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Cloning and analysis of a NBS-LRR disease resistance gene candidate *PnAG$_1$* from peanut (*Arachis hypogaea* L.)

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Abstract

Background: Based on the conserved sequences of a known NBS resistance gene, a pair of degenerate primers was designed to amplify the NBS-LRR resistance gene from peanut using PCR and RACE methods. **Results:** Analyzing the amino acid sequence by BLAST on NCBI, which was deduced from the 1088bp-long gene named PnAG1-2, showed that it had a certain homology with some resistance proteins, among which *Arachis cardenasii* resistance protein gene had the highest homology (66%). Relative quantification PCR analysis indicated that PnAG1-2 gene expresses more in J11 (an *A. flavus*-resistant variety) than in JH1012 (an *A. flavus*-susceptible variety) when the harvest time was coming. **Conclusions:** In this study, the NBS-LRR resistance sequence was successfully cloned from peanut and prokaryotic expression was done on the gene, which provided a foundation for cultivating anti-*A. flavus* peanut varieties.

Keywords: bioinformatics, NBS-LRR, peanut, real-time fluorescence quantitative PCR

INTRODUCTION

Peanut (*Arachis hypogaea* L.) is one of the four major oil crops in the world, and also the highest yield of oil crops in China with the total output accounting for 45% of the world's peanut production. Over the years, peanut has been the highest amount of export oil crops in China. However, pests and diseases, in particular aflatoxin contamination in peanut have become more common, often resulting in the deterioration of yield and quality. Preharvest aflatoxin contamination has been identified by the peanut industry as a serious issue in food safety and human health because of the carcinogenic toxicity (Guo et al. 2005). In recent years, 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. Aflatoxin contamination cannot be resolved in many Chinese peanut-producing areas, and excessive levels of aflatoxin have become an important limiting factor in Chinese peanut export. Therefore, studies on resistant varieties of peanut should improve the understanding of resistance mechanisms to *A. flavus*.

Plants have evolved elegant and complex systems in the evolutionary process to defend themselves against the invading of microbial pathogens, nematodes and insects. The current study focused on resistance gene cloning (Bertioli et al. 2003). There are five main classes of *R* genes have been defined based on the structural characteristics of cloned resistant genes (Dangi and Jones, 2001). The majority of functionally described *R* genes are the nucleotide binding site-leucine rich repeat (NBS-LRR) type. The NBS domains are characteristic of various proteins with ATP/GTP binding activity and

comprise the P-loop, kinase 2a, kinase 3a and GLPL motifs (Traut, 1994), while LRR domains play roles in the interaction of protein-protein (Kobe and Deisenhofer, 1994). The NBS domains may be the combination of disease resistance signal transduction and play an important role in plant disease resistance. Features include anti-fungal (Ellis and Jones, 1998), anti-bacterial (Meyers et al. 1999), anti-virus (Hammond-Kosack and Jones, 1997), anti-nematode (Williamson, 1999) factors. At present, resistance genes have been cloned successfully from corn, grapefruit, rice, wheat, sweet potatoes and other plants. Through the amino acid sequence analysis of these resistance genes, it has been found that most plant disease resistance genes encode NBS-LRR resistance protein, such as leaf rust resistance genes of wheat (Wang et al. 2009a, downy mildew resistance genes (Huang et al. 2004) and others (Wang et al. 2005; Chen et al. 2006; Wang et al. 2009b).

With the complexity of the peanut genome and lack of polymorphism of developed molecular markers of peanut, using transposable element tagging and map-based cloning to clone resistance genes is impossible at the present. However, from the above analysis it can be feasible to design degenerate primers according to the conserved region of NBS domains to clone resistance genes from peanut. Through this method, *A. flavus*-resistant genes have been cloned from soybean, sweet potato, corn and other plants. These genes in peanut have high homology with soybean, sweet potato, and corn, and it can be speculated that there might be NBS-LRR class resistance genes related to *A. flavus*-resistance existing in peanut. In this study, through analyzing a conserved region of NBS domain and other resistant genes cloned previously, degenerate primers were designed to clone peanut NBS resistant genes. Gene function was primarily analyzed and characterized. This should be useful for developing new varieties resistant to *A. flavus*.

MATERIALS AND METHODS

Materials

Peanut (*A. hypogaea* L.) lines, J-11 and JH1012 were used in this study. J-11 (an *A. flavus*-resistant variety) and JH1012 (an *A. flavus*-susceptible variety) were grown in the field at the Shan Dong Peanut Institute. The *Escherichia coli* BL21, DH5α, the pEASY-T1, PEGX-4T-1 and PMD-18-T vectors were all bought from TianGen Biotech (BeiJing) limited company. DNA Markers (500-15,000 bp) were all bought from TaKaRa company.

Isolation of genomic DNA and designing degenerate primers

Genomic DNA was isolated from testa of J-11 using CTAB method as described by Rogers and Bendich (Rogers and Bendich, 1988). 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 used in amplification.

primers	conserved domains	amino acid sequences	primer sequences（5’→3’）
Pf1	P-loop	GMGGVGKTT	GGNATGGGNGGNGTNGGNAARACNAC
Pr1	GLPL	GLPLALKV	NACYTTNAGNGCNAGNGGNAGNCC

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

PnAG$_1$ gene fragment cloning by PCR

Polymerase chain reaction (PCR analysis) was performed using 1 µL of genomic DNA as template, 0.2 µM of each degenerate primer, 0.1 µM of each dNTP, and 2.5 U of HotStarTaq DNA Polymerase in a final volume of 50 µL.

PCR was carried out using a Perkin-Elmer 9600 thermal cycler with an initial heat activation step at 94ºC for 5 min, and amplifications were achieved through 32 cycles at 94ºC for 60 sec, 48ºC for 60

sec, and 72ºC for 60 sec. A final extension reaction was carried out for 10 min at 72ºC. Negative controls for PCR were included. The PCR products were then purified and sequenced. The sequence of the fragment named *PnAG$_1$* was then used to design gene-specific primers for 5' and 3' rapid amplification of cDNA ends (RACE).

RNA extractions and reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNAs were extracted from liquid nitrogen-frozen test of J-11 with TRZOL-Reagent (Invitrogen, Carlsbad, CA), according to manufacturer's instructions. RT-PCR amplifications were performed using SMART RACE cDNA Amplification kit (Clontech). 1 µg of total RNA were used for first-strand cDNA synthesis at 42ºC in a 10 µL volume with 1 µL of 5'-or 3'-CDS primer A, 1 µL of oligo(dT), 2 µL of 5 x first-strand buffer, 1 µL of DTT (20 mM), 1 µL of dNTP Mix (10 mM) and 1 µL of MMLV reverse transcriptase according to manufacturer's instructions.

Rapid amplification of cDNA ends (RACE)

Full-length peanut *PnAG$_1$* cDNA was obtained by 5' and 3' RACE using the SMART RACE cDNA amplification kit (Invitrogen, Carlsbad, CA). To obtain the 5' and 3' termini of the *PnAG$_1$* gene, the 5'- and 3'-rapid amplification of cDNA ends (RACE) was performed using the SMART RACE cDNA Amplification kit (Clontech) following the manufacturer's instructions.

The gene-specific primer AGPR1 (5'-GTC GGC ATC ATC ACT CCA AAC ATC GTC C-3') from the antisense strand was designed for 5'-RACE, and the gene specific primer AGPR2 (5'-GGA TCT GAC GAA GGG ATT CGA TCT G-3') from the sense strand was used for 3'-RACE. 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'). Two gene-specific primers [designed from the 5' and 3'-untranslated region (UTR) of AG_1 cDNA], AGPR3 (forward), and AGPR4 (reverse), were used to obtain the full-length AG_1 cDNA. All PCRs were performed using the Advantage 2 PCR enzyme system (TaKaRa, Dalian, PR China) in a TaKaRa PCR Thermal Cycler. Negative controls for both cDNA synthesis, and PCR were always included. After electrophoresis on 1% agarose gels, RACE PCR products were excised and purified using a QIAEXII gel extraction kit (QIAGEN).

Bioinformatics analysis of *PnAG$_1$* gene

The purified fragments were then cloned into a pGEM-T Easy vector. The plasmid DNA was purified by using Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI).

DNA sequencing was performed by Invitrogen. The search for homologous sequences at the GenBank was carried out using the BLAST (Altschul et al. 1997). Multiple sequence alignments were performed using DNASIS v2.5 (MiraiBio, Alameda, CA) for Windows software. Trees were generated by using ClustalW and the Bootstrap method to create distance matrices. Protein conserved domains were searched from the conserved domain database (Marchler-Bauer et al. 2005) using web based tools of CDART (Geer et al. 2002) and CD-Search (Marchler-Bauer and Bryant 2004).

Preparation for the fluorescent quantitative standard samples and standard curve

RT-PCR amplifications were performed using PrimeScript RT reagent kit (TAKALA, LiaoNing, CN) to synthesize cDNA. Real-time fluorescence quantitative PCR amplifications were performed according to F-value method (Zhang et al. 2005), using 2 µL of cDNA product as template, 0.5 µL of each primer (Table 2), 10 µL of SYBR GREEN, 7 µL of sterile distilled water, and 2.5 U of HotStarTaq DNA Polymerase in a final volume of 50 µL. PCR was carried out using a LightCycler 480 Real-time PCR System with an initial heat activation step at 94ºC for 2 min, and amplifications were achieved through 40 cycles for fluorescence collection at 94ºC for 15 sec, 57ºC for 15 sec, and 72ºC for 20 sec. Then the final extension reaction was achieved for collecting fluorescence every 0.2ºC from the 60ºC gradually to 94ºC for melting curve analysis. Then, the PCR product was cooled at 40ºC for 30 sec and the target fragments were collected using Gel Extraction Kit (TAKALA, LiaoNing, CN) after PCR products detected by 1% agarose gel electrophoresis.

Table 2. Primers used in real-time fluorescence quantitative PCR.

primers	primer sequences（5'→3'）
AL	TGT GGA GTG TGC TTG TAG GG
AR	GCT TCG TGT CGT CAC CAG TA
DFL	GAG GAG AAG CAG AAG CAA GTT G
DFR	AGA CAG CAT ATC GGC ACT CAT C

Note: AL and AR: specific primers of AG1 gene; DFL and DFR: primers of House-keeping gene DF-actin.

Linked the target fragments to vector pMD-18T with ligase, and transformed it into *E. coli* DH5α competent cells, which cultured in LB medium (included AMP) at 37ºC overnight. The positive clones were detected by colony PCR and the plasmid was digested. The DNA plasmid was extracted using a plasmid extraction kit. The DNA was diluted 50 times to be used as real-time fluorescence quantitative PCR template, the concentration of the template sample is determinated by diluting 10 times of the last one in turn. The standard curves of the AG_1 gene and DF-actin gene were mapped according to CP values and gene copy number logarithm.

Relative quantification PCR and statistics analysis

Total RNAs were extracted from testa and embryos of J-11 and JH1012, respectively, at different times, and RT-PCR was carried out. Then, the real-time fluorescence quantitative PCR was carried out using the cDNA as template, and AG_1 and DF-actin primers as shown in Table 2. Each sample was repeated 3 times. The treatment CP value is the 3 times mean value. Different treatment's data is uniform by the internal reference gene. The hyperbolic method was used to calculate the F value ($F = 10^{\Delta C_{T,T}/A_T - \Delta C_{T,R}/A_R}$).

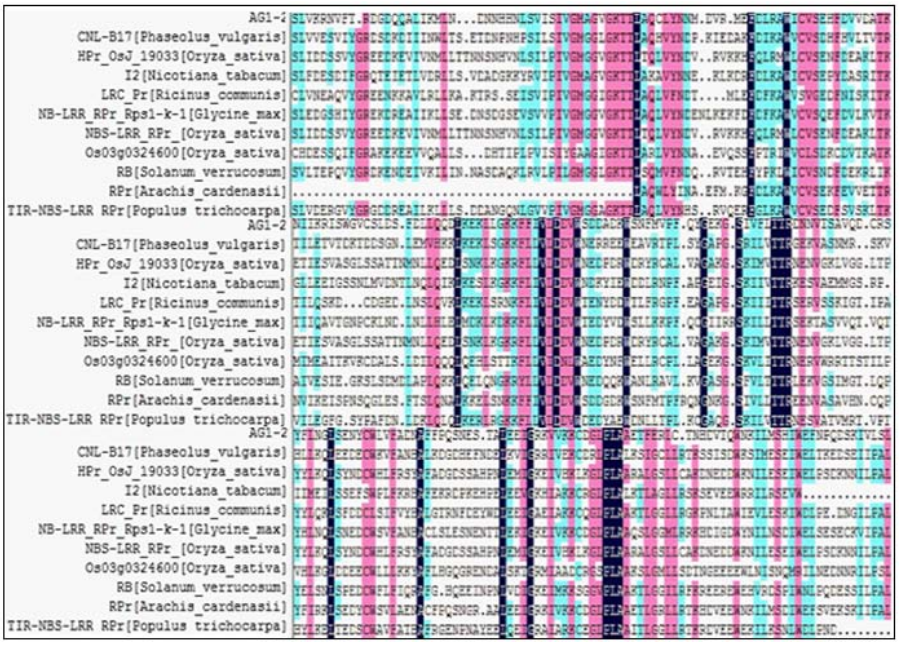

Fig. 1 Comparison of the deduced amino acid sequence of *PnAG$_1$* with10 known disease resistance proteins. Sequences were aligned using the CLUSTAL W program. Gaps have been introduced to optimize the alignment. Identical or conserved amino acids are shaded in black and grey, respectively. The sources of the proteins and GenBank accession numbers are as follows, *Arachis cardenasii* (AAN85395.1), *Ricinus communis* (gbEEF32840.1), *Glycine max* (AAX89382.1), *Phaseolus vulgaris* (ACZ74713.1), *Solanum verrucosum* (ABO28718.1), *Oryza sativa Japonica Group* (AAT69649.1), *Oryza sativa Japonica Group* (EEE64201.1), *Nicotiana tabacum*(ABB00542.1), *Populus trichocarpa* (ABF81464.1), *Oryza sativa Japonica Group* (NP 001049979.1).

Real-time fluorescence quantitative PCR products were detected by 1% agarose gel electrophoresis. LightCycler 2.0 and SPSS softwares were used for statistical analysis and statistical significance test.

RESULTS

Cloning and identifying of *PnAG$_1$* gene

By comparing amino acid sequences of resistance genes of NBS structural domains, deduced from full-length cDNA sequences of the genes available in GenBank, consensus regions were identified. Degenerate primers were designed, and peanut genomic DNA were used as template. PCR amplification of the DNA resulted in a single band of about 500 bp in size on a 1% agrose gel. The 500-bp PCR product named *PnAG$_1$*-fragment was cloned into pCRR 4-TOPO vector and sequenced with 520 bp in length. The open reading frame could be found in the sequence of *PnAG$_1$*-fragment. BLAST search (Altschul et al. 1997) showed that the deduced amino acid sequence shares high identity with some known plant disease resistance genes listed in the GenBank.

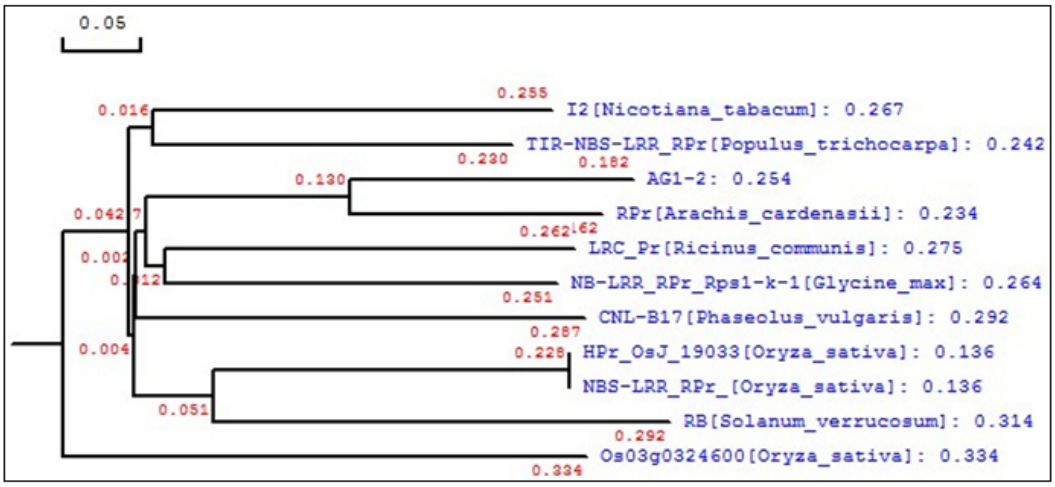

Fig. 2 Phylogenetic tree of deduced amino acid sequence of *PnAG$_1$* gene.

The gene-specific primers for 5' and 3' end RACE were then designed based on the sequence of *PnAG$_1$*-fragment to amplify full-length cDNA. The complete peanut *PnAG$_1$* cDNA sequence is 1,088 bp in length (accession no. 1435133) encoding a putative protein of 283 amino acids. Comparison of the *PnAG$_1$* amino acid sequence with known proteins demonstrates a homology. The deduced amino acid sequence shares high identity with known plant disease resistance genes such as *Arachis cardenasii* resistance protein, *Ricinus communis* leucine-rich repeat containing protein, *Glycine* max NBS-LRR type disease resistance protein, and *Phaseolus vulgaris* CNL-B17 (Figure 1).

The deduced amino acid sequence of peanut *PnAG$_1$* gene was aligned with other disease resistance gene sequences in plants using DNASIS software, and the phylogenic trees were generated by using ClustalW and the Bootstrap method (Figure 2). The amino acid sequence alignment of *PnAG$_1$* revealed that it has the highest sequence identity (66%) with *Arachis cardenasii* resistance protein (accession no. AAN85395.1). The *PnAG$_1$* protein shows a sequence identity of 49% to *Ricinus communis* leucine-rich repeat containing protein (accession no. gbEEF32840.1) and *Glycine max* NBS-LRR type disease resistance protein Rps1-k-1 (accession no. AAX89382.1), 51% to *Phaseolus vulgaris* CNL-B17 (accession no. ACZ74713.1) and *Solanum verrucosum* RB (accession no. ABO28718.1), 48% to *Oryza sativa* Japonica Group hypothetical protein OsJ_19033 (accession no. EEE64201.1), *Oryza sativa* Japonica Group NBS-LRR type resistance protein (accession no. AAT69649.1) (Figure 2).

Determination of target fragment copy number with ultraviolet spectrophotometry

We determined copy numbers of DF-actin gene and *PnAG₁* 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}	10^{-7}
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}	9.58×10^{5}

Table 4. Copy number of AG1 gene.

Diluted multiple	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Copy number	3.21×10^{11}	3.21×10^{10}	3.21×10^{9}	3.21×10^{8}	3.21×10^{7}	3.21×10^{6}	3.21×10^{5}

Construction of real-time fluorescence quantitative PCR standard curve

The real-time fluorescence quantitative PCR standard curves of *PnAG₁* gene and DF-actin gene were constructed using LightCycler 4.05 software.

As for the standard curve, we drew a line by employing copy number, determined by ultraviolet spectrophotometry, as horizontal ordinates, and CP 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.

Determination of *PnAG₁* gene expression by relative quantifying

Figure 3 showed that real-time qPCR amplification through response latency, exponential increase period and plateau. According to the method referred to "A Novel and Convenient Relative Quantitative Method of Fluorescence Real Time RT-PCR Assay Based on Slope of Standard Curve", it is specified that in contrast tissues, the expression of target gene is 1 relative to control gene, so that in treated tissues, the relative expression of target gene can be expressed as $F = 10^{\Delta C_{T.T}/A_T - \Delta C_{T,R}/A_R}$ ($\Delta C_{T,T}$: the difference of CT value of target gene between experiment and contrast; $\Delta C_{T,R}$: the difference of CT value of control gene between experiment and contrast; A_T and A_R: the slope of standard curve of target gene and control gene respectively).

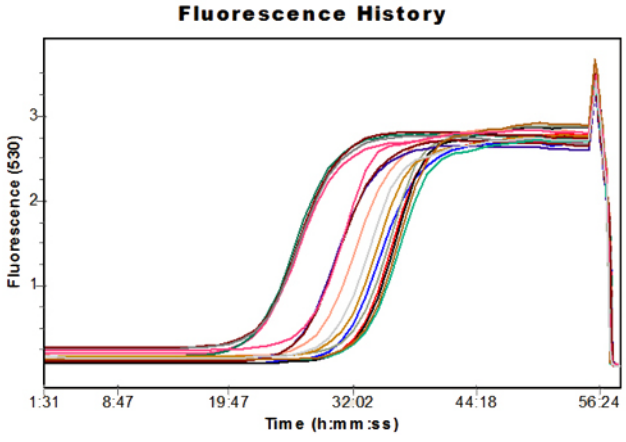

Fig. 3 The amplification curves of *PnAG₁* gene and control gene by real-time qPCR.

Figure 4 showed that each melting curve has only one peak, which indicated that it is specific amplification with simplex products.

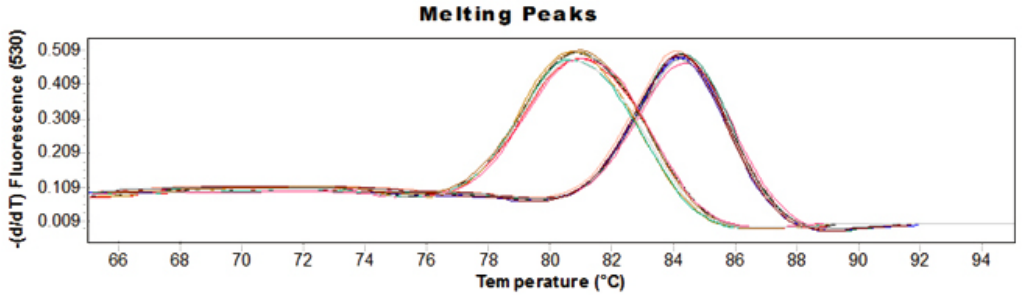

Fig. 4 The melting curves of *PnAG*$_1$ gene and control gene by real-time qPCR.

To determine the changes of *PnAG*$_1$ gene expression during the pod-maturing phase in testae of JH1012 and J-11, sampling was done in 12 weeks, 13 weeks, 14 weeks and 15 weeks respectively after sowing. The *PnAG*$_1$ gene expression decreasing before 13 weeks after sowing and then increasing in testae was recorded for up to 15 weeks and compared for JH1012 and J-11. The increase along time in J-11 plants was significantly higher than in JH1012 plants (Figure 5). A similar pattern was observed in the embryo (Figure 6). In general, *PnAG*$_1$ gene expresses more in J-11 than in JH1012 especially near the harvest time. Perhaps high temperatures and drought conditions around harvest time induce the gene expression. J-11 is *A. flavus*-resistant variety, and JH1012 is *A. flavus*-susceptible. Drought stress is the most important environmental factor exacerbating *Aspergillus* infection and aflatoxin contamination in peanut. Therefore, this could be attributed somewhat to the expression of *PnAG*$_1$ gene induced by drought stress in *A. flavus*-resistant plants.

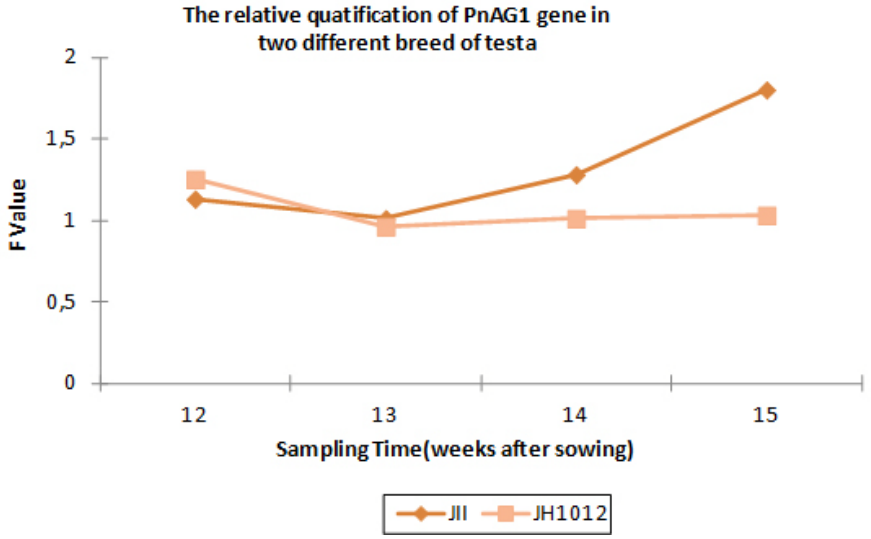

Fig. 5 The changes of *PnAG*$_1$ gene expression during the pod-maturing phase in testa of JH1012 and J-11. Values are means ± SD (n = 3) from one representative of three independent experiments with similar results. *Significant at $P < 0.05$ compared with the wild type based on Student's t test.

DISCUSSION

Since the first plant disease resistance gene Hm1 was cloned successfully (Johal and Briggs, 1992), more than 70 disease resistance genes and their analogues have been cloned from a variety of plants using the position cloning, transposon tagging and homology cloning techniques (Liu et al. 2007). Amino acid analysis showed that these genes that have been cloned are highly conserved in some domains. The majority of them belong to NBS-LRR class disease resistance protein. Peanut is one of the most vulnerable crops to aflatoxin contamination, and there is no effective anti-*Aspergillus* species available currently. Therefore, the study and characterization of peanut disease resistance genes to *A. flavus* and aflatoxin contamination is a major emphasis of many programs.

In this study, degenerate primers were designed based on consensus regions of NBS structural domains. Two disease resistance gene fragments about 500 bp have been cloned from peanut using homology cloning method. These are named *PnAG*$_1$-fragment and *PnAG*$_3$-fragment respectively. The gene-specific primers for 5' and 3' end RACE were designed based on the sequence of *PnAG*$_1$-fragment to amplify full-length cDNA, and three different full-length cDNAs were obtained. One of them, designated *PnAG*$_1$-2 (accession no. 1435133) was selected to analyze in this study, the cloned peanut *PnAG*$_1$-2 cDNA sequence is 1,088 bp in length encoding a putative protein of 283 amino acids. The deduced amino acid sequence shares high identity (all over 40%) with known plant disease resistance genes such as *Arachis cardenasii* resistance protein (AAN85395.1), *Ricinus communis* leucine-rich repeat containing protein, putative (gbEEF32840.1), *Glycine max* NB-LRR type disease resistance protein Rps1-k-1 (AAX89382.1), *Phaseolus vulgaris* CNL-B17 (ACZ74713.1), *Solanum verrucosum* RB (ABO28718.1), *Oryza sativa Japonica Group* putative NBS-LRR type resistance protein (AAT69649.1), *Oryza sativa Japonica Group* hypothetical protein OsJ_19033 (EEE64201.1), *Populus trichocarpa* TIR-NBS-LRR type disease resistance protein (ABF81464.1), *Nicotiana tabacum I2* (ABB00542.1) and *Oryza sativa* Japonica Group Os03g0324600 (NP 001049979.1). It has the highest sequence identity (66%) with *Arachis cardenasii* resistance protein (accession no. AAN85395.1). In addition, the deduced amino acid sequence has the conserved features, as noted by Bertioli et al. (2003), for P-loop, GLPL and kinase-3(GSRVLVTTR), which are highly conserved in all known NBS-LRR genes. So *PnAG*$_1$-2 gene can be placed in the NBS-LRR class. Further analysis is needed to determine whether the three genes of different length described in this study belong to the same family.

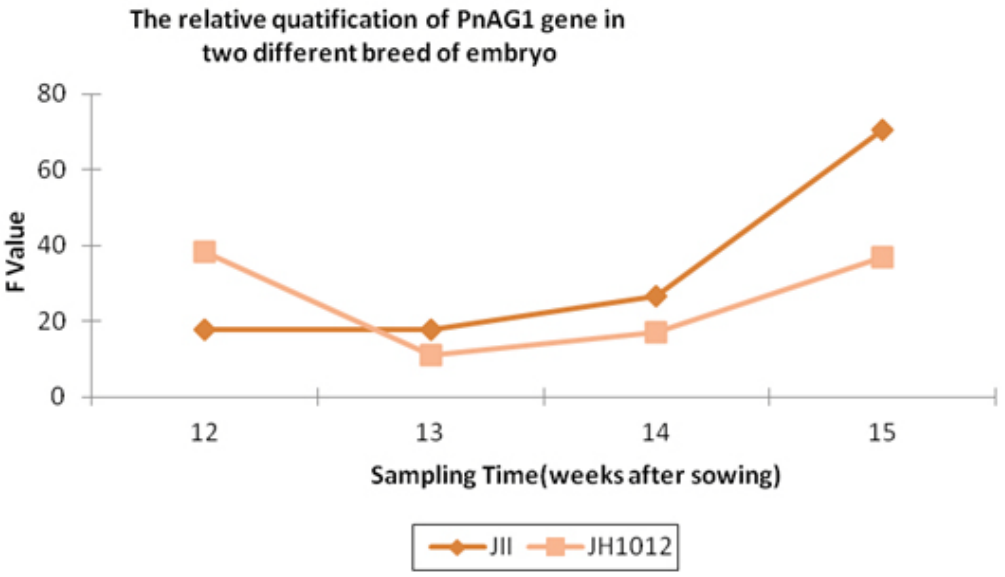

Fig. 6 The changes of *PnAG*$_1$ gene expression during the pod-maturing phase in embryo of JH1012 and J-11. Values are means ± SD (n = 3) from one representative of three independent experiments with similar results. *Significant at P < 0.05 compared with the wild type based on Student's t test.

Each NBS–LRR protein encodes a conserved NBS for ATP binding and hydrolysis (Tameling et al. 2002). NBS domain is necessary for the function of ATP or GTP binding and exists in eukaryotic proteins with ATP and GTP binding activity, such as the ATP synthetase β subunit, ribosomal extended elements, adenylate kinase and proteins encoded by resistance genes and so on. These proteins play a key role for cell growth and differentiation, cytoskeleton formation, vesicular transport and defence response (He et al. 2001). NBS domain plays an important role in signal transduction of plant defence responses, which were described in the study on I-2 and Mi-1 of potato (Tameling et al. 2002), PR5 of tobacco (Mestre and Baulcombe, 2006), and Arabidopsis (Ade et al. 2007). The majority of functionally described *R* genes are the nucleotide binding site-leucine rich repeat (NBS-LRR) type. The NBS domains are characteristic of various proteins with ATP/GTP binding activity, and comprise the P-loop, kinase 2a, kinase 3a and GLPL motifs (Traut , 1994), while LRR domains play roles in the interaction of protein-protein (Kobe and Deisenhofer, 1994); Bertioli et al. (2003) isolated a total of 78 complete NBS-encoding regions, of which 63 had uninterrupted ORFs. Yuksel et al. (2005) 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 improve 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 the binding of NBS domain and nucleotide triphosphate change the interaction between disease resistance protein and its defensive signals. As *PnAG*$_1$-2 belongs to NBS-LRR class gene and shows higher expression in resistant variety, especially along with maturing and dehydration during seed development, we speculate that *PnAG*$_1$-2 may be involved in the resistance to *A. flavus* in J-11.

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REFERENCES

ADE, J.; DE YOUNG, B.J.; GOLSTEIN, C. and INNES, R.W. (2007). Indirect activation of a plant nucleiotide binding site-leucine-rich repeat protein by a bacterial protease. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 7, p. 2531-2536. **[CrossRef]**

ALTSCHUL, S.F.; MADDEN, T.L.; SCHÄFFER, A.A.; ZHANG, J.; ZHANG, Z.; MILLER, W. and LIPMAN, D.J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research*, vol. 25, no. 17, p. 3389-3402. **[CrossRef]**

BERTIOLI, D.J.; LEAL-BERTIOLI, S.C.M.; LION, M.B.; SANTOS, V.L.; PAPPAS, G.; CANNON, S.B. and GUIMARÃES, P.M. (2003). A large scale analysis of resistance gene homologues in *Arachis. Molecular Genetics and Genomics*, vol. 270, no. 1, p. 34-45. **[CrossRef]**

CHEN, G.-S.; ZHOU, Y.; LIN, S. and PAN, D. (2006). Isolation and sequence analysis of NBS-type resistance gene analogues in sweet potato (*Ipomoea batatas* (L.) Lam.). *Journal of Tropical and Subtropical Botany*, vol. 14, no. 5, p. 359-365.

DANGI, J.L. and JONES, J.D.G. (2001). Plant pathogens and integrated defence responses to infection. *Nature*, vol. 411, no. 6839, p. 826-833. **[CrossRef]**

ELLIS, J. and JONES, D. (1998). Structure and function of proteins controlling stain-specific pathogen resistance in plants. *Current Opinion in Plant Biology*, vol. 1, no. 4, p. 288-293. **[CrossRef]**

GEER, L.Y.; DOMRACHEV, M.; LIPMAN, D.J. and BRYANT, S.H. (2002). CDART: Protein homology by domain architecture. *Genome Research*, vol. 12, no. 10, p. 1619-1623. **[CrossRef]**

GUO, B.Z.; HOLBROOK, C.C.; YU, J.; LEE, R.D. and LYNCH, R.E. (2005). Application of technology of gene expression in response to drought stress and elimination of preharvest aflatoxin contamination. In: ABBAS, H.D. ed, *Aflatoxin and Food Safety*. CRC, Boca Raton, p. 313-331.

HAMMOND-KOSACK, K.E. and JONES, J.D.G. (1997). Plant disease resistance genes. *Annual Review of Plant Physiology and Plant Molecular Biology*, vol. 48, p. 575-607. **[CrossRef]**

HE, C.; ZHANG, Z. and CHEN, S. (2001). Isolation and characterization of soybean NBS analogs. *Chinese Science Bulletin*, vol. 46, no. 23, p. 1984-1988. **[CrossRef]**

HUANG, D.-Q.; WANG, P. and LÜ, L.-X. (2004). Isolation of NBS-LRR class resistance gene analogs from stigma cDNA of pomelo (*Citrus grandis* cv. guanxi). *Scientia Agricultura Sinica*, vol. 37, no. 10, p. 1580-1584.

JOHAL, G.S. and BRIGGS, S.P. (1992). Reductase activity encoded by the HM1 disease resistance gene in maize. *Science*, vol. 258, no. 5084, p. 985-987. **[CrossRef]**

KOBE, B. and DEISENHOFER, J. (1994). The leucine-rich repeat: A versatile binding motif. *Trends in Biochemical Sciences*, vol. 19, no. 10, p. 415-421.

LIU, J.; LIU, X.; DAI, L. and WANG, G. (2007). Recent progress in elucidating the structure, function and evolution of disease resistance genes in plants. *Journal of Genetics and Genomics*, vol. 34, no. 9, p. 765-776. **[CrossRef]**

MARCHLER-BAUER, A. and BRYANT, S.H. (2004). CD-Search: Protein domain annotations on the fly. *Nucleic Acids Research*, vol. 32, no. 2, p. W327-W331. **[CrossRef]**

MARCHLER-BAUER, A.; ANDERSON, J.B.; CHERUKURI, P.F., DEWEESE-SCOTT, C.; GEER, L.Y.; GWADZ, M.; HE, S.; HURWITZ, D.I.; JACKSON, J.D.; KE, Z.; LANCZYCKI, C.J.; LIEBERT, C.A.; LIU, C.; LU, F.; MARCHLER, G.H.; MULLOKANDOV, M.; SHOEMAKER, B.A.; SIMONYAN, V.; SONG, J.S.; THIESSEN, P.A.; YAMASHITA, R.A.; YIN, J.J.; ZHANG, D. and BRYANT, S.H. (2005). CDD: A conserved domain database for protein classification. *Nucleic Acids Research*, vol. 33, no. 1, p. D192-D196. **[CrossRef]**

MESTRE, P. and BAULCOMBE, D.C. (2006). Elicitor-mediated oligomerization of the tobacco N disease resistance protein. *The Plant Cell*, vol. 18, no. 2, p. 491-501. **[CrossRef]**

MEYERS, B.C.; DICKERMAN, A.W.; MICHELMORE, R.W.; SIVARAMAKRISHNAN, S.; SOBRAL, B.W. and YOUNG, N.D. (1999). Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. *The Plant Journal*, vol. 20, no. 3, p. 317-332.

ROGERS, S.O. and BENDICH, A.J. (1988). Extraction of DNA from plant tissues. In: GELVIN, S.B. and SCHILPEROORT, R.A. (eds) *Plant Molecular Biology Manua*l, p. A6:l-10. Boston, MA: Kluwer, Academic Publishers.

TAMELING, W.I.L.; ELZINGA, S.D.J.; DARMIN, P.S.; VOSSEN, J.H.; TAKKEN, F.L.W.; HARING, M.A. and CORNELISSEN, B. (2002). The tomato *R* gene products I-2 and Mi-1 are functional ATP binding proteins with ATPase activity. *The Plant Cell*, vol. 14, no. 11, p. 2929-2939. **[CrossRef]**

TRAUT, T.W. (1994). The functions and consensus motifs of nine types of peptide segments that form different types of nucleiotide-binding sites. *European Journal of Biochemistry*, vol. 222, no. 1, p. 9-19. **[CrossRef]**

WANG, S.; ZHANG, D.; LI, P.; WANG, X.D.; LI, S.G.; ZHU, L.H. and ZHAI, W.X. (2005). Cloning and analysis of a new NBS-LRR resistance gene family in rice. *Acta Genetica Sinica*, vol. 32, no. 7, p. 704-711.

WANG, H.-Y.; LIU, D.-G.; YANG, W.-X.; LI, Z.-F.; ZHANG, L.-R. and ZHANG, N. (2009a). Identification and cloning of a NBS resistance gene homology cDNA sequence from wheat. *Acta Phytopathologica Sinica*, vol. 39, no. 5, p. 507-513.

WANG, J.; JIANG, H.; ZHAO, Y.; XIANG, Y.; ZHU S.-W. and CHENG, B.-J. (2009b). Genome-wide analysis of NBS-encoding disease resistance genes in maize inbred line B73. *Acta Agronomica Sinica*, vol. 35, no. 3, p. 566-570. **[CrossRef]**

WILLIAMSON, V.M. (1999). Plant nematode resistance genes. *Current Opinion in Plant Biology*, vol. 2, no. 4, p. 327-331. **[CrossRef]**

YUKSEL, B.; ESTILL, J.C.; SCHULZE, S.R. and PATERSON, A.H. (2005). Organization and evolution of resistance gene analogs in peanut. *Molecular Genetics and Genomics*, vol. 274, no. 3, p. 248-263. **[CrossRef]**

ZHANG, C.-Y.; XU, S.-G. and HUANG, X.-X. (2005). A novel and convenient relative quantitative method of fluorescence real time RT-PCR assay based on slope of standard curve. *Progress in Biochemistry and Biophysics*, vol. 32, no. 9, p. 883-888.

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