Development of groundnut (Arachis hypogaea L.)
.transgenics for resistance to Aspergillus flavus

Sowmini Sunkara¹,², Pooja Bhatnagar-Mathur¹, Kiran K. Sharma¹

¹Genetic Transformation Laboratory, International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh 502 324.

²Jawaharlal Nehru Technological University Hyderabad, Kukatpally, Hyderabad, Andhra Pradesh 500085
Groundnut- Importance

- Groundnut- an economically important oil and protein rich crop
- seed propagating and self-pollinating legume crop.
- good sources of high quality edible oil (~50%),
- easily digestible protein (~25%) and carbohydrates (~20%),
- good animal feed and fodder
- Shells are used for fuel, soil conditioners, fodder, chemicals, resin extenders, cork substitute and for hard board.
Area & Production (FAOSTAT 2012)

- Peanuts rank 3rd in production among oilseeds
- Developing countries contribute 97% of the world’s groundnut area and 94% of total production largely grown by the smallholder farmers in SAT tropical and sub tropical areas of the world (ERS 2001).
- India - largest peanut growing area - 4.90 million ha (20.46 %)
- 2nd in the production at 5.78 million tons (15 %)
- Average yield -11794 Hg/Ha.
Aspergillus flavus

Groundnut- one of the most susceptible crops- *A. flavus* invasion and aflatoxin contamination.

These fungi - weak facultative pathogens and can exist as saprophytes-capable of surviving on many organic nutrient sources.

ubiquitous- reduces yield of agricultural crops and decreases the quality of the harvested grains- tremendous loss
Aflatoxins are toxic polyketide-derived secondary metabolites - *A. flavus* and *A. parasiticus*.

These toxins are associated with both acute and chronic toxicity in animals and humans.

- Carcinogenic – liver cirrhosis and liver damage
- Teratogenic – Tumour causing agents
- Immuno-suppressive substances

Stringent limits on the levels of aflatoxins permissible in groundnut and groundnut-products

- USDA - 20µg kg\(^{-1}\)
- European Union - 2 to 4 µg kg\(^{-1}\)
- Japan- None
Hammond-Kosack & Jonathan, 1996
Plant responses on attack with pathogens

• Host Resistance gene- pathogen avirulence gene of (R-Avr gene approach)

• Hypersensitive response where host cells die within few hours of pathogen attack-thus limiting availability of nutrients for further pathogen colonization

3 reasons for pathogen failure to cause a disease

1) Plant is either unable to support the nutritional requirements for the pathogen to grow

2) Plant possess structural barriers or toxic compounds which prevent infections from pathogens

3) Upon pathogen attack, defense mechanisms get activated which will prevent infection
Pathogenesis-related (PR) genes

- Plants express a wide variety of genes referred to as pathogenesis-related (PR) genes in response to pathogen/pest infection.

Integrated scheme for plant molecular breeding using biotechnology

Dita et al, 2006
Approaches

- Use of resistant varieties against fungi or resistant varieties for the toxin after fungal colonization

- Traditional breeding methods: Not sufficient to meet the increasing demands - limited germplasm variability, time consuming and labor intensive

- Antifungal genes such as rice chitinase, defensin, 13S and 9S LOX (lipoxygenase) via genetic engineering techniques to combat fungal diseases.

- Biotechnological approach with traditional techniques proves promising

- AFLATOXIN free GROUNDNUTs
Depending on the source of the genes used, there are two approaches for development of genetically engineered fungal resistance in plants.

1) pathogen-derived resistance (PDR) - a part or a complete fungal gene is introduced into the plant, which subsequently, interferes with one or more essential steps in the life cycle of the fungus thereby inhibiting the production of aflatoxin.

2) Non-pathogen-derived resistance, on the other hand, is based on utilizing host resistance genes and other genes responsible for adaptive host processes elicited in response to pathogen attack, to obtain transgenics resistance to fungus.
Lipoxygenase (LOX)

- Plant lipoxygenases (LOXs) are hypothesized to play an important role in mediating host-pathogen interactions by initiating the octadecanoic branch in response to fungal attack.

- LOX catalyzes the oxidation of polyunsaturated fatty acids such as linoleic acid (18:2) and α-linolenic acid (18:3) to produce unsaturated fatty acid hydroperoxides.

- Lipoxygenase genes - increase the resistance to *Aspergillus* through jasmonic acid (JA) from conversion of linolenic acid to 13-hydroperoxy octadecatrienoic acid (HPOTE).

- Jasmonic acid (JA), a derivative of α-linolenic acid has been reported as a potent inhibitor of aflatoxin biosynthesis.

Overview of the lipoxygenase pathway. (Loiseau et al, 2001).
- *PnLOX2* and *PnLOX3* are both 13S-HPODE producers (13-LOX) and are specifically expressed in seed.

- 13S-HPODE provide in vitro evidence that specific seed lipoxygenase activity could provide resistance to mycotoxin contamination by *Aspergillus* spp-directly or indirectly repress AF biosynthesis.

- Hence 13S-HPODE molecules act as putative resistance factors.

*Invitro* observation - exogenous 9S-HPODE extended the time of aflatoxin gene transcription whereas exogenous 13S-HPODE and 13S-HPOTE inhibited aflatoxin gene transcription
Objectives

Sub-cloning of Lipoxygenase gene (*PnLOX3*) from pTMK 12.6 (Tsitsigiannis et al., 2005) under 35S promoter into marker free binary vector pPZP200.

Genetic transformation of groundnut genotypes by using binary vector containing lipoxygenase gene through *Agrobacterium*-mediated transformation.

Identification of transgenic plants showing high expression through molecular characterization by PCR, RT-PCR, Southern blotting.

Confirmation of resistance through fungal bioassays and ELISA techniques.
Sub-cloning strategy

Step 1: pTMK12.6 restricted with *Kpn*I- *Xho*I and subcloned into *Kpn*I-*Xho*I site of pTOPO>TSV CP

Step 2: *Pst*I fragment of pRT 103 subcloned into *Pst*I site of pGEM-T easy
Step 3: pTOPO-\textit{PnLOX3} restricted with \textit{KpnI}-\textit{XbaI} and subcloned into \textit{KpnI}-\textit{XbaI} site of pGEMT-35S:polyA

Step 4: pGEMT-35S:\textit{PnLOX3}:polyA restricted with \textit{SpeI}-\textit{SalI} and subcloned into pPZP200-d35S:\textit{PBNV-ASNP}:nosT
Restriction digestion analysis of pPZP200>35S:PnLOX3:polyA binary construct

Groundnut Transformation protocol

- **Genotype used:** JL24
- **Agrobacterium strain:** C58 strain - marker free binary vector pPZP200\(>\)35S::PnLOX3::polyA.
- **Explant:** De-embryonated, split cotyledonary explants. (Sharma & Anjaiah, 2000)

A. Mature JL24 seeds; B-D. Decoated, de-embryonated and split cotyledonary explants; for Agrobacterium-mediated transformation kept on shoot induction medium (SIM) containing MS supplemented with 20 µM BA and 10 µM 2,4-D; E-F. Explants turned green and enlarged kept for Induction of adventitious multiple shoot buds on SIM with 250 µg ml\(^{-1}\) cefotaxime; G-I. Multiple shoots elongated on shoot elongation medium, MS supplemented with 2 µM BA; J. Rooting of the elongated shoots on root induction medium, MS supplemented with 5 µM NAA; K-L. Initial transfer into jiffy pots containing sterile sand for the establishment of the roots covered in perforated poly bag and incubated in growth chamber for 4 days; M-N. Fully established healthy plants transferred into bigger pots containing sand: soil mixture and maintained in the P2 greenhouse.
Molecular characterization of $T_0$ and $T_1$ generation transgenics carrying pPZP200>35S:PnLOX3:polyA

PCR amplification of $PnLOX3$ in $T_0$ transgenic peanut plants with junction primers.

PCR analysis of $PnLOX3$ in $T_1$ transgenic plants
Molecular characterization of T₁ and T₂ generation transgenics carrying pPZP200>35S:PnLOX3:polyA

RT-PCR of T₁ transgenic peanut plants

PCR analysis of T₂ transgenic events using junction primers assuring the stable integration of transgene.
Molecular characterization of \( T_3 \) and \( T_4 \) generation transgenics carrying pPZP200>35S:*PnLOX3*:polyA binary construct

A: PCR analysis of \( T_3 \) groundnut transgenics with junction primers amplifying 714 bp amplicon; Lane B- Blank; Lane C- untransformed groundnut control; Lane 1-16- \( T_3 \) groundnut transgenics; M-100 bp ladder; Lane +- plasmid.

B. RT-PCR analysis of \( T_3 \) groundnut transgenics with internal primers amplifying 1356 bp *PnLOX3* gene; Lane 1-Blank; Lane 2-12- \( T_3 \) groundnut transgenics; Lane 13-untransformed groundnut control; Lane 14-100bp ladder; Lane 15- plasmid.

PCR analysis of \( T_4 \) groundnut transgenics with junction primers amplifying 714 bp amplicon; Lane B- Blank; Lane C- untransformed groundnut control; ; Lane 1-24- \( T_4 \) groundnut transgenics; M-100bp ladder; Lane +- plasmid.
Southern blotting of T3 transgenics carrying pPZP200>35S:PnLOX3:polyA binary construct

Southern blotting analysis of genomic DNA restriction of T3 groundnut transgenics carrying binary construct pPZP200>35S:PnLOX3:polyA with HindIII enzyme. A: Lane 1, 2: Blank; Lane 3: Sample 2-1-5-5; Lane 4: Sample 3-1-1-6; Lane 5: Sample 5-2-2-6; Lane 6: Sample 6-4-1-25; Lane 7: JL24 control DNA; Lane 8: Plasmid pPZP200>35S: PnLOX3: polyA.
Southern blotting of T₃ transgenics carrying pPZP200>35S:PnLOX3:polyA binary construct

Southern blotting analysis of genomic DNA restriction of T₃ groundnut transgenics carrying binary construct pPZP200>35S:PnLOX3:polyA with HindIII enzyme. A: Lane 1: Sample 2-1-1-10; Lane 2: Sample 2-1-5-5; Lane 3: Sample 3-1-1-6; Lane 4: Sample 5-2-1-34; Lane 5: Sample 5-2-2-6; Lane 6: Sample 6-4-1-25; Lane 7: Blank; Lane 8 JL24 control DNA; Lane 9: Plasmid pPZP200>35S: PnLOX3: polyA. B. Lane 1: Sample 2-2-1-1; Lane 2: Sample 3-1-1-6; Lane 3: Sample 5-2-2-6; Lane 4: Control JL24; Lane 5: Plasmid pPZP200>35S: PnLOX3: polyA.
Inheritance pattern of *peanut lipoxygenase* gene (*PnLOX3*) in $T_0$ generation transgenics carrying pPZP200>35S:*PnLOX3*:polyA

<table>
<thead>
<tr>
<th>Event #</th>
<th>No of plants tested</th>
<th>PCR positives</th>
<th>Negatives</th>
<th>Chi square ($\chi^2$)</th>
<th>Mendelian ration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0.666667</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>3.266667</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>5.555556</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td>0.222222</td>
<td>Yes</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>Yes</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0.666667</td>
<td>Yes</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0.666667</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* $\chi^2$ value at 0.05% probability at 1 df is 3.84. Calculated values above 3.84 were non-significant, and the samples did not fit for 3:1 segregation ratio.*
Inheritance pattern of *peanut lipoxygenase* (*PnLOX3*) gene in T1 & T2 generation transgenics carrying pPZP200>35S:*PnLOX3*:polyA

<table>
<thead>
<tr>
<th>Event #</th>
<th>No of plants tested</th>
<th>PCR positives</th>
<th>Negatives</th>
<th>Chi square (χ²)</th>
<th>Mendelian ration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>14</td>
<td>10</td>
<td>4</td>
<td>0.095238</td>
<td>Yes</td>
</tr>
<tr>
<td>1-2</td>
<td>14</td>
<td>8</td>
<td>6</td>
<td>2.380952</td>
<td>Yes</td>
</tr>
<tr>
<td>2-1</td>
<td>14</td>
<td>9</td>
<td>5</td>
<td>0.857143</td>
<td>Yes</td>
</tr>
<tr>
<td>2-2</td>
<td>14</td>
<td>11</td>
<td>3</td>
<td>0.095238</td>
<td>Yes</td>
</tr>
<tr>
<td>2-4</td>
<td>7</td>
<td>5</td>
<td>2</td>
<td>0.047619</td>
<td>Yes</td>
</tr>
<tr>
<td>4-1</td>
<td>14</td>
<td>8</td>
<td>6</td>
<td>2.380952</td>
<td>Yes</td>
</tr>
<tr>
<td>1-1-1</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>Yes</td>
</tr>
<tr>
<td>1-1-2</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>0.222222</td>
<td>Yes</td>
</tr>
<tr>
<td>1-1-3</td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>5.555556</td>
<td>No</td>
</tr>
<tr>
<td>1-1-4</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>0.222222</td>
<td>Yes</td>
</tr>
<tr>
<td>1-1-5</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td>0.222222</td>
<td>Yes</td>
</tr>
<tr>
<td>2-2-1</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0.111111</td>
<td>Yes</td>
</tr>
<tr>
<td>3-1-1</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td>0.222222</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Fungal Bioassay

**Inoculum preparation**

**A:** Inoculum preparation

**B:** Inoculum

**Inoculum**
- Sorghum seeds infected with *A. flavus* (AF II-4)

**Inoculation**
- Before sowing
- 3rd week after sowing
- 5th week after sowing
- 7th week after sowing
- 9th week after sowing

**Drought imposition**
- At 75 days of sowing only in pod and peg zone
- Root zone watered until harvest

**Preharvest screening**
- *In vitro* seed colonization for *A. flavus* infection
- ELISA for aflatoxin estimation
Post harvest invivo seed colonization- 30% seed moisture for 7 days at 28°C

- A. flavus population studies in soil- AFPA sowing- 0 CFU/gm, flowering- 8000 CFU/gm, harvest -20-25k CFU/gm

- Indirect competitive ELISA analysis

- >90% of PCR positive transgenics screened under GH conditions contained AFB₁ between 0.01-20 µg/kg

A-B: Post harvest seed colonization in control & transgenics; C: A. flavus population studies; D: ELISA Analysis
<table>
<thead>
<tr>
<th>Event</th>
<th>% Visual infection</th>
<th>Aflatoxin content (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-4-1</td>
<td>27.6</td>
<td>17.9*</td>
</tr>
<tr>
<td>5-2-1</td>
<td>46.9</td>
<td>70.1</td>
</tr>
<tr>
<td>J11</td>
<td>9</td>
<td>58.7</td>
</tr>
<tr>
<td>JL24</td>
<td>15</td>
<td>78.6</td>
</tr>
<tr>
<td>GM</td>
<td>12</td>
<td>58.8</td>
</tr>
<tr>
<td>SE</td>
<td>7.55</td>
<td>6.23</td>
</tr>
<tr>
<td>LSD</td>
<td>26.11</td>
<td>21.56</td>
</tr>
<tr>
<td>CV%</td>
<td>48.3</td>
<td>15</td>
</tr>
<tr>
<td>F- value</td>
<td>0.034</td>
<td>0.001</td>
</tr>
<tr>
<td>Significance</td>
<td>Not Significant</td>
<td>Significant at 5%</td>
</tr>
</tbody>
</table>
Protein expression studies

SDS-PAGE analysis of bacterially (BL21<sub>DE3</sub> pLysS) expressed peanut lipoxygenase protein. Lane UI: bacterial protein of un-induced bacterial protein of clones 1, 2 & 3; Ladder: NEB Protein marker; Lane 0.1 M: bacterial protein of clones 1, 2 & 3 induced with 0.1M IPTG; Lane 1M: bacterial protein of clones 1, 2 & 3 induced with 1M IPTG.
Pepsin digestibility studies

LOX Protein + Simulated gastric fluid (SGF) containing 0.3% pepsin

Temperature - 37°C

Sampling at different time intervals ranging from 5s-2h
Legume - Seed Specific promoters

**Sequence analysis**- NCBI BLAST, Gibb’s sampling, Melina software

**Motif search**- MEME (multiple expectation maximization for motif elicitation), PlantCARE, Genomatix-MatInspector - PLACE database

**GenBank Accession ID’s**

Groundnut Seed specific Promoter (GSP)- **HM215006**

Chickpea Lectin Promoter (CPLP)- **EU60424**.
Electro Mobility Shift Assay (EMSA) for the confirmation of promoter regions from peanut and chickpea. (A) Peanut seed-specific promoter (GSP) binding assay on 0.8% agarose gel. Lane 1 contains unbound GSP fragment, Lanes 2-4 carry GSP fragment bound with peanut seed nuclear extracts in presence of EDTA and KCl, Lanes 5-7 carry GSP fragment bound with leaf, immature seed, and testa extracts, respectively, Lane 8 is blank and the Lane 9 carries the 100 bp ladder; (B) Chickpea lectin promoter (CPLP) binding assay on 6% native PAGE. Lane 1 contains the unbound CPLP fragment, Lane 2 carries CPLP fragment bound with chickpea seed nuclear extracts.
a) pPZP200 > 35S:GUS:polyA

b) pPZP200 > CPLP:GUS:polyA

c) pCAMBIA2300 > GSP:GUS:polyA
Histochemical GUS analysis in *Arabidopsis* & tobacco


Sunkara et al., 2014
Molecular characterization of $T_0$ and $T_1$ generation tobacco transgenics

Molecular characterization of tobacco transgenics amplifying the 1213 bp fragment of $uidA$ (GUS) gene. a-c: PCR analysis of tobacco transgenics transformed with the binary plasmid pPZP200>CPLP:$uidA$:polyA and pCAMBIA2300>GSP:$uidA$:polyA in $T_0$ and $T_1$ generations respectively; d-e: RT-PCR analysis of tobacco transgenics in $T_1$ generation transformed with the binary plasmid pPZP200>CPLP:$uidA$:polyA and pCAMBIA2300>GSP:$uidA$:polyA respectively; Lane B-Blank; Lane C-untransformed tobacco control; +-positive control plasmid.
Summary

- Binary vector construction by sub-cloning peanut lipoxygenase gene (*PnLOX3*) under constitutive and tissue (seed) specific promoters.

- Developed 25 marker free groundnut transgenics carrying peanut lipoxygenase gene.

- Molecular confirmation of presence and integration of transgene over generations (T₀-T₄) by PCR, RT-PCR & Southern blotting.

- Novel protocol (mimicking micro-sick plots) of fungal bioassay used for green house evaluation of transgenics to understand *A. flavus*-drought interactions.

- Isolated and validated two novel seed specific promoters from groundnut and chickpea.
Future prospects

Production of transgenics with the marker free binary constructs containing lipoxygenase gene under the control of chickpea lectin promoter and groundnut seed specific promoter.

Molecular characterization of putative groundnut transgenics produced with the two constructs-by PCR, RT-PCR, Southern Blotting, ELISA analysis and fungal bioassays.

Purification of lipoxygenase protein from pET induced bacterial culture and standardization of lipoxygenase enzyme assay.

Real Time PCR analysis of these transgenics for copy number estimation and quantitative expression studies.
Acknowledgements

Dr K. K. Sharma (Supervisor), Director-PTTC, CEO- AIP, ICRISAT

Dr Farid Waliyar (Co-Supervisor), Director (WCA), ICRISAT

Dr Pooja Bhatnagar-Mathur, Senior Scientist, GTL, ICRISAT

Scientific, Administration & Supporting Staff

- Genetic Transformation Laboratory (GTL), ICRISAT
- Mycotoxin Diagnostics and Virology Laboratory (MDVL), ICRISAT

University Grants Commission, New Delhi - JRF & SRF.

My Family…