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Research article

Comparative transcriptomic analysis reveals genes encoding polygalacturonase inhibitors and lectins as promising candidates conferring bruchid (*Callosobruchus chinensis*) resistance in moth bean (*Vigna aconitifolia*)

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Abstract

Importance of the work: Yield loss caused by the seed beetles (also known as bruchids or seed weevils) *Callosobruchus chinenesis* and *Callosobruchus maculatus* is a serious problem in several legume crops. Enhancing seed resistance to these bruchids is a major goal in legume breeding programs. Moth bean (*Vigna aconitifolia*) is an underutilized crop with only a few reports on genomic research. The current study used comparative RNA-seq-based transcriptomic analysis to identify candidate genes for bruchid resistance in moth bean.

Objectives: To identify candidate genes for bruchid resistance in moth bean (*Vigna aconitifolia*), a hardy minor legume crop.

Materials & Methods: The moth bean accessions TN67 (wild and resistant) and IPCMO056 (cultivated and susceptible) were grown under controlled conditions. RNA-seq-based transcriptomic analysis was performed on the immature seeds and semi-mature seeds of the two cultivars. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was used to confirm the genes with probable function for bruchid resistance that showed different expression.

Results: The transcriptomic analysis showed that a gene encoding a polygalacturonate inhibitor and two genes producing lectins had differential expression. These genes were up-regulated at both the immature and semi-mature stages, albeit in each case, their expression was significantly higher in the mature stage. RT-qPCR analysis confirmed the up-regulated expression of these genes. Altogether, these results suggested that genes encoding a polygalacturonase inhibitor and lectins could be candidate genes for bruchid resistance in moth bean.

Main finding: Genes relating to plant defense in the seeds of moth bean were identified. Genes encoding a polygalacturonase inhibitor and lectins were identified as candidate genes conferring bruchid resistance in the moth bean accession TN67.

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Introduction

The moth bean [*Vigna aconitifolia* (Jacq.) Marechal] belongs to the genus *Vigna* within the family Fabaceae. It is an underutilized crop mainly grown in semi-arid and arid areas of South Asia, with the dry seeds being used to prepare several types of confectionary items due to their higher proportions of albumin and glutamine fractions (Siddhuraju, 2006). The moth bean green pods are used as vegetables and after harvesting, the other plant parts are used as fodder, hay and green manure.

The moth bean is a hardy legume that can survive in dry, arid and sandy soils under tropical climates due to its distinctive traits of drought and heat tolerance (Garg et al., 2001; Tiwari et al., 2018), which have made it a priority crop in legume breeding where the adaptation to climate change is of growing importance in order to meet the dietary protein requirements in drier parts of the world. However, susceptibility to pests is the major drawback for its commercial cultivation.

Bruchids (also known as seed beetles and seed weevils) are serious post-harvest insect pests that cause severe yield losses in several legume crops, with the azuki bean weevil (*Callosobruchus chinensis* L.) and cowpea weevil (*Callosobruchus maculatus* Fab.) being the most serious bruchids of moth bean (Somta et al., 2018). In fact, these bruchids are the most important stored-insect pests of legumes in the tropical and sub-tropical regions and have become cosmopolitan insect pests due to international seed trading, albeit their origins are in the specific areas in the world (Srinives et al., 2007). Bruchids can lead a complete loss of seed lot within 3–4 mth during storage (Srinives et al., 2007; Tripathy, 2016). The damage is due to continued feeding on the seeds by bruchid larvae and pupae until the adult emerges from the seed (Srinives et al., 2007). Bruchid infestation occurs in two stages: primary infestation takes place in the field and secondary infestation during storage, with the latter causing much more serious economic damage than the primary infestation (Srinives et al., 2007). Therefore, bruchid pest management is a crucial component in the production chain of legume crops. Several methods have been developed and proposed to reduce infestation, including biological, chemical and physical controls (Mishra et al., 2018). However, these methods are not suitable or applicable for smallholders. In addition, the use of pesticides can be hazardous to users, consumers and the environment; therefore the use of resistant cultivars is the most preferable method to control bruchids.

Molecular breeding is a promising approach in crop improvement and has been applied in several major crops, especially cereals, although it is less practiced in legume crops, especially minor legumes. Since the moth bean is an unfertilized and orphan crop, there is a lack of genetic studies and knowledge regarding breeding. The lack of genetic information is a major challenge for breeding efforts in moth bean. At present, advanced sequencing technologies, such as next-generation sequencing (NGS) and third-generation sequencing, play a pivotal role in revealing genetic information and molecular breeding of crops because NGS technologies contribute to obtaining transcriptome sequence data and molecular markers and to genetic and physical mapping of traits and marker-assisted breeding to direct the development of large-scale genomic resources (Varshney et al., 2009). NGS is becoming a cost effective and efficient technology to create a platform for more precise molecular studies and it is helpful for breeding programs through large-scale genomic resources (Raizada and Souframanien, 2019). Microarray analysis and RNA-seq are transcriptome sequencing techniques applied to understand genetic mechanisms and metabolic pathways at the transcriptional level (Xie et al., 2016). RNA-seq uses advanced sequencing technologies for sequencing and quantifying transcripts and is a highly useful tool to decipher and quantify gene expression and regulatory pathways and to find candidate genes (Gong et al., 2020), which can be applied even if the genome sequence of the organism is not known (Raizada and Souframanien, 2019). In addition, identification of novel genes and molecular markers, such as single nucleotide polymorphisms (SNPs) and simple sequence repeats, can be achieved through RNA-seq (Trick et al., 2009). Functions of differentially expressed genes (DEGs) detected using RNA-seq can provide valuable insights into plant defense mechanisms, which ultimately can be used for molecular breeding of resistant cultivars.

Somta et al. (2018) reported quantitative trait locus (QTL) mapping of seed resistance to *C. chinensis* in wild moth bean accession “TN67”. They found that the resistance was mainly controlled by one major QTL (*qVacBrc2.1*). Recently, fine mapping dissected the QTL *qVacBrc2.1* into two closely QTLs—*VacBrc2.1-A* and *VacBrc2.1-B* (Gamage et al., 2022). The current study aimed to identify candidate genes for the bruchid resistance in pods (including seeds) at different maturity stages of moth bean based on RNA-seq analysis using the resistant accession TN67 and the susceptible accession ICPMO056.

Materials and methods

Planting materials

The moth bean accessions TN67 and IPCMO056 were used in the RNA sequencing analysis. These two accessions have been used as mapping parents for the QTL analysis of bruchid resistance (Somta et al., 2018; Gamage et al., 2022). The plants were grown in a growth chamber (11 hr of light at an intensity of 33,300 lux at 28°C and 13 hr of darkness at 22°C) at Nagoya University, Japan. Fifty plants from each accession were maintained in pots; flowering occurred at about 28–30 d after planting.

RNA isolation, library construction and sequencing

RNA was extracted from the immature leaves, mature seeds (green colored, without pericarp) and five different stages of pods (including seeds and pericarps) in the maturation process as three biological replications. Different batches of samples were collected until the plants were aged 45 d after planting. Pod sample collection commenced 3 d after pod emergence and continued with another three samples at 2 daily intervals (on 3, 7 and 9 d after pod emergence). The mature pod samples were collected at 13 d after pod emergence.

RNA extraction was performed using a Nucleospin® RNA Plant and Fungi kit (TaKaRa Bio; Shiga Japan) following the manufacturer's guidelines. The RNA concentration and purity were quantified using a Nanodrop 1000 spectrometer (Thermo Fisher Scientific; Waltham, CA, USA). The RNA integrity was verified using the High Sensitivity RNA ScreenTape system (Agilent Technologies; Santa Clara, CA, USA). The RNA-seq library was constructed using to an Illumina RNA Seq library kit (Illumina, Inc.; San Diego, CA, USA) and RNA sequencing was performed using the Illumina HiSeqTM2000 platform (Illumina, Inc.; San Diego, CA, USA).

Sequencing analysis and mapping of differentially expressed genes and analysis of their functional annotation

The RNA-seq were trimmed using the trim_galore 0.6.4 software (Krueger et al., 2021; https://github.com/FelixKrueger/TrimGalore/releases) using default parameters and then the trimmed reads were mapped to the azuki bean genome (*Vigna angularis* (Willd.) Ohwi and Ohashi) (Sakai et al., 2015; https://viggs.dna.affrc.go.jp) using the STAR software (Dobin et al., 2013). Since the reference genome was not the same species of moth bean, the Stringtie 2.1.2 (https://github.com/gpertea/stringtie/releases/tag/v2.1.2) software was used to assemble novel transcripts before estimating the abundance using the Salmon software (Patro et al., 2017) which provides rapid and bias-aware quantification of transcript expression. Differential expression detection was performed using the DEseq2 software (Love et al., 2014).

Analysis of gene ontology pathway

Gene ontology (GO) annotation was performed using the GO seq software (Young et al., 2012) to describe biological processes, molecular functions and cellular components with category reference to DEGs (Ashburner et al., 2000; Conesa et al., 2005). In addition, gene functions were annotated using the NCBI databases. The WEGO 2.1 software (Ye et al., 2006) was used to describe functional analysis.

Gene regulatory network analysis

Gene network analysis of the DEGs was performed using the Cytoscape software (https://cytoscape.org). All the up-regulated and down-regulated The DEGs were analyzed to describe the pathways of the networks responsible for insect resistance in plants (defense response pathway, phytohormones, signaling pathways and peptidase pathway). Genes identified as being involved in insect resistance were used to develop a heatmap to describe the normalized gene expression values using the "Pheatmap" function in the R software package (Kolde, 2018). Expression data related to insect defense functions were used to predict candidate genes for the bruchid resistance in TN67.

Validation of candidate gene expressions using quantitative reverse transcription polymerase chain reaction

To validate the candidate genes presumed to be involved in bruchid resistance, expression analysis was performed using quantitative reverse transcription polymerase chain reaction (RT-qPCR). Immature (3 d after pod emergence) and semi-matured (9 d after pod emergence) pods (including seeds and pericarps) were used for the RT-qPCR procedure. Three biological and three technical replicates were conducted. The RT-qPCR was carried using the StepOnePlus™ Real-Time PCR system (Thermo Fisher Scientific; Waltham, MA, United States) using SYBR Premix ExTaqTMII

(TaKaRa Bio; Shiga Japan). Amplification was programmed as: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. *ACTIN* and *PTB* (Chi et al., 2016) were used as reference genes. The comparative CT method (ΔΔCT method) of quantification was used to quantify the relative expression of the candidate genes (Livak and Schmittgen, 2001). Statistical analysis was performed to determine differences in the expression level between the two accessions based on a t test with a p-value threshold of 0.05 using the R program version 2.10.0 (R Core Team, 2012).

Results

Mapping of sequencing data and functional annotation of mapped transcripts

In total, 24,308 clean reads resulted from RNA sequencing were mapped to the azuki bean genome. All the mapped high-quality transcripts were annotated using BLASTX against the azuki bean reference genome [*Vigna angularis* (Ohwi) Ohwi and Ohashi] at the NCBI (Johnson et al., 2008; https://blast.ncbi.nlm.nih.gov). According to the BLAST results 2,104 transcripts were recorded as hypothetical proteins, while another 951 transcripts represented non-coding genes and other transcripts were functionally annotated in both moth bean genotypes referenced to the azuki bean genome.

Differentially expression analysis of mapped transcripts

Moth bean RNA-seq samples of immature leaves, seeds and pods were mapped to the azuki genome, due to the lack of an available moth bean genome sequence. Despite the reference genome belonging to a different species, mapping rates of 90.8% on average were obtained, with 82.7% mapping uniquely to a single locus. These high mapping rates indicated that the azuki genome was reasonably similar to the moth bean genome and could be used for RNA-seq analysis. A de-novo assembly step was included in the analysis to account for sequence differences between the two organisms. Of the 33,735 genes annotated in the adzuki genome, 22,973 (68%) were expressed in the moth bean samples, besides 1,335 novel genes that were not present in the adzuki annotation. In total, 6,095 DEGs were detected in the moth bean genotypes. Among these DEGs, 3,210 exhibited higher expression in the resistant genotype (up regulation) while 2,885 exhibited higher expression in the susceptible genotype (down regulation), with 13% and 12% of all expressed genes, respectively. Of the total genes, 899 were not expressed during the pod maturation period (Fig. 1A).

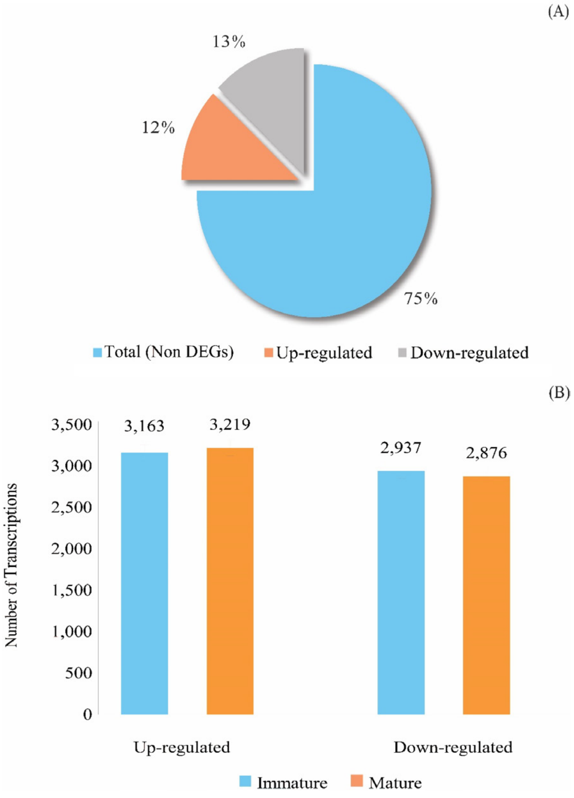

Fig. 1 Distribution of differentially expressed genes (DEGs) in bruchid-resistant moth bean accession TN67: (A) distribution of DEGs in total transcriptome assembly; (B) distribution of DEGs in immature and mature stages of pods

Table 1 Primers developed for polygalacturonase inhibitor protein (*PGIP*) and *Lectin*

Primer name	Forward primer	Reverse primer
PGIPMSTRG.10164_1	GACACAGATTGCTGCCAATG	AAATGACGAGGGAGGTGATG
PGIPMSTRG.10164_2	CAACCCACACGACAAGAAAG	CATTCGACGCAGTACCATTG
PGIPMSTRG.10164_3	CCCAGTTGCATTGTCTGAAC	CATTCGACGCAGTACCATTG
PGIPMSTRG.10164_4	AATGGTACTGCGTCGAATGC	TTGTGGAATTCGAGGGTCTC
LectiMSTRG.23200_1	ACTTATGGTGGCGAATCCAG	AGCTGCTCCTTCCCAAATTC
LectiMSTRG.23200_2	GGAATTCATGGCGGAGATAG	GATCGAAAACCCACCTGTTG
LectiMSTRG.23200_3	CGAGGAAGAAATGGAGATGG	CGAACCAAATCGGACAAGAC
LectiMSTRG.23200_4	ACTTATGGTGGCGAATCCAG	ATTCCCCTGTTGCAGAACTC
LectiMSTRG.23200_5	CATCGACCTCAACAACATCG	AACAGCAGATTCTGGCCATC

In addition, 3,163 and 3,219 genes were up-regulated with higher fold change values in the immature and mature stages, respectively, while 2,937 and 2,876 genes were down regulated in the immature and mature stages, respectively (Fig. 1B). There were no significant differences in the numbers of up regulated genes and down regulated genes between the immature and mature stages. The expression clustered patterns of defense response DEGs were generated on a heat map based on the log2 values of their expression ratios (Fig. 2). However, 16 genes with possible roles in plant defense showed different expression of which genes encoding the polygalacturonase-inhibiting protein and the lectin domain-containing protein showed up-regulation (Fig. 3).

Gene ontology analysis

Gene ontology (GO) functional annotations and categorizations of the annotations were performed using the WEGO 2.0 tool based on the BLASTX results against the *Vigna angularis* database. GO classification revealed 1,131 biological functions, 540 cellular functions and 1,630 molecular functions with higher expression in the TN67 bruchid-resistant accession in the immature stage. In the mature stage, 1,159 genes were classified with biological functions, 546 with cellular functions and 1,669 with molecular functions (Figs. 4A and 4B).

Fig. 2 Heatmap of significant differentially expressed genes between moth bean accessions IPCMO056 (bruchid-susceptible) and TN67 (bruchid-resistant), where TI, PGIP, Jas/Sil and Lec are trypsin inhibitors, polygalacturonate inhibitors, jasmine acid and salicylic acid signaling, and lectin, respectively, blue color gradient represents up regulated expression level (highest expression represents by dark blue and lowest expression represents by light blue color) and red color gradient represents down regulated expression level (highest expression represents by dark red and lowest expression represents by light pink color)

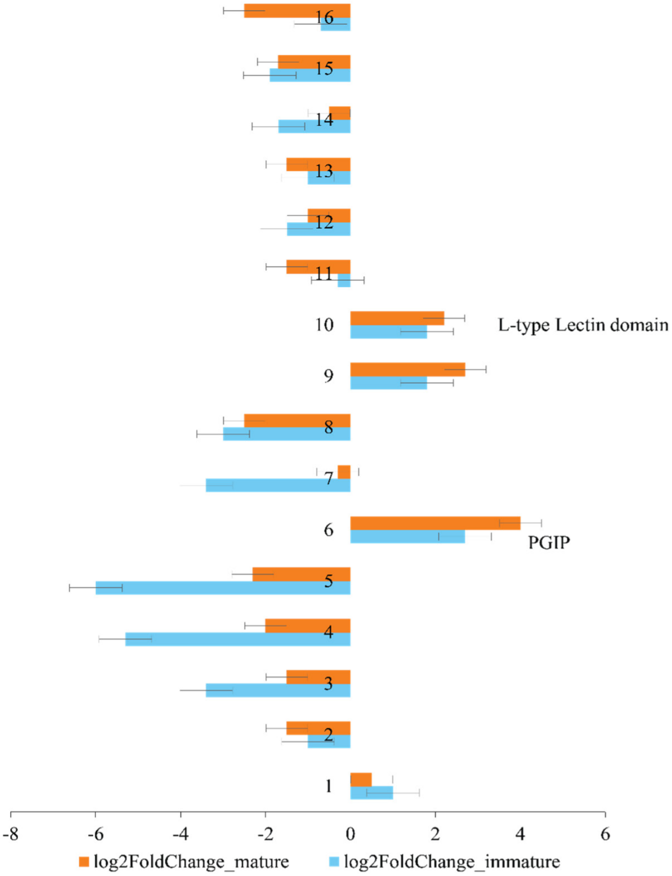

Fig. 3 Differentially expressed genes related to plant defense in seeds of moth bean IPCMO056 (bruchid-susceptible) and TN67 (bruchid-resistant) determined based on RNA-seq, where old numbers 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16 represent inter-alpha-trypsin inhibitor heavy chain H4, inter-alpha-trypsin inhibitor heavy chain H3, trypsin inhibitor DE-3, chymotrypsin inhibitor 3, trypsin inhibitor DE-3, polygalacturonase inhibitor, polygalacturonase inhibitor 2-like, polygalacturonase inhibitor 2, L-type lectin-domain containing receptor kinase S.1, L-type lectin-domain containing receptor kinase S.1, L-type lectin-domain containing receptor kinase VII.2, L-type lectin-domain containing receptor kinase S.5, L-type lectin-domain containing receptor kinase VIII.1, L-type lectin-domain containing receptor kinase VIII.1-like, L-type lectin-domain containing receptor kinase VIII.2-like and L-type lectin-domain containing receptor kinase S.7, respectively, and error bar indicates standard error of mean.

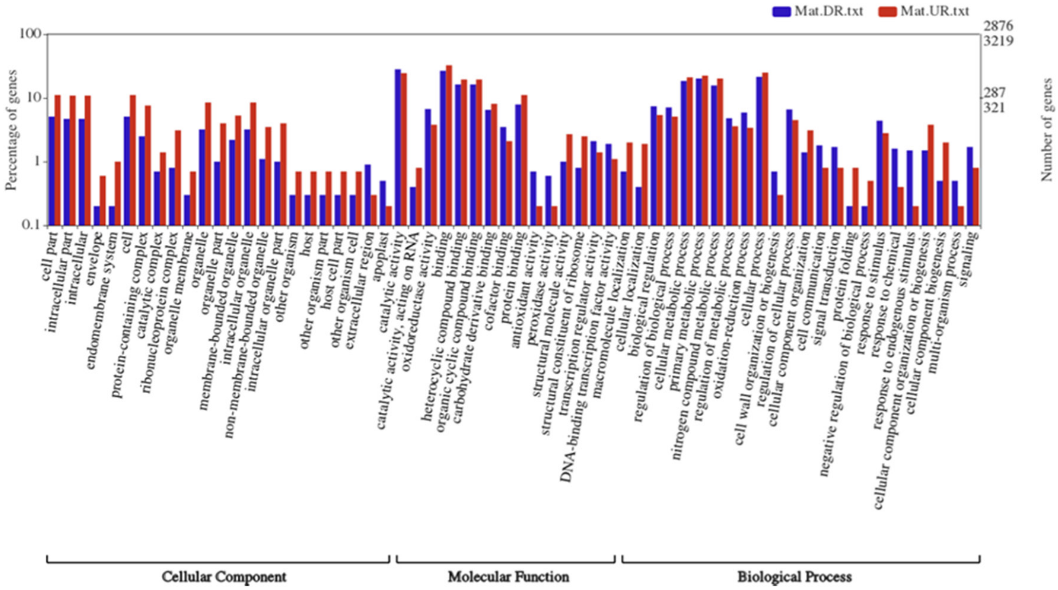

Fig. 4 Histogram of gene ontology classification in bruchid-resistant moth bean accession TN67, where BP = biological process, CG = cellular component and MF = molecular function

Gene regulatory network analysis

Gene regulatory network analysis was performed using the Cytoscape 3.8.1 software. The most direct and indirect genes relating to insect resistance were utilized in the gene regulatory network analysis. Plant defense response pathways, phytohormones, signaling pathways and peptidase activity pathways were generated (Figs. 5A, 5B and 5C). The defense response pathways describing all defense response activities were down-regulated (Fig. 5A), while systemic-acquired resistance was up-regulated (Fig. 5B). The highest expressed response to abscisic acid in the phytohormone and signaling pathways was not related to insect resistance. However, all other phytohormones were down-regulated. Gene regulation related to the peptidase activity pathway had significantly higher up-regulated activity of serine-type endopeptidase inhibitors (Fig. 5C). Cystine-type endopeptidase inhibitor activity was down-regulated.

Validation of differentially expressed genes using quantitative reverse transcription polymerase chain reaction

To validate the changes in gene expression detected based on the RNA-seq, three potential candidate genes, consisting of a polygalacturonase inhibitor and two L-type lectin domains, were selected for RT-qPCR. The RT-qPCR results indicated that expression of these genes was consistent with the RNA-Seq data (Fig. 6). Furthermore, the results indicated that the RNA-Seq data were reliable.

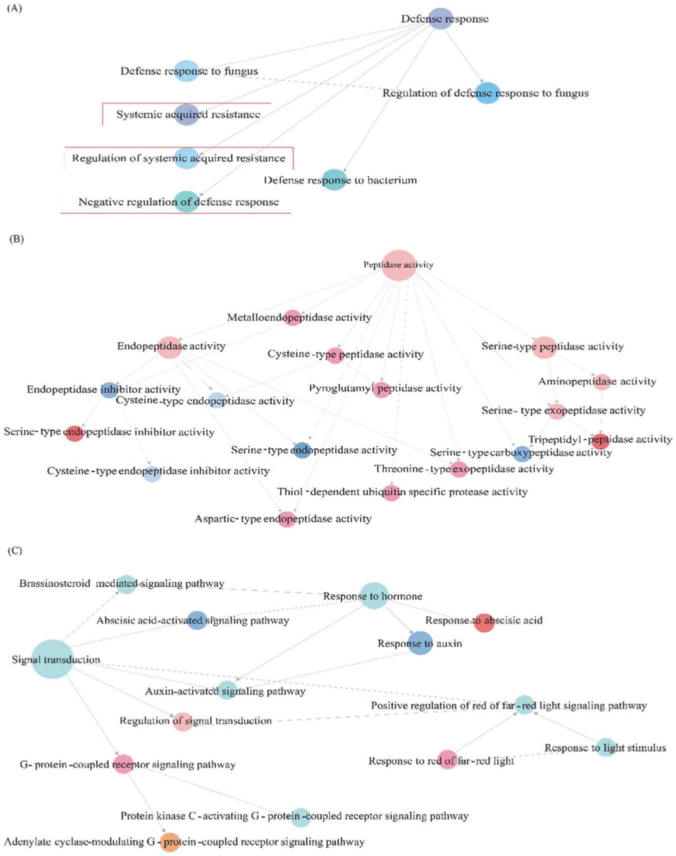

Fig. 5 Three gene regulatory networks of bruchid-resistant moth bean accession TN67 determined from transcripts associated with defense mechanisms: (A) defense response network; (B) peptidase activity network; (C) signaling pathway network, where blue circles represent down regulated genes, red/pink circles represents up regulated genes, with the size of circles representing the number of genes involved and a low color intensity represents lower expression, while a high color intensity represents higher expression.

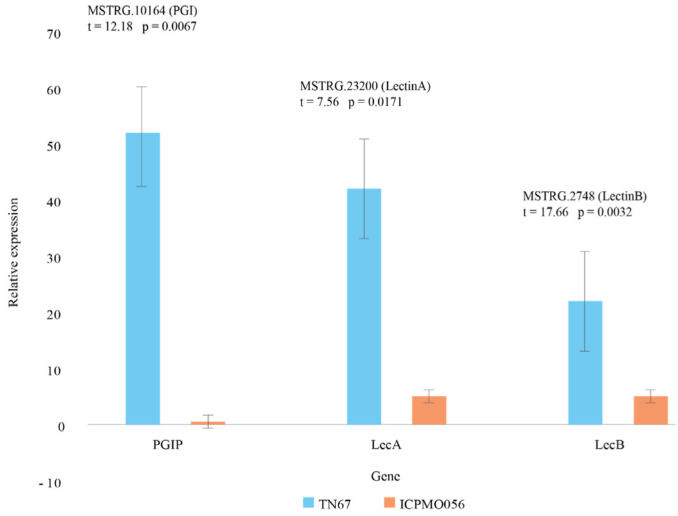

Fig. 6 Relative expression of a *polygalacturonase-inhibiting protein* (*PGIP*) and two *lectin* genes—*Lectin A* (*LecA*) and *Lectin B* (*LecB*) based on quantitative real-time polymerase chain reaction in moth bean accessions TN67 (bruchid-resistant) and ICPMO056 (bruchid-susceptible)

Discussion

C. chinensis is one of the most serious insect pests of stored grain legumes. Plant scientists have long been interested in identifying one or more genes for resistance to these insects. Since bruchids consume both immature and mature seeds, especially the latter, the chemicals produced and the genes expressed during seed growth and development are believed to play important roles in the resistance. Other studies have explained seed proteins and plant inhibitors (PIs) are mostly responsible for bruchid resistance in legumes. For example, PGIP (polygalacturonase inhibitor protein) in mung bean (Chotechung et al., 2016; Zhang et al., 2021) and moth bean (Gamage et al., 2022) and *α*- amylase inhibitor (cowpea) are different PIs responsible for resistance reported in different *Vigna* species. PGIPs could inhibit the activity of polygalacturonase enzymes in insects (Chotechung et al., 2016; Zhang et al., 2021) while playing an important role in plant defense against insects and pathogens. PGIPs activate during early infestation, producing cell-wall-associated PGIPs against invading agents, including insects. The *α*-amylase halted the growth and development of both *C. chinensis* and *C. masculatus* reported in cowpea (Huesing et al., 1991) and common bean (Ishimoto and Chrispeels, 1996). Lectin receptor kinase and chitinase are factors in bruchid resistance in black gram (Somta et al., 2019)

In the current study, RNA-seq revealed dissimilarities between up-regulated and down-regulated DEGs in different seed maturity stages (Fig. 1B). Comparison of gene expression during the immature and mature pod stages revealed that the 10-highest up-regulated genes differed in the two stages (Table 2). However, in both stages, none of the genes among the highest-expressed 10 genes had a defense-related function. GO enrichment analysis showed that defense or insect resistance-related genes were involved in the biological process group (Fig. 3).

Table 2 Highest up-regulated gene at immature and mature stages of pod

Stage	Gene ID	Annotated function
Immature stage	MSTRG.9880	Protein of unknown function DUF639
	MSTRG.22476	Cytochrome P450 superfamily
	MSTRG.1885	Conserved hypothetical protein CHP02058
	MSTRG.19043	Universal stress protein A family | UspA
	MSTRG.13897	Cupin 1 | RmlC-like cupin domain superfamily
	MSTRG.18190	Cupin 1 | RmlC-like cupin domain superfamily | 11-S seed storage protein,
	MSTRG.22475	Cytochrome P450, conserved site
	MSTRG.3775	NAD(P)-binding domain superfamily | Short-chain dehydrogenase/reductase SDR
	MSTRG.15273	Lipoxygenase, iron binding site | PLAT/LH2 domain superfamily
	MSTRG.17159	Cupin 1 | RmlC-like cupin domain superfamily
Mature stage	MSTRG.1885	Conserved hypothetical protein CHP02058
	MSTRG.15864	Unknown seed protein
	MSTRG.22346	Low temperature-induced protein
	MSTRG.6184	Uncharacterized
	MSTRG.13720	Extensin-3
	MSTRG.4183	DNA-binding domain superfamily | AP2/ERF domain
	MSTRG.12787	18 kDa seed maturation protein
	MSTRG.10181	Late embryogenesis abundant protein, LEA_1 subgroup
	MSTRG.17934	NAD(P)-binding domain superfamily | Short-chain
	MSTRG.18717	Uncharacterized

There are different regulatory network systems related to a defense response, including transcription factors, proteases and signaling pathways related to hormones (Fig. 4). The defense response network system (Fig. 4A) showed that all genes related to defense mechanisms (systemic acquired resistance, regulation of systemic acquired resistance and negative regulation of defense response) were significantly down-regulated, suggesting that these genes were highly expressed and present in the IPCMO056 cultivar. The protease regulatory network demonstrated that more than 70% of up-regulated activities were related to genes expressed in the TN67 resistant genotype. Serine-type endopeptidase inhibitor activity had the highest up-regulated expression of the regulated activities. Genes with endopeptidase inhibitor activity and cysteine-type endopeptidase activity were highly expressed in the susceptible genotype (Fig. 4B). These results suggested that the serine endopeptidase inhibitor may be one of the factors providing resistance to *C. chinensis* in moth bean. Serine endopeptidases (serine proteinase) is one of the enzymes found in the digestive system of *Callosobruchus* species (Ishimoto and Chrispeels, 1996; Silva et al., 2001). The existence of serine proteinase in the larval midgut of the bruchid species *Zabrotes subfasciatus* may be involved in the hydrolysis of the common bean (*Phaseolus vulgaris*) α -amylase inhibitor 1 (α-AI1) to detoxify the protein. However, the serine endopeptidase inhibitor has not been reported as a major factor in bruchid resistance. Genes relating to jasmonic acid (JA) biosynthesis process were down-regulated in the mature pod stage. JA is a major hormone responsible for insect resistance in plants as it activates the production of chemical and physical barriers against herbivorous insects after the infestation (Yang et al., 2019) due to genes in the phosphorylation cascade leading to JA biosynthesis being trigged by insect attack and eliciting signals (Ruan et al., 2019) and the fact that the plant parts (pods and seeds) used in the current study were free from insect attack meant that genes relating to JA biosynthesis were not involved in the bruchid resistance.

RNA sequencing revealed the presence of the DEGs of trypsin inhibitors, polygalacturonase inhibitors (PGIPs), lectins and lectin-like proteins (Fig. 6). Among these genes, one PGIP and two L-type lectin–domain containing receptor kinase S.1 genes were up-regulated (Fig. 6). However, only three DEGs related to seed proteins exhibited up-regulated behavior in both the immature and mature stages (Fig. 5). The two lectins and a PGIP followed the same expression pattern, with their expression being s significantly higher in the mature stage (Fig. 5). This result indicated that mature seeds were rich in those seed proteins. PGIP genes have been shown to promote bruchid resistance in mung bean (Chotechung et al., 2016; Kaewwongwal et al., 2017, 2020; Zhang et al., 2021) and moth bean (Gamage et al., 2022). PGIPs are believed to provide resistance to bruchid by inhibiting bruchid polygalacturonases, which are enzymes used to hydrolyze seed starch (Chotechung et al., 2016). In addition, PGIPs from the common bean have been reported to play roles in resistance to mirid bugs (Frati et al., 2006). Lectins are related to cell-to-cell interaction in their biological processes and they are heterogenous group of carbohydrate-binding proteins. Genes encoding for lectins have been reported as candidate genes for resistance to *C. maculatus* in black gram (Somta et al., 2019). The current results supported another study that reported the PGIP1 and PGIP2 genes were resistance genes for bruchid resistance in moth bean (Gamage et al., 2022). Furthermore, the current RT-qPCR results confirmed the up regulated expression of PGIP and the duplicated L-type lectin domain containing the receptor kinase S-1, revealing them as candidate genes for bruchid resistance in the TN67 moth bean.

In conclusion, the current study identified genes that related to seed resistance to bruchids based on comparative transcriptomic analysis of moth bean seeds. The transcriptomic analysis and RT-qPCR revealed that a gene encoding PGIP and two genes encoding lectin-containing protein that were up-regulated in seeds showed differential expression between the resistant and susceptible moth bean accessions. The genes encoding PGIP and encoding the lectin-containing protein should be considered as candidate genes for bruchid resistance in moth bean.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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References

Ashburner, M., Ball, C.A., Blake, J.A., et al. 2000. Gene ontology: Tool for the unification of biology. Nat. Genet. 25: 25–29. doi.org/10.1038/75556

Chi, C., Shen, Y., Yin, L., Ke, X., Han, D., Zuo, Y. 2016. Selection and validation of reference genes for gene expression analysis in *Vigna angularis* using quantitative real-time RT-PCR. PLoS One 11: e0168479. doi.org/10.1371/journal.pone.0168479

Chotechung, S., Somta, P., Chen, J., Yimram, T., Chen, X., Srinives, P. 2016. A gene encoding a polygalacturonase-inhibiting protein (PGIP) is a candidate gene for bruchid (Coleoptera: Bruchidae) resistance in mungbean (*Vigna radiata*). Theor. Appl. Genet. 129: 1673–1683. doi.org/10.1007/s00122-016-2731-1

Conesa, A., Götz, S., García-Gómez, J.M., Terol, J., Talón, M., Robles, M. 2005. Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21: 3674–3676. doi.org/10.1093/bioinformatics/bti610

Dobin, A., Davis, C.A., Schlesinger, F., et al. 2013. STAR: Ultrafast universal RNA-seq aligner. Bioinformatics 29: 15–21. doi.org/10.1093/bioinformatics/bts635

Krueger, F., James, F., Ewels, P., Afyounian, E., Weinstein, M., Schuster-Boeckler, B., Hulselmans, G. 2021. FelixKrueger/TrimGalore: v0.6.7. doi.org/10.5281/zenodo.5127899

Frati, F., Galletti, R., De Lorenzo, G., Salarino, G., Conti, E. 2006. Activity of *endo*-polygalacturonases in mirid bugs (Heteroptera: Miridae) and their inhibition by plant cell wall proteins (PGIPs). Eur. J. Entomol. 103: 515–522. doi: 10.14411/eje.2006.067

Gamage, S.I.R., Kaewwongwal, A., Laosatit, K., Yimram, T., Lin, Y., Chen, X., Nakazono, M., Somta, P. 2022. Tandemly duplicated genes encoding polygalacturonase inhibitors are associated with bruchid (*Callosobruchus chinensis*) resistance in moth bean (*Vigna aconitifolia*). Plant Sci. 323: 111402. doi.org/10.1016/j.plantsci.2022.111402

Garg, B.K., Kathju, S., Burman, U. 2001. Influence of water stress on water relations, photosynthetic parameters and nitrogen metabolism of moth bean genotypes. Biol. Plant. 44: 289–292. doi.org/10.1023/A:1010215812791

Gong, L., Han, S., Yuan, M., Ma, X., Hagan, A., He, G. 2020. Transcriptomic analyses reveal the expression and regulation of genes associated with resistance to early leaf spot in peanut. BMC Res. Notes 13: 381. doi.org/10.1186/s13104-020-05225-9

Huesing, J.E., Shade, R.E., Chrispeels, M.J., Murdock, L.L. 1991. α-Amylase inhibitor, not phytohemagglutinin, explains resistance of common bean seeds to cowpea weevil. Plant Physiol. 96: 993–996. doi.org/10.1104/pp.96.3.993

Ishimoto, M., Chrispeels, M.J. 1996. Protective mechanism of the Mexican bean weevil against high levels of α-amylase inhibitor in the common bean. Plant Physiol. 111: 393–401. doi.org/10.1104/pp.111.2.393

Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S., Madden, T.L. 2008. NCBI BLAST: A better web interface. Nucleic Acids Res. 36: W5–W9. doi.org/10.1093/nar/gkn201

Kaewwongwal, A., Chen, J., Somta, P., Kongjaimun, A., Yimram, T., Chen, X., Srinives, P. 2017. Novel alleles of two tightly linked genes encoding polygalacturonase-inhibiting proteins (VrPGIP1 and VrPGIP2) associated with the br locus that confer bruchid (*Callosobruchus* spp.) resistance to mungbean (*Vigna radiata*) accession V2709. Front. Plant Sci. 28: 1692. doi.org/10.3389/fpls.2017.01692

Kaewwongwal, A., Liu, C., Somta, P., Chen, J., Tian, J., Yuan, X., Chen, X. 2020. A second *VrPGIP1* allele is associated with bruchid resistance (*Callosobruchus* spp.) in wild mungbean (*Vigna radiata* var. *sublobata*) accession ACC41. Mol. Genet. Genomics 295: 275–286. doi.org/10.1007/s00438-019-01619-y

Kolde, R. 2018. Package ‘pheatmap’. R Package 1. p. 10. https://cran.r-project.org/web/packages/pheatmap/pheatmap.pdf

Livak, K.J., Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25: 402–408. doi.org/10.1006/meth.2001.1262

Love, M.I., Huber, W., Anders, S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15: 550. doi.org/10.1186/s13059-014-0550-8

Mishra, S.K., Macedo, M.L.R., Panda, S.K., Panigrahi, J. 2018. Bruchid pest management in pulses: Past practices, present status and use of modern breeding tools for development of resistant varieties. Ann. Appl. Biol. 172: 4–19. doi.org/10.1111/aab.12401

Patro, R., Duggal, G., Love, M.I., Irizarry, R.A., Kingsford, C. 2017. Salmon provides fast and bias-aware quantification of transcript expression. Nat. Methods 14: 417–419. doi.org/10.1038/nmeth.4197

R Core Team. 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. http://www.R-project.org/

Raizada, A., Souframanien, J. 2019. Transcriptome sequencing, de novo assembly, characterisation of wild accession of blackgram (*Vigna mungo* var. *silvestris*) as a rich resource for development of molecular markers and validation of SNPs by high resolution melting (HRM) analysis. BMC Plant Biol. 19: 358. doi.org/10.1186/s12870-019-1954-0

Ruan, J., Zhou, Y., Zhou, M., Yan, J., Khurshid, M., Weng, W., Cheng, J., Zhang, K. 2019. Jasmonic acid signaling pathway in plants. Int. J. Mol. Sci. 20: 2479. doi.org/10.3390/ijms20102479

Sakai, H., Naito, K., Ogiso-Tanaka, E., et al. 2015. The power of single molecule real-time sequencing technology in the de novo assembly of a eukaryotic genome. Sci. Rep. 5: 16780. doi.org/10.1038/srep16780

Siddhuraju, P. 2006. The antioxidant activity and free radical-scavenging capacity of phenolics of raw and dry heated moth bean (*Vigna aconitifolia*) (Jacq.) Marechal) seed extracts. Food Chem. 99: 149–157. doi.org/10.1016/j.foodchem.2005.07.029

Silva, C.P., Terra, W.R., Lima, R.M. 2001. Differences in midgut serine proteinases from larvae of the bruchid beetles *Callosobruchus maculatus* and *Zabrotes subfasciatus*. Arch. Insect Biochem. Physiol. 47: 18–28. doi.org/10.1002/arch.1031

Somta, P., Chen, J., Yundaeng, C., Yuan, X., Yimram, T., Tomooka, N., Chen, X. 2019. Development of an SNP-based high-density linkage map and QTL analysis for bruchid (*Callosobruchus maculatus* F.) resistance in black gram (*Vigna mungo* (L.) Hepper). Sci. Rep. 9: 3930. doi.org/10.1038/s41598-019-40669-5

Somta, P., Jomsangawong, A., Yundaeng, C., Yuan, X., Chen, J., Tomooka, N., Chen, X. 2018. Genetic dissection of azuki bean weevil (*Callosobruchus chinensis* L.) resistance in moth bean (*Vigna aconitifolia* [Jaqc.] Maréchal). Genes 9: 555. doi.org/10.3390/genes9110555

Srinives, P., Somta, P., Somta, C. 2007. Genetics and breeding of resistance to bruchids (*Callosobruchus* spp.) in *Vigna* crops: A review. NU Science Journal Sci. 4: 1–17.

Tiwari, B., Kalim, S., Bangar, P., Kumari, R., Kumar, S., Gaikwad, A., Bhat, K.V. 2018. Physiological, biochemical, and molecular responses of thermotolerance in moth bean (*Vigna aconitifolia* (Jacq.) Marechal). Turk. J. Agric. For. 42: 176–184. doi.org/10.3906/tar-1709-1

Trick, M., Long, Y., Meng, J., Bancroft, I. 2009. Single nucleotide polymorphism (SNP) discovery in the polyploid *Brassica napus* using Solexa transcriptome sequencing. Plant Biotechnol. J. 7: 334–346. doi.org/10.1111/j.1467-7652.2008.00396.x

Tripathy, S.K. 2016. Bruchid resistance in food legumes-an overview. Res. J. Biotechnol. 11: 97–105.

Varshney, R.K., Nayak, S.N., May, G.D., Jackson, S.A. 2009. Next-generation sequencing technologies and their implications for crop genetics and breeding. Trends Biotechnol. 27: 522–530. doi.org/10.1016/j.tibtech.2009.05.006

Xie, L., Li, F., Zhang, S., Zhang, H., Qian, W., Li, P., Zhang, S., Sun, R. 2016. Mining for candidate genes in an introgression line by using RNA sequencing: The anthocyanin overaccumulation phenotype in *Brassica*. Front. Plant Sci. 7: 1245. doi.org/10.3389/fpls.2016.01245

Yang, J., Duan, G., Li, C., Liu, L., Han, G., Zhang, Y., Wang, C. 2019. The cross talks between jasmonic acid and other plant hormone signaling highlight the involvement of jasmonic acid as a core component in plant response to biotic and abiotic stresses. Front. Plant Sci. 10: 1349. doi.org/10.3389/fpls.2019.01349

Ye, J., Fang, L., Zheng, H., et al. 2006. WEGO: A web tool for plotting GO annotations. Nucleic Acids Res. 34: W293–W297. doi.org/10.1093/nar/gkl031

Young, M.D., Wakefield, M.J., Smyth, G.K., Oshlack, A. 2010. Gene ontology analysis for RNA-seq: Accounting for selection bias. Genome Biol. 11: R14. doi.org/10.1186/gb-2010-11-2-r14

Zhang, Q., Yan, Q., Yuan, X., et al. 2021. Two polygalacturonase-inhibiting proteins (VrPGIP) of *Vigna radiata* confer resistance to bruchids (*Callosobruchus* spp.). J. Plant Physiol. 258–259: 153376. doi.org/10.1016/j.jplph.2021.153376