Journal of Agricultural Science; Vol. 4, No. 7; 2012
ISSN 1916-9752 E-ISSN 1916-9760
Published by Canadian Center of Science and Education

Cloning, Expression and Characterization of *PnLOX*$_2$ Gene Related to *Aspergillus flavus*-resistance from Peanut (*Arachis hypogaea* L.) Seed Coat

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Received: March 2, 2012 Accepted: March 23, 2012 Online Published: May 22, 2012

doi:10.5539/jas.v4n7p67 URL: http://dx.doi.org/10.5539/jas.v4n7p67

Financial support came from the following research projects, (1) Modern Agro-industry Technology Research System (CARS-14); (2) Agro-industry Technology Research System of Shandong Province; (3)Bio-resource Innovation and Research Project of Shandong Province.

Abstract

Aspergillus flavus is one of the major diseases of peanut. The objective of the study was to clone *PnLOX2* gene from peanut seed coat and analyze its expression. Real-time RT-PCR analysis indicated that the gene was overexpressed in the challenge of *Aspergillus flavus* and the greatest expression occurred 10 days after inoculation. The full length of *PnLOX2* gene was 2592bp and encoded a 97.5kDa protein containing 863 amino acids. The fusion protein (apparent 121.5kDa) only existed in the precipitation and the maximum was obtained by inducing at 37ºC for 3.5h. As a result of response to the challenge of *Aspergillus flavus*, *PnLOX2* gene was more greatly expressed in the resistant genotype than in the susceptible genotype. It indicated that *PnLOX2* gene was closely related to *Aspergillus flavus*-resistance of peanut. Further investigations were needed to bacteriostatically identify the gene in vitro.

Keywords: peanut seed coat, *Aspergillus flavus*, *PnLOX2* gene, cloning, prokaryotic expression

Peanut (*Arachis hypogaea* L.) is an important economic crop and oil crop in the world. Seeds have close relationship with the quality and production of peanut. *Aspergillus flavus* is only second to *Aspergillus fumigatus* as a cause of human invasive *Aspergillosis* (Hedayati, et al., 2007). Aflatoxin is an secondary metabolites after *Aspergillus flavus* infecting peanuts, corn, tree nuts as well as other crops and was a very strong carcinogenic to human and animals (Bhatnagar, et al., 2006; Diener, et al., 1987). Aflatoxin contamination caused by *Aspergillus flavus* is one of the major destructive legume diseases of peanut worldwide, which has been affecting the peanut production, processing and trade of China. Aflatoxin contamination appears in the whole process of peanut, from planting, harvesting to the storage. The development of resistant cultivars could be effective in decreasing production costs and improving product quality.

The peanut seed coat is a crucial barrier in the process of *Aspergillus flavus* infection. It is essential to breed disease-resistant varieties isolating and cloning the genes of peanut seed coat related to *Aspergillus flavus*-resistance. Lipoxygenases (EC 1.13.11.12; LOXs) are non-heme, non-sulfur iron dioxygenases, are encoded by a multi-gene family and widely distributed in higher plants (Porta and Rocha-Sosa, 2002). Its metabolic products as Jasmonic acid, salicylic acid (SA), *etc*., are anti-insect or antibiotic active substances in which active oxygen radicals can destroy cytomembrane (Fritig & Legend, 1993; Juttner & Slusarenko, 1993; Ohta et al., 1991; Shukle & Murdock, 1983) and inhibit fungus and aflatoxin generation (Vergopoulou et al., 2001; Zeringue, 1996). *LOX* gene expression is regulated by different forms of stress, such as wounding (Porta et al., 1999), water deficiency (Porta et al., 1999), or pathogen attack (Melan et al., 1993).

In peanuts, the studies about the association of LOX gene with *Aspergillus flavus*-resistance are made progress now. *PnLOX1*, *PnLOX2* and *PnLOX3* were three primary genes coding for LOXs in the seeds of peanut (Siedow, 1991). Burow et al. (2000) found that the catalyzed products of *PnLOX1* in mature peanut seeds, 9-HPOD and 13-HPOD, are the inhibitor and inducer of mycotoxin synthesis respectively. 9S-HPODE was promptly induced from 8 h to 48 h after infected with *Aspergillus flavus* but decreased gradually then. Therefore, they inferred that both 9-HPOD and 13-HPOD were participated in the interaction of seed and *Aspergillus flavus*, furthermore, 9-HPOD could restrain the expression of 13-HPOD. Recently, Tsitsigiannis et al. (2005) isolated two LOX genes (*PnLOX2* and *PnLOX3*) encoding 13-LOXs which has identical biochemical properties and highly expressed in mature peanut seed, but both genes, differently from *PnLOX1*, are repressed during *Aspergillus flavus* infection. It was predicted that 9-HPOD, the product of *PnLOX2*, was susceptible to *Aspergillus flavus* and 13-HPOD, product of *PnLOX3,* was resistant to *Aspergillus flavus*.

At present, concern about the interaction of *LOX* and *Aspergillus flavus* infection was focused on peanut kernels and the research about seed coat was still in the blank. In view of the importance of peanut seed coat in defending disease, it was essential to insight into seeds resistance mechanisms at the molecular level and to develop specific gene probes for using in breeding disease-resistant cultivars. In this paper, we took advantage of four genes isolated which have NBS structural domain, cloned *PnLOX2* in seed coat, analyzed the expression pattern of the gene, and further detected protein activity. It will provide new thought for the exploration of *Aspergillus flavus*-resistance molecular mechanism and the development of disease-resistant peanut cultivar.

2. Material and Methods

2.1 Plant Genotypes and Treatments

Peanuts cultivars J11 (highly resist *Aspergillus flavus* infection, incidence is below 13%) and Jinhua1012 (highly susceptive to *Aspergillus flavus* infection, incidence is 100%) were used. *Aspergillus flavus* strains were cultured on the czapek's medium at 30ºC for 7d and the spore suspension (1×10^6 spores per ml sterile water) were inoculated into corn meal. The peanut roots were infected by the corn meal once 10 days for 3 times a month before harvesting. The immature peanut seeds were collected 5 days after every inoculation and striped seeds coat were stored at -70ºC until required.

2.2 Cloning and Sequencing of PnLOX2 Gene

The gene-specific primers LoxF1 and LoxR1 were designed for PCR amplification according to the published LOX gene sequence (Database accession number DQ068249.1). Based on the ORF of *PnLOX2* gene, forward primer LoxF2 containing a *Sma*I site and reverse primer LoxR2 containing a *Xho*I site were designed for RT-PCR amplification. A 25 µl reaction mixture contained 3 µl of template, 5 µl of 10×PCR buffer (Mg^{2+} plus), 2 µl of 25mM $MgCl_2$, 2 µl of 10mM dNTP mix, 0.5 µl of 5U *Taq* DNA polymerase, 0.5 µl of 10 µM primer. The PCR conditions were: 94ºC for 5 min; 94ºC for 30s, 58ºC for 30s, and 72ºC for 3min, for 30 cycles; and a final extension at 72ºC for 10min. After sequenced and identified, RT-PCR products were purified from the melted agarose gel using DNA gel extraction kit (BIOER, Nanjing), cloned to the linearized vector pBS-T (Invitrogen, Shanghai), and transformed into *E.coli* DH5α following the manufacturer's instructions. Transformed cells were plated on medium with IPTG/Xgal and grown overnight at 37ºC. The positive recombinant clones were screened and identified by colony PCR directly, and then digested with *Sma*I and *Xho*I and sequenced. BLAST program (http://www.ncbi.nlm.nih.gov/) and DNAMAN software were analyzed the homology. The ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) was used to analyze the open reading frame. EXPASY (http://www.expasy.cn/tools/) was used to predict the property of protein.

2.3 Real-time RT-PCR

The primers RTF and RTR were synthesized based on a conservative region of *PnLOX2* sequence obtained. The primers *Actin*-F and *Actin*-R were designed according to 3'EST of peanut *Actin* gene fragment (380bp, unpublished) as internal control for calculating relative transcript abundance.

Total RNA was isolated from J11 and Jinhua1012 seeds coat infected by *Aspergillus flavus* 30, 20 and 10 days before harvesting using plant RNA kit (OMEGA Company). First-stand complementary DNA was synthesized by RNA PCR Kit (AMV) Ver.3.0 kit, and then Real-time PCR was performed from cDNA with TaKaRa SYBR® PrimeScript™ RT-PCR Kit according to the manufacture's instructions (Takara, Shanghai). PCR assay was carried out with SYBR Green system in Light Cycler 2.0 Carousel. Cycling parameters were set up as the recommendation of SYBR® PrimeScript™ RT-PCR Kit. Melt curves were run immediately after the last PCR cycle to examine if the measurements were influenced by primer-dimer pairs.

The internal reference gene β-*actin* and target gene *PnLOX2* were analyzed in one plate, and each reaction was repeated three times to access the reproducibility. The amplification curve was generated after analyzing the raw data and adjusting the cycle threshold (C_T) value. The model $2^{-\triangle\triangle C_T}$ for comparing relative expression results between treatments in real-time PCR was applied. The amount of target, normalized to the reference control and relative to a calibrator, is given by $R = 2^{\triangle\triangle C_T}$, where $\Delta\Delta C_T = \Delta C_T$sample - ΔC_Tcontrol. The final value obtained was a measure of the fold change in gene expression for the particular gene of interest between the treated samples and the untreated samples.

2.4 Prokaryotic Expression of PnLOX2 Gene in E. coli

Positive clones pBS-T::*PnLOX2* were digested with *Sma*I and *Xho*I restriction enzymes and separated on a 1% (w/v) agarose gel. The target fragment were extracted from the gel and directionally subcloned into the *Sma*I and *Xho*I sites of the expression vector pGEX-4T-1 (TIANGEN, Beijing). The resultant construct pGEX-4T-1::*PnLOX2* was transformed into *E. coli* strain BL21 (DE3) competent cells. Positive colonies were selected in Luria-Bertani plates containing ampicillin (50μg/mL).

To determine the optimal induction time, transformed *E. coli* BL21(DE3) cells were grown in small flasks until the *A*600 reached 0.5, and IPTG was added at a final concentration of 1 mM. Aliquots were analyzed every 1 h for a total of 5 h and a final aliquot was taken after 5 h and solubilized by sonication. The inclusion bodies and the supernatant were collected respectively from the crude extract after centrifugation in 12,000g for 5min at 4ºC. Both of them were resuspended in buffer containing SDS and electrophoresed in 12% (w/v) polyacrylamide denaturing gels.

3. Results

3.1 Isolation, Cloning and Identification of PnLOX2 Gene from Seed Coat

To obtain genome and cDNA of the *PnLOX2* gene, DNA and RNA was extracted from immature peanut seeds coat of J11 and Jinhua1012, and an attempt was then made to amplify the gene using the PCR and RT-PCR method, which showed about 3500bp and 2600bp specific band respectively (Figure 1A). The complete nucleotide sequence of *PnLOX2* gene and the corresponding deduced amino acid sequence were presented in supplemental figures (for example J11). The DNA was 3491bp long containing 8 introns of 900bp and an open reading frame of 2592bp, which is sufficient to encode a predicted 97.5kDa mature polypeptide of 863 amino acids. The deduced amino acid sequence of *PnLOX2* showed from 74% to 99% identity to lipoxygenase from other species (Figure 2). A phylogenetic tree based on the amino acid sequences of plant lipoxygenase showed that *PnLOX2* protein was highly homologous (99%) with that published by Keller N.P. (2000). The protein was predicted to be insoluble and isoelectric point was 4.66 by the EXPASY program which located in the periplasm of the cell.

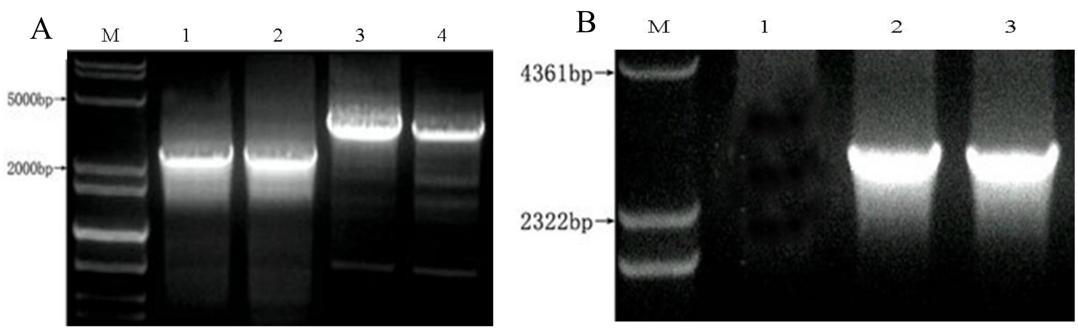

Figure 1. (A) PCR and RT-PCR amplification of *PnLOX2* gene from the resistant and susceptible genotypes. The lanes 3 and 4 are the PCR products of Jinhua1012 and J11 separately. The lanes 1 and 2 were RT-PCR products of Jinhua1012 and J11 separately. (B) The individual bacterial colonies PCR identification of the recombinant plasmid pBS-T-*PnLOX2*. M, molecular weight marker; Lane 1, negative control; lane 2, the product of Jinhua1012; lane 3, the product of J11

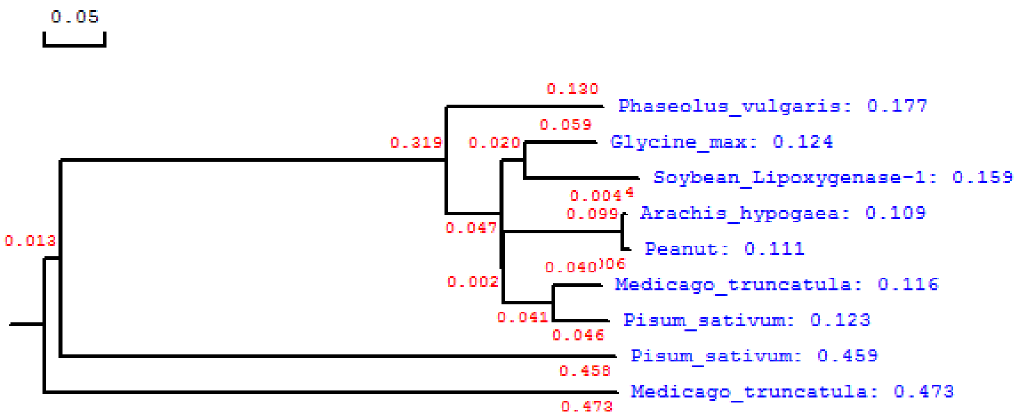

Figure 2. A phylogenetic tree of nine lipoxygenase based on the similarities of their amino acid sequences. The phylogenetic tree was constructed with DNAMAN software (Lynnon Corporation). The relative phylogenetic distance is indicated by the numbers

The RT-PCR amplified product was purified and directionally inserted into cloning vector pBS-T. The recombinant pBS-T::*PnLOX2* was transformed into bacterial host strain *E.coli* DH5α. By blue-white screening with IPTG-Xgal plates and the colony PCR identifying, a strong intensity band about 2600bp was observed in the bacterial lysates which was absent in induced cells containing the empty vector (Figure 1B). The band were extracted and further verified by sequencing. It disclosed that *PnLOX2* gene had successfully transformed into *E.coli* DH5α.

2.2 Gene Expression of J11 and Jinhua1012 in the Challenge of Aspergillus Flavus

Real-time RT-PCR was used to profile the gene expression patterns and to characterize the difference between fungal-challenged samples and control samples of resistant and susceptible genotypes. The two peanut genotypes inoculated 30d, 20d and 10d before harvesting were used for quantitative gene expression analyses by real-time PCR. The relative quantity comparisons based on C_T values (cycle threshold) from challenged and control samples in each genotype were conducted as the algorithm $R=2^{-\Delta\Delta C_T}$. The results indicated that the expression levels of *PnLOX2* gene in J11 samples challenged exceeded significantly the levels of the control samples. Especially, the expression challenged 30d before harvesting was the greatest which was 11.16 times of the control. After that, the expression of the gene kept low levels which were 2.55 times and 2.62 times of the control respectively. However, few differences in Jinhua1012 were observed between the induced and the control samples challenged 30d before harvesting (1.659 times). Instead, the gene was expressed at an even greater level in the control samples than in the induced samples challenged 20d, 10d before harvesting (0.287 times and 0.178 times separately). It may be the experiment errors caused by template concentration and further verification was needed. For all that, the expression levels of the genes had obvious difference between the resistant and the susceptible genotypes in the same infection period (Figure 3). It illustrated that the expression of *PnLOX2* gene was inducible in peanut seed coat.

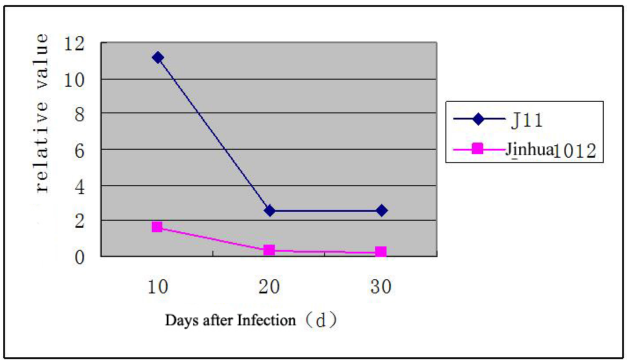

Figure 3. Comparison of the expression levels of *PnLOX2* gene from J11 and Jinhua1012 after inoculation with *Aspergillus flavus* for 10d, 20d, and 30d before harvesting. The expression levels of J11 in induced samples all significantly exceeded that of the control samples. There were obvious difference between the induced and control samples at three different periods. The expression level after first inoculation was the greatest. After that, the expression of two genotypes kept a low level

2.3 Prokaryotic Expression of PnLOX2 Gene

To confirm the function of *PnLOX2* gene, we characterized its expression in bacterial cells. The PCR products of pBS-T::*PnLOX2* were directionally cloned into the *Sma*I and *Xho*I sites of expression vector pGEX-4T-1 and used to transform *E.coli* BL21 (DE3). Identically treated *E.coli* BL21 (DE3) cells transformed with vector pGEX-4T-1 were used as a control (Figure 4A, 4B). Analysis by SDS-PAGE of cells lysates showed induced expression of an approximately 121.5kDa fusion protein in J11 and Jinhua1012 samples inoculated 30 d before harvesting for a period of 1 to 5 h. Except for tag GST, the molecular mass of expression product was approximately 97.5kDa. The result showed that, when induced for 3.5 to 5 hours, the maximum recombinant protein was obtained and the optimum induced time was 3.5 h (Figure 5A). The bacterial crude was centrifugated in 9000 g for 15 min, and then the precipitation and the supermanant were separately applied to SDS-PAGE. The dense, 121.5kDa band was observed in the precipitation, indicating that the protein is insoluble and existed in the inclusion body of bacterial cells (Figure 5B). The subcellular localization is necessary to further perform for thorough research.

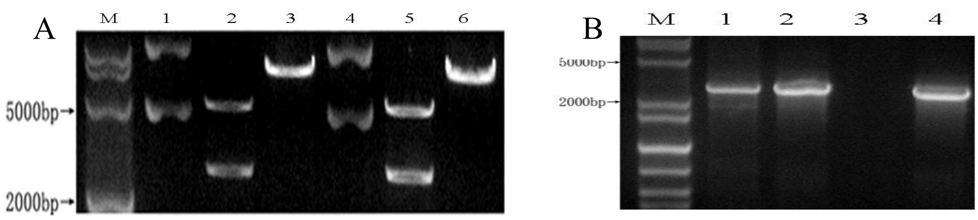

Figure 4. (A) Identification of pGEX-4T-1::*PnLOX2* by enzymatic digestion of *Sma*I and *Xho*I. M, Molecular weight maker; 1, the recombinant of Jinhua1012; 2, the recombinant of Jinhua1012 by double enzymatic digestion; 3, the recombinant of Jinhua1012 by single enzymatic digestion; 4, the recombinant of J11; 5, the recombinant of J11 by double enzymatic digestion; 6, the recombinant of J11 by single enzymatic digestion. (B) The individual bacterial colonies PCR identification of *E.coli* BL21 (DE3) transformed by the recombinant plasmid pGEX-4T-1::*PnLOX2*. M, molecular weight marker; lane1, 2, the colony PCR product of Jinhua1012; lane 3, 4, the colony PCR product of J11

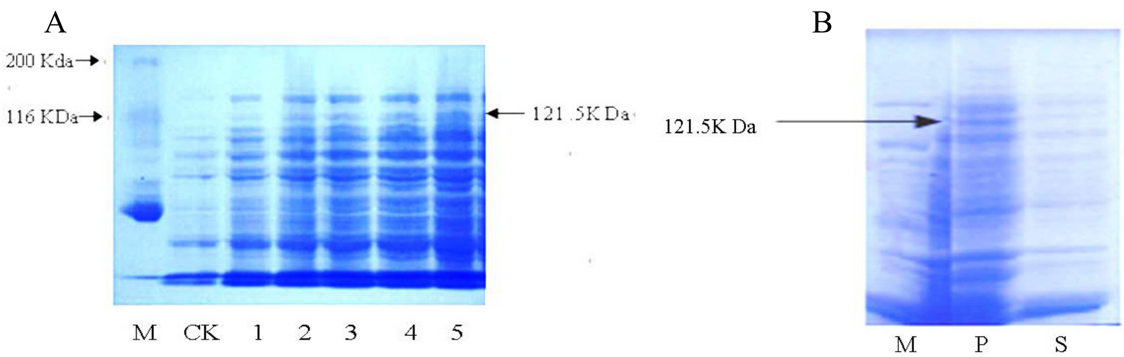

Figure 5. (A) SDS-PAGE analysis of the expression products of J11 in *E.coli* BL21. M: Protein marker; CK: Control cells bearing empty vector pGEX-4T-1; 1-5: Bacterial cells with pGEX-4T-1::*PnLOX2* were induced for 1 h, 2 h, 3 h, 4 h, 5 h. (B) The primary localization of fusion protein in *E.coli* BL21 cells. M, protein marker; P, the precipitation; S, the supermanant

3. Discussion

The focus of this study was to identify the association of *PnLOX2* gene from the peanut seeds coat with the resistance to *Aspergillus flavus* infection using real-time PCR by comparing the expression difference of resistant and susceptible genotypes which was further validated on protein levels by prokaryotic expression. Real-time PCR, a simple and effective method for gene quantification, can detect quickly the expression difference of target gene in various plant biological processes, such as plant disease resistance, environmental stress responses, fruit and seed development, signaling in photomorphogenesis, and nitrate assimilation. By comparison of the expression levels in the two genotypes infected 30 day before harvesting, *Aspergillus flavus* infection was demonstrated to stimulate *PnLOX2* gene expression in the resistant genotype which produced massive antifungal active material LOX responding to biotic stresses. It indicated that *PnLOX2* gene existed in peanut seed coat and was a defense-related gene. We will further detect the gene expression peak from 0 to 10 d after infection in the next growing season. It may be one of the causes for peanut seed coat as important barrier of resisting *Aspergillus flavus* infection. Further investigations were needed to bacteriostatically identify the gene in vitro.

Recent studies in LOXs largely mainly centre on the physiological function, but attempt is being made to clarify their mechanism in plant defense against fungal infection. Jensen et al. (1992) verified that there was a density-dependent conidia-sclerotia switch in *Aspergillus flavus*. This swith could be attenuated by LOX-derived metabolite encoded by Af*lox* and further activate conidium formation to indirectly refrain infection (Horowitz Brown et al., 2008). Oxylipins, produced through the lipoxygenase (LOX) pathway, are important components in plant defense responses to pathogens and pests (Rosahl and Feussner, 2005). The products of oxylipin metabolism including 9-HPOD and 13-HPOD, jasmonates (JA), OPDA, 6- and 9-carbon aldehydes, oxoacid and divinyl ether fatty acids, bioactive compounds, *etc*. could act either in defence signaling or as direct antimicrobials (Blee, 2002; Feussner and Wasternack, 2002). Moreover, Calvo et al. (1999) reported that 13S-LOX could inhibit mycotoxin biosynthesis in *Aspergillus spp.*. Presently, several members of the *LOX* gene families were cloned or analysed at the protein level from major crop species such as barley, wheat, and rice (Peng et al., 1994; Bohland et al., 1997; Mauch et al., 1997; Mizuno et al., 2003; Agrawal et al., 2004). The functional analysis indicated that overexpression of *13-LOX* gene RCI-1 increased the transcripts level of pathogenesis-related protein PR-1 in the rice. This study suggested that 13-LOXs may be involved in the activation of acquired resistance (Zabbai et al., 2004). The author predicted that seeds LOXs could have an important role in seed/fungi interaction and oxylipins produced by this pathway could be important molecular mediators in this interaction. All of them verified the close association of LOX gene with *Aspergillus flavus*-resistance. Thus, we purposed that *PnLOX2* gene, similar to other member of the LOX gene families, indirectly involved in interaction with the fungus by the metabolite.

Induction of LOX genes and it's metabolite during plant-pathogen interactions has been reported in several species. In tobacco, 9-LOX activity and *Lox1* mRNA expression are induced upon infection by *Phytophtora parasitica var nicotianae*. Interestingly, both 9-LOX activity and *Lox1* mRNA expression appear earlier in an incompatible plant-pathogen interaction than in a compatible one (Helena Porta and Mario Rocha-Sosa, 2002), which supporting a role of this 9-LOX in plant defense against fungal infection (Rance´ et al., 1998). In infected almond seeds by *A. carbonarius*, 9-HPOD could be converted into other antifungal oxylipins, for example, the amount of C9-aldehydes increased, as were LOX and hydroperoxide lyase (HPL) appear to be concomitantly expressed. It indicated that both *LOX* gene expression and activity are up-regulated during pathogen infection (Giovanni Mita1 et al., 2007). In the maize genome, cssap92, a predominantly 9-LOX, is up-regulated during infection with *Aspergillus flavus* and *Fusarium verticillioides* (Kolomiets et al., 2004; Wilson et al., 2001; Kim et al., 2003). ZmLOX10, attributing to a linoleate 13-LOX in maize, was strongly induced into defence-related hormones as jasmonic acid (JA), salicylic acid (SA) when inoculation with an a virulent strain of *Cochliobolus carbonum*, which suggested this gene and its products are primarily involved in defensive responses to insects and pathogens (Andriy Nemchenko et al., 2006). In conclusion, all these results indicated that LOX-derived metabolite could be induced in seed defence response and considered an interesting biotechnological target in program aimed at improving plant resistance toward pathogen infection.

4. Conclusions

In this study, we revealed the expression profile of *PnLOX2* gene from peanut seed coat challenged by *Aspergillus flavus* using real-time RT-PCR, further cloned and identified the cDNA and amino acid sequence, and finally, a recombinant prokaryotic expression vector of the gene was successfully constructed and expressed stably in *E.coli*. Although *PnLOX2* gene had been proven to be involved in peanut fungal resistance, further functional evaluation as bacteriostatic identification in vitro and subcellular localization will be needed in more genotypes to confirm the function. Patterns of the *PnLOX*2 expression after challenged by *Aspergillus flavus* provide an interesting insight into the regulation on the fungal infection. The accumulation of the *PnLOX*2 mRNA and protein after inoculation indicate that expression of this gene is linked with the *Aspergillus flavus* infection and plays an important role in the *Aspergillus flavus*-resistance of peanut.

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Table 1. Oligonucleotide primers used in the study

Primer	sequence (5′-3′)*
RTF	5′-GTC CTG GAC GTT GAC ACC TT-3′
RTR	5′-GTT GCC ATT CTC ATC GGA TT-3′
Actin-F	5′- GTC CAT CAG GCA ACT CGT AGC -3′
Actin-R	5′- GCC CTC GAC TAT GAG CAA GAG -3′
LoxF	5′-ATG TTT TCA GGG GTA ACC GGA AT-3′
LoxR	5′-TTA GAT AGA GAT GCT GTT TGG AAC TC-3′
LoxF-*Sma*I	5′-ATA CCC GGG (*Sma*I)ATG TTT TCA GGG GTA ACC GGA-3′
LoxR-*Xho*I	5′-CGC TCG AG(*Xho*I)T TAG ATA GAG ATG CTG TTT GGA-3′

* Restriction sites are underlined.

Supplemental materials

Nucleotide and deduced amino acid sequence of the cDNA clone *PnLOX2* from J11 seed coat. Nucleotides and amino acids are numbered on the left, beginning with the translation start codon, ATG. Single-letter amino acid designations are used. The initiation and stop codons are shown in boldface. The predicted protein sequence (863 amino acids) was the methionine (M) and stop (Z) codons are shown in boldface.

1 **atg**ttttcaggggtaaccggaatgctcaaccgtggccacaagatcaaagggactgtggtcttgatgcgcaagaatgtcctggacgttgacacctttactgatgttgttgccaccgccaac

1 **M** F S G V T G M L N R G H K I K G T V V L M R K N V L D V D T F T D V V A T A N

121 atcggaggcctcattggcaccggcatcaacgtcatt ggctccaccgttgacgccctcaccgccttcttaggccgcagtgtctccctccagctcatcagttctactcaatccgatgagaat

41 I G G L I G T G I N V I G S T V D A L T A F L G R S V S L Q L I S S T Q S D E N

241 ggcaacggaaaagttgtcaaggatacatttctggaaggtattattgcgtcgttaccaaccttaggagctggagaatctgcattcagcattcattttgaatgggacgatagcatgggaatc

81 G N G K V V K D T F L E G I I A S L P T L G A G E S A F S I H F E W D D S M G I

361 cctggtgcattttacatcaagaactatatgcaagttgagtttttcctcaagaccttaactcttgaagatgttccaaaccaaggaaccatccatttgtttgcaactcttgggtttacaac

121 P G A F Y I K N Y M Q V E F F L K T L T L E D V P N Q G T I H F V C N S W V Y N

481 tctaaactctacaaatccccacgcattttcttctccaacaagccatatcttccaagtgaaacaccagctccacttgttaagtacagagaagaagacctgaagaatttaagaggtgatgga

161 S K L Y K S P R I F F S N K P Y L P S E T P A P L V K Y R E E D L K N L R G D G

601 aaaggggagcgtcaggaacacgaaagaatttatgattatgatgtctacaatgatttggggaatccggatcggaacgaaaaccatgctcgccccatccttggaggttctaccactttccct

201 K G E R Q E H E R I Y D Y D V Y N D L G N P D R N E N H A R P I L G G S T T F P

721 taccctcgcaggggaagaactggtagatatcctgcaagaaatgatcctaacagtgagaaaccaggggatgtttatgttcctagagatgaaaactttggacacttgaaatcttcggacttt

241 Y P R R G R T G R Y P A R N D P N S E K P G D V Y V P R D E N F G H L K S S D F

841 cttgcaaattcaataaagtttttgactcggtatgtgctgccagcttttgaatctgtgttcgatttgaatttgaccccaaatgagtttgatagcttccaagatgttcgtgatctctatgaa

281 L A N S I K F L T R Y V L P A F E S V F D L N L T P N E F D S F Q D V R D L Y E

961 ggcggaattaggctacctacggaagtaattagcacaattagccccttacctgtcatcaaagaactcttccgtaccgatggcgaacaagtcctcaagtttccaccacctcacatcattcaa

321 G G I R L P T E V I S T I S P L P V I K E L F R T D G E Q V L K F P P P H I I Q

1081 gtgaataaatctgcatggatgactgatgaagaattcgcaagagaaatgattgctggtgtaaatccttgcatgattcgtagtcttcaagagtttcctcccaaaagcactttggatcccaca

361 V N K S A W M T D E E F A R E M I A G V N P C M I R S L Q E F P P K S T L D P T

1201 atctatggtgatcaaaacagtaagataactgcagaagttcttgatcttgaagggtgctca ctagaagaggcaattaatggtcggagactgtttatattagattaccatgatgtgttcatg

401 I Y G D Q N S K I T A E V L D L E G C S L E E A I N G R R L F I L D Y H D V F M

1321 ccatttgtgaggcgaataaatgagacccatgcaaaagcatatgccactaggactatcctttttctgagagaggatggaacattgaagccagtggccattgaattaagcttgccacatcct

441 P F V R R I N E T H A K A Y A T R T I L F L R E D G T L K P V A I E L S L P H P

1441 gatggagataaatcaggtgctatcagtgaagttatcttacctgcaaaggaaggtgttgaaagcacaatttggctactagccaaagcttatgtcatagtaaatgactcatgctaccatcaa

481 D G D K S G A I S E V I L P A K E G V E S T I W L L A K A Y V I V N D S C Y H Q

1561 ctcatgagccattggttgaatactcatgcagttattgagccatttgtgatagcaacaaatagacagctaagtgtgattcacccaatttataaacttttatctccacactaccgtgacact

521 L M S H W L N T H A V I E P F V I A T N R Q L S V I H P I Y K L L S P H Y R D T

1681 atgaacatcaatgcacttgctaggcagaatctgattaattctgatggcataatagaaagaactttcttgccctccaagttttctctggagatgtcttcagctgtttataagaactgggtt

561 M N I N A L A R Q N L I N S D G I I E R T F L P S K F S L E M S S A V Y K N W V

1801 ttcactgatcaagcactacctgctgatctcatcaagagaggaatggcagtggaggattcatcttctccttatggaattcgtcttgtaatagaagactacccttatgctgttgatggacta

601 F T D Q A L P A D L I K R G M A V E D S S S P Y G I R L V I E D Y P Y A V D G L

1921 gagatatggtttgc cattaagacatgggtccaagattatgtctcattgtactatccaacagacaatgatctcagaaaaggccctgaactccaaaattggtggaaagaagctgttgaggta

641 E I W F A I K T W V Q D Y V S L Y Y P T D N D L R K G P E L Q N W W K E A V E V

2041 ggtcatggtgatttgaaagataagccatggtggccaaagatgcagacagttgaagagttagttgaatcatgcacaaccataatatggacggcgtcggcgctccatgcagccgttaatttt

681 G H G D L K D K P W W P K M Q T V E E L V E S C T T I I W T A S A L H A A V N F

2161 ggacagtatccatatggagggcttatactgaaccgtccaacacttagcagaagattgcttcctgaacaaggcactgcagagtatgaagagatggtgaagagtcaccaaaaggcttatctg

721 G Q Y P Y G G L I L N R P T L S R R L L P E Q G T A E Y E E M V K S H Q K A Y L

2281 agaacaattacaccgaaattggagactcttattgaccttacaaccatagaaatcttatcaaagcatgcttctgatgaggtgtatcttggagagagggataatccacattggacatttgat

761 R T I T P K L E T L I D L T T I E I L S K H A S D E V Y L G E R D N P H W T F D

2401 tcaagagcattacaagcatttcagagatttgggaacaaactgagtgagattgaggagaagctaacagagaagaacaaagatgggagactgagtaatagaattgggccagttgaattgcca

801 S R A L Q A F Q R F G N K L S E I E E K L T E K N K D G R L S N R I G P V E L P

2521 tacactctgcttcatcctactagcaatgaagggttgacttttagaggagttccaaacagcatctctatc**taa**

841 Y T L L H P T S N E G L T F R G V P N S I S I **Z**