Gene Mapping of Bruchid Resistance in Moth Bean (*Vigna aconitifolia*)

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INTRODUCTION

Vigna crops are widely cultivated as food legumes with their excellent nutrition properties and adaptability to different cropping systems worldwide. Moth bean is an underutilized *Vigna* crop that is cultivated in drier parts of Asia due to its extreme traits of drought tolerance, heat tolerance, and survival ability in marginal lands. Moth bean can be utilized and improved as future *Vigna* crops adapt to climate change. While these crops possess several useful traits, susceptibility to pests is the major drawback when at the commercial cultivation level. Bruchids, *Callosobruchus* species (Coleoptera; Bruchidae) is a severe post-harvest pest that lead to critical losses in *Vigna* crops. Several drawbacks in the conventional bruchid management approach, worldwide interest makes on applying biotechnological tools in breeding for bruchid pest management in *Vigna* crops. Therefore, the approach of MAS has become more prevalent in modern plant breeding. The basis of MAS is the identification of markers linked with the interesting trait, through gene mapping. QTL analysis through gene mapping functions, identification of candidate gene(s), and gene expression analysis are significant applications that are widely used in modern plant breeding with recent advances in genomics. With this background, genetic analysis should be carried on through the exploitation of bruchid resistant trait(s) among different genotypes /cultivars of these two crops for developing bruchid resistant varieties using advanced genomic tools to obtain a sustainable yield without complete loss due to bruchid infestation.

Therefore, the study will be comprised of the following objectives.

1. Narrow down the genomic region of *Br* locus (*qBr5.1*) to *C. chinensis* species in moth bean
2. Identification of candidate gene(s) and analysis of gene expression for the resistance to *C. chinenesis* species in moth bean

MATERIALS AND METHODS

The study was carried out at the field and laboratory of legume breeding at the Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Thailand, and Laboratory of Genetics and Plant Breeding at the Graduate School of Bio-Agricultural Science, Nagoya University Japan.

3.1 Experiment 1: Fine gene mapping of the *qBr5.*1 locus for *C. chinenesis* resistance moth bean

3.1.1 Plant materials and genomic DNA extraction

We studied two moth bean F_2 populations: F_2OA (188 plants) and F_2NB (521 plants and parents). We crossed TN67, a wild moth-bean resistant to C. chinensis, with a susceptible cultivated accession from India. All plants were grown at Kasetsart University and had their DNA extracted from young leaves using the method in (Lodhi et al., 1994).

3.1.2 Evaluation of bruchid resistance and segregation analysis

Seeds of *C. chinensis* were tested for resistance by introducing adult insects for a week. Infested seeds were counted for damage after being kept at 30°C and 60% relative humidity for 60 days. Based on the percentage of damaged seeds, F_2 plants were classified as resistant, partially resistant, or susceptible. A chi-square test was performed to test for a single-gene model.

3.1.3 Development and analysis of new SSR markers from related legume species

A gene called QTL *qVacBrc2.1* gives C. chinensis resistance to TN67. To study it, researchers designed primers and used markers to determine genotypes in F_2OA and F_2NB populations. Linkage maps were made for each population with QTL IciMapping 4.2 software.

3.1.5 Identification and sequencing of *PGIP1* and *PGIP2*

The search for genes that resist bruchids in *qVacBrc2.1* led to the identification of PGIP1 and PGIP2 as potential candidates. The nucleotide and amino-acid sequences of VacPGIP1 from TN67 and ICPMO056 were aligned to identify polymorphisms using Clustal Omega.

3.1.6 Expression analysis

RNA was extracted from TN67 and ICPMO056 seeds at two stages. qRT-PCR was done using ACTIN as reference for PGIP1 and PGIP2 expression. Statistical differences were tested using a t-test at *P*-value of 0.05 and R program version 2.10.0.

3.1.7 Phylogenetic analysis of *PGIP1*

The amino-acid sequence of *VacPGIP1* and those of PGIPs of different *Vigna* crops were used to construct a phylogenic tree.

3.2 Experiment 2: Identification of candidate gene(s) using RNA seq technology and analysis of gene expression for the resistance to *C. chinenesis* species in moth bean

3.2.1 Planting materials

Moth bean accessions TN67 and IPCMO056 were used for RNA sequencing analysis.

3.2.2 RNA isolation, library construction and Sequencing

RNA was extracted from three plant sources with three replications: immature leaves, green mature seeds, and pods with seeds and pericarps. TaKaRa's Nucleospin® RNA kit was used for extraction, and DEGs were analyzed for functional annotation through sequencing.

3.2.3 Analysis of GO Pathway and analysis of gene regulatory network were performmed

3.2.4 Validation of candidate gene expressions by real time quantitative PCR (RT-qPCR)

RT-qPCR was used to analyze candidate genes for bruchid resistance in immature and semi-matured pods using nine primers for PGIP and Lectin. We used the ΔΔCT method for quantification and performed statistical analysis with a *p*-value threshold of 0.05 using R program.

RESULTS

4.1 Fine gene mapping of the *qBr5.*1 locus for *C. chinenesis* resistance moth bean

4.1.1 Resistance against *C. chinensis* in the F_2NB population and its parents

Our study of 497 F_2 individuals showed a range of resistance to C. chinensis, with TN67 exhibiting high resistance and ICPMO056 being completely susceptible. The PDS values had a bimodal distribution but skewed towards TN67. Based on PDS, 409 plants were classified as resistant, and 88 as susceptible. The trait is controlled by two genes exhibiting dominant and recessive epistasis.

4.1.2 Chromosomal location of, and increased marker density at, *qVacBrc2.1* in *Vigna indica* and azuki bean

qVacBrc2.1-B was localized between Vind-SSR016 and Vind-SSR011, accounting for 39.93–44.14% PDS in the population, depending on seasons/generations, and exhibited additive and dominant effects of 20.41–26.30 and − 3.65 –1.12 .

4.1.3 Fine mapping and identification of candidate genes in *qVacBrc2.1-B*

We've examined and adjusted the QTL *qVacBrc2.1-B* with the F_2NB population. Using ICIM analysis, we've identified qVacBrc2.1-B at 0.60 cM between Vind-SSR004 and VrBrSSR013. It has an LOD score of 170.9, explaining 79.95% PDS, with an additive effect of 36.39 and a dominant effect of -0.03..

Based on the functions of these genes in the two species, we identified two genes—*VacPGIP1* and *VacPGIP2*—as candidate genes for resistance to *C. chinensis* in moth bean.

4.1.4 Expression analysis and phylogeny of *VacPGIP1* and *VacPGIP2*

The expression levels of *VacPGIP1* and *VacPGIP2* in the seeds at green- and yellow-pod stages of TN67 and ICPMO056 were determined using qRT-PCR. We observed statistically insignificant difference (up to two-fold) in the expression of *VacPGIP1* and *VacPGIP2* between TN67 and IPCMO056. A phylogenic tree of *VacPGIP1* and PGIPs from several *Vigna spp*., common bean, soybean, chickpea, and *M. truncatula* revealed that *VacPGIP1* was closely related with *VindPGIP1* from *V. indica* and *VrPGIP1* from mungbean.

Table 1 QTLs detected for percentage of damaged seeds (% damaged seeds) and area under disease progress curve (AUDPC) caused by *Callosobruchus chinenis* and 100-seed weight in moth

bean F_2 and $F_{2:3}$ populations of the cross IPCMO056 x TN67 by inclusive composite interval mapping

Population	QTL name	Position[a]	Flanking markers	LOD	PVE[b] (%)	Add[c]	Dom[d]
F_2	*qVacBrc2.1-A*	86.0	CEDG261 – Vind-SSR016	30.51	32.19	17.88	-19.44
	qVacBrc2.1-B	95.7	Vind-SSR016 – Vind-SSR011	37.27	43.73	26.30	-2.36
$F_{2:3}$-A	*qVacBrc2.1-A*	81.1	CEDG261 – Vind-SSR016	12.99	25.27	16.10	0.07
	qVacBrc2.1-B	93.3	Vind-SSR016 – Vind-SSR011	23.12	41.03	20.40	-0.12
$F_{2:3}$-B (Set I)	*qVacBrc2.1-A*	82.9	CEDG261 – Vind-SSR016	16.77	28.63	19.89	-0.91
	qVacBrc2.1-B	93.3	Vind-SSR016 – Vind-SSR011	28.21	44.14	24.50	-3.19
$F_{2:3}$-B (Set II)	*qVacBrc2.1-A*	83.1	CEDG261 – Vind-SSR016	20.57	33.86	21.63	-1.00
	qVacBrc2.1-B	93.2	Vind-SSR016 – Vind-SSR011	26.80	39.93	23.30	-3.65

[a] Position on the linkage group (centimorgan; cM) [b] Phenotypic variance explained by the QTL
[c] Additive effect [d] Dominant effect

4.2 Identification of candidate gene(s) using RNA seq technology and analysis of gene expression for the resistance to *C. chinenesis* species in moth bean

4.2.1 Mapping of sequencing data and functional annotation of mapped transcripts

Transcripts were annotated using BLASTX. Hypothetical proteins and non-coding genes were found, as well as functional annotations in both moth bean genotypes.

We studied moth bean RNA-seq samples from immature leaves, seeds, and pods. We mapped them to the azuki genome and found high mapping rates. We included a de-novo assembly step in the analysis to account for sequence differences. We found 68% of annotated genes expressed in the samples, 1335 novel genes, and 6095 differentially expressed genes (DEGs). We observed up regulation in resistant genotypes and down regulation in susceptible genotypes, and 899 genes were not expressed during pod maturation. We generated a heat map to visualize the expression clustered patterns of defense response DEGs.

4.2.3 Gene ontology (GO) analysis

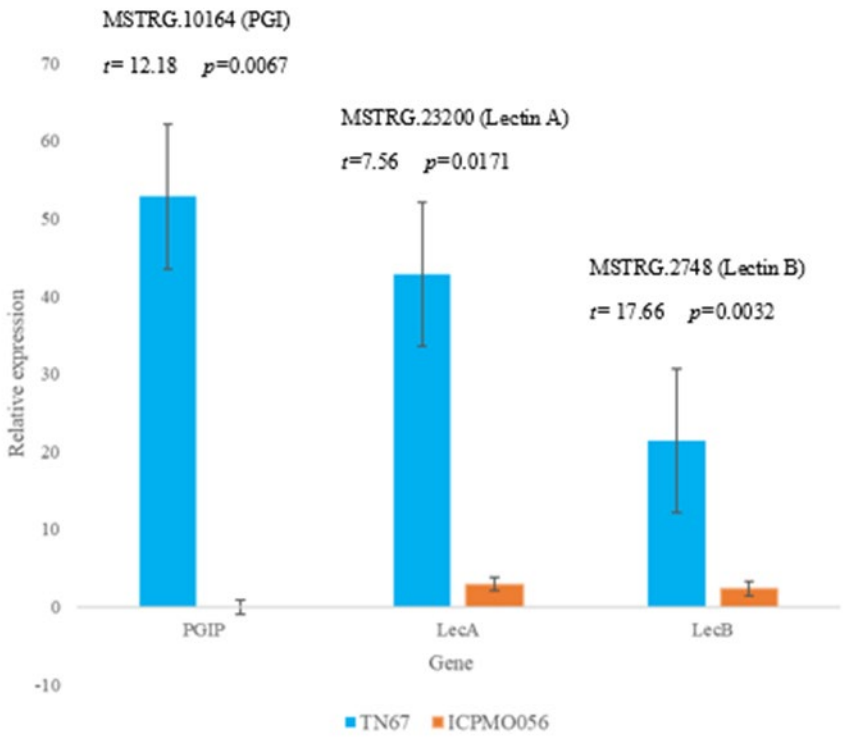
WEGO 2.0 tool categorized Gene Ontology based on BLASTX results against Vigna angularis database. At immature stage, bruchid-resistant TN67 had higher expression of 1,131 biological functions, 540 cellular functions, and 1,630 molecular functions. At mature stage, 1,159 genes had biological functions, 546 genes had cellular functions, and 1,669 genes had molecular functions.

4.2.4 Gene regulatory network analysis

I analyzed insect resistance gene networks using Cytoscape 3.8.1 software. Direct and indirect genes were studied, producing pathways for plant defense response, phytohormones, signaling, and peptidase activity. The defense response pathway showed all defense response activities decreasing, while systemic acquired resistance increased. In the peptidase activity pathway, serine-type endopeptidase inhibitors increased, while cystine-type endopeptidase inhibitor activity decreased.

4.2.5 Validation of differentially expressed genes using RT-qPCR

To validate the changes in gene expression detected by the RNA-Seq, three potential candidate genes including polygalacturonase inhibitor, and two L-type lectin domains were selected for RT-qPCR (Figure 18). The RT-qPCR demonstrated that expression of these genes was consistent with the RNA-Seq data (Figure 19). The results indicated that the RNA-Seq data is reliable.



CONCLUSION

In the present study, fine mapping of qVacBr2.1 revealed two linked QTLs— *VacqBrc2.1-A* and *VacqBrc2.1-B*—that regulated resistance to *C. chinensis* in the wild moth-bean accession TN67. Using comparative genomics, gene mapping, sequencing, and expression analysis, we demonstrated an association between the mutations in VacPGIP1 and *C. chinensis* resistance in TN67. These mutations altered the amino-acid sequence of the LRR domains present in VacPGIP1. To the best of our knowledge, this is the first report of VacPGIP1 as a candidate gene for bruchid resistance in moth bean. Therefore, the findings of this study will improve the understanding of legume-plant resistance against bruchids.