILs from a cross between BKB and LYD3 were used for resequencing. The DNA samples of sufficient quality were randomly interrupted into 350-bp fragments using a Bioruptor Pico ultrasonic crushing machine, and a genomic DNA library construction kit was used for library preparation, including DNA fragment end flattening, adding an 'A' to 3' end, connecting an adaptor with DNA end and PCR amplification. Quality control was performed with the constructed libraries. The libraries of sufficient quality were sequenced on an Illumina HiSeq2500 for high-throughput sequencing, generally using PE150 sequencing mode, that is, double-end sequencing with 150 bp measured at each end. The raw image data files obtained by high-throughput sequencing were analysed by CASAVA base calling and converted into original sequencing sequences, which were called raw data or raw reads. After filtering, clean and effective reads were obtained. The clean reads of the two parents and 157 RILs were compared with the reference genome G19833 v1.0 [59] using BWA software [1]. The single nucleotide polymorphisms (SNPs) of each sample were detected using GATK 3.8 software [19], and tens of thousands of SNP markers were developed.

We combined the SNP markers that were continuously unrecombined in the genome into a bin. First, the sliding window method was used to construct a recombination map. Each sliding window contained 15 SNPs. According to the proportion of SNP markers in the sliding window from the male and female parents, the genotype of the segment in which the sliding window was located was determined, and the location of the recombination breakpoint of the chromosome fragment was identified. A recombination map was then drawn. Second, a bin map was constructed based on the recombination map of all the families in the group. Each bin was used as a marker for genetic marker screening and genetic linkage map construction. Taking the linkage group as a unit, JoinMap 4.0 software [70] was used to construct the genetic linkage map, and the Kosambi algorithm was used to calculate the genetic distance between markers.

QTL mapping for bruchid resistance

Quantitative trait loci (QTLs) were identified by inclusive composite interval mapping (ICIM) using QTL IciMapping 4.2 software [40]. A 1-cM scan window was employed, and the likelihood ratio statistic was computed every 2 cM. The LOD (log of odds) values and PVE were determined based on likelihood ratio tests under a hypothesis allowing both additive and dominance effects. QTLs with LOD values of 3.0 and higher were called for candidate loci.

DNA markers development

The candidate region for bruchid resistance was verified using the analysis of SSR (simple sequence repeats) markers and InDel (insertion-deletion) markers. The SSR markers and InDel markers were designed in primer5 (Premier Biosoft International, USA). According to the location of candidate intervals in the reference genome G19833v1.0 [59], forty-four new SSR markers and 5 InDel markers on chromosome 6 were developed to screen for polymorphisms between BKB and LYD3 (Table S1). Polymerase chain reaction (PCR) was performed in 10 µl reactions containing 20-25 ng of template DNA, 0.2 µmol L⁻¹ forward and reverse primers each 0.5 µL, 2×Taq DNA polymerase Mix 5 µL and suitable nuclease-free water in a T100 Thermal Cycler (Bio-Rad Research, USA). The amplification profile was followed by 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 50 °C-60 °C for 30s, 72 °C for 1min/1kb, and final extension for 10 min at 72 °C. The amplified products were electrophoresed on 8 % nondenaturing polyacrylamide gels, and the bands were visualized by using silver nitrate staining.

Association study of candidate region

One natural population (a total of 625 common bean accessions representing broad genetic diversity, including 504 accessions in China and 124 elsewhere) was used in the association study for candidate region (Table S2). Genotypic data were generated from the whole genome resequencing project of 683 accessions as previously described by Wu [74], and nucleotide variations were further filtered based on

the missing rates ($\geq 10\%$) and a minor allele frequency of < 0.05 . Two traits of 625 common bean accessions, PDS and NP, were assessed in this study. The general linear model (GLM) [17] was used to detect the associations between markers and phenotypes using Tassel 5.0. The P value threshold was 1×10^{-6} , and the markers that exceeded the threshold (1×10^{-6}) were considered to be associated with PDS or NP.

Annotation of candidate genes

The genes of candidate regions were annotated using the annotation file of common bean accession G19833v1.0 [59]. The homologous genes of common bean candidate genes were searched in *Arabidopsis thaliana* and other crops according to the NCBI (<https://www.ncbi.nlm.nih.gov/>) and UniProt (<https://www.uniprot.org/>) databases.

Sequence amplification of candidate genes for bruchid resistance

Based on the results from QTL mapping, gene-specific primer pairs (Table S4) were developed and used to amplify the genomic DNA (gDNA), complementary DNA (cDNA) and promoter sequences of the candidate genes in BKB and LYD3. PCR amplification was performed in 50- μ L volumes containing 100 ng of template DNA, 1 μ L of each forward and reverse primer ($10 \mu\text{mol L}^{-1}$), 25 μ L of 2 $\times$ Trans Start[®] KD Plus Super Mix (-dye) and the corresponding amount of nuclease-free water in a T100 Thermal Cycler (Bio-Rad Research, USA). The thermal cycling programme was as follows: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 53–60 °C for 30 s, and 68 °C for 1 kb/min, and 72 °C for 8 min. The amplified products were electrophoresed on a 1.0% agarose gel, and the target bands were cut and purified using a TIANGel Midi Purification Kit, connected with the *pEASY*[®]-Blunt Cloning vector and transferred into the newly thawed *Trans1*-T1 Phage Resistant Chemically Competent Cell. The connected product was coated on an LB plate containing IPTG, X-gal and ampicillin antibiotics and cultured overnight. White monoclones were selected for detection and sequencing.

Bioinformatics analysis

The domains of the proteins encoded by the candidate genes were identified and annotated using a search of the SMART (Simple Modular Architecture Research Tool) Database (<https://smart.embl.de>). A BLAST search against the NCBI and UniProt databases was performed to find homologous proteins from the common bean G19833 reference genome (*Phaseolus vulgaris*), soybean (*Glycine max*), wild soybean (*Glycine soja*), cowpea (*Vigna angularis* var. *angularis*), mung bean (*Vigna radiata* var. *radiata*), lupine (*Lupinus albus*), wheat (*Triticum aestivum*), maize (*Zea mays*), rice (*Oryza glumipatula*), barley (*Hordeum vulgare* subsp. *vulgare*), *Medicago truncatula*, *Arabidopsis thaliana* and others. Phylogenetic analysis of

the candidate genes from common bean was performed by comparison with homologous sequences from other crops. Phylogenetic trees were constructed using the neighbour-joining method as implemented in MEGA 6.0 software [55, 66] with 1,000 bootstrap replicates. MEME software [7] was used to identify the motifs of conserved sequences.

Candidate gene expression analysis by qRT-PCR

We selected seeds and fresh leaves of BKB and LYD3 for quantitative real-time PCR (qRT-PCR) detection using three biological replicates and three technical replicates. Total RNA was extracted from the seeds using an improved method combining the cold phenol method [69] and TRIzol reagent [33] and reverse transcribed into cDNA using a reverse transcription kit (Tiangen, Beijing, China). qPCR was performed in a 20- μ L volume consisting of 2 μ L of cDNA (1 ng), 0.4 μ L of each gene-specific forward and reverse primer (10 μ M) (Table S4), 10 μ L of 2 $\times$ *TransStart*[®] Top Green qPCR Super Mix (+dye) and a suitable amount of nuclease-free water. The reaction was run on an ABI 7500 real-time PCR machine (Bio-Rad Corporation, USA) using the following programme: 94 °C for 30 s, 40 cycles of 94 °C for 5 s and 56 °C for 15 s and 72 °C for 10 s. The $2^{-\Delta\Delta C_t}$ method [37] was used to analyse the gene expression data from three biological replicates and three technical replicates.

Abbreviations

APA, Arcelin, phytohemagglutinin, and α -amylase

LOD, Logarithm of odds

QTL, Quantitative trait loci

RIL, Recombinant inbred line

LG, Linage group

SNP, Single nucleotide polymorphism

SSR, Simple sequence repeats

InDel, Insertion-deletion

PDS, The percentage of damaged seeds

NP, The number of perforations

AAI, α -amylase inhibitor

qRT-PCR, Quantitative real-time reverse transcription-polymerase chain reaction

Declarations

Ethics approval and consent to participate

The data collection of plants and all methods were carried out with permission of related institution, and complied with national or international guidelines and legislation.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest

Availability of data and material

The data supporting the findings of this study are available from the corresponding author (Jing Wu) upon request.

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Author contributions

XM L performed the map construction, QTL analysis and the characterization of candidate genes. XM L, YS T, YJ C, LF W, J W and SM W provided guidance and conducted the phenotype investigation assays. XM L and J W wrote and revised the manuscript. SM W and J W planned and organized this study. All authors contributed to and approved the final manuscript.

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Tables

Table 1 Description of the basic characteristics of the genetic linkage map

LG ID ^a	Bin number ^b	Effective Bin Number ^c	SNP Number	Map length ^d (cM)	Average distance ^e (cM)	Max Gap ^f (cM)	cM/Mb ^g
LG01	444	364	246412	95.36	0.63	6.46	1.67
LG02	403	288	264367	104.37	0.56	6.01	1.93
LG03	443	366	289659	159.84	0.65	7.81	2.79
LG04	380	328	252752	128.37	0.60	7.05	2.52
LG05	337	198	78079	116.43	0.58	10.56	2.54
LG06	261	191	160566	96.32	0.57	4.71	2.61
LG07	381	286	196439	141.40	0.66	5.77	2.49
LG08	449	417	297249	166.73	0.71	13.39	2.58
LG09	329	134	110344	79.23	0.43	5.36	1.87
LG010	355	177	109441	100.12	0.52	3.19	2.07
LG011	432	357	229461	95.51	0.80	7.63	1.76
Total	4214	3106	2234769	1,283.68	0.61	17.56	2.25

The high-density genetic linkage map in this study contained 11 linkage groups. a, LG ID represents the number of linkage groups. b, Bin number represents the total number of bin markers in each group. c, Effective bin number represents the total number of bin markers after filtering out biased separation markers. d, Map length represents genetic distance of a linkage group. e, Average distance(cM) represents the average genetic distance of bin markers on each linkage group. f, Max gap(cM) represents the biggest gap in each linkage group. g, cM/Mb represents the conversion ratio between genetic distance and physical distance.

Table 2 Location and effects of QTLs controlling resistance to *Acanthoscelides obtectus* detected in RILs

trait	QTL name	Chromosome	Marker interval ^a	Interval size (cM)	LOD ^b	PVE ^c (%)	Add ^d
PDS	qCBr6	6	Bin1565-Bin1566	0.23	5.49	16.10	-0.11
NP		6	Bin1565-Bin1566	0.23	4.31	16.37	-27.48

a, The QTL was located between left and right markers. b, The LOD threshold was determined using 1000 permutations. c, PVE was the phenotypic variation explained by the QTL. d, Add represents the additive effect.

Table 3 Gene annotation of the reference genome G19833 on chromosome 6

Gene name	Region ^a	Gene annotations
<i>Phvul.006G003700</i>	1376123-1378343	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
<i>Phvul.006G003800</i>	1406616-1408098	Carbohydrate-binding X8 domain superfamily protein
<i>Phvul.006G003900</i>	1417873-1419245	-
<i>Phvul.006G004000</i>	1454577-1457697	homolog of yeast ADA2 2B
<i>Phvul.006G004100</i>	1486729-1491368	homolog of yeast ADA2 2A

Horizontal line indicates that no putative conserved domains have been detected.

a, Region represents the physical location of candidate gene in G19833 genome.

Figures

Figure 1

Phenotypic characteristics and frequency distribution of PDS and NP in the $F_{2:3}$ families. The $F_{2:3}$ families were derived from a cross between LYD3 (bruchid-susceptible parent) and BKB (bruchid-resistant parent). **(a)** Phenotypic characteristics of the two parents before infection with bean weevils. **(b)** Phenotypic characteristics of the two parents after infection with bean weevils. The large-yellow seeds on the left are the susceptible parent LYD3, and the small black seeds on the right are from the resistant parent BKB. **(c)** Frequency distribution of the percentage of seeds damaged by *Acanthoscelides obtectus* (Say) and in the $F_{2:3}$ families from a cross of LYD3 and BKB.

Figure 2

Genetic map of RILs from a cross between LYD3 and BKB. A high-density genetic map containing 11 chromosomes was constructed using 157 RILs derived from a cross between LYD3 (bruchid-susceptible parent) and BKB (bruchid-resistant parent). The bar on the left represents the genetic distance. The total genetic distance of the high-density map was 1,283.68 cM, with an average interval of 0.61 cM between the bin markers. Chromosome 8 was the longest with a map length of 166.73 cM, contained 417 bin

markers with 297249 SNPs and had an average genetic distance of 0.71 cM. Chromosome 9 was the shortest, with a map length of 79.23 cM, which included 110344 SNPs with an average genetic distance of 0.42 cM. The black lines represent the relative position of the markers on the genetic linkage group. More of the black lines indicate denser markers.

Figure 3

Genetic and physical maps of QTL loci for resistance to *Acanthoscelides obtectus* (Say) and candidate gene analysis. (a) Genetic map of the QTL for both the reduced percentage of seeds damaged by *A. obtectus* and the number of infected holes in linkage group 6 using 157 RILs derived from LYD3 × BKB. The markers list at the right and left are the genetic positions (cM). (b) Physical map of the bin markers on chromosome 6. The markers list on the left and right are the physical positions in base pairs (bp). (c) Delimitation of the qCBr6 locus to a 122.3 kb region of chromosome 6, and segregation of the PDS and NP trait. (d) Predicted candidate genes for QTL loci associated with bruchid resistance between markers I6-4 and I6-16 according to the physical position on the reference genome common bean accession G19833v1.0.

Figure 4

Sequence differences in promoter region of gene *Phvul.006G003700* between BKB (resistant) and LYD3 (susceptible). There was a 5bp difference on 800bp upstream of the start codon in the promoter region of gene *Phvul.006G003700* between LYD3 and BKB, locating on the essential cis-regulatory element TATA box of the promoter, resulting in the absence of TATA box in the susceptible parent. The TATA box was marked in red box. The red asterisks indicate the different base sites between two sequences.

Figure 5

Relative quantitative expression analysis of the predicted gene *Phvul.006G003700* in seed and leaf tissue of BKB and LYD3 by quantitative real-time PCR (qRT-PCR). The black bars represent BKB (bruchid-resistant parent), and the grey bars represent LYD3 (bruchid-susceptible parent). The horizontal axis of the graph indicates the different tissues, and the vertical axis indicates the relative expression levels. The data are presented as the means of three biological and technical replicates $\pm$ standard errors. ** indicates $P < 0.01$ and *** indicates $P < 0.001$ determined by a *t*-test.

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