Cloning and analysis of a NBS-LRR disease resistance gene candidate 
\textit{PnAG}_1 from peanut (\textit{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 \textit{Arachis cardenasii} resistance protein gene had the highest homology (66%). Relative quantification PCR analysis indicated that PnAG1-2 gene expresses more in J11 (an \textit{A. flavus}-resistant variety) than in JH1012 (an \textit{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-\textit{A. flavus} peanut varieties.

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

INTRODUCTION

Peanut (\textit{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 \textit{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 \textit{R} genes have been defined based on the structural characteristics of cloned resistant genes (Dangi and Jones, 2001). The majority of functionally described \textit{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>
<thead>
<tr>
<th>primers</th>
<th>conserved domains</th>
<th>amino acid sequences</th>
<th>primer sequences (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pf1</td>
<td>P-loop</td>
<td>GMGGVGKT</td>
<td>GGNATGGGGNGNNTGNAARACNAC</td>
</tr>
<tr>
<td>Pr1</td>
<td>GLPL</td>
<td>GLPLALK</td>
<td>NACYTTNAGNGCNAGNGNAGNCC</td>
</tr>
</tbody>
</table>

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

PnAG1 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 94°C for 5 min, and amplifications were achieved through 32 cycles at 94°C for 60 sec, 48°C for 60
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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 42°C 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 PnAG1 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 PnAG1 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.

Bioinformatics analysis of PnAG1 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 DNASTIS v2.5 (MiralBio, 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 94°C for 2 min, and amplifications were achieved through 40 cycles for fluorescence collection at 94°C for 15 sec, 57°C for 15 sec, and 72°C for 20 sec. Then the final extension reaction was achieved for collecting fluorescence every 0.2°C from the 60°C gradually to 94°C for melting curve analysis. Then, the PCR product was cooled at 40°C 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.
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 37°C 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 determined by diluting 10 times of the last one in turn. The standard curves of the AG1 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 AG1 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 \( F = 10^{\frac{\Delta C_T}{A_R} - \frac{\Delta C_T}{A_T}} \).

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

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

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

Fig. 1 Comparison of the deduced amino acid sequence of *PnAG1* with 10 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* (AA679649.1), *Oryza sativa Japonica Group* (EEE64201.1), *Nicotiana tabacum* (ABB00542.1), *Populus trichocarpa* (ABF81464.1), *Oryza sativa Japonica Group* (NP_001049979.1).
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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% agarose 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.

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 phylogenetic 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 \( PnAG_1 \) gene with ultraviolet spectrophotometry as shown in Table 3 and Table 4.

<table>
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<tr>
<th>Diluted multiple</th>
<th>( 10^{-1} )</th>
<th>( 10^{-2} )</th>
<th>( 10^{-3} )</th>
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<th>( 10^{-5} )</th>
<th>( 10^{-6} )</th>
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<tr>
<td>Copy number</td>
<td>( 9.58 \times 10^{11} )</td>
<td>( 9.58 \times 10^{10} )</td>
<td>( 9.58 \times 10^{9} )</td>
<td>( 9.58 \times 10^{8} )</td>
<td>( 9.58 \times 10^{7} )</td>
<td>( 9.58 \times 10^{6} )</td>
<td>( 9.58 \times 10^{5} )</td>
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<tr>
<th>Diluted multiple</th>
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<th>( 10^{-3} )</th>
<th>( 10^{-4} )</th>
<th>( 10^{-5} )</th>
<th>( 10^{-6} )</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Copy number</td>
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<td>( 3.21 \times 10^{10} )</td>
<td>( 3.21 \times 10^{9} )</td>
<td>( 3.21 \times 10^{8} )</td>
<td>( 3.21 \times 10^{7} )</td>
<td>( 3.21 \times 10^{6} )</td>
<td>( 3.21 \times 10^{5} )</td>
</tr>
</tbody>
</table>

Construction of real-time fluorescence quantitative PCR standard curve

The real-time fluorescence quantitative PCR standard curves of \( PnAG_1 \) 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 \( PnAG_1 \) 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 \( F = 10^{A_T \Delta C_{T,R}} \) (\( A_T \): the slope of standard curve of target gene and control gene respectively).

![Fluorescence History](image)

Fig. 3 The amplification curves of \( PnAG_1 \) 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.

To determine the changes of PnAG1 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 PnAG1 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, PnAG1 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 PnAG1 gene induced by drought stress in A. flavus-resistant plants.

Fig. 4 The melting curves of PnAG1 gene and control gene by real-time qPCR.

Fig. 5 The changes of PnAG1 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 PnAG1-fragment and PnAG3-fragment respectively. The gene-specific primers for 5' and 3' end RACE were designed based on the sequence of PnAG1-fragment to amplify full-length cDNA, and three different full-length cDNAs were obtained. One of them, designated PnAG1-2 (accession no. 1435133) was selected to analyze in this study, the cloned peanut PnAG1-2 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 (AAAX9382.1), Phaseolus vulgaris CNL-B17 (ACZ74713.1), Solanum verrucosum RB (ABO28718.1), Oryza sativa Japonica Group putative NBS-LRR type resistance protein (AAD69644.1), 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 Bertoli et al. (2003), for P-loop, GLPL and kinase-3(GSRVLVTTR), which are highly conserved in all known NBS-LRR genes. So PnAG1-2 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.

![The relative quantification of PnAG1 gene in two different breed of embryo](image)

Fig. 6 The changes of PnAG1 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-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-2 may be involved in the resistance to A. flavus in J-11.

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