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Received: 10 June 2025

Accepted: 12 February 2026

Published online: 18 February 2026

Cite this article as: Amkul K., Laosatit K., Chaisaen P. *et al.* Validation and fine mapping of *qVmunBr6.2* locus reveal a gene encoding hevamine-A, a defense protein with chitinase activity, is associated with bruchid (*Callosobruchus maculatus*) resistance in black gram (*Vigna mungo*). *Sci Rep* (2026). <https://doi.org/10.1038/s41598-026-40341-9>

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Validation and fine mapping of *qVmunBr6.2* locus reveal a gene encoding hevamine-A, a defense protein with chitinase activity, is associated with bruchid (*Callosobruchus maculatus*) resistance in black gram (*Vigna mungo*)

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Abstract

Callosobruchus maculatus (cowpea weevil) is an insect pest that causes significant yield loss in cultivated black gram (*Vigna mungo* var. *mungo*) during storage. A previous study showed that seed resistance to *C. maculatus* in wild black gram (*V. mungo* var. *silvestris*) accession ‘TC2210’ is controlled by two linked QTLs, *qVmunBr6.1* and *qVmunBr6.2*. However, none of these QTL have been validated, and the molecular basis of these QTLs is not yet known. The objectives of this study are to validate *qVmunBr6.2* and identify candidate gene(s) for bruchid resistance at this locus using wild black gram accession ‘TVNu1076’ as the source of resistance. QTL mapping using a F₂ population of the cross ‘Chai Nat 80’ (cultivated black gram) × TVNu1076 showed that *qVmunBr6.2* controls the resistance in TVNu1076. Fine mapping using a large F_{2:3} population of 1,144 plants located

the *qVmunBr6.2* to the marker interval VmBru-SSR74 and VmBru-SSR98. The markers VmBru-SSR74 and VmBru-SSR98 corresponded to a genome region of 9.27 Kb containing two genes including *VmunHev* encoding hevamine-A, a known enzyme involved biotic stress resistance. Compared with the wild black gram TVNu1076, sequence alignment of *VmunHev* revealed two one-base pair substitutions that cause amino acid changes in hevamine-A in the cultivated black grams. Gene expression analysis revealed that *VmunHev* expressed in the seeds. These results showed that *VmunHev* is a strong candidate gene conferring seed resistance to *C. maculatus* in wild black gram. The results present in this study provide insight into the genetic basis of *C. maculatus* resistance in wild black gram.

Keywords Bruchid, Black gram, host-plant resistance, Chitinase, *Vigna mungo*

Bruchids (also known as seed weevils, pulse beetles and seed beetles) (Coleoptera: Bruchidae) is a group of insect pests that feed on the seeds and grains of legume crops during storage, causing significant yield and economic loss worldwide¹⁻³. Among many bruchid species, *Callosobruchus chinensis* (azuki bean weevil) and *Callosobruchus maculatus* (cowpea weevil) are among the most serious bruchid species in tropical and subtropical regions throughout the world. The primary infestation occurs in the field when adult bruchids lay eggs on developing pods; larvae then bore through the pod wall, penetrate into young seeds, and feed and develop concealed inside the seeds³. This infestation causes loss of seed weight, viability, and seed chemical changes⁴. While this primary infestation leads to minimal yield loss, when infested pods and seeds are harvested and stored, adult bruchids emerge from the seeds and initiate secondary infestation by laying eggs directly on the seeds³. Without protection, a seed lot of legumes can be completely infested and destroyed by secondary infestation within 2–3 months due to their short life cycle of only about 22–30 days⁴. In commercial production, bruchids are generally controlled by fumigation with phosphine. However, fumigation can leave chemical residue in legume seeds and food products, increase production costs, and is environmentally unfriendly³. The best way to manage bruchids is through the use of resistant cultivars.

Black gram is an important legume crop in Asia, with a planting area of > 7 million hectares (Mha). India is the largest producer, followed by Myanmar, which has a planting area of 5.60 and 0.95 Mha, respectively⁵. It is grown in rotation with cereal crops such as rice, maize, and wheat or as a component in other cropping systems⁶. Mature seeds of black gram contain approximately 25% protein and 65% carbohydrates⁷. Whole or split seeds of black gram are used to prepare various foods, including soups, cakes, biscuits, snacks, cookies, and doughnuts⁷.

Cultivated black gram (*V. mungo* var. *mungo*) is completely susceptible to *C. maculatus*, although it is perfectly resistant to *C. chinensis*. In contrast, the wild progenitor of black gram (*Vigna mungo* var. *silvestris*) is highly resistant to both *C. maculatus* and *C. chinensis*^{8,9}. Seed resistance to *C. maculatus* in the wild black gram is likely due to antibiosis¹⁰. Wild black gram exhibits three modes of resistance against *C. maculatus*: (i) decreased number of emerged adults, (ii) reduced number of damaged seeds, and (iii) prolonged developmental period of adults⁸⁻¹⁰. A Mendelian genetics analysis based on the percentage of adult emergence (PAE) suggested that resistance to *C. maculatus* in wild black gram is controlled by two dominant duplicate genes (*Cmr1* and *Cmr2*)¹¹. Quantitative trait loci mapping revealed that two QTLs (*Cmrae1.1* and *Cmrae1.2*) controlled PAE, while six QTLs (*Cmrdp1.1*, *Cmrdp1.2*, *Cmrdp1.3*, *Cmrdp1.4*, *Cmrdp1.5*, and *Cmrdp1.6*) controlled the developmental period of the bruchid in the wild black gram accession ‘INGR10133’¹⁰. However, we demonstrated in a recent study that the damage to seeds and the severity of infestation (developmental period) of the bruchid in wild black gram accession ‘TC2210’ are controlled by the same two QTLs, *qVmunBr6.1* and *qVmunBr6.2*¹². These QTLs are intricately linked, being approximately 10.0 centimorgan (cM) apart¹².

Although several QTLs controlling *C. maculatus* resistance have been identified in wild black grams^{10,12} and those QTLs can be used to develop new black gram cultivar(s) with enhanced *C. maculatus* resistance through marker-assisted selection (MAS), none of the QTLs have been validated due to a lack of genomic resources⁶. QTL validation and/or fine mapping are required for accurate and efficient MAS¹³. In addition, the genetic basis of those QTLs is not yet known. Nonetheless, recently, four reference genome sequences of black gram have become publicly available¹⁴⁻¹⁷. These genome sequences are useful for fine mapping and identification of gene(s) responsible for bruchid resistance in black gram. The objectives of this study were to validate the *qVmunBr6.2* and identify candidate gene(s) for

the bruchid resistance at this locus using wild black gram accession ‘TVNu1076’ as the source of resistance.

Methods

Mapping populations and DNA extraction

The F_2 and $F_{2:3}$ populations developed from the hybridization between cultivated black gram accession ‘Chai Nat 80’ (female parent; hereafter referred to as ‘CN80’) and wild black gram accession TVNu 1076 (male parent) were used in this study. CN80 is an improved cultivar from Thailand and is completely susceptible to *C. maculatus*, whereas TVNu1076 is from India and is highly resistant to *C. maculatus*. CN80 was used to construct a reference genome sequence for black gram¹⁴.

The F_2 population comprised 198 plants. These plants and their parental plants were grown in an experimental field at Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand, from March to July 2021. Cultural practices were performed according to Park¹⁸. Mature pods and seeds were harvested from each plant for evaluation of seed resistance to *C. maculatus*. Total genomic DNA was extracted from the young leaves of each plant following the CTAB method described by Lodhi et al.¹⁹. Quantity and quality of the DNA were assessed by gel electrophoresis and spectrophotometric measurements.

The $F_{2:3}$ population comprised 1,144 plants derived from seven F_2 plants that were resistant to *C. maculatus* with percentage of damaged seeds (PDS) value of 0–20% and heterozygous at the *qVmunBr6.2* as determined by markers VmBr-SSR74 and VmBru-SSR77 flanking this QTL (see details in Results). The $F_{2:3}$ plants, along with CN80 and TVNu 1076, were grown in the same field used for the F_2 population from March to July 2023. Cultural practices were performed according to Park¹⁸. Mature pods and seeds of each plant were harvested separately for *C. maculatus* resistance evaluation. DNA extraction of the $F_{2:3}$

population and its quantity and quality assessment were performed in the same manner as described for the F₂ population.

Evaluation of *C. maculatus* resistance

Evaluation of *C. maculatus* resistance in the F₂ and F_{2:3} populations was carried out as described by Somta et al.²⁰ with minor modifications. In brief, cultures of *C. maculatus* were reared on seeds of CN80 at 30°C and 75% relative humidity. Forty seeds harvested from each plant were placed in a transparent plastic box. Twenty-five pairs (25 males and 25 females) of 1-3-day-old adults of *C. maculatus* were introduced and kept in the box for 7 days for egg laying. The infested seeds were maintained at 30°C and 75% relative humidity. Eighty days after insect introduction, the number of seeds damaged by the bruchids (seeds with holes) was counted and converted into the PDS.

Development of New DNA markers and validation of *qVmunBr6.2* in the F₂ population

Previously, *qVmunBr6.2* in wild black gram TC2210 was located between single-nucleotide polymorphism (SNP) markers, Marker28338/Marker14881 and Marker9422/Marker9514¹². These SNP markers were generated using the specific-locus amplified fragment sequencing (SLAF-seq) technique. To develop markers for verifying *qVmunBr6.2* in TVNu1076, we performed a BLASTN search of the sequences of the markers Marker28338, Marker14881, Marker9422, and Marker9514 against the reference genome sequence of CN80¹⁴ (NCBI accession number GCA_013427195.1 and Bioproject accession number PRJNA623719). Once the locations of these markers on the CN80 chromosome were identified, a genomic region covering these four markers was downloaded and searched for simple sequence repeats (SSRs) using Simple Sequence Repeat Identification Tool (SSRIT)²¹. Subsequently, primers for the SSRs were designed using Primer3²². Primers (Supplementary Table S1) were

screened for polymorphisms between CN80 and TVNu1076. Polymerase chain reaction (PCR) and gel electrophoresis were performed as described by Laosatit et al.²³. In brief, PCR was conducted in a total volume of 5 µl containing 5 ng of DNA template, 1 × *Taq* buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 1 U *Taq* DNA polymerase (Thermo Fisher Scientific, United States), and 2.5 µM each of forward and reverse primers. Amplification was performed at 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds using a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, United States). PCR products were separated by 5% polyacrylamide gel electrophoresis and visualized by silver staining. Seven polymorphic markers were selected and used to analyze the F₂ population.

A linkage map was constructed for the F₂ population using QTL IciMapping 4.2²⁴. The markers were grouped using a logarithm of the odds (LOD) value of 5.0 and ordered based on their physical locations on the reference genome sequence of CN80¹⁴. The distance between markers in cM units was calculated using Kosambi's mapping function²⁵. The location of *qVmunBr6.2* on the linkage map was determined by inclusive composite interval mapping (ICIM)²⁶ using IciMapping 4.2. ICIM was performed at every 0.01 cM with the probability in stepwise regression (PIN) of 0.001. The significant LOD score threshold for the QTL was determined by running a 3,000-permutation test at the probability of 0.01.

Narrowing down *qVmunBr6.2* and identification of candidate gene(s) for resistance

After verifying the *qVmunBr6.2* in the F₂ population, the QTL was further narrowed down using the F_{2:3} population. Seven polymorphic SSR markers were used to analyze the population. Among these markers, two were the same as those were used in the F₂ population. SSR marker analysis, linkage map construction, and QTL mapping were conducted in the same manner as described for the F₂ population.

Once *qVmunBr6.2* was narrowed down, the black gram reference genome sequences of CN80¹⁴ were explored to identify candidate gene(s). Annotated gene(s) located between the markers flanking *qVmunBr6.2* were considered candidate gene(s) for *C. maculatus* resistance.

Sequencing of candidate gene *VmunHev*

The candidate gene *VmunHev* at *qVmunBr6.2* was sequenced. The coding sequence (CDS), 5'-untranslated region (5'UTR), and 3'-untranslated region (3'UTR) sequences of *VmunHev* were amplified from the genomic DNA of TVNu1076 and CN80 using the primers listed in Supplementary Table 1. PCR was carried out in a total volume of 10 µl containing 5 ng of DNA template, 1 × Taq buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 1 U KOD-Plus-Neo DNA polymerase (TOYOBO, China), and 0.5 µM each of forward and reverse primers. PCR was performed on a SimpliAmp thermal cycler (Thermo Fisher Scientific, United States) programmed as follows: 94°C for 2 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, and 72°C for 10 minutes. PCR products were run on 1.5% agarose gel electrophoresis to confirm the amplification of a single DNA fragment. The fragment was eluted from the gel and cleaned up. Subsequently, the fragments were sequenced using an ABI 3730xl DNA Analyzer (Applied Biosystems, United States) by Tsingke (Beijing, China). The sequences of TVNu1076 and CN80 were aligned with the reference sequences CN80¹⁴, JP256029 (Subsmotod)¹⁷ and Pant U-31¹⁵ to identify polymorphisms using Clustal Omega²⁷. The CDSs of TVNu1076 and CN80 were translated into protein sequences using Expasy (2003; <https://web.expasy.org/translate>), and the resulting sequences were aligned to find amino acid polymorphisms using Clustal Omega.

Expression analysis of *VmunHev*

TVNu1076 and CN80 were grown in a crossing block under open field condition. Total RNA was extracted from the seed coat and seeds (cotyledons and embryos) of these two accessions using procedures reported by Laksana and Chanprame²⁸. The RNA was then converted into complementary DNA (cDNA) using the RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Scientific, United States). The cDNA was used for quantitative real-time PCR (qRT-PCR) to determine the expression level of the *VmunHev* gene. qRT-PCR was performed with three biological and technical replicates using the ViiA 7 Real-Time PCR System (Applied Biosystems, United States). Primers for qRT-PCR of the *VmunHev* gene and the reference gene *VmACTIN* are listed in Supplementary Table 1. The qRT-PCR was conducted according to Laositit et al.²³. The $2^{-\Delta\Delta CT}$ method²⁹ was used to calculate relative levels of gene expression. Statistical difference in gene expression between TVNu1076 and CN80 was tested by *t*-test using the R program³⁰.

Phylogenetic analysis of hevamine/chitinase proteins

The *VmunHev* protein sequence, along with chitinase protein sequences from soybean (*Glycine max* (L.) Merr.) (32 sequences) and *Arabidopsis thaliana* L. (24 sequences) reported by Lv et al.³¹, were subjected to phylogenetic analysis using Molecular Evolutionary Genetics Analysis (MEGA) 11.0³². The sequences were aligned using the CLUSTAL W function present in the software MEGA, and a phylogenetic tree was constructed using the maximum likelihood method with a bootstrap value of 1,000. The phylogenetic tree was drawn using the Interactive Tree of Life (iTOL) version 6³³.

Results

Validating *qVmunBr6.2* controlling *C. maculatus* resistance

In this study, we determined whether *qVmunBr6.2*, which governs *C. maculatus* resistance previously detected in wild black gram accession TC2210, also controls resistance in the wild black gram accession TVNu1076. The PDS caused by *C. maculatus* in the F₂ population from the cross CN80 × TVNu1076 was assessed. 80 days after insect introduction (DAI), PDS varied between 0.0 and 100.0%, with a mean of 16.0%. CN80 was highly susceptible, with a PDS of 79.8%, whereas TVNu1076 was nearly perfectly resistant, with a PDS of 2.0% (Fig. 1A). The frequency distribution of PDS in the F₂ population showed a continuous distribution and skewed toward TVNu1076 (Fig. 1A).

The *qVmunBr6.2* in TC2210 was previously localized between SNP markers Marker28338/Marker14881 and Marker9422/Marker9514. In this study, a BLASTN search revealed that these markers were located at positions 43,617,315, 43,156,600, 43,096,644, and 43,155,494 on chromosome 3 of the reference genome of CN80 (Supplementary Table S2). Thus, these markers occupied a genome region of 520.67 Kb (43,096,644..43,617,315). A total of 99 SSR markers were developed within and around this genome region (Supplementary Table S1). Marker polymorphism screening between CN80 and TVNu1076 identified 50 polymorphic markers (Supplementary Table S1). However, nine polymorphic markers were selected for analysis of the F₂ population. A genetic linkage map constructed for the F₂ population using nine polymorphic SSR markers was only 1.26 cM in length (Figs. 2A and 3). Markers VmBr-SSR66 and VmBr-SSR69 were mapped to the same location, while markers VmBr-SSR74 and VmBr-SSR98 were also mapped to the same location. The average map distance between marker positions was 0.25 cM. QTL analysis using the ICIM method identified *qVmunBr6.2* between the markers VmBr-SSR74/VmBru-SSR98 and VmBru-SSR77 (Figs. 2A and Table 1). The *qVmunBr6.2* accounted for 28.90% of the PDS variation in the F₂ population and exhibited additive and dominant effects of 16.09 and -3.39,

respectively. Nonetheless, QTL mapping confirmed that *qVmunBr6.2* controls the *C. maculatus* resistance in wild black gram.

Narrowing down the *qVmunBr6.2*

To narrow down *qVmunBr6.2* controlling the *C. maculatus* resistance, a large F_{2:3} population (1,144 plants) was developed by combining F₃ plants derived from seven F₂ plants showing heterozygous *qVmunBr6.2* detected by markers VmBr-SSR74, VmBru-SSR98, and VmBru-SSR77 (Supplementary Table S3). In the F_{2:3} population, PDS caused by *C. maculatus* varied between 0.0 and 84.0%, with a mean of 9.1% (Figure 1B). CN80 was highly susceptible, with a PDS of 82.0%, whereas TVNu1076 was highly resistant, with a PDS of 1.9%. The frequency distribution of the PDS in the F_{2:3} population exhibited the same distribution pattern as in the F₂ population (Fig. 1B).

A linkage map was constructed for the F_{2:3} population using seven SSR markers. The map was 0.52 cM in length (Figs. 2B and 3) with an average distance between markers of 0.09 cM. ICIM detected *qVmunBr6.2* between the markers VmBru-SSR74 and VmBru-SSR98 (Fig. 2B). These two markers were only 0.05 cM apart. The QTL accounted for 8.30% of the PDS variation in the F_{2:3} population and exhibited an additive effect of 5.25 and a dominant effect of -1.87 (Table 1). It is worth noting that the physical locations of VmBru-SSR74 and VmBru-SSR98 on the CN80 genome were 43,321,875 and 43,331,149 on chromosome 3, respectively, covering a genome region of only 9.274 Kb.

***VmunHev* Encoding Hevamine is the Candidate Gene for Bruchid Resistance**

Based on the results from QTL mapping and the reference genome sequence of CN80, there were only two open reading frames between the markers VmBru-SSR74 and VmBru-SSR98, ORF1 (*evm.model.Scaffold_5190_HRSCAF=5891.8250*) and ORF2

(*evm.model.Scaffold_5190_HRSCAF=5891.8251*) (Fig. 3). These two ORFs were designated *VmunHev* and *VmunPKM*, respectively. *VmunHev* was predicted to encode hevamine-A (Supplementary Table S4), a defense protein known for its role in resistance to biotic stresses. In contrast, *VmunPKM* was predicted to encode pyruvate kinase (Supplementary Table S4), an enzyme that catalyzes the conversion of phosphoenolpyruvate and ADP to pyruvate and ATP in glycolysis, playing a role in regulating cell metabolism. Thus, *VmunHev* was selected as the candidate gene for *C. maculatus* resistance.

The *VmunHev* is an intronless gene with a total length of 1,331 bp (Supplementary Figure S1). *VmunHev* was sequenced in TVNu1076 and CN80 using the Sanger sequencing method, and the sequences were aligned to identify nucleotide polymorphism(s). The *VmunHev* sequences from cultivated black gram cultivars Pant U-31 and Subsmotod (Supplementary Data 1) were also included in the sequence alignment. The *VmunHev* sequence obtained from Sanger sequencing of CN80 (GenBank accession PQ790604) was identical to that of the CN80 reference sequence. The sequence alignment revealed that all three cultivated black grams were identical in the *VmunHev* sequence (Fig. 4). However, the alignment demonstrated that compared to the wild black gram TVNu1076, there were five single-base substitutions and a 4-bp (CATG) insertion in the cultivated black grams (Fig. 4). Three of the substitutions were in the coding sequence (CDS): A101G, A325C, and G712T. The other two substitutions and the insertion were in the 3' UTR.

Alignment of the predicted *VmunHev* protein sequences revealed two amino acid polymorphisms between the wild and cultivated black grams, specifically T25A and K227N (Fig. 5). These amino acid polymorphisms are caused by the substitutions A101G and G712T, respectively.

Expression of *VmunHev* gene in seeds

Gene expression analysis by qRT-PCR revealed that *VmunHev* is expressed in the seed coat and seeds (cotyledons and embryos) of both accessions at both green and yellow pod stages of CN80 and TVNu1076. At the green-pod stage, the expression of *VmunHev* in the seed coat and seeds without seed coat was not significantly different between the two accessions (Figs. 6A and 6B). At the yellow-pod stage, the expression of *VmunHev* was statistically different between the two accessions in both the seed coat and seeds without seed coat (Figs. 6A and 6B). However, the expression of *VmunHev* in the seed coat contrasted with that in the seeds without the seed coat. In the seed coat, the expression in TVNu1076 was about 18-fold greater than that in CN80, while in the seeds without seed coat, the expression in TVNu1076 was less than that in CN80.

Phylogenetic analysis of *VmunHev* proteins and chitinases

A phylogenetic analysis of chitinases revealed five major groups, namely groups I, II, III, IV, and V (Fig. 7). The analysis confirmed that *VmunHev* is a chitinolytic enzyme (Fig. 7) and indicated that *VmunHev* belongs to chitinase cluster III. *VmunHev* was closely related to Glyma.20G164600 and Glyma.10G227700 and clustered with hevamine from the rubber tree (P23472) (Fig. 7). Notably, 11 chitinases previously identified as members of chitinase “cluster II,” including Glyma.02G007400, Glyma.08G259200, Glyma.09G038500, Glyma.10G138400, Glyma.15G143600, Glyma.18G283400, Glyma.19G221800, AT1G02360, AT1G05850, AT3G16920, and AT4G01700, were separated into two different groups (six in groups IV and five in group V) (Fig. 7).

Discussion

Seed yield loss in legume crops during storage due to infestation by bruchid species of the genus *Callosobruchus* is a significant problem contributing to food insecurity in developing countries^{4,34}. Plant breeders have long been interested in understanding the genetics and molecular basis of seed resistance to bruchids. Generally, cultivated legume seeds are susceptible to bruchid infestation, while their wild progenitors/relatives exhibit resistance^{3,9,36}. Wild black gram is known for its high resistance to *C. maculatus*. Thus, unraveling the molecular basis of the resistance in wild black gram can be useful for the molecular breeding of cultivated black gram and other related legume crops. In this study, we demonstrated that the QTL *qVmunBr6.2* conferring *C. maculatus* resistance in wild black gram accession TC2210, as reported previously by Somta et al.¹² also control the resistance in wild black gram accession TVNu1076 (Figs. 2 and 3). Our results validated that the role of *qVmunBr6.2* in different wild black gram germplasm in conferring *C. maculatus* resistance, and thus the *qVmunBr6.2* can be useful in improving *C. maculatus* resistance in cultivated black gram.

The *qVmunBr6.2* locus was previously mapped between the SNP markers: Marker28338 and Marker9514¹². Based on the black gram reference genome sequence¹⁴, these flanking markers occupied a large genome region of 520.76 Kb, harboring many genes. However, by utilizing a large F_{2:3} population and exploiting reference genome information, we narrowed down the *qVmunBr6.2* locus using SSR markers to a small genomic region of only 9.27 Kb containing only two genes (Fig. 3). The effect of the *qVmunBr6.2* on PDS estimated for TVNu1076 in this study (phenotypic variance explained by QTL (PVE) = 8.30% in the F_{2:3} population) is comparable to that estimated for wild black gram TC2210 reported by Somta et al.¹² (PVE = 10.61% in the recombinant inbred line (RIL) population). Nonetheless, the PVE of the *qVmunBr6.2* in the F_{2:3} population was much lower than that in the F₂ population (Table 1). In general, the effect of a QTL is overestimated when small

populations are used for QTL mapping^{37,38}. So, the reduction of QTL effect at the *qVmunBr6.2* detected in the F_{2:3} population is likely due to a much population size compared with the F₂ population.

Seed defense chemicals are among the factors contributing to resistance to bruchids^{3,35-36,39}. Compared to *C. maculatus* feeding on seeds of cultivated black gram, those feeding on seeds of wild black gram exhibited longer metamorphosis from egg to adult and a lower percentage of adult emergence from seeds^{9-10,12,36}. This indicates that *C. maculatus* resistance in wild black gram is due to seed antibiosis. In this study, *VmunHEV* encoding hevine-A was identified as the candidate gene for the *C. maculatus* resistance at the locus *qVmunBr6.2*, although there was another gene in the *qVmunBr6.2* region (Fig. 3). Hevine is an endochitinase first isolated from vacuoles in the latex of the rubber tree (*Hevea brasiliensis* L.)⁴⁰. Chitinases are hydrolytic enzymes that cleave β -1,4-glycosidic bonds in chitin, a polymer of β -1,4-linked N-acetyl-D-glucosamine (GlcNAc) that constitutes a major component of the cell wall of many fungi, as well as the exoskeleton (cuticle) and peritrophic membrane/matrix (PM) of insects⁴¹. The PM plays important roles in digestion in the insect gut and protects insects from invasion by microorganisms and parasites⁴². In fact, all insects produce chitinases in different tissues throughout their life cycles for cuticle turnover⁴³. Throughout the life cycle of insects, they periodically shed their old exoskeleton and PM either continuously or periodically and resynthesize new ones by using their chitinases⁴⁴⁻⁴⁵. Nonetheless, plant chitinases can exhibit insecticidal activity by targeting and degrading the chitin component of the insect cuticle and PM, resulting in arrested growth, development, and death of the insects⁴⁴⁻⁴⁵. Therefore, chitinases are plant defense proteins that play a key role in insect resistance. Gomes et al.⁴⁶ showed that a chitinase from cowpea seeds significantly negatively affected the larval development of *C. maculatus*, although its effect on larval survival was not high. Ferreira et al.⁴⁷ showed that chitin-binding proteins (CBP), including

chitinases and vicilins from cowpea seeds, significantly decreased larval mass and length of *C. maculatus*. Silva et al.⁴⁸ showed that a chitinase from soybean seeds (*Glycine max* (L.) Merr.) greatly reduced larval survival and weight of *C. maculatus*. Additionally, Khan et al.⁴⁹ demonstrated that a chitinase-homolog protein from mungbean seeds is associated with resistance/susceptibility to bruchid species *C. chinensis*. These findings highlight the insecticidal effect of plant chitinases on *C. maculatus* and related bruchid species, supporting our conclusion that *VmunHev* is a strong candidate gene for the *C. maculatus* resistance in the wild black gram. The differences in the *VmunHev* protein sequence may account for the contrasting resistance/susceptibility to *C. maculatus* between cultivated and wild black grams (Fig. 5).

At the yellow-pod stage (maturation), TVNu1076 and CN80 showed opposite expression patterns of *VmunHev* in the seed coat and seed without seed coat (Figs. 6A and 6B). In the seed coat, the resistant TVNu1076 had much higher expression compared to the susceptible CN80 (Fig 6A), suggesting that TVNu1076 plants concentrates hevine-A in the seed coat. When bruchid larvae hatch on the surface of the legume seeds or pods, they must bore through the seed coat to reach the nutrients inside. So, the high concentration of hevine-A in the seed coat of TVNu1076 likely kills or inhibits the larvae before they can penetrate the seed. Conversely, inside the seed (cotyledons/embryo), TVNu1076 had lower expression than CN80 (Fig 6B). This suggests an efficient allocation of metabolic resources in TVNu1076. Because the TVNu1076 seed coat acts as an impenetrable barrier, it does not need to waste energy producing a high level of hevine-A inside the cotyledon.

Previous studies on bruchid resistance in *Vigna* species showed that genes encoding protein inhibitors provide resistance to bruchids; *PGIPs* encoding polygalacturonase inhibitors in mungbean and moth bean (*Vigna aconitifolia* (Jacq.) Verdc.)⁵⁰⁻⁵² and *TaXI* encoding a xylanase inhibitor in zombi pea (*Vigna vexillata* (L.) A. Rich)³⁶. In this study, we

showed that *VmunHEV* encoding hevine-A is a strong candidate gene conferring bruchid resistance in black gram (Figs. 3–6). Altogether, these studies indicate that diverse genes/chemicals provide bruchid resistance in legume species of the genus *Vigna*. These genes can be manipulated to develop cultivars with durable resistance to bruchids in legume crops through biotechnology approaches. However, it is noteworthy that all previous reports on the biotic defense properties of hevine were based solely on the prediction of its protein structure or *in vitro* functions of lysozymes/chitinases^{53–57} without supportive genetic evidence. Our finding that *VmunHev* was genetically associated with *C. maculatus* resistance (Fig. 3) provides the first line of evidence on biotic defense role of hevine. Nonetheless, additional studies should be conducted to determine the insecticidal effect of hevine-A (*VmunHev*) in wild black gram toward *C. maculatus*.

MAS can save 5–7 months in each selection cycle for black gram seed resistance to bruchids compared with phenotypic selection. SSR marker analysis is a standard and widely used method for MAS because SSRs are highly reproducible, co-dominant in inheritance, simple and inexpensive to use, and highly polymorphic¹³. In this study, several SSR markers locating physically remarkably close to the *qVmunBr6.2* locus and the candidate gene *VmunHEV* for the resistance were developed (Supplementary Table S1 and Fig. 3). These markers will be useful for MAS for bruchid resistance in black gram.

In conclusion, we developed novel SSR markers by exploiting the reference genome sequence of black gram and used these SSR markers to validate and finely map the *qVmunBr6.2* locus conferring seed resistance to *C. maculatus* in wild black gram accession TVNu1076. QTL mapping using the F₂ population from the cross between Chai Nat 80 and TVNu1076 validated that *qVmunBr6.2* controls *C. maculatus* resistance in wild black grams. Fine mapping revealed *VmunHEV* encoding hevine-A as the candidate gene for this

resistance. To our knowledge, these are the first findings at the molecular genetics level, on the involvement of the hevine protein in plant resistance to biotic factors. *VmunHEV* sequence variations in *VmunHEV* between wild black gram (bruchid-resistant) and cultivated black grams (bruchid-susceptible) revealed in this study provide opportunities to develop functional or perfect DNA markers for MAS of *VmunHEV* from wild black gram conferring *C. maculatus* resistance.

Data availability statement

All data generated during this study are included in this published article and its supplementary information files. The datasets used and/or analysed during the current study available from the corresponding author on reasonable request. The sequences of *VmunHev* are deposited to the National Center for Biotechnology Information (NCBI; <https://www.ncbi.nlm.nih.gov>), accession numbers PQ790604 and PQ790605 (email to info@ncbi.nlm.nih.gov).

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Acknowledgements

This research was supported by Kasetsart University Research and Development Institute (KURDI). We are thankful to Miss Tarika Yimram for field assistance.

Author contributions

PS and KL conceived and designed the studies. KA, PC, and KL carried out field experiments and phenotyping. KA, KL, PC, YL, and XY conducted DNA marker analysis, gene expression analysis and DNA sequencing. KA, KL and PS analyzed data. PS, KA, XY and XC secured research funding. PS and XY supervised and coordinated the study. PS, KA and KL wrote and revised the manuscript. All authors read and approved the final version of the manuscript.

Funding

This research was supported by NSRF via the Program Management Unit for Human Resources and Institutional Development, Research and Innovation (grant number

B16F640185 and B13F670075), and China Agriculture Research System of MOF and MARA-Food Legumes (grant number CARS-08-G15).

Declarations**Completing interests**

The other authors declare no competing interests.

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Figure captions

Fig. 1 Frequency distribution of the percentage of damaged seeds caused by *Callosobruchus maculatus* in black gram F₂ and F_{2:3} populations from the cross Chai Nat 80 (CN80) × TVNu1076.

Fig. 2 Log of odds (LOD) graph of QTL *qVmunBr6.2* identified by inclusive composite interval mapping in F₂ (A) and F_{2:3} (B) populations from the cross Chai Nat 80 × TVNu1076. The line parallel to the x-axis represents the significant LOD threshold for the QTL.

Fig. 3 A comparative map showing the location of *qVmunBr6.2* detected on the linkage group in the F₂ (top) and F_{2:3} (middle) populations and its position on the reference genome sequence of the black gram cultivar Chai Nat 80 (bottom). DNA markers flanking *qVmunBr6.2* are highlighted in red. The dotted line connects common DNA marker between linkage groups and indicates its physical location on the chromosome. Predicted genes (open-reading frames (ORF)) exist between markers VmBru-SSR69 and VmBru-SSR77 are shown.

Fig. 4 *VmunHev* sequence alignment between wild black gram TVNu1076 and cultivated black grams Chai Nat 80, Pant U-31, and Subsmotod. TVNu1076 is resistant to *C. maculatus*, while Chai Nat 80, Pant U-31 and Subsmotod are susceptible to *C. maculatus*. The number in parenthesis upper/under nucleotide(s) indicates its position from the first nucleotide of the start codon.

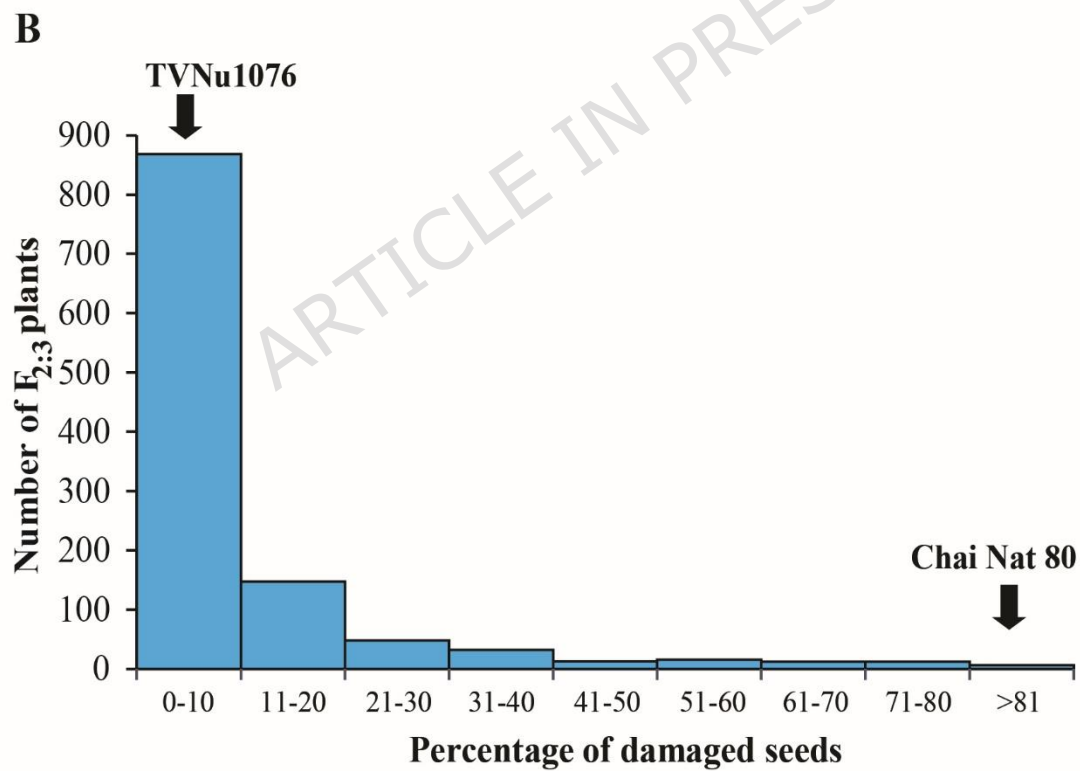
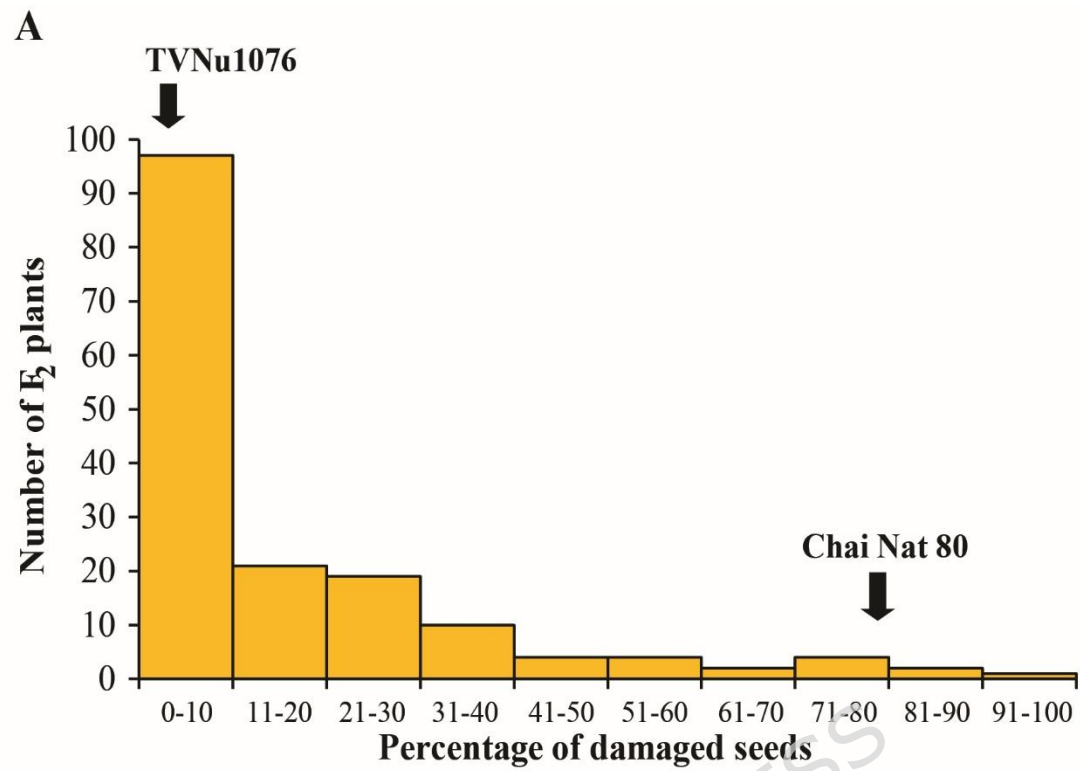
Fig. 5 *VmunHev* protein sequence alignment between wild black gram TVNu1076 and cultivated black grams Chai Nat 80, Pant U-31, and Subsmotod. TVNu1076 is resistant to *C.*

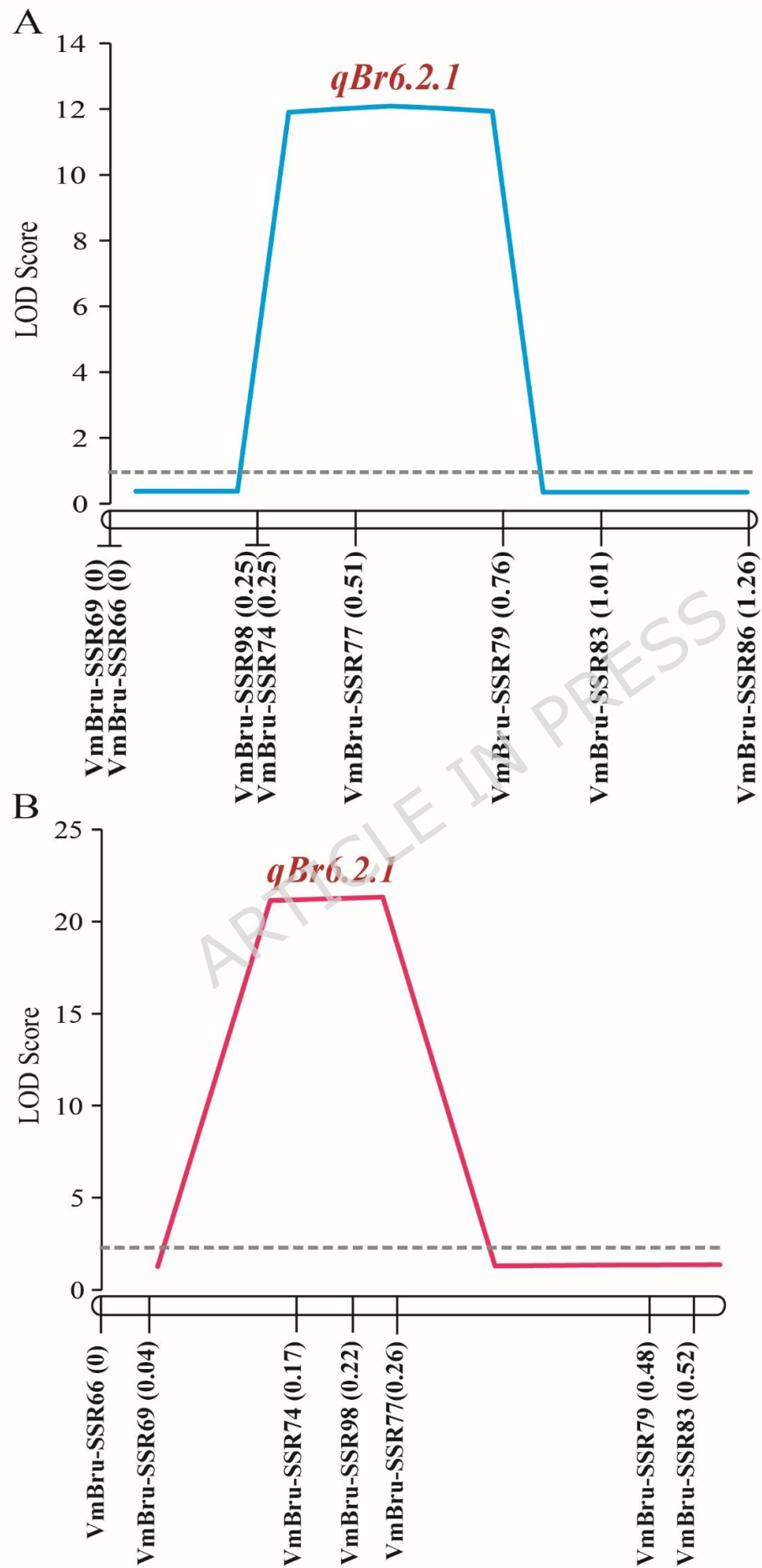
maculatus, while Chai Nat 80, Pant U-31 and Subsmotod are susceptible to *C. maculatus*.

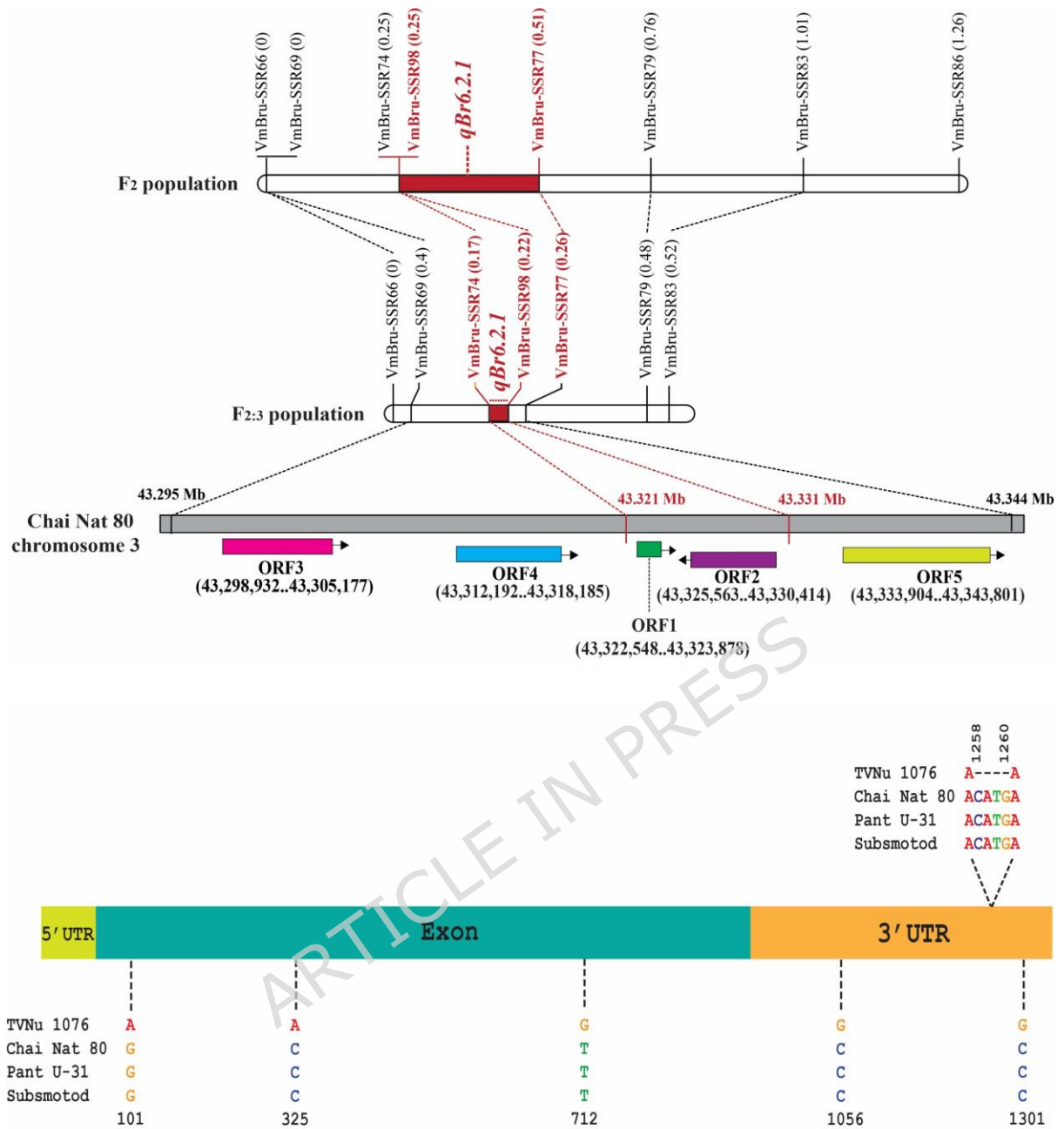
Asterisk (*) indicates a conserved amino acid. Polymorphism sites are highlighted in yellow.

Fig. 6 Relative gene expression level of *VmunHev* determined by qRT-PCR in seed coat (A) and seeds without seed coat (B) at green-pod and yellow-pod stages of CN80 and TVNu1076. The difference in expression level is determined by *t*-test.

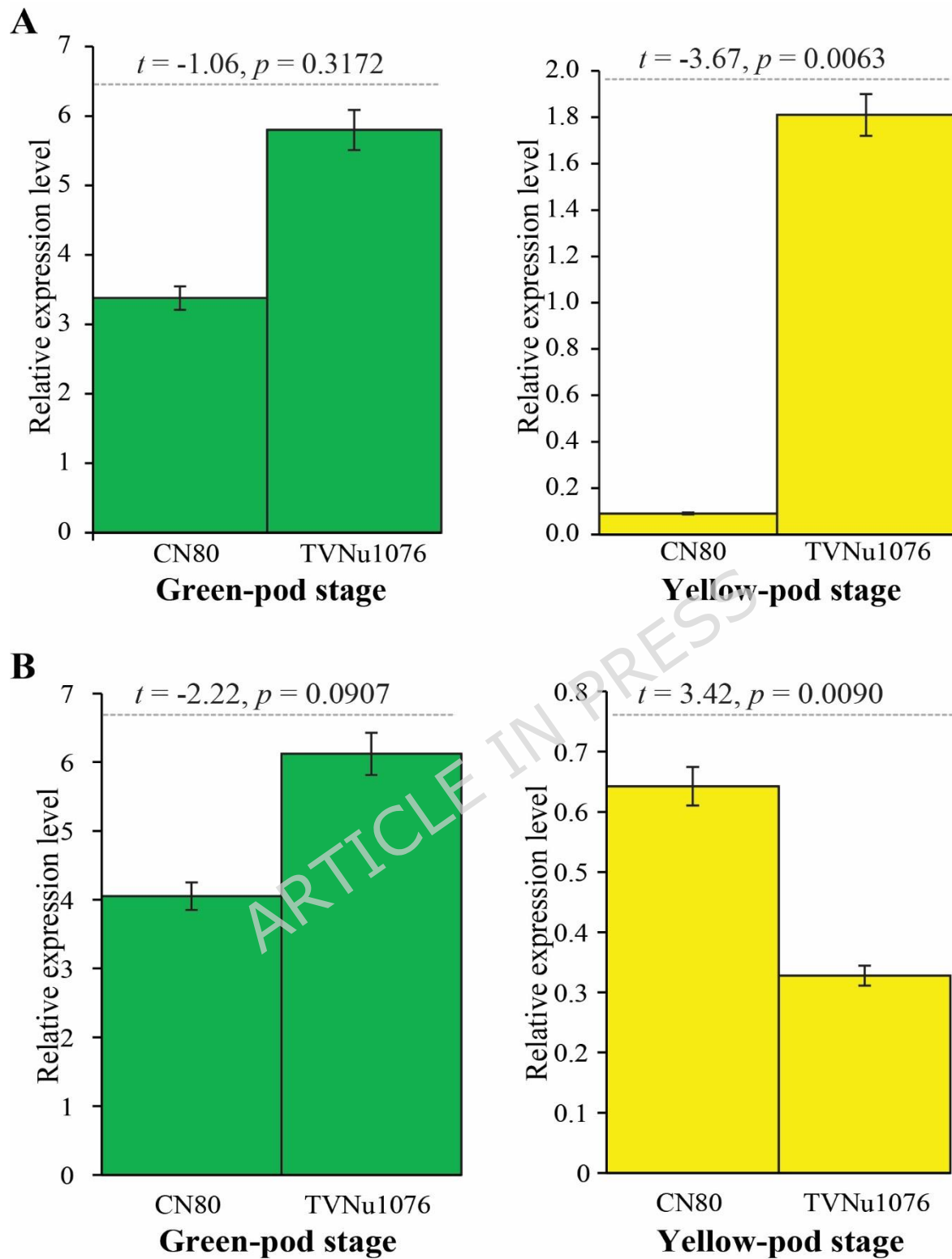
Fig. 7 A phylogenetic tree depicting the relationship of hevamines from black gram (*VmunHev*) and rubber tree (P23472) and chitinases from soybean and *Arabidopsis thaliana*. Chitinases with the prefix Glyma and AT are from soybean and *Arabidopsis thaliana*, respectively. The tree is constructed using the maximum likelihood method. Groups (I to V) are named as per Lv et al.³¹. Chitinases that were previously grouped into Group II by Lv et al.³¹ but are grouped into Groups IV or V in this study are underlined.







TVNu 1076	MGKSKNSDDFSLLLPLFFFTLVGTSHAGGIAIYWQNGNEGTLSEACATGRYTHVNIAFL	60
Chai Nat 80	MGKSKNSDDFSLLLPLFFFTLVGASHAGGIAIYWQNGNEGTLSEACATGRYTHVNIAFL	60
Pant U-31	MGKSKNSDDFSLLLPLFFFTLVGASHAGGIAIYWQNGNEGTLSEACATGRYTHVNIAFL	60
Subsmotod	MGKSKNSDDFSLLLPLFFFTLVGASHAGGIAIYWQNGNEGTLSEACATGRYTHVNIAFL *****:*****	60
TVNu 1076	NKFGNGQTPEMNLAGHCNPATNSCTKFSAEIKDCQSKNIKVLLSIGGGIGSYTLASVEDA	120
Chai Nat 80	NKFGNGQTPEMNLAGHCNPATNSCTKFSAEIKDCQSKNIKVLLSIGGGIGSYTLASVEDA	120
Pant U-31	NKFGNGQTPEMNLAGHCNPATNSCTKFSAEIKDCQSKNIKVLLSIGGGIGSYTLASVEDA	120
Subsmotod	NKFGNGQTPEMNLAGHCNPATNSCTKFSAEIKDCQSKNIKVLLSIGGGIGSYTLASVEDA *****	120
TVNu 1076	RNVSTFLWNTFLGGKSSTRPLGDAVLGDGIDFDIEQGSTENYEHLARFLKAYSRKGRVYL	180
Chai Nat 80	RNVSTFLWNTFLGGKSSTRPLGDAVLGDGIDFDIEQGSTENYEHLARFLKAYSRKGRVYL	180
Pant U-31	RNVSTFLWNTFLGGKSSTRPLGDAVLGDGIDFDIEQGSTENYEHLARFLKAYSRKGRVYL	180
Subsmotod	RNVSTFLWNTFLGGKSSTRPLGDAVLGDGIDFDIEQGSTENYEHLARFLKAYSRKGRVYL *****	180
TVNu 1076	GAAPQCFIPDRFLGTALDTGLFDFVWVQFYNNPPCQYADGNVTNLLKSWNRWTSTVPAGK	240
Chai Nat 80	GAAPQCFIPDRFLGTALDTGLFDFVWVQFYNNPPCQYADGNVTNLLNSWNRWTSTVPAGK	240
Pant U-31	GAAPQCFIPDRFLGTALDTGLFDFVWVQFYNNPPCQYADGNVTNLLNSWNRWTSTVPAGK	240
Subsmotod	GAAPQCFIPDRFLGTALDTGLFDFVWVQFYNNPPCQYADGNVTNLLNSWNRWTSTVPAGK *****:*****	240
TVNu 1076	IFLGLPAAPAAAGSGFIPADVLTSQILPVIKESPKYGGVMLWSRFFDVQNGYSTSIIGSV	300
Chai Nat 80	IFLGLPAAPAAAGSGFIPADVLTSQILPVIKESPKYGGVMLWSRFFDVQNGYSTSIIGSV	300
Pant U-31	IFLGLPAAPAAAGSGFIPADVLTSQILPVIKESPKYGGVMLWSRFFDVQNGYSTSIIGSV	300
Subsmotod	IFLGLPAAPAAAGSGFIPADVLTSQILPVIKESPKYGGVMLWSRFFDVQNGYSTSIIGSV *****	300



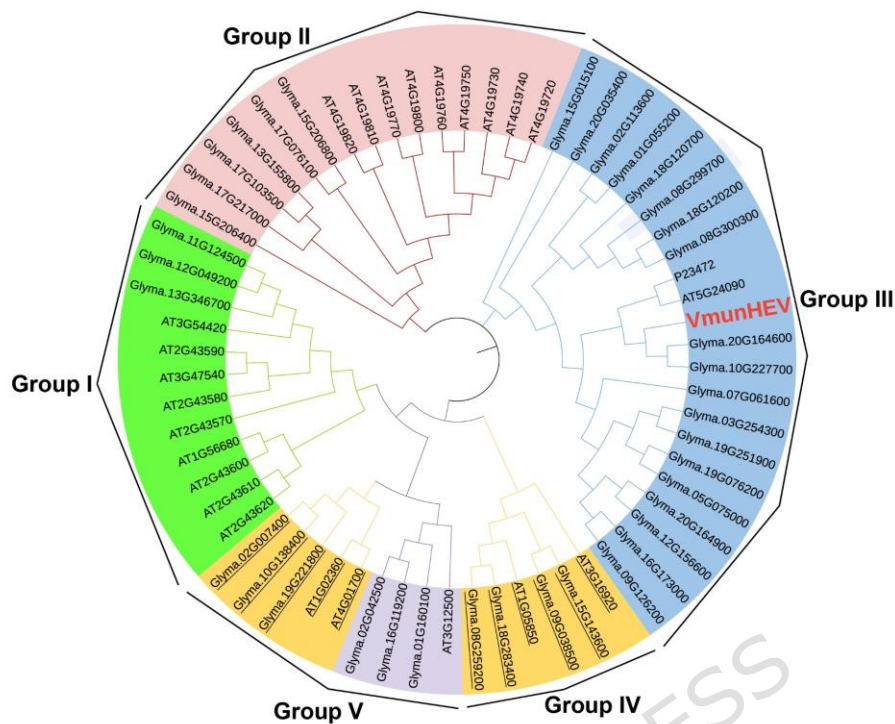


Table 1 Location and effect of *qVmunBr6.2* controlling bruchid resistance detected in F₂ and F_{2:3} populations from the cross Chai Nat 80 × TVNu1076 by the inclusive composite interval mapping method.

Popula tion	QTL name	Position on	Marker interval	LO D	PVE ¹ (%)	Addit ive	Domin ant
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		linkage		sco		effect	effect
		map (cM)		re			
F ₂	<i>qBr6.2.</i>	0.5	VmBru-SSR98–	12.	28.90	16.09	-3.39
	<i>1</i>		VmBru-SSR77	09			
F _{2:3}	<i>qBr6.2.</i>	0.2	VmBru-SSR74–	21.	8.30	5.25	-1.87
	<i>1</i>		VmBru-SSR98	33			

¹PVE percentage of variance explained by QTL