Identification of a candidate gene for bruchid resistance by combining fine mapping and transcriptome profiling in mung bean (*Vigna radiata* L.)

Honglin Chen
Chinese Academy of Agricultural Sciences Institute of Crop Sciences
Liangliang Hu
Chinese Academy of Agricultural Sciences Institute of Crop Sciences
Lixia Wang
Chinese Academy of Agricultural Sciences Institute of Crop Sciences
Suhua Wang
Chinese Academy of Agricultural Sciences Institute of Crop Sciences
Xuzhen Cheng (chengxuzhen5543@163.com)
Chinese Academy of Agricultural Sciences Institute of Crop Sciences https://orcid.org/0000-0002-5737-9336

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Abstract

Bruchids or seed weevils are serious storage insect pests of mung bean and other pulses. Though bruchid-resistant mung bean germplasm accessions are screened out, the molecular mechanisms of bruchid resistance in mung bean are still unclear. In this study, a segregating population with 182 RILs plants was developed; delimit the controlling gene to a 111-kb physical interval, in which 11 genes were predicted. *Vr04g00919* encoding the function of a polygalacturonase inhibitor, was the most likely candidate genes. Here, sequence analysis of the candidate gene coding regions revealed that it has six SNPs between the parental lines and three SNPs resulted in amino acid changes. Sequence alignment revealed that one of these three SNPs are located in a conserved leucine rich repeat (LRR) domain, which is essential for the function of the protein. Subcellular localization of the VrPGIP2-GFP fusion protein indicated that the candidate gene *PGIP2* is located in the nucleus and cytosol. RNA-seq and quantitative real-time PCR (qRT-PCR) analysis indicated that many defense responses, cell wall synthesis, biotic and abiotic stresses, and hormone synthesis were greatly activated in the bruchid resistance plants. These findings contribute to the molecular marker assisted selection of bruchid resistance cultivars.

Introduction

Mung bean (*Vigna radiata* [L.] Wilczek) belongs to the *Vigna* genus within the *Phaseoleae* tribe of crop legumes, which genome size is approximately 550 Mb. Mung bean is an important grain legume, is widely planted in South and Southeast Asia, and they provide an energy and protein source for many populations, particularly in the developing countries, and can be also used to improve soil fertility and increasing nutrients inputs through nitrogen fixation (Nair et al. 2013). Besides, mung bean vegetable sprouts are popular in Asian cuisine, and they are good sources of essential fatty acids, fiber, vitamin C and minerals (Chen et al. 2015; Somta et al. 2007). Bruchid beetles (*Coleoptera: Chrysomelidae: Bruchinae*) are among the most important and well-known as major insect pests of stored grains, can cause significant losses in some legumes during storage. *Callosobruchus chinensis* (L.) is one of the most destructive insect pests of stored legume seeds, which often causes damage to the seed of mung bean (*Vigna radiata* L.), adzuki bean (*Vigna angularis* L.), cowpea (*Vigna unguiculata* L.), pea (*Pisum sativum* L.), faba bean (*Vicia faba* L.), chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris* L.), soybean (*Glycine max* L. Merr.) and lotus (*Nelumbo nucifere Gaertn*.) (Duan et al. 2014). The weevils could firstly lay eggs on the pods in the field, but the damage of the crop is generally low, and then the emerging adults deposit eggs on the seed during storage, causing rapid multiplication of the pest, and therefore, even if only a small amount seed were infected can cause considerable damage during storage. Generaly, this weevil causes 33% *Vigna* seeds loss during storage, which may results in up to 100% of legume grain loss with 3-5 months of storage, economic losses can also reach up to 100% (Boeke et al. 2004; Somta et al. 2006). World-wide crop yield losses without the use of pesticides and other non-chemical control strategies is estimated to be about 70% of crop production, which amounts to US $ 400 billion (Lawrence and Koundal 2002).

There are mainly three ways in controlling storage insect pests; one way is environmental manipulations to prevent their growth, development and reproduction by using of chemical insecticides methods. Chemical pesticides such as pesticides, organophosphate, carbamates, synthetic pyrethroids and insect growth regulators most widely used to protect legumes against this insects and invasive pests are effectively (Daglish et al. 1993), but have a large number of disadvantages including high costs and environmental pollution, with risks to consumer health, and tolerance to insecticide in pest (Gbaye et al. 2011; Sales et al. 2000). The other way to control storage insect pests is physical control methods, including low temperature treatment and airtight storage. In addition, improving the genetic resistance of the host plant would be the most economical and sustainable way to preserve legume seeds against destruction by weevils during storage (Meiners et al. 1980).

The mung bean germplasm resources of bruchid resistance have been identified and characterized for many years, but the resistant germplasm is scarce, and only a few bruchid-resistant lines are available (Hong et al. 2015). Wild mung bean (*Vigna radiata* var. *sublobata*) accession TC1966 is completely resistant to a number of bean weevil species, such as *Callosobruchus chinensis*, *Callosobruchus maculatus*, *Callosobruchus phaseoli*, and *Zabrotes subfasciatus* (Tomooka et al. 2006). The bruchid-resistant accession TC1966 is crossed with *Vigna radiata*, and the buchid resistance gene was introduced into the cultivars (Fujii et al. 1989).

Bruchid resistance in mungean TC1966 is controlled by a single dominant gene. Gene mapping in TC1966 indicated that the bruchid resistance gene (*Br*) is located on linkage group IV between restriction fragment length polymorphism (RFLP) markers, pM151a and pA882 , to a location about 3.6 centimorgans (cM) from the pA882 (Young et al. 1992). The *Br* locus was narrowed down to 0.7 cM between RFLP markers, Bng143 and Bng110, with Bng143 0.2 cM away from *Br* locus (Kaga and Ishimoto 1998a). Cleaved amplified polymorphism (CAPs) markers linked with the *Br* were also developed (Chen et al. 2007). Based on QTL mapping, a SSR marker DMB158 was tightly linked with the *Br*, with less than 0.1 cM (Chen et al. 2013).

Although these DNA markers closely linked to the *Br* locus have been reported, the candidate gene and molecular basis are remaining unknown. The whole-genome sequences of mung bean were assembled (Kang et al. 2014), which is convenient for development of DNA markers and gene identification. In this study, we reported fine mapping of the *Br* locus in wild mung bean. The aim of this study was to identify the candidate gene responsible for the bruchid resistance and reveal the molecular basis.

Materials And Methods

Plant materials

The RIL population consisting of 182 F_7 lines was derived from a cross between two inbred lines, C618 (female parent, resistant) and Zhonglv 5 (male parent, susceptible) using single-seed descent. Two

bruchid-resistant mung bean lines C618 and N618 (isogenic line of Zhonglv 5), and 13 bruchid-susceptible mung bean accessions, including Zhonglv 5, Zhonglv 10, Zhonglv 12, Jilv 5, Jilv 7, Bailv 11, VC2917, VC3890A, VC1628A, VC2778A, and CN60 were used to analyze the mutation sites of the target gene in this study (Supplementary Table S1). All mung bean seeds were obtained from the National Crop Genebank of China (NCGC).

Evaluation of bruchid resistance

Bruchids of *C. chinensis* used for identification of bruchid resistance. Bruchid were obtained by feeding the adults with the susceptible mung bean seeds of Zhonglv 5, and the parental plants and each of the RILs were individually harvested and used to evaluate for bruchid resistance. A total of 40 mature seeds from patents, the seeds of RILs were evaluated by exposure to *C. chinensis* in three replicates as described previously (Mei et al. 2009). When each of the susceptible control seeds of Zhonglv 5 is about 5 eggs, the adult insects were removed. Assays were maintained until all these seeds showed typical bruchid symptoms. Ratios of the number of damaged seeds to the total number of seeds tested were used for calculating the bruchid infestation. Highly resistant (HR) exhibited 0–20 % damaged seeds, highly susceptible (HS) exhibited 81–100 % damaged seeds, and moderately resistant (MR) exhibited 21–80 % damaged seeds. A Chi-square analysis (χ^2) goodness-of-fit test was used to determine the goodness-of-fit to a 3 resistant (R) : 1 susceptible ration (S).

Simple Sequence Repeat (SSR) analyses and fine mapping

Flanking SSR markers on mung bean chromosome 4 were used to screen for polymorphism between parental lines. Polymorphism in each synthesized marker was firstly assessed using parental DNA. The physical positions of developed SSR markers were determined by BLAST searches of the primer sequences against the Zhonglv 5 genome. The polymorphic markers were then used to determine the genotype of recombinant plants.

Molecular cloning and sequencing

The total genomic DNA was extracted from mung bean young leaf tissue using a modified cetyl-trimethyl-ammonium bromide method (CTAB) method (Chen et al. 2015; Mei et al. 2009). Total RNA was isolated from two-week-old mung bean seedling using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was amplified by using reverse transcription PCR (RT-PCR) with MLV Reverse Transcriptase (Promega, Tokyo, Japan). Both the genomic DNA and cDNA of the candidate gene were cloned from bruchid-resistant and susceptible lines in this study. Primer pairs (VrPGIP2-F/VrPGIP2-R and VrPGIP2JC-F/VrPGIP2JC-R in Supplementary Table S2) were designed to amplify the whole and partial genomic DNA or cDNA sequences of bruchid-resistant and susceptible lines. The PCR reactions were performed and monitored in 20 μL of total volumes and the products were electrophoresed on 1% agarose gels and extracted by using a gel extraction kit (TsingKe, China).

Localization of the VrPGIP-GFP fusion protein

To assess the subcellular localization of the *VrPGIP2* protein, the *VrPGIP2* ORF (open reading frame) without the termination codon was amplified using primers (VrPGIP-GFP-F and VrPGIP-GFP-R) that incorporated *BamHI* and *KpnI* sites: 5'-GGATCC ATGCGAAGCCTGTTAATGAT-3' (*BamHI* site underlined) and 5'-GGTACCCTTGGTGCAGGGCAGAAGTG-3' (*KpnI* site underlined). In the present study, the PCR products were cloned into the pE3025-*GFP* vector to generate the pE3025-*VrPGIP-GFP* construct, which under the control of the CaMV dual 35S promoter, as well as a TEV enhancer. The plasmid was introduced to onion epidermal cells via a gene gun transformation (Bio-Rad, California, USA). At last, GFP fluorescence was analyzed by confocal fluorescence microscopy (Nikon, Tokyo, Japan).

Protein sequence, phylogenetic and 3D-modelling analyses

Protein sequences of PGIP orthologs were retrieved from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/entrez). The PGIP alignment was performed by using multiple amino acid sequence alignment software CLUSTALX 1.83 (Thompson et al. 1997), and the phylogenetic tree was constructed using MEGA 5.0 (Tamura et al. 2007). The distances between the branches were computed via the neighbor-joining method with 1000 bootstrap replicates (Saitou and Nei 1987). Three-dimensional (3D) structures of VrPGIP2 were predicted using the SWISS-MODEL protein structure homology-modelling server (http://swissmodel.expasy.org) operating in automated mode (Arnold et al. 2006). Full length amino acid sequences of VrPGIP were uploaded and visualized using Swiss-PdbViewer (Guex and Peitsch 1997).

RNA-seq and qRT-PCR analysis

Total RNA was extracted from the immature seeds of bruchid-resistant and susceptible parent lines using a TRIzol RNA extraction kit (Invitrogen, CA, USA). The total RNAs were treated with RNase-free DNase I. Reverse transcription was performed with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). The cDNA libraries of C618 and N618 were sequenced with Illumina platform The FASTQ files containing raw data for all the reads were submitted to the sequence read archive (SRA) database at Genbank (www.ncbi.nlm.nih.gov), and given the accession number SRP043316. qRT-PCR amplification and analysis were performed using specific primers (Primer pairs sequences were listed in Supplementary Table S3) with fluorescence dye SYBR Green (SYBR Green Supermix kit, Bio-Rad, USA) which was carried out on CFX96 qRT-PCR detection system (Bio-Rad, USA). Actin was used as an internal control for normalization. The relative expression level was determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Results

Phenotypic evaluation of bruchid-resistant and susceptible lines

To further confirm the inheritance of bruchid resistance, these mature seeds of C618, Zhonglv 5, N618 (isogenic line of Zhonglv 5), and these seeds of RILs was evaluated against *C. chinensis*, approximately 60 days post-infestation, the seeds of C618 (Figure 1a), Zhonglv 5 (Figure 1c), and the seeds of F_1

generation of (Zhonglv 5 × C618) appeared symptom-free, whereas the susceptible parent Zhonglv 5 showed worm-eaten symptoms (Figure 1b). Based on *C. chinensis* inoculation tests, 133 exhibited resistant (R), and 49 exhibited susceptible (S) (Figure 1d), it showed a 3:1 ratio (R : S) (χ^2 = 2.374, P > 0.1), which suggesting that bruchid resistance is a single dominant trait.

Map-based cloning of bruchid resistance gene

In our previous studies, the *Br* gene was mapped to a 380-kb physical region on chromosome 4 between two simple sequence repeat (SSR) markers Vr04-221 and Vr04-604 (Figure 2a). To further delineate this bruchid resistance gene, we further used 182 RILs plants for fine mapping analyses. According to new developed genome-wide SSR marker resources, 7 SSR markers between Vr04-221 and Vr04-604 between the parents were polymorphic. By screening the population with SSR markers Vr04-246 and Vr04-531, 12 recombination events were detected, and 7 recombination events were detected by SSR markers Vr04-249 and Vr04-483, which delimited *Br* to a 111-kb physical region (Figure 2a), a total of 11 candidate genes were predicted in this delimited region (Table 1), and only *Vr04g00918* and *Vr04g00919* were reported to be related with resistance to diseases and pests. The full-length of DNA sequence of these 11 candidate genes in the bruchid-resistant and susceptible lines were sequenced, and we found that the *Vr04g00919* gene with six single-nucleotide substitutions (Figure 2b), among them, G-to-T, T-to-C, and A-to-C changes at positions 958, 995 and 1003, causing three amino acids changes from alanine (A) to serine (S) at position 320, leucine (L) to proline (P) at position 332, and threonine (T) to proline (P) at position 335. *Vr04g00919* was found to be intronless with 1011 nucleotides encoding a protein of 336 amino acids. It is a potential candidate of *Br* (Figure 2c), and it encoded a receptor-like kinase. The putative amino acid sequences were aligned with those of the homologous proteins in the NCBI database, and polygalacturonase inhibitor (*PGIP*) genes were identified.

To better understand these variations that may affect gene function, the sequence differences of bruchid-resistant and susceptible lines were aligned. The full-length genome DNA sequences from six lines (two resistant to *C. chinensis* and four susceptible to *C. chinensis*) were PCR amplified and sequenced. Both the full-length cDNAs and deduced amino acid sequences were also aligned, respectively. Alignment analysis indicated that six different SNPs and three amino acids were identified in the cDNAs and amino acids from C618, N618, Zhonglv 1, Zhonglv 5, Zhonglv 10, and CN60, respectively (Figure 2b, 2c). Moreover, we used another 13 mung bean lines to investigate the utility of the primer sequences of VrPGIPJC for MAS, and found that genotypes be co-segregated with bruchid resistance in all lines.

Subcellular localization of VrPGIP2

To determine the subcellular localization of VrPGIP2, GFP was fused to the C-terminus of *VrPGIP2* under the control of the CaMV35S constitutive promoter. The *VrPGIP2* CDS was isolated from the Zhonglv 5. The cellular location of *VrPGIP2* proteins was investigated in onion epidermal cells transiently expressing gene fusions to the green fluorescent protein (GFP). Microscopic analyses detected fluorescence of GFP was present throughout the entire cell (Figure 2a-c), indicating the VrPGIP2-GFP protein accumulated

primarily in both the nucleus and cytosol (Figure 3 d-f). Thus, VrPGIP2 was a nuclear and cytosol-localized protein.

The protein structure of VrPGIP2

To find the domain essential for the function of the VrPGIP protein, the amino acid sequences of VrPGIP2 were searched against the protein database of NCBI. The protein is composed of leucine-rich repeats (LRRs). Analysis of protein structure indicated that the VrPGIP2 polypeptide contains a typical LRR domain and other conserved motifs (N-glycosylation site, protein kinase C phosphorylation site, casein kinase II phosphorylation site, LDL-receptor class B (LDLRB) repeat site, and plant-specific LRR proteins. The N-terminal and C-terminal contain cysteine-rich domains. VrPGIP protein is composed of four parts: a signal peptide domain, LRR domain, B domain, and D domain (Figure 4a). The N-terminus of VrPGIP includes a signal peptide (M1-A34) and B domain (V35-D82). B-domain contains four homologous Cys residues (C39, C69, C70, and C80) and two conserved Trp residues (W63 and W74), which might be necessary for PGIPs activity. The leucine rich repeat N-terminal domain (LRRNT) (H_{40}-D_{81}), the LDLRB domain (K_{284}-I_{297}), the plant-specific LRR domain (P_{97}-L_{169}), and the typical LRR domain (L123-L193) is highly conserved among the plant PGIPs. The main domain included 10 LRRs (each about 23 amino acids long), which is thought to mediate protein-protein interactions.

To understand the protein structure, the 3D structure of VrPGIP2 was predicted using SWISS-MODEL, it indicated that VrPGIP2 contains one α-helix and two β-sheets (Figure 4b). One β-sheet occupies the concave inner side of the molecule and contains residues crucial for PG recognition, which is a determinant of PGIPs specificity. The 10 LRRs of PGIPs are of the extracytoplasmic type (LxxLxLxxNxL) (Figure 4a), which are organized to form two β-sheets (Figure 4b). Compared to the amino acid sequence of susceptible mung bean lines, the mutation site (A_{320} to S_{320}) is located in the conserved LRR domain (Figure 4a), which could be related to the PGIP functions in plant defense responses, and the non-polar alanine was changed to polar serine, the conformation was changed, which finally result in functional change of LRR domain (Figure 4b). The other two mutation sites (P_{332} to L_{332}, P_{335} to T_{335}) are located in the conserved D domain, and polar threonine was changed to non-polar proline (Figure 4a and 4b). These data revealed that these SNPs variation are responsible for resistance/susceptible to bruchids.

Sequence alignment indicated that VrPGIP2 shares the highest similarity (73.0% identity) with CcPGIP (*Cajanus cajan*), and relatively high homology (65.0% identity) with GmPGIP1 (*Glycine max*) through an alignment analysis of sequences from leguminous plants. It also shares 62.0% identity with CaPGIP1 (*Cicer arietinum*, XP_012569258.1), but relatively low homology (30-56% identities) with other plant PGIPs (Figure 4c), suggesting that the genetics relationship among leguminous plants is more closely than other plants and the function of PGIP in leguminous plants is conserved.

Differential gene expression analysis of the bruchid-resistant and susceptible lines

To study the influence of *VrPGIP2* on bruchid resistance, RNA sequencing analysis was performed to detect differentially expressed genes (DEGs. A total of 83,542 transcript profiles of bruchid-resistant line

N618 and susceptible line Zhonglv 5, and 894 differentially expressed genes were identified, of which 436 and 458 genes were up-regulated and down-regulated in N618 compared to Zhonglv 5. Some of these DEGs were further confirmed by using qRT-PCR. A functional cluster analysis of the DEGs indicated that the genes that were involved in many defense responses, cell wall synthesis, biotic and abiotic stresses, and hormone synthesis were greatly activated in the N618 plants. For example, resistant specific protein (*Vr04g00908*), serine/threonine-protein kinase (*Vr01g01268*), Mitogen-activated protein kinase (*Vr09g01762*), auxin transporter protein (*Vr02g02682*), cellulose synthase (*Vr04g02415*, *Vr06g01105*), lipid-transfer protein (*Vr05g02418*, *Vr08g01103*), and MYB114 (*Vr04g00047*), were significantly induced in the bruchid-resistant line N618 (Figure 5). However, the RNA sequencing analysis shows that many defense-related genes are activated in the bruchid-resistant lines, such as serine/threonine-protein kinase, mitogen-activated protein (MAPK), cellulose synthase, and lipid-transfer protein. No candidate genes in the mapped regions were found differentially expression in N618 and Zhonglv 5 in the mapped regions except the resistant specific protein (*Vr04g00908*), which was very close to candidate genes. *VrPGIP2* is also not great differently expressed in resistant lines and susceptible lines due to the mutation sites of *VrPGIP2* is located in the coding region.

Discussion

Storage insect pests could cause serious damage to all legume crops (Keneni et al. 2011). Bruchid beetles are the most important pests of stored grain legume seeds, including *Callosobruchus maculatus* (F.) and *Callosobruchus chinensis* (L.) (Bushara 1988; Kashiwaba et al. 2003), and which are the most important pests for stored grain legumes in *Vigna* crops and cause tremendous damage. Legume breeders have long been interested in breeding bruchid-resistant cultivars and understanding the genetic control of the bruchid resistance. However, study on bruchid resistance is difficult due to the genetics of seed components are different in both generation and ploidy level, and bruchid beetles are easy to escape, need to maintain live colonies, and appropriate conditions of temperature, humidity, and light (Yencho et al. 2000; Blair et al. 2010).

Characterization of bruchid resistance genes would help understand the molecular mechanism of bruchid resistance. Although, fine mapping of the bruchid-resistant locus has been reported many years ago (Kaga and Ishimoto 1998b), identifying of the genes or candidate genes responsible for the bruchid resistance is difficult due to the lack of availability of large genomic sequences for mung bean, and the efficiency of genetic transformation of mung bean is still relatively low at present. Previously, much effort toward the identification of factors are responsible for bruchid resistance present in mung bean (Liu et al. 2016; Lin et al. 2005; Chen et al. 2002), cowpea (Luthi et al. 2013; Ahn et al. 2013; Solleti et al. 2008), rice bean (Pavithravani et al. 2013; Venkataramana et al. 2016), common bean (Blair et al. 2010; Nishizawa et al. 2007), and chickpea (Luthi et al. 2013). Metabolism and transposable elements (TEs) may be modifier factors for bruchid resistance by transcriptomic comparison of mung bean bruchid-resistant and susceptible parental lines and their offspring (Liu et al. 2016). Transgenic cowpeas expressing alphaAI-1 strongly inhibited the development of *C. chinensis* and *C. maculatus* (Solleti et al. 2008). The activity of APA (arcelin, phytohemagglutinin and α-amylase inhibitor) plays important role bruchid

resistance (Pavithravani et al. 2013). The α-amylase inhibitor negatively affects the growth and development of *C. chinensis* and *C. maculatus* in common bean seeds was reported (Ishimoto et al. 1996; Chen et al. 2009). The alphaAI-1 in transgenic cowpea and chickpea lines significantly increases resistance to *C. chinensis* and *C. maculatus* (Luthi et al. 2013). The role of proteinase and amylase inhibitor activity in legumes bruchid resistance remains unknown (Ishimoto et al. 1996; Piergiovanni et al. 1994; Fernandes et al. 1993). Some of the putative *Br* factors of mung bean bruchid lines were thought to be harmful for human consumption (Miura et al. 1996). Subchronic oral toxicity studies of bruchid-resistant mung bean demonstrated that bruchid-resistant mung bean is as safe as conventional mung bean (Yao et al. 2015). The whole-genome sequence of mung bean enabled complete cloning and characterization of genes. In this study, a candidate gene responsible for bruchid resistance was mapped in mung bean, and the candidate gene has sequence similarity to glycine max polygalacturonase inhibitor gene (XP_014499188). However, the function of the gene in mung bean remains unclear.

Plant polygalacturonase-inhibiting proteins (PGIPs) are one of the plant protease inhibitors (PIs), which could inhibit the activtiy of polygalacturonase (PG) enzymes produced by fungal bacterial pathogens (Ryan 1990; Kim et al. 2009), and insects (Lawrence and Koundal 2002; Ryan 1990; Green and Ryan 1972; Darvill et al. 1994). PGIPs are extracellular plant proteins bound to the plant cell wall containing LRR glycoproteins of approximately 360 amino acids in length (Cook et al. 1999), which play essential roles in plant defense against pathogens and insects. Polygalacturonases (PGs) are an important pathogenicity factors, produced by pathogens and insects during early infection that depolymerize the major component of homogalacturonan and pectin , which are the major components of plant cell wall (Lionetti et al. 2010). Plants produce cell wall-associated PGIPs against fungal, microbial, and insect PGs (Spadoni et al. 2006). The overexpression of PGIPs in transgenic plants improves the resistance to bacterial and fungal pathogen (Aguero et al. 2005; Ferrari et al. 2012). PGIPs counteract fungal PGs by forming specific complexes with them in the cell wall of many plants (Torki et al. 2000; De Lorenzo et al. 2001), the accumulation of partially digested fragments of polygalacturonic acid and oligogalacturonides induce the plant defense responses (De Lorenzo et al. 2001; Martin et al. 2003; Mohammadzadeh et al. 2015). PGIP reduces the susceptibility to bacterial and fungal attack in different transgenic plants, such as Arabidopsis (Ferrari et al. 2003), wheat (Janni et al. 2008), sugar beet (Powell et al. 2000), pear (Powell et al. 2000), and apple (Mohammadzadeh et al. 2015). The resistance of PGIPs to fungal and bacterial has been extensively studied, but their resistance to insects is much less known.

The LRR involved in recognition of the resistance gene products in many plant (Martin et al. 2003), and several receptors involved in perception of hormones (Szekeres 2003), elicitors (Gomez-Gomez et al. 2001), defense responses against insects (Szekeres 2003), and fungal and bacterial symbiosis (Kistner and Parniske 2002). The LRRs of PGIPs are organized into β1 and β2 sheets (Kalunke et al. 2014), and a single amino acid change in β1 sheet confers the ability to recognize a novel PG, which imply that the LRR domain is critical for determining recognition specificity toward insects or pathogens (Leckie et al. 1999). In our study, the mutation sites are located in the conserved LRR domain, which change the polarity of amino acids, results in the conformational and functional change of LRR domain. Most of

plant disease resistance (*R*) genes encode proteins contained a C-terminal LRR domain. The mutation sites are related to the functions of PGIP against for bruchid resistance.

Abbreviations

Br, bruchid resistance gene; QTL, quantitative trait locus; PG, polygalacturonases; PGIP, polygalacturonase-inhibiting protein; RFLP, restriction fragment length polymorphism; CAP, cleaved amplified polymorphism; SSR, simple sequence repeat; SNP, single-nucleotide polymorphism; ORF, open reading frame; DEG, differentially expressed gene; qRT-PCR, quantitative real-time PCR; PI, protease inhibitors; MAPK, mitogen-activated protein kinase; LRR, leucine rich repeat; GFP, green fluorescent protein; RFP, red fluorescent protein.

Declarations

Author contribution statement

HL C and XZ C designed the project. LL H performed the experiments, HL C analyzed the data, wrote the paper, XZ C reviewed drafts of the paper. LX W and SH W performed some of the fieldwork and *C. chinensis* infestation test. All authors contributed to and approved the final manuscript.

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Declaration of competing interest

Authors declare that there are no conflicts of interest.

References

Aguero CB, Uratsu SL, Greve C, Powell ALT, Labavitch JM, Meredith CP, Dandekar AM (2005) Evaluation of tolerance to Pierce's disease and Botrytis in transgenic plants of Vitis vinifera L. expressing the pear PGIP gene. Mol Plant Pathol 6:43-51

Ahn JE, Zhou X, Dowd SE, Chapkin RS, Zhu-Salzman K (2013) Insight into hypoxia tolerance in cowpea bruchid: metabolic repression and heat shock protein regulation via hypoxia-inducible factor 1. PLoS One 8 :e57267

Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22:195-201

Blair MW, Munoz C, Buendia HF, Flower J, Bueno JM, Cardona C (2010) Genetic mapping of microsatellite markers around the arcelin bruchid resistance locus in common bean. Theor Appl Genet 121:393-402

Boeke SJ, Baumgart IR, van Loon JJA, van Huis A, Dicke M, Kossou DK (2004) Toxicity and repellence of African plants traditionally used for the protection of stored cowpea against Callosobruchus maculatus. J Stored Prod Res 40:423-438

Bushara AG (1988) Insect depredation during storage. In: Summerfield RJ (ed) World crops: Cool season food legumes: A global perspective of the problems and prospects for crop improvement in pea, lentil, faba bean and chickpea. Springer Netherlands, Dordrecht, pp 367-378

Chen H, Bodulovic G, Hall PJ, Moore A, Higgins TJ, Djordjevic MA, Rolfe BG (2009) Unintended changes in protein expression revealed by proteomic analysis of seeds from transgenic pea expressing a bean alpha-amylase inhibitor gene. Proteomics 9:4406-4415

Chen H, Wang L, Wang S, Liu C, Blair MW, Cheng X (2015) Transcriptome sequencing of mung bean (Vigna radiate L.) genes and the identification of EST-SSR markers. PLoS One 10:e0120273

Chen HM, Ku HM, Schafleitner R, Bains TS, Kuo CG, Liu CA, Nair RM (2013) The major quantitative trait locus for mungbean yellow mosaic Indian virus resistance is tightly linked in repulsion phase to the major bruchid resistance locus in a cross between mungbean [Vigna radiata (L.) Wilczek] and its wild relative Vigna radiata ssp sublobata. Euphytica 192:205-216

Chen HM, Liu CA, Kuo CG, Chien CM, Sun HC, Huang CC, Lin YC, Ku HM (2007) Development of a molecular marker for a bruchid (Callosobruchus chinensis L.) resistance gene in mungbean. Euphytica 157:113-122

Chen KC, Lin CY, Kuan CC, Sung HY, Chen CS (2002) A novel defensin encoded by a mungbean cDNA exhibits insecticidal activity against bruchid. J Agric Food Chem 50:7258-7263

Cook BJ, Clay RP, Bergmann CW, Albersheim P, Darvill AG (1999) Fungal polygalacturonases exhibit different substrate degradation patterns and differ in their susceptibilities to polygalacturonase-inhibiting proteins. Mol Plant Microbe In 12:703-711

Daglish GJ, Erbacher JM, Eelkema M (1993) Efficacy of protectants against Callosobruchus phaseoli (Gyll.) and C. maculatus (F.) (Coleoptera: Bruchidae) in mungbeans. J Stored Prod Res 29 (4):345-349

Darvill A, Bergmann C, Cervone F, De Lorenzo G, Ham KS, Spiro MD, York WS, Albersheim P (1994) Oligosaccharins involved in plant growth and host-pathogen interactions. Biochem Soc Symp 60:89-94

De Lorenzo G, D'Ovidio R, Cervone F (2001) The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. Annu Rev Phytopathol 39:313-335

Duan CX, Li DD, Sun SL, Wang XM, Zhu ZD (2014) Rapid development of microsatellite markers for Callosobruchus chinensis using Illumina paired-end sequencing. PLoS One 9 (5):e95458

Fernandes KV, Sabelli PA, Barratt DP, Richardson M, Xavier-Filho J, Shewry PR (1993) The resistance of cowpea seeds to bruchid beetles is not related to levels of cysteine proteinase inhibitors. Plant Mol Biol 23:215-219

Ferrari S, Sella L, Janni M, De Lorenzo G, Favaron F, D'Ovidio R (2012) Transgenic expression of polygalacturonase-inhibiting proteins in Arabidopsis and wheat increases resistance to the flower pathogen Fusarium graminearum. Plant Biol (Stuttg) 14 Suppl 1:31-38

Ferrari S, Vairo D, Ausubel FM, Cervone F, De Lorenzo G (2003) Tandemly duplicated arabidopsis genes that encode polygalacturonase-inhibiting proteins are regulated coordinately by different signal transduction pathways in response to fungal infection. Plant Cell 15:93-106

Fujii K, Ishimoto M, Kitamura K (1989) Patterns of resistance to bean weevils (Bruchidae) in Vigna radiata-mungo-sublobata complex inform the breeding of new resistant varieties. Appl Entomol Zool 24:126-132

Gbaye OA, Millard JC, Holloway GJ (2011) Legume type and temperature effects on the toxicity of insecticide to the genus Callosobruchus (Coleoptera: Bruchidae). J Stored Prod Res 47 (1):8-12

Gomez-Gomez L, Bauer Z, Boller T (2001) Both the extracellular leucine-rich repeat domain and the kinase activity of FLS2 are required for flagellin binding and signaling in arabidopsis. Plant Cell 13:1155-1163

Green TR, Ryan CA (1972) Wound-Induced Proteinase Inhibitor in Plant Leaves: A possible defense mechanism against insects. Science 175 (4023):776-777

Guex N, Peitsch MC (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 18:2714-2723

Hong M-G, Kim K-H, Ku J-H, Jeong J-K, Seo M-J, Park C-H, Kim Y-H, Kim H-S, Kim Y-K, Baek S-H (2015) Inheritance and Quantitative Trait Loci Analysis of Resistance Genes to Bruchid and Bean Bug in Mungbean (Vigna radiata L. Wilczek). Plant Breed Biotechnol 3:39-46

Ishimoto M, Sato T, Chrispeels MJ, Kitamura K (1996) Bruchid resistance of transgenic azuki bean expressing seed alpha-amylase inhibitor of common bean. Entomol Exp Appl 79:309-315

Janni M, Sella L, Favaron F, Blechl AE, De Lorenzo G, D'Ovidio R (2008) The expression of a bean PGIP in transgenic wheat confers increased resistance to the fungal pathogen Bipolaris sorokiniana. Mol Plant Microbe Interact 21:171-177

Kaga A, Ishimoto M (1998a) Genetic localization of a bruchid resistance gene and its relationship to insecticidal cyclopeptide alkaloids, the vignatic acids, in mungbean (Vigna radiata L. Wilczek). Mol Gen

Genet 258:378-384

Kaga A, Ishimoto M (1998b) Genetic localization of a bruchid resistance gene and its relationship to insecticidal cyclopeptide alkaloids, the vignatic acids, in mungbean (Vigna radiata L. Wilczek). Mol Gen Genetics 258 (4):378-384

Kalunke RM, Cenci A, Volpi C, O'Sullivan DM, Sella L, Favaron F, Cervone F, De Lorenzo G, D'Ovidio R (2014) The pgip family in soybean and three other legume species: evidence for a birth-and-death model of evolution. BMC Plant Biol 14:189

Kang YJ, Kim SK, Kim MY, Lestari P, Kim KH, Ha BK, Jun TH, Hwang WJ, Lee T, Lee J, Shim S, Yoon MY, Jang YE, Han KS, Taeprayoon P, Yoon N, Somta P, Tanya P, Kim KS, Gwag JG, Moon JK, Lee YH, Park BS, Bombarely A, Doyle JJ, Jackson SA, Schafleitner R, Srinives P, Varshney RK, Lee SH (2014) Genome sequence of mungbean and insights into evolution within Vigna species. Nat Commun 5

Kashiwaba K, Tomooka N, Kaga A, Han OK, Vaughan DA (2003) Characterization of resistance to three bruchid species (Callosobruchus spp., Coleoptera, Bruchidae) in cultivated rice bean (Vigna umbellata). J Econ Entomol 96:207-213

Keneni G, Bekele E, Getu E, Imtiaz M, Damte T, Mulatu B, Dagne K (2011) Breeding Food Legumes for Resistance to Storage Insect Pests: Potential and Limitations. Sustainability 3 (9):1399

Kim JY, Park SC, Hwang I, Cheong H, Nah JW, Hahm KS, Park Y (2009) Protease inhibitors from plants with antimicrobial activity. Int J Mol Sci 10:2860-2872

Kistner C, Parniske M (2002) Evolution of signal transduction in intracellular symbiosis. Trends Plant Sci 7:511-518

Lawrence PK, Koundal KR (2002) Plant protease inhibitors in control of phytophagous insects. Electron J Biotechno 5 (1) pp.5-6

Leckie F, Mattei B, Capodicasa C, Hemmings A, Nuss L, Aracri B, De Lorenzo G, Cervone F (1999) The specificity of polygalacturonase-inhibiting protein (PGIP): a single amino acid substitution in the solvent-exposed β-strand/β-turn region of the leucine-rich repeats (LRRs) confers a new recognition capability. EMBO J 18:2352-2363

Lin C, Chen CS, Horng SB (2005) Characterization of resistance to Callosobruchus maculatus (Coleoptera: Bruchidae) in mungbean variety VC6089A and its resistance-associated protein VrD1. J Econ Entomol 98:1369-1373

Lionetti V, Francocci F, Ferrari S, Volpi C, Bellincampi D, Galletti R, D'Ovidio R, De Lorenzo G, Cervone F (2010) Engineering the cell wall by reducing de-methyl-esterified homogalacturonan improves saccharification of plant tissues for bioconversion. Proc Natl Acad Sci U S A 107 (2):616-621

Liu MS, Kuo TCY, Ko CY, Wu DC, Li KY, Lin WJ, Lin CP, Wang YW, Schafleitner R, Lo HF, Chen CY, Chen LFO (2016) Genomic and transcriptomic comparison of nucleotide variations for insights into bruchid resistance of mungbean (Vigna radiata [L.] R. Wilczek). BMC Plant Biol 16

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25:402-408

Luthi C, Alvarez-Alfageme F, Ehlers JD, Higgins TJ, Romeis J (2013) Resistance of alphaAI-1 transgenic chickpea (Cicer arietinum) and cowpea (Vigna unguiculata) dry grains to bruchid beetles (Coleoptera: Chrysomelidae). Bull Entomol Res 103:373-381

Martin GB, Bogdanove AJ, Sessa G (2003) Understanding the functions of plant disease resistance proteins. Annu Rev Plant Biol 54:23-61

Mei L, Cheng XZ, Wang SH, Wang LX, Liu CY, Sun L, Xu N, Humphry ME, Lambrides CJ, Li HB, Liu CJ (2009) Relationship between bruchid resistance and seed mass in mungbean based on QTL analysis. Genome 52:589-596

Meiners JP, Elden TC, Summerfield RJ, Bunting AH (1980) Resistance to insects and diseases in Phaseolus. Advances in Legume Science pp359-364

Miura K, Ishimoto M, Yamanaka N, Miyazaki S, Hiramatsu M, Nakajima Y, Hirano K, Kitamura K (1996) Effects of bruchid-resistant mungbean meal on growth and blood-biochemical values in mice. JIRCAS Journal (3):23-31

Mohammadzadeh R, Motallebi M, Zamani M, Jahromi ZM, Norouzi P, Benedetti M, de Lorenzo G (2015) Generation of transgenic sugar beet (Beta vulgarism L.) overexpressing the polygalacturonase inhibiting protein 1 of Phaseolus vulgaris (PvPGIP1) through Agrobacterium-mediated transformation. Turk J Agric For 39:429-438

Nair RM, Yang RY, Easdown WJ, Thavarajah D, Thavarajah P, Hughes J, Keatinge JD (2013) Biofortification of mungbean (Vigna radiata) as a whole food to enhance human health. J Sci Food Agric 93 (8):1805-1813

Nishizawa K, Teraishi M, Utsumi S, Ishimoto M (2007) Assessment of the importance of alpha-amylase inhibitor-2 in bruchid resistance of wild common bean. Theor Appl Genet 114:755-764

Pavithravani BV, Gowda R, Bhanuprakash K, Ramesh S, Rao MA, Subramanya S, Gireesh C (2013) Biochemical Components: An index of bruchid resistance in rice bean [Vigna Umbellata (Thunb.) Ohwi and Ohashi]. Legume Res 36:582-588

Piergiovanni A, Della Gatta C, Sergio L, Perrino P (1994) High antinutrient levels and bruchid resistance of cowpea (Vigna unguiculata) seeds. Euphytica 80:59-62

Powell AL, van Kan J, ten Have A, Visser J, Greve LC, Bennett AB, Labavitch JM (2000) Transgenic expression of pear PGIP in tomato limits fungal colonization. Mol Plant Microbe Interact 13:942-950

Ryan CA (1990) Protease inhibitors in plants: genes for improving defenses against insects and pathogens. Annu Rev Phytopathol 28:425-449

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425

Sales MP, Gerhardt IR, Grossi-De-Sa MF, Xavier-Filho J (2000) Do legume storage proteins play a role in defending seeds against bruchids? Plant Physiol 124:515-522

Solleti SK, Bakshi S, Purkayastha J, Panda SK, Sahoo L (2008) Transgenic cowpea (Vigna unguiculata) seeds expressing a bean alpha-amylase inhibitor 1 confer resistance to storage pests, bruchid beetles. Plant Cell Rep 27:1841-1850

Somta P, Ammaranan C, Ooi PAC, Srinives P (2007) Inheritance of seed resistance to bruchids in cultivated mungbean (Vigna radiata, L. Wilczek). Euphytica 155:47-55

Somta P, Talekar NS, Srinives P (2006) Characterization of Callosobruchus chinensis (L.) resistance in Vigna umbellata (Thunb.) Ohwi & Ohashi. J Stored Prod Res 42:313-327

Spadoni S, Zabotina O, Di Matteo A, Mikkelsen JD, Cervone F, De Lorenzo G, Mattei B, Bellincampi D (2006) Polygalacturonase-inhibiting protein interacts with pectin through a binding site formed by four clustered residues of arginine and lysine. Plant Physiol 141:557-564

Szekeres M (2003) Brassinosteroid and systemin: two hormones perceived by the same receptor. Trends Plant Sci 8:102-104

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596-1599

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876-4882

Tomooka N, Lairungreang C, Nakeeraks P, Egawa Y, Thavarasook C (2006) Development of bruchid-resistant mungbean line using wild mungbean germplasm in Thailand. Plant Breeding 109:60-66

Torki M, Mandaron P, Mache R, Falconet D (2000) Characterization of a ubiquitous expressed gene family encoding polygalacturonase in Arabidopsis thaliana. Gene 242:427-436

Venkataramana PB, Gowda R, Somta P, Ramesh S, Rao AM, Bhanuprakash K, Srinives P, Gireesh C, Pramila CK (2016) Mapping QTL for bruchid resistance in rice bean (Vigna umbellata). Euphytica

207:135-147

Yao Y, Cheng X, Ren G (2015) A 90-day study of three bruchid-resistant mung bean cultivars in Sprague-Dawley rats. Food Chem Toxicol 76:80-85

Yencho GC, Cohen MB, Byrne PF (2000) Applications of tagging and mapping insect resistance loci in plants. Annu Rev Entomol 45:393-422

Young ND, Kumar L, Menancio-Hautea D, Danesh D, Talekar NS, Shanmugasundarum S, Kim DH (1992) RFLP mapping of a major bruchid resistance gene in mungbean (Vigna radiata, L. Wilczek). Theor Appl Genet 84:839-844

Figures

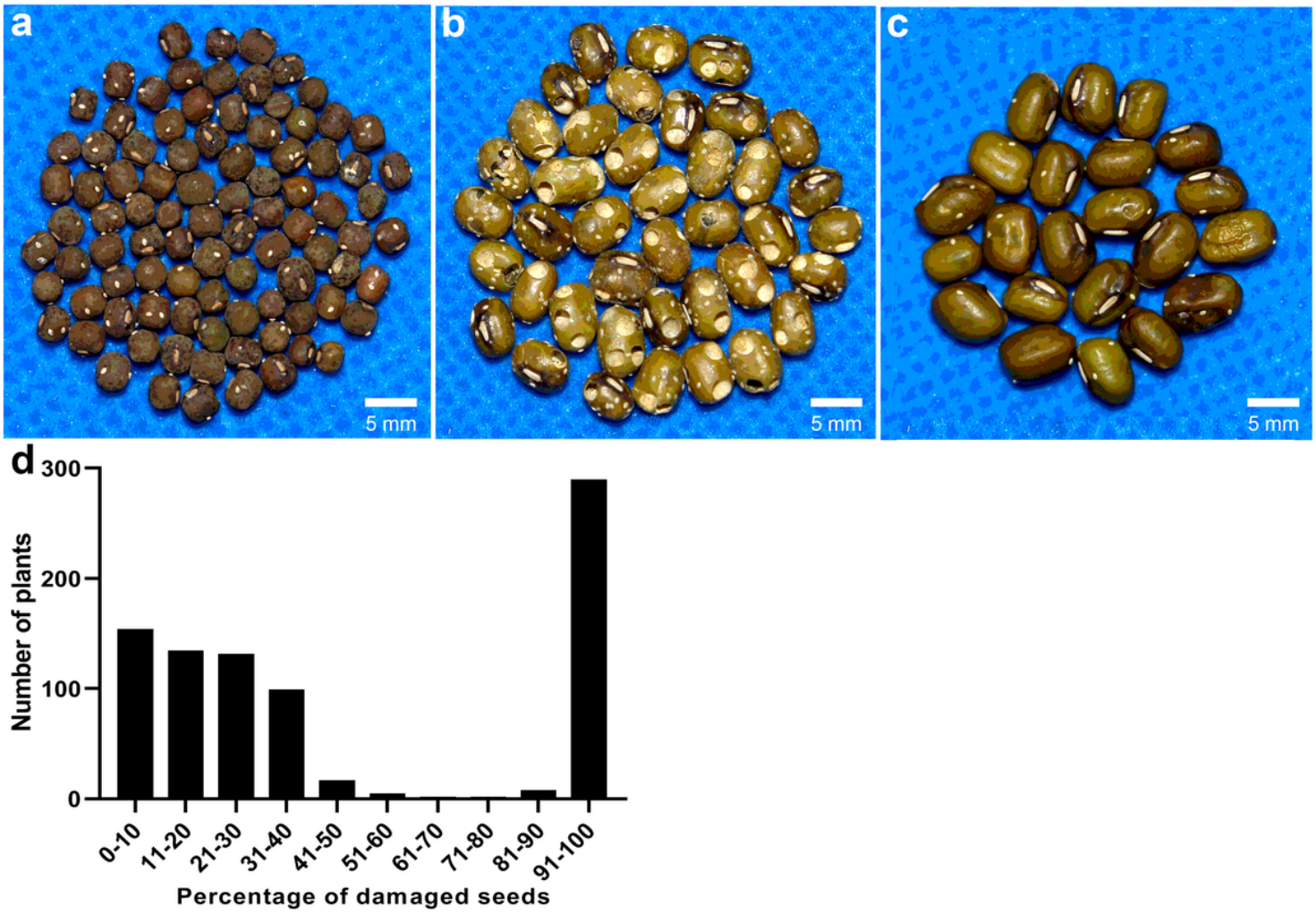

Figure 1

Phenotypic evaluation of bruchid resistance in C618, Zhonglv 5, and N618 (isogenic line of Zhonglv 5). (a) The undamaged seeds of C618, white oviposition are visible on seeds, but no damaged seeds. (b) The

completely damaged seeds of Zhonglv 5, bruchid holes: typical bruchid symptoms. (c) The undamaged seeds of N618, white oviposition are visible on seeds, but no damaged seeds.

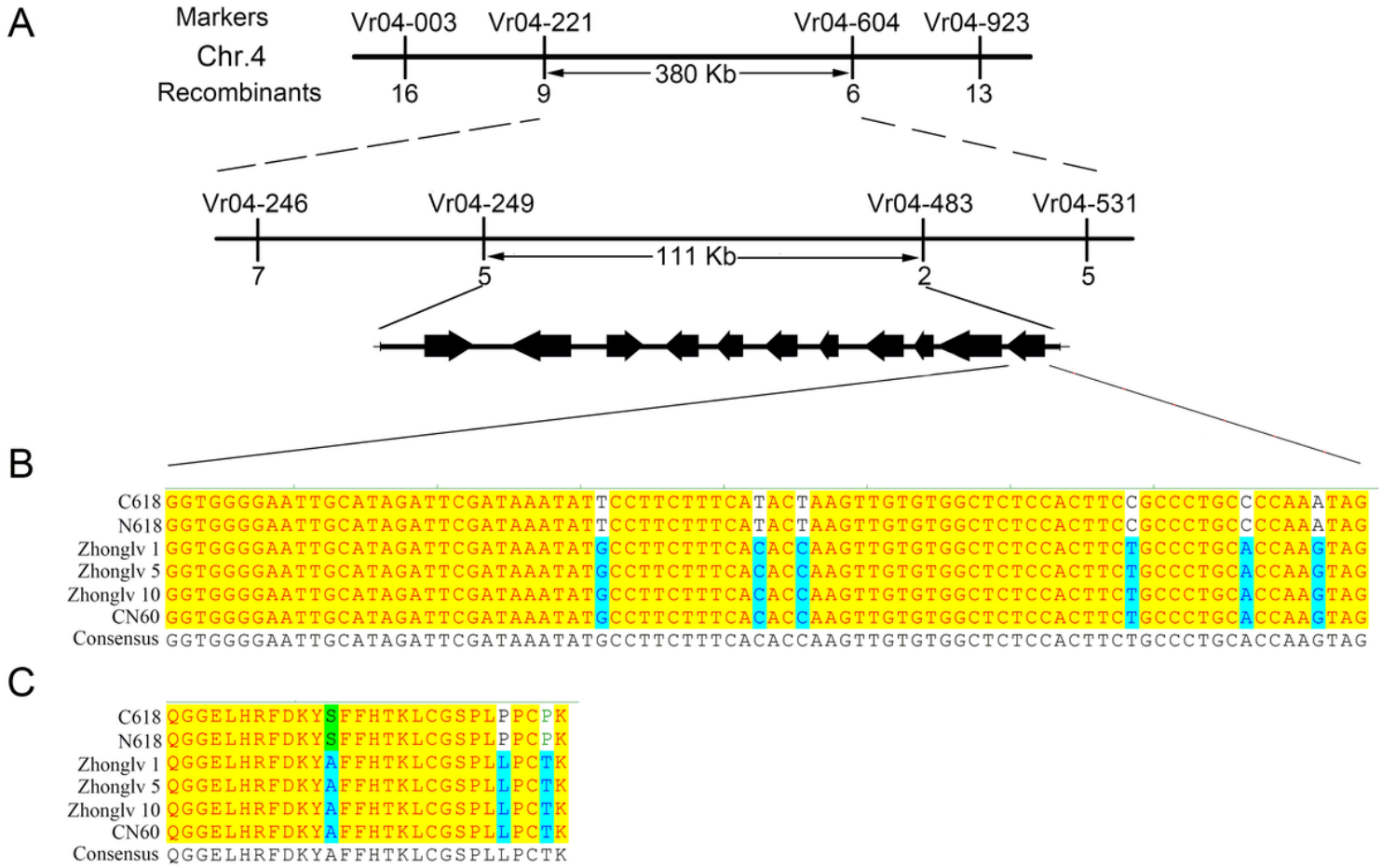

Figure 2

Map-based cloning of the Br gene. (a) The Br gene was mapped to a 111-kb region between SSR markers Vr04-249 and Vr04-483 on the chromosome 4 with 10 candidate genes. The Vr04g00919 gene encoding polygalacturonase inhibitor with a six single-base substitution is the best candidate gene. (b) Two bruchid resistant lines (C618 and N618) and four bruchid susceptible lines (Zhonglv 1, Zhonglv 5, Zhonglv 10, and CN60) were used for homologous analyses. The six nucleotides substitutions are consistent in bruchid-resistant and susceptible lines, respectively. (c) The three amino acids substitutions are consistent in bruchid-resistant and susceptible lines, respectively.

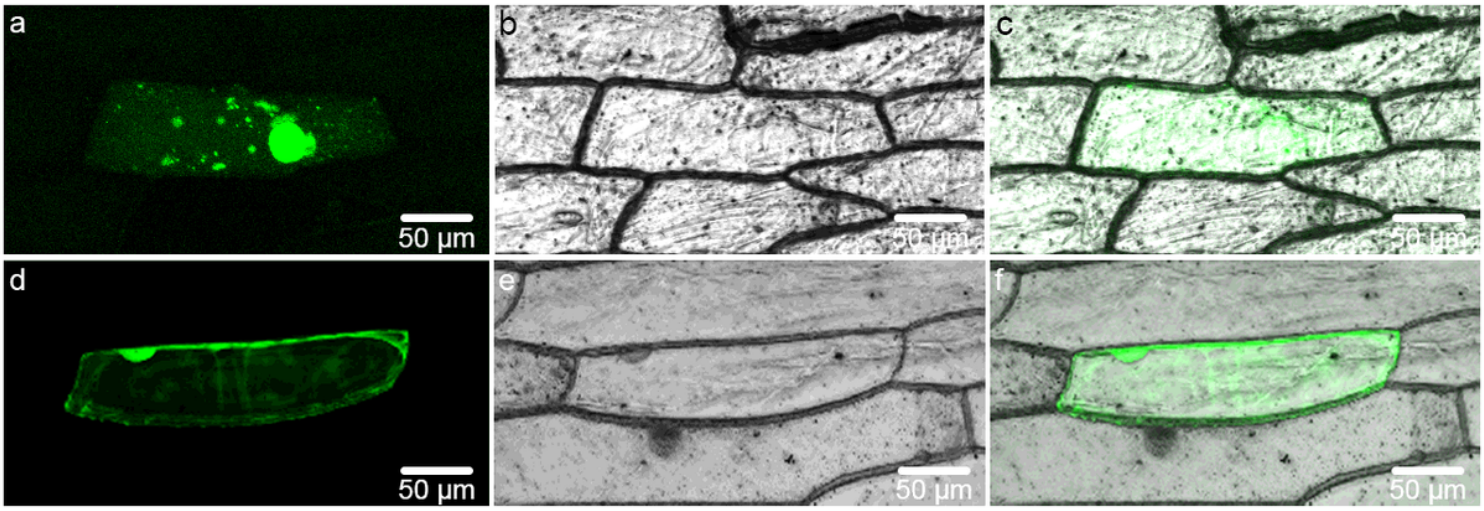

Figure 3

Subcellular localization analysis of VrPGIP2. (a) a-c Subcellular localization of pE3025-GFP. d-f Subcellular localization of pE3025-VrPGIP2-GFP. Cells expressing GFP alone were used as a control. a, d GFP fluorescence (green), b, e Bright field images; c, f merged images.

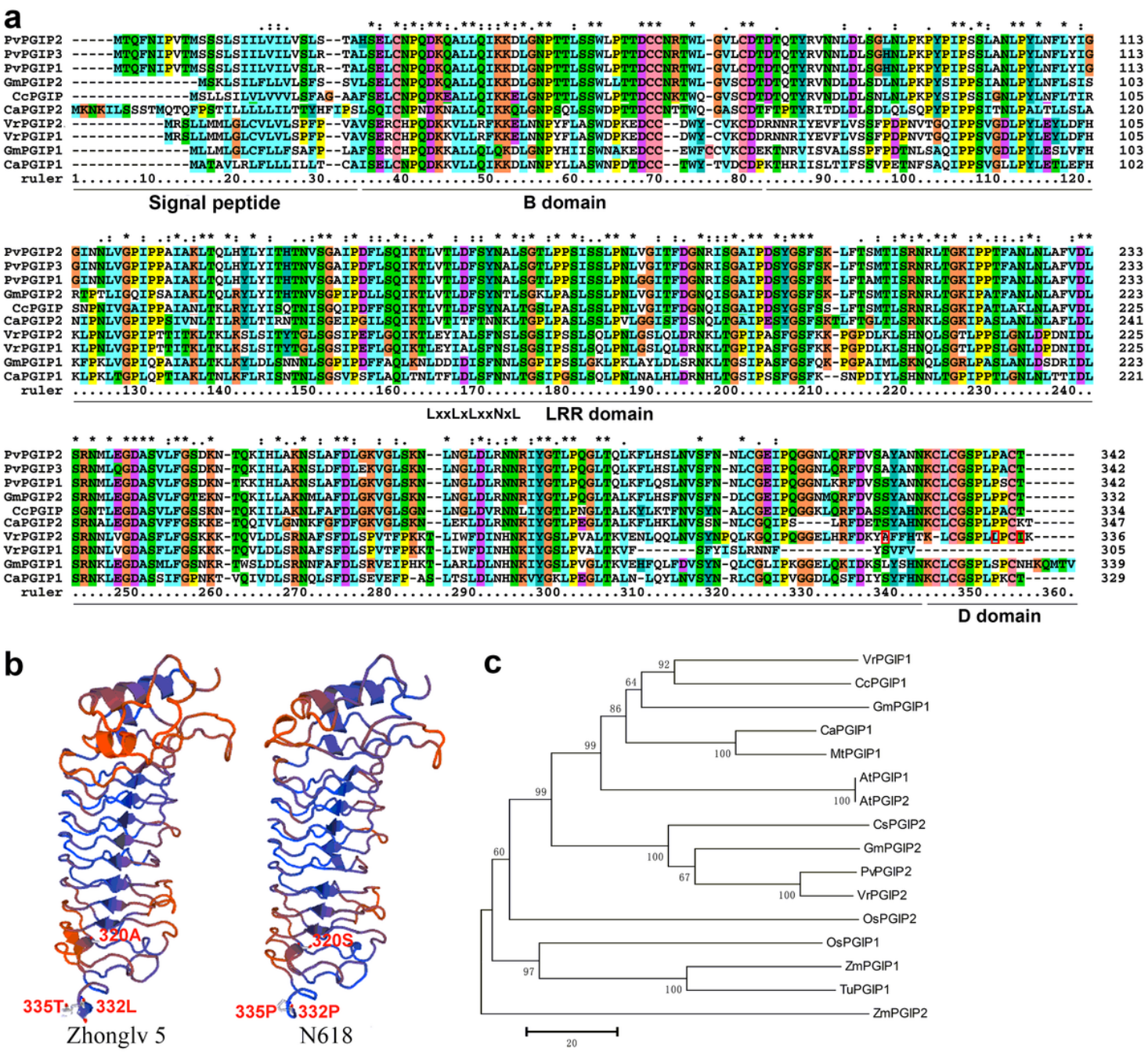

Figure 4

Sequence comparison of PGIPs. (a) Alignment of the mung bean PGIP amino acid sequence with other members in legume plants: CaPGIP1 in Cajanus cajan, GmPGIP1 in Glycine max, CaPGIP1 in Cicer arietinum, MtPGIP in Medicago truncatula, PvPGIP1 in Phaseolus vulgaris, VrPGIP1, VrPGIP2 in Vigna radiata, GmPGIP2 in Glycine max, and CcPGIP2 in Cajanus cajan (the mutation sites are in the LRR domain and D domain, indicated by the red boxs). (b) Prediction of 3D structures of Zhonglv 5 and N618 proteins using SWISS-MODEL and visualization using Swiss-PdbViewer (the mutation sites are indicated in red boxes. (c) Phylogenetic analysis of PGIPs in plants.

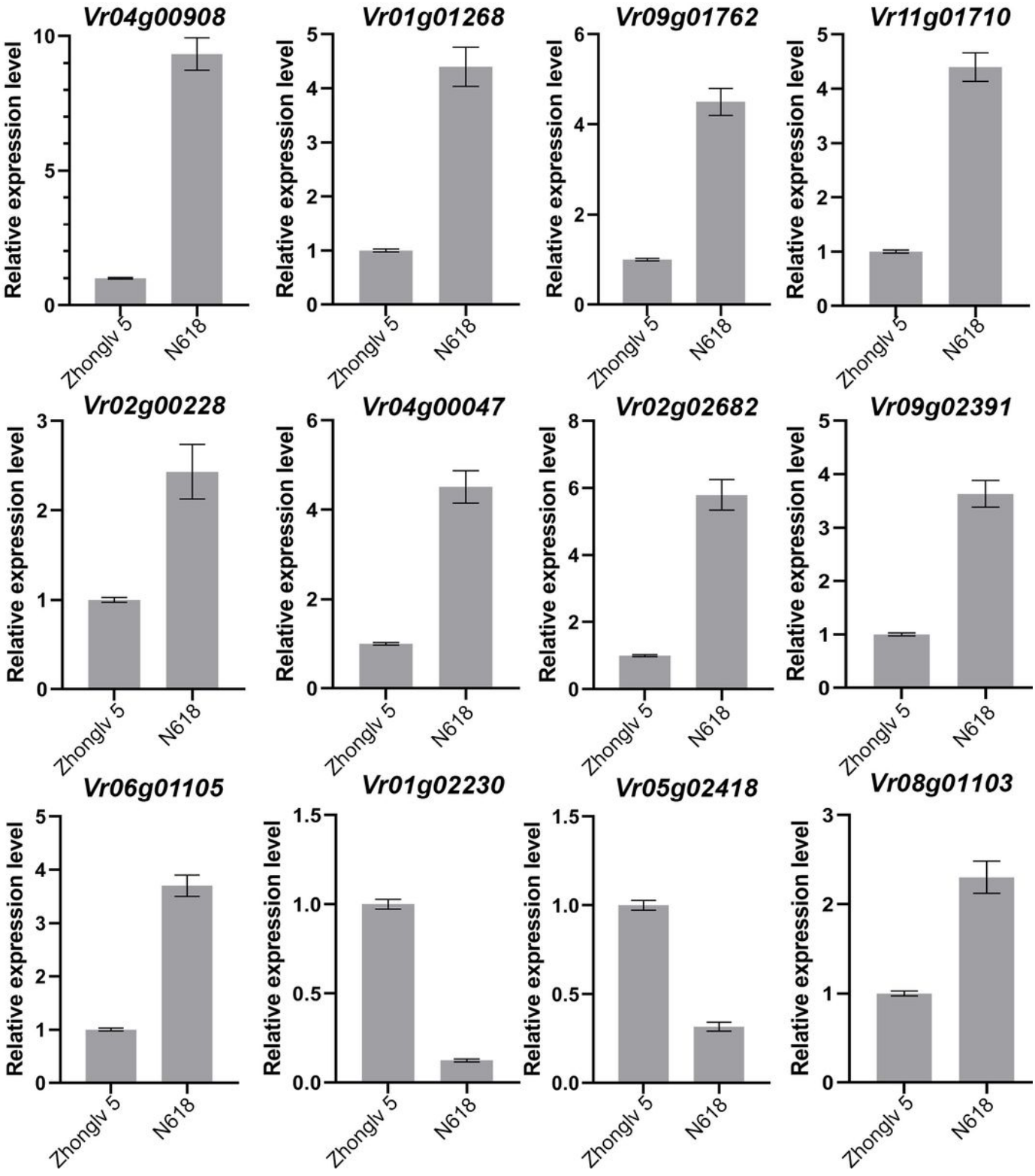

Figure 5

qRT-PCR validation of differentially expressed transcripts between bruchid-resistant line N618 and susceptible line Zhonglv 5. Error bars represent standard errors derived from three replications.

Supplementary Files

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