

Cloning and Characterization of a NBS-LRR Resistance Gene from Peanut (*Arachis hypogaea* L.)

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Received: July 24, 2012 Accepted: September 10, 2012 Online Published: November 15, 2012

doi:10.5539/jas.v4n12p243

URL: <http://dx.doi.org/10.5539/jas.v4n12p243>

Financial support came from National Natural Science Foundation of China (30771361); Modern Agro-industry Technology Research System (CARS-14)

Abstract

The nucleotide-binding site (NBS)-Leucine-rich repeat (LRR) gene family accounts for the largest number of known disease resistance genes, and is one of the largest gene families in plant genomes. In the present study, based on the NBS domain, resistance gene analogues (RGAs) have been isolated from peanut, which named *PnAG₃*. A full-length cDNA, *PnAG₃* was obtained by rapid amplification of cDNA ends (RACE) method. Sequence analysis indicated that the length of *PnAG₃* was 1 882 bp, including a complete open reading frame of 1 335 bp encoding *PnAG₃* protein of 444 amino acids. Multiple analysis showed that it had a certain homology with known resistance proteins, among which *Arachis cardenasii* resistance protein had the highest homology (48.01%). The polypeptide has a typical structure of nonTIR-NBS-LRR genes. Real-time fluorescence quantitative PCR analysis showed that after *A. flavus* infection, the expression of *PnAG₃* gene in J11 (*A. flavus* resistance species) has increased 16.68, 11.16 and 25.96 times in seed coat, kernel and pericarp, respectively. But it only increased 2-3 times in JH1012 (*A. flavus* sensitive species). The cloning of putative resistance gene from peanut provides a basis for studying the structure and function of peanut disease-resistance relating genes and disease resistant genetic breeding in peanut.

Keywords: peanut, NBS-LRR, bioinformatics, real-time fluorescence quantitative PCR, *A. flavus* resistance

1. Introduction

Peanut (*Arachis hypogaea* L.) is one of the four major oil crops in the world. For years, peanut has been the highest export crop in china. However, peanut is susceptible to *A. flavus* infect and pests which cause yield reduction and quality decline problems, especially by the aflatoxin contamination. It is known that, peanut *A. flavus* infection and its subsequent accumulation of toxic and carcinogenic secondary metabolites, aflatoxin, are serious agricultural problems, especially in dry conditions. In recent years, therefore the European Union (EU), Association of Southeast Asian Nations (ASEAN), Japan and other importing countries have made standards of peanut aflatoxin and pesticide residues more stringent and peanut production areas of aflatoxin contamination can not be resolved a long-term, there are excessive of aflatoxin in peanut, which had been a limiting factor of our peanut exports. Therefore, studies on resistant varieties of peanut should improve the understanding of resistance mechanisms to *A. flavus* and cultivating *A. flavus* resistance peanut varieties.

In the evolutionary process, plants had gradually formed their own defense mechanisms to defend themselves against the invading microbial pathogens, nematodes and insects which include structural defenses (such as thick cuticle), inhibitors (phenolic compounds, tannins, lectins, etc.), enzymes (chitinase and glucanase enzymes) and

specific pathogen recognition mechanisms. The identified mechanism included cell wall thickening, resistance gene (R gene) expression and apoptosis. Current studies are focusing on R genes cloning (Bertioli et al., 2003). The genetic analysis of genetic resistance to rust and flax rust fungus pathogenicity made it the first time to recognize the gene for gene interaction theory (Jeff et al., 2000). According to Flor's gene-for-gene hypothesis, there is a co-evolution relationship between plants and pathogens. Each R gene of the host plant has a corresponding "non-toxic gene" in the pathogen. Also in pathogen there exists "virulence genes" corresponding to "non-toxic genes". The pathogens which contained "non-toxic genes" or "virulence genes" have non affinity and affinity to the host plant which has R genes, respectively (Eric et al., 2001). The study (Eric et al., 2001) showed that avirulence gene has diversity, but the corresponding R genes are only five types. And the NBS-LRR family is the most abundant type which includes anti-fungi, bacteria, viruses, nematodes, etc (Ellis & Jones, 1998; Meyers et al., 1999; Hammond-Kosack & Jonathan, 1997; Williamson, 1999). At present, there has been successfully cloned R genes from maize (Wang et al., 2009), grapefruit (Huang et al., 2004), rice (Wang et al., 2005), wheat (Wang et al., 2009) and potato (Chen et al., 2006). Regarding the analysis of amino acid sequences of these R genes, most of the plant R genes encode NBS-LRR resistance protein, such as wheat leaf rust R gene and downy mildew. NBS (Nucleotide Binding Site) R genes is the largest category of plant disease R genes. NBS conserved domain contains many conserved motifs, such as p-loop, Kinase 2, Kinase 3a and transmembrane domain GLPL (Traut, 1994) and may be combined to the disease resistance signals' transduction and played an important role in plants disease resistance.

Because of the complexity of the peanut genomics, using of transposon tagging and map-based cloning to clone R genes was very difficult in peanut. From the above analysis, it is a simple, effective and feasible method to clone NBS R gene in peanut by designing a pair of degenerate primers according to the NBS conserved regions. Using this method, the *A. flavus* resistance gene had been cloned in soybean, sweet potato, maize and other crops. However, peanut has high homology to soybean, sweet potato, and maize. Therefore, it can be deduced that the NBS-LRR R gene may exist in peanut. Based on the analysis of NBS conserved domain and some cloned R genes, we designed a pair of degenerate primers to cloned peanut NBS R gene and started the initiation of breeding of peanut *A. flavus* resistance varieties.

2. Materials and Methods

2.1 Materials

The peanut cultivar J11 (an *A. flavus*-resistant variety) and JH1012 (an *A. flavus*-susceptible variety) were grown at test field of Shandong Peanut Research Institute, Lai'xi, China. 45 days before harvest, started to *A. flavus* infection and sampling every 10 days, and there were 3 times in total. Samples, including seed coat, pericarp and kernel, were taken from those two peanut varieties respectively and kept in -80°C.

The *Escherichia coli* DH5 α and BL21, pEASY-T and pMD-18-T vector were purchased from TIANGEN Biotech Company (Beijing). The SMARTer™ RACE cDNA Amplification Kit was purchased from BD Bioscience Clontech Company (Palo Alto, California, USA). M-MLV transcriptase, SYBR Premix Ex Taq, restriction enzymes and rest tool enzymes were purchased from TaKaRa Biotechnology Company (Dalian, China). All other chemical reagents were analytic purity.

2.2 Genomic DNA Extraction and Amplification of Genomic NBS Fragment

Genomic DNA was extracted with Cetyltrimethyl ammonium bromide (CTAB), as described by Rogers (1988). The quality and concentration of DNA was measured with the electrophoresis and spectrophotometer. As shown in Table 1, degenerate primers were designed based on amino acid sequences MGGVGKT and GLPLALK of proteinic conserved domains P-loop and GLPL respectively, which were coded by resistance genes of NBS structural domains (Bertioli et al., 2003).

Table 1. Degenerate primers

| Primer name | Conserved domain | conservative motif | Primer sequence(5'-3') |
|-------------|------------------|--------------------|----------------------------|
| Pf1 | P-loop | GMGGVGKTT | GGNATGGGNGGNGTNGGNAARACNAC |
| Pr1 | GLPL | GLPLALKV | NACYTTNAGNGCNAAGNGGNAAGNCC |

Note : f, forward primer; r, reverse primer; N=A/T/C/G; R=AG, Y=CT.

PCR was performed using 25 ng of genomic DNA as template, 0.4 μ M of each degenerate primer, 0.2 mM of each dNTP, 1 U Taq polymerase and 2.5 μ l 10 \times PCR buffer (Mg^{2+} plus) in a final volume of 25 μ L. Cycling conditions were as follows: The template was denatured at 94°C for 5 min; 35 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 90 s; followed by 10 min at 72°C. The PCR products were then purified and sequenced. The sequence of the fragment named *PnAG₃* was then used to design gene-specific primers for 5' and 3' rapid amplification of cDNA ends (RACE).

2.3 Total RNA Extraction and First-strand cDNA Synthesis

Total RNA was extracted with Plant RNA Kit (OMEGA, USA) according to the manufacturer's instructions. RT-PCR amplifications were performed using SMART RACE cDNA Amplification kit (Clontech). 1 μ g of total RNA were used for first-strand cDNA synthesis at 42°C in a 10 μ l volume with 1 μ l of 5'-or 3'-CDS primer A, 1 μ l of oligo (dT), 2 μ l of 5 \times first-strand buffer, 1 μ l of DTT (20 mM), 1 μ l of dNTP Mix (10 mM) and 1 μ l of MMLV reverse transcriptase according to manufacturer's instructions.

2.4 Rapid Amplification of cDNA Ends (RACE)

Based on the DNA fragment obtained above, two gene-specific primers GSP1 for 5'-RACE and GSP2 for 3'-RACE were designed (Table 2). The sequence of the universal primer for 5'-RACE and 3'-RACE was given in the user manual of the kit (Long: 5'-CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT-3' and Short: 5'-CTA ATA CGA CTC ACT ATA GGG C-3'). The 3'-cDNA ends and 5'-cDNA ends were amplified using SMARTer™ RACE cDNA Amplification Kit following the user manual (Clontech, Palo Alto, California, USA). The amplification profile was as follows: The template was denatured at 94°C for 5 min; 5 cycles of 94°C for 30 s, 72°C for 3 min; 5 cycles of 94°C for 30 s, 70°C for 30s, 72°C for 3 min; 27 cycles of 94°C for 30 s, 60°C for 30s, 72°C for 3 min; followed by 10 min at 72°C.

For 5'-RACE and 3'-RACE, the PCR products were smear, so designed two anchor primers GSP1-2 and GSP2-2 for 5'-RACE and 3'-RACE nest PCR respectively. Using the smear products as template to amplified the 5'-cDNA ends and 3'-cDNA ends.

The PCR products were purified by gel extraction and cloned into the pMD-18T vector. Recombinant clones were sequenced by TaKaRa Biotechnology Company (Dalian, China).

Table 2. Primers used in the study

| Primer name | Primer sequence(5'-3') | Company |
|-------------|-----------------------------|---------|
| GSP1 | GCAGCAACTCTCAATGGCAAGCACAAC | TaKaRa |
| GSP2 | ATTGTTGCCACTGCTTCTCCTTACCAG | TaKaRa |
| GSP1-2 | AGACAAGTTGAGCAAGAGTAGT | TaKaRa |
| GSP2-2 | ATTGTTGCCACTGCTTCTCCTTACCAG | TaKaRa |
| AG3-F | TGTGGAGTGTGCTTGTAGGG | TaKaRa |
| AG3-R | GCTTCGTGTCGTCACCAGTA | TaKaRa |
| DF-F | GAGGAGAAGCAGAAGCAAGTTG | TaKaRa |
| DF-R | AGACAGCATATCGGCACTCATC | TaKaRa |
| PRAG3 | TGGTCGACATCAAGAGCAATAGGC | TaKaRa |
| PFAG3 | GCGGATCCATGGAAAGTGTCTGTT | TaKaRa |

2.5 Full-length cDNA Amplification of *PnAG₃*

By comparing and aligning DNA internal fragment, the 5'-RACE and 3'-RACE product sequences with BIOXM software (ver.2.6) package, the full-length cDNA was deduced and obtained through RT-PCR amplification, which was performed with gene-specified primers PRAG3 and PFAG3 (Table 2) and named *PnAG₃*.

2.6 Bioinformatics Analysis of *PnAG₃* Gene

Database searches for similarity were performed using BLASTN and BLASTX algorithms against the GenBank database. The sequences were translated to suitable open reading frame using the ORF Finder in the NCBI

(<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). Multiple alignments of deduced amino acid sequences and the phylogenetic tree were performed using DNAMAN.

2.7 Functional Analysis of *PnAG₃* Gene with Real-time Fluorescence Quantitative PCR

Real-time fluorescence quantitative PCR was used to investigate *PnAG₃* gene expression profiling in various tissues of peanut before and after *A. flavus* infection. Total RNA was extracted separately from tissues including seed coat, kernel and pericarp of two kinds of peanut varieties, and quantified with electrophoresis and spectrophotometer.

The reverse transcription PCR was done by PrimeScript™ RT reagent Kit (Perfect Real Time) (TaKaRa). The system volume was 10 μL. It contained 2 μL of 5×PrimeScript™ Buffer (for Real Time), 0.5 μL PrimeScript™ RT Enzyme Mix 1, 0.5 μL of Oligo dT Primer (50 μmol/L), 0.5 μL of Random 6 mers (100 μmol/L), 500 ng total RNA as template. The reverse transcription was programmed at 37°C for 15 min, 85°C for 5 s.

Real-time fluorescence quantitative PCR analysis was performed by SYBR® Rremix Ex Taq™ (Perfect Real Time) (TaKaRa) with the cDNA products obtained as the templates. The 25 μL of reaction system consisted of 12.5 μL of SYBR Rremix Ex Taq, 0.5 μL of the forward and the reverse primers each, 4 μL of cDNA template, and 7.5 μL of sterilized water. Each sample was amplified thrice as replicates. PCR was carried out using a LightCycler480 Real-time PCR System with an initial heat activation step at 94°C for 2 min, and amplifications were achieved through 45 cycles for fluorescence collection at 94°C for 15 s, 62°C for 15 s, and 72°C for 20 s. A final extension reaction was collected fluorescence every 0.2°C from the 62°C gradually to 94°C for melting curve analysis. Then it is cooled at 40°C for 30 s.

2.7.1 Standard Curve Established

Total RNA was extracted from the seed coat of J11. The reverse transcription PCR and real-time PCR were done as above. The PCR products of *PnAG₃* gene and DF-actin gene were purified by gel extraction and cloned into the pMD-18T vector and transformed into *E. coli* DH5α competent cells respectively. Recombinant clones were identified by colony PCR and sequencing. Then using plasmid extraction kit extracted plasmid. The plasmid was diluted 50 times to determinate the concentration. Gradient 10 times diluted the plasmid as the template of real-time fluorescence quantitative PCR. According to CP values and gene copy number logarithm, mapping the standard curves of the *PnAG₃* gene and DF-actin gene.

2.7.2 Relative Quantification Analysis of *PnAG₃* Gene

Total RNA from different peanut varieties and different times were prepared as described above. According to sequence of *PnAG₃* gene, two primers AG3-F and AG3-R (Table 2) were designed to amplify a product of 202 bp. A constitutive expression gene, the DF-Actin, was used as internal control to verify the quantitative real-time PCR reaction. Two primers DF-F and DF-R were used to amplify a 106bp fragment of Peanut DF-Actin gene cDNA (Table 2). The real-time fluorescence quantitative PCR was carried out as previously described in detail. Each sample repeated 3 times. The treatment CP value is the 3 times mean value. Different treatment's data is uniform by the internal reference gene. Using the hyperbolic method calculate the F value ($F=10^{\frac{\Delta C_{T,T}}{\Delta C_{T,R}} - \frac{\Delta C_{T,F}}{\Delta C_{T,R}}}$). Real-time fluorescence quantitative PCR products were detected by 1% agarose gel electrophoresis. LightCycler 2.0 and SPSS software were used for statistical analysis and statistical significance test.

3. Results

3.1 Cloning of the *PnAG₃* Internal DNA Fragment

By comparing amino acid sequences of resistance genes of NBS structural domains, and deduced from full-length cDNA sequences of the genes available in GenBank, consensus regions were identified. Degenerate primers were designed, and peanut genomic DNA was used as template. PCR amplification of the DNA resulted in a single band of about 500 bp in size on a 1% agarose gel. The 500 bp PCR product named *PnAG₃* gene fragment was cloned into pEASY-T vector and sequenced with 539 bp in length. The open reading frame could be found in the sequence of *PnAG₃* gene fragment. BLAST search result showed that the deduced amino acid sequence shares high identity with some known plant disease resistance genes listed in the GenBank (Figure 1).

3.2 Isolation and Characterization of the Full-length cDNA of *PnAG₃* Gene

The gene-specific primers for 5' and 3' end RACE were then designed based on the sequence of *PnAG₃*-fragment to amplify full-length cDNA. The complete peanut *PnAG₃* cDNA sequence was 1,876 bp, which was predicted to have an initiation codon ATG at position 47bp and a stop codon TGA at position 1 381bp using the ORF Finder in the NCBI (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) (data not shown). The cDNA sequence contained a 1

335bp open reading frame (ORF) encoding a protein of 444 amino acid residues with a polyadenylation tail, an isoelectric point (pI) of 5.46 and calculated molecular weight of about 50.36 kDa.

3.3 Gene Sequence Analysis

Comparison of the *PnAG₃* amino acid sequence with known proteins demonstrates a homology. Sequence analysis showed the deduced *PnAG₃* protein contained conserved NBS motifs such as P-loop, Kinase-2, Kinase-3a, GLPL and RNBS-C (YEVxxLSDEEAWELFCKxAF) motif (Figure 2). According to the research of Meyers, NBS-LRR genes are further subdivided into two categories, based on conserved domains at the N-terminus of their products. The first group comprises genes encoding a conserved leucine-zipper-like pattern (NonTIR). The second group was made up of genes whose products contain a structure homologous to human interleukin-1 and *Drosophila* Toll-like receptor regions (TIR). The last acid of Kinase-2 of TIR was any amino acids, usually aspartic acid(D). But the last acid of Kinase-2 of nonTIR was tryptophan (W). The last acid of Kinase-2 of *PnAG₃* was tryptophan (W). So *PnAG₃* gene had typical structure of nonTIR-NBS-LRR genes, with RNBS-A-nonTIR (FnLxAWVCvSQxF) domains. The deduced amino acid sequence for the ORF of *PnAG₃* gene showed 15.81, 15.52, 12.55, 17.12, 48.01, 15.93, 15.40 and 15.77% similarity with *rga3* (AAP45181.2) from *Solanum bulbocastanum*, B149 (AAR29073.1) from *Solanum bulbocastanum*, RGA2 (XP_002513098.1) from *Ricinus communis*, protein-like (AAU89637.1) from *Poncirus trifoliata*, C8_V_253 (AAN85399.1) from *Arachis cardenasii*, cc-nbs-lrr (XP_002297751.1) from *Populus trichocarpa*, leucine-rich_rep (XP_002513078.1) from *Ricinus communis* and Rpi-bt1 (ACI16480.1) from *Solanum bulbocastanum*, respectively. Phylogenetic analysis showed *PnAG₃* gene from peanut was more similar to C8_V_253 from *Arachis cardenasii* (Figure 3).

Sequences producing significant alignments:

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident | Links |
|----------------|---|-----------|-------------|----------------|---------|-----------|-------|
| AY157738.1 | Arachis cardenasii clone C8_V_253 resistance protein gene, partial c | 1160 | 1160 | 36% | 0.0 | 98% | |
| HP029196.1 | TSA: Arachis duranensis DurSNP_c29221.Ardu mRNA sequence | 324 | 324 | 13% | 1e-84 | 90% | |
| EZ757288.1 | TSA: Arachis hypogaea CL12329Contig1.Arhy mRNA sequence | 237 | 237 | 12% | 2e-58 | 86% | |
| EZ739061.1 | TSA: Arachis hypogaea CL14821Contig1.Arhy mRNA sequence | 209 | 209 | 12% | 4e-50 | 84% | |
| AB111399.1 | Amia calva GARS-AIRS-GART mRNA for glycinamide ribonucleotide sy | 71.3 | 71.3 | 2% | 2e-08 | 95% | |
| AB210849.1 | Lemna gibba LqLHY H1 mRNA for LHY homologe1, complete cds | 65.8 | 65.8 | 2% | 1e-06 | 93% | |
| AB121202.1 | Macrothelae gigas Magi 2 mRNA, complete cds | 63.9 | 120 | 2% | 3e-06 | 100% | |
| BT080532.1 | Caligus clemensi clone ccle-ovs-514-047 Androgen-induced protein | 62.1 | 62.1 | 1% | 1e-05 | 100% | |
| BT080313.1 | Caligus clemensi clone ccle-ovs-503-049 Nuclear nucleic acid-bindin | 62.1 | 62.1 | 1% | 1e-05 | 100% | |
| AB330453.1 | Bombyx mori BNGR-A32 mRNA for neuropeptide receptor A32, comple | 62.1 | 62.1 | 1% | 1e-05 | 100% | UG |
| AY319415.3 | Odontesthes bonariensis transcription factor SOX9 mRNA, complete | 62.1 | 62.1 | 1% | 1e-05 | 97% | |
| AM850110.1 | Artemia franciscana mRNA for beta-actin (act gene), isolated from G | 62.1 | 62.1 | 2% | 1e-05 | 97% | |
| AB280349.1 | Humulus lupulus DFR mRNA for dihydroflavonol 4-reductase, comple | 62.1 | 62.1 | 2% | 1e-05 | 93% | |
| AB243685.1 | Lemna gibba LqPRRH59 mRNA for pseudo-response regulator 59 hom | 62.1 | 62.1 | 1% | 1e-05 | 100% | |
| AB243682.1 | Lemna paucicostata LpPRRH59 mRNA for pseudo-response regulator | 62.1 | 62.1 | 1% | 1e-05 | 100% | |
| AB175675.1 | Scyliorhinus canicula Gcm-2 mRNA for transcription factor Glial cells | 62.1 | 62.1 | 1% | 1e-05 | 100% | |
| FB657020.1 | Cancer pagurus mRNA for putative prophenoloxidase (propo gene) | 60.2 | 60.2 | 1% | 5e-05 | 97% | |
| FN672804.1 | Psammecinus miliaris mRNA for citrate synthase (cs gene) | 60.2 | 60.2 | 1% | 5e-05 | 100% | |
| FJ588638.3 | Chlamys farreri glutathione S-transferase pi-class mRNA, complete | 60.2 | 60.2 | 1% | 5e-05 | 100% | |
| FM986355.1 | ChorThippus parallelus mRNA for putative CAAx prenyl protease (cq9) | 60.2 | 60.2 | 1% | 5e-05 | 100% | |
| AB236909.1 | Xenopus (Silurana) tropicalis mRNA for Runx2, complete cds | 60.2 | 60.2 | 1% | 5e-05 | 100% | UG |
| NM_001112751.1 | Bombyx mori cytochrome P450 monoxygenase (Cyp306a1), mRNA | 60.2 | 60.2 | 1% | 5e-05 | 100% | UG |
| AY445913.2 | Locusta migratoria heat shock protein 90 mRNA, complete cds | 60.2 | 60.2 | 1% | 5e-05 | 100% | |
| AM114539.1 | Sus scrofa mRNA for integrin beta 6 subunit precursor (ITGB6 gene), | 60.2 | 60.2 | 1% | 5e-05 | 100% | UG |

Figure 1. Blastn search results of *PnAG₃* gene by NCBI

3.4 Expression Analysis of *PnAG₃* Gene in Peanut

3.4.1 Determination of Target Fragment Copy Number with Ultraviolet Spectrophotometry

We determined copy numbers of DF-actin gene and *PnAG₃* gene with ultraviolet spectrophotometry as shown in Table 3 and Table 4.

Table 3. Copy number of *DF-actin* gene

| Diluted multiple | 10 ⁻¹ | 10 ⁻² | 10 ⁻³ | 10 ⁻⁴ | 10 ⁻⁵ | 10 ⁻⁶ |
|------------------|-----------------------|-----------------------|----------------------|----------------------|----------------------|----------------------|
| Copy number | 9.58×10 ¹¹ | 9.58×10 ¹⁰ | 9.58×10 ⁹ | 9.58×10 ⁸ | 9.58×10 ⁷ | 9.58×10 ⁶ |

Table 4. Copy number of *PnAG₃* gene

| Diluted multiple | 10 ⁻¹ | 10 ⁻² | 10 ⁻³ | 10 ⁻⁴ | 10 ⁻⁵ | 10 ⁻⁶ |
|------------------|-----------------------|-----------------------|-----------------------|----------------------|----------------------|----------------------|
| Copy number | 5.38×10 ¹² | 5.38×10 ¹¹ | 5.38×10 ¹⁰ | 5.38×10 ⁹ | 5.38×10 ⁸ | 5.38×10 ⁷ |

3.4.2 Establishment of Real-Time Fluorescence Quantitative PCR Standard Curve

The real-time fluorescence quantitative PCR standard curves of *PnAG₃* gene and DF-actin gene were established using LightCycler 4.05 software.

By the standard curve, we drew a line by employing copy number, determined by ultraviolet spectrophotometry, as horizontal ordinates, and C_t value as longitudinal ordinates. This curve was described by a curvilinear equation. According to this equation, we can calculate the copy numbers of same gene in different samples and get the absolute expression quantity. The C_t value and the logarithm of template concentration had a good correlation with correlation coefficient (R^2) larger than 0.99. The Standard curves for amplification was in accordance with the PCR exponential amplification and had no undesirable influencing components (Figure 4 and Figure 5).

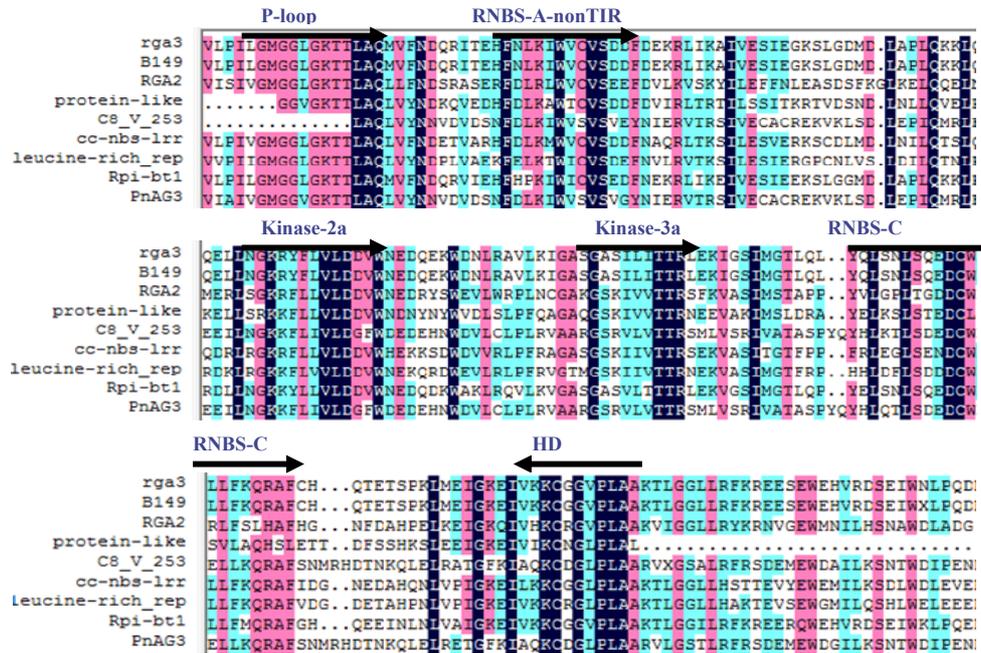


Figure 2. The conserved domain comparison between the deduced amino acid sequence of PnAG3 and 8 known disease resistance proteins

Note: Sequences were aligned using the CLUSTAL W program. Gaps have been introduced to optimize the alignment. Identical or conserved amino acids are shaded in blue and pink, respectively. The sources of the proteins and GenBank accession numbers are as follows, rga3(AAP45181.2) from *Solanum bulbocastanum*, B149(AAR29073.1) from *Solanum bulbocastanum*, RGA2(XP_002513098.1) from *Ricinus communis*, protein-like(AAU89637.1) from *Poncirus trifoliata*, C8_V_253(AAN85399.1) from *Arachis cardenasii*, cc-nbs-lrr(XP_002297751.1) from *Populus trichocarpa*, leucine-rich_rep(XP_002513078.1) from *Ricinus communis* and Rpi-bt1(ACI16480.1) from *Solanum bulbocastanum*.

3.4.3 Expression Analysis of *PnAG₃* Gene Resistance to *A. flavus* Infection

To evaluate the function of *PnAG₃* gene resistance to *A. flavus* infection, Real-time fluorescence quantitative PCR was performed with AG3-F and AG3-R to detect the transcripts of *PnAG₃* gene in seed coat, kernel and pericarp of two kinds of peanut species after *A. flavus* infection.

As shown in Table 5, *PnAG₃* gene could be expressed in different parts of peanut and the expression of gene *PnAG₃* increased in different parts and different peanut varieties after *A. flavus* infection. With the increase of resistance to *A. flavus*, the expression of *PnAG₃* gene will also notably increase, with more significant difference in different parts among different varieties. After *A. flavus* infection, gene expression amount in *A. flavus*-resistant variety J11 was higher than that in *A. flavus*-susceptible variety JH1012. Compared to JH1012, J11 has relatively higher gene expression amount in seed coat, kernel and pericarp. After *A. flavus* infection, the expression amount of *PnAG₃* gene in J11 has increased 16.68, 11.16 and 25.96 times in seed coat, kernel and pericarp respectively. But it only increased 2 to 3 times in JH1012. From the results we can speculate that the function of *PnAG₃* gene has positive correlation with peanuts resistance to *A. flavus* infection, or the gene can be induced by *A. flavus* infection.

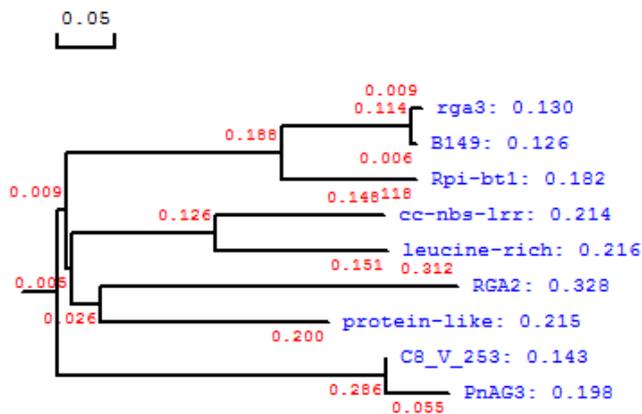


Figure 3. Phylogenetic tree of deduced amino acid sequence of *PnAG₃* gene

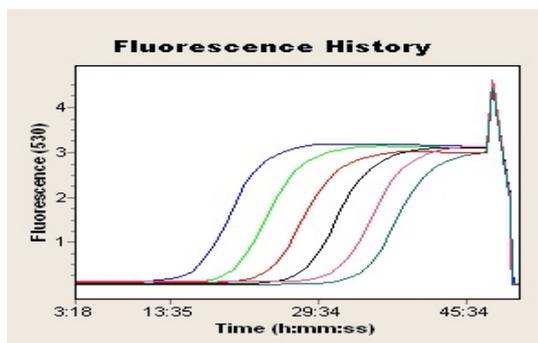


Figure 4. The amplification curves of DF-actin gene by RT-PCR

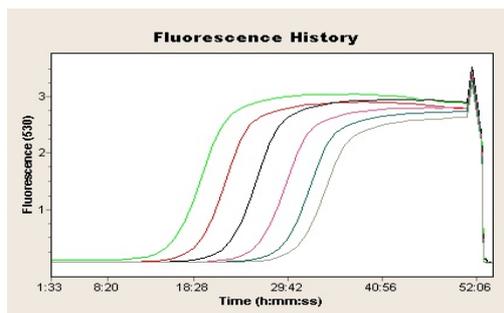


Figure 5. The amplification curves of *PnAG₃* gene by RT-PCR

Table 5. The relative quantification expression of *PnAG₃* gene in different species and part

| Parts | Varieties | CK | Infection | Difference | Multiple |
|-----------|-----------|-------------|---------------|------------|----------|
| seed coat | J11 | 1.07±0.042 | 17.85±0.815 | * | 16.68 |
| | JH1012 | 1.59±0.069 | 3.58±0.117 | * | 2.25 |
| kernel | J11 | 26.2±1.092 | 292.48±12.043 | * | 11.16 |
| | JH1012 | 23.94±0.873 | 166.17±6.026 | * | 6.94 |
| pericarp | J11 | 2.78±0.092 | 72.16±2.351 | * | 25.96 |
| | JH1012 | 1.98±0.732 | 6.52±0.395 | * | 3.29 |

Note: Each value is the mean of three replicates with the standard deviation. *indicate significant difference between control and *A. flavus* infection at 5% level of probability.

4. Discussion

Study of R genes is one of the key subjects of plant science at present. Cloning and characterization of R genes can not only facilitate the understanding of molecular mechanism of interaction between host and pathogen but also provide opportunities for disease resistance crop breeding (Wang et al., 2003 & 2004). Since the first plant R gene *Hm1* cloned at 1992, people have used position cloning, transposon tagging and homology cloning technology labeled plant R genes and their analogues more than 70 one after another from many varieties (Liu et al., 2007). Analysis of these amino acids encoding by cloned genes revealed that they are highly conserved in some segments. And most of them were NBS-LRR disease resistance proteins. It makes the homology cloning technology under the spotlight. Peanut is one of the crops which vulnerable to *A. flavus* infection. Currently, no effective *A. flavus*-resistant peanut species has been cultivated. So, cloning of R genes from peanut has become the focus now.

In view of the homology cloning method is simple and has a high success rate, we used NBS-LRR conserved region to design a pair of degenerate primers, and combined with RACE technology, successfully cloned a NBS-LRR R gene from peanut. The gene named *PnAG₃*. The ORF is 1335 bp and encoding 444 amino acids. The deduced amino acid sequence for the ORF of *PnAG₃* gene showed 15.81%, 15.52%, 12.55%, 17.12%, 48.01%, 15.93%, 15.40% and 15.77% similarity with *rga3* (AAP45181.2) from *Solanum bulbocastanum*, B149 (AAR29073.1) from *Solanum bulbocastanum*, RGA2 (XP_002513098.1) from *Ricinus communis*, protein-like (AAU89637.1) from *Poncirus trifoliata*, C8_V_253 (AAN85399.1) from *Arachis cardenasii*, cc-nbs-lrr (XP_002297751.1) from *Populus trichocarpa*, leucine-rich_rep (XP_002513078.1) from *Ricinus communis* and Rpi-bt1 (ACI16480.1) from *Solanum bulbocastanum*, respectively. Phylogenetic analysis showed *PnAG₃* gene has more similar to C8_V_253 from *Arachis cardenasii*. The amino acid encoding by *PnAG₃* gene is not only has NBS-LRR conserved sequence P-loop and GLPL, but also contains kinase-2, kinase-3a (GSRVLTTR) and RNBS-C (YEVxxLSDEEAWELFCKxAF). So, it can be confirmed that gene *PnAG₃* was a NBS-LRR gene.

The NBS domains are characteristic of various proteins with ATP/GTP binding activity, and comprise of the P-loop, kinase 2a, kinase 3a and GLPL motifs (Traut, 1994), while LRR domains play roles in the interaction of protein to protein (Kobe & Deisenhofer, 1994). These proteins have an important role in cell growth and differentiation, cytoskeleton formation, vesicle transport, and defense reaction (He et al., 2001). I-2 and Mi-1 are two CC-NBS-LRR R proteins in Tomato. I-2 is resisted to blight and Mi-1 is resisted to root-knot nematode and Potatoes aphids. But they all have the ATP combining ability. It had been proved that P-loop is the key to ATP combining ability (Tameling et al., 2002). In tobacco (Mestre et al., 2006) and *Arabidopsis thaliana* PR5 (Ade et al., 2007) also found a similar conclusion. NBS region of resistance proteins have three characteristic conserved regions. P-loop is take part in phosphate and Mg²⁺ binding (Saraste et al., 1990) and kinase-3 combined with the purine or ribose (Traut et al., 1994). The NBS domain of highly conserved disease R gene product indicate that nucleoside triphosphate is necessary to these proteins' function. Yuksel et al. (2005) had isolated 234 resistance gene analogs (RGAs) by using primers designed from conserved regions of different classes of resistance genes including NBS-LRR, and LRR-TM classes. They identified 250 putative resistance gene loci, and the BACs isolated here would help improved our understanding of the evolution and organization of these genes in the peanut genome (Yuksel et al., 2005). The mechanism of NBS domains for plant disease resistance is not clear, but may be related to the binding of NBS domain and nucleotide triphosphate changing the interaction between disease resistance protein and its defensive signals.

Under the changes of external environment, the related genes expression will change either. In this study, the expression changes of *PnAG₃* gene were different in different peanut species under infection by *A. flavus*. After *A. flavus* infection, the expression amount in *A. flavus* resistant species J11 was higher than that in *A. flavus* sensitive species JH1012. After *A. flavus* infection, the expression amount of *PnAG₃* gene in J11 has increased 16.68, 11.16 and 25.96 times in seed coat, kernel and pericarp respectively. But it only increased 2 to 3 times in JH1012. From the results we can speculate that the function of gene *PnAG₃* has positive correlation with peanuts resistance to *A. flavus* infection. Or the gene can be induced by *A. flavus*. As *PnAG₃* gene belongs to NBS-LRR class gene and shows higher expression in peanut *A. flavus*-resistant variety after *A. flavus* infection, we speculate that *PnAG₃* have something to do with the resistance to *A. flavus* in J11.

Acknowledgement

The authors would express their thanks to Agro-industry Technology Research System of Shandong Province and Bio-resource Innovation and Research Project of Shandong Province.

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