ROOTS TRANSFORMATION OF *Glycine max* WITH RESPONSIVE PROMOTER FOR NEMATODES INFECTION

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Agricultural crops worldwide lost Billions of dollars by destruction caused every year because by Nematodes infection. The crop rotation does not remove nematodes, but merely declines crop damage (Klink *et al.*, 2009). However, it is important to find and characterize resistance genes in plants against nematodes feeding site (Kandoth *et al.*, 2011), it is more important to identify promoters that continue to express those genes well in and around syncytial cells. In this regards, Narayanan *et al.* (1999) demonstrated that expression of *GUS* by CaMV 35S promoter in soybean transgenic hairy roots was strong in root tips and in younger segments, while older root sections were *GUS* negative. Furthermore, when transgenic roots were challenged with cyst nematode (SCN), there was no *GUS* staining at SCN infection sites at nine days post inoculation.

Moreover, transgenic potato plants produced green fluorescent protein (GFP) at the developing feeding site of the potato cyst nematode (*G. rostochiensis*) less than four days after infection using the CaMV 35S promoter. In *Arabidopsis*, the CaMV 35S promoter was active in roots, when inoculated with *Heterodera schachtii*. However the cells around the feeding sites retained constant GFP fluorescence after infection, GFP fluorescence declined at the feeding site over time (Urwin *et al.*, 1997). There was some GFP present in the syncytium at least until 27 days after inoculation of the roots with *H. schachtii*. In contrast, GFP fluorescence was present in the gall formed around the feeding site of *Meloidogyne incognita*. Hence, it appears that the CaMV 35S promoter varies in its activity according to the plant, the nematode, and the time of assay. Thus, this concluded that CaMV 35S promoter is not suitable for SCN control

The activation of peroxidase genes upon nematode infection stress was reported (Reardon, 2010). The microarray data collected from infected plant tissue, syncytia, after 3, 6 and 9 days of continuous infection with cyst nematode *H. glycines* using Laser capture microdissection (LCM) as described in (Klink *et al.*, 2009) showed the activation of Peroxidase class III gene in all studied time points. Peroxidases are haem-containing

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enzymes which consume hydrogen peroxide as the electron acceptor to catalyze a number of oxidative reactions (Paes de Sousa *et al.*, 2011). Peroxidases detoxify hydrogen peroxide at the expense of thiol (Hudson *et al.*, 2011). It is thought that catalase-peroxidase provides protection to cells under oxidative stress (Welinder, 1991). The prompt and continuous activation of Peroxidase class III gene nominated its promoter to test where it suitable for expressing resistant genes at the site of nematode feeding that may inhibit the life cycle cyst nematode *H. glycines*.

The Experiments of this study was designed to determine the temporal and spatial gene expression of the Peroxidase class III gene promoters in roots of soybean to confirm that they express well in roots and remain expressing at the sites of invasion and feeding by cycle cyst nematode (SCN). The peroxidase class III gene isolated promoters aimed to provide scientists with a promoter useful for expressing genes and gene products at the site of nematode feeding that may inhibit the life of SCN (*H. glycines*).

MATERIALS AND METHODS

Preparing samples for microarray data (MA)

RNA Samples were collected from infected plant tissue, syncytia, after 3, 6 and 9 days of infection with cyst nematode (SCN) (*H. glycines*) using Laser Capture Microdissection (LCM) as described in (Klink *et al.*, 2009). LCM is a procedure that can specifically isolate syncytia from *G. max* roots so that mRNA can be used for quantitative gene expression experiments (Klink *et al.*, 2005). In the experiments presented here, LCM is used to specifically isolate syncytia for transcriptomic analyses. Syncytia are easy to identify by their darkened cytoplasm as compared to adjacent, unaffected cells. The presence of the nematode is used as an *in situ* physical marker for the syncytium. These samples are used for RNA isolation and subsequent microarray to identify ESTs that are induced during those three time points.

Bioinformatics analysis of microarray data (MA)

ESTs from Microarray analyses and *G. max* probe set annotations were used as described in (Klink *et al.*, 2009). Briefly, the Gene Chip_Soybean Genome Array (Affymetrix, Cat. # 900526) containing 37, 744 *G. max* transcripts (35, 611 transcripts) were used for the MA. Details of the Gene Chip_Soybean genome array can be obtained (http://affymetrix.com/index. affx). All microarray hybridizations were performed at the Laboratory of Molecular Technology, SAIC-Frederick, National Cancer Institute at Frederick, Frederick, MD 21701, USA. The soybean GeneChip_ data was imported and analyzed using the MATLAB Bioinformatics Toolbox (Mathworks Inc.; Natick, MA, USA) and Array Assist (Stratagene) to do RNA normalization on the probe sets before taking the log 2 of expression values. Tak-

ing the log base 2 was done for scaling and compressing the data sets, as usually is done with microarray data, and not for normalizing the data sets. Volcano plots were produced using samples having a fold change of C|±1.5| and also having a P value B 0.05 as compared to the control (Alkharouf *et al.*, 2006). The t-test was used to calculate P values. The differential expression analysis outcome was tested by false discovery rate (FDR) set to 10% unless otherwise stated. Significance analysis of microarrays (SAM) (Tusher *et al.*, 2001) 3.0 was used to perform FDR tests.

Alignment of expressed sequence tags (ESTs) of soybean after infection with cyst nematode (SCN) (*Heterodera glycines*) produced separate contiguous sequences (contigs). The *Glycine Max* roots with Peroxidase class III gene responsive promoter was selected based on its criteria to be expressed after 3, 6 and 9 days after nematode (SCN) (*H. glycines*) infection (Table 1).

Mathematical modeling of gene expression

Mathematica7 (Wolfram) program, the expression data were fitted the data by using this command: $\mathrm{Fitquadratic} = \mathrm{Fit}[\mathrm{data}, \{1, t, t^2\}, t]$; The resulting equation is: 2.29747 +246.914 t-24.7839 t2.

Then, from above equation FC from t= 0 to 30 days by using Mathematica program by this command: $[\{\{"t", "FC"\}, \{t, 2.2974749999997734 + 246.91415833333343t - 24.78393055555556t^2\}\}, \mathrm{Dividers} \to \mathrm{All}, \mathrm{ItemSize} \to 8], \{\{t, 0\}, 0, 30, 1\}]$. The results were tabulated and plotted as shown in (Fig. 2).

K-means clustering

The K-means clustering unsupervised learning algorithm was used for gene clustering of the three time point courses in order to find patterns in the data sets. The procedure was performed according to Klink *et al.* (2009). Twenty clusters (k) were chosen for grouping the 37, 744 *G. max* probe-set dataset. Those 20 clusters were used to identify centroids, defined as an average point specific to a cluster of points. With centroids moved into a position such that an optimum separation of objects into groups occurred, clustering was run for 1,000 repetitions using squared-Euclidian as the algorithm to measure pairwise distance between data points. Having 20 clusters fit the dataset so that all the values were affiliated with a specific cluster. Using more or fewer clusters resulted in either over-fitting or under-fitting the dataset to a respective centroid. The K-means clustering were executed in MATLAB, using both the Bioinformatics and Statistics toolboxes. All replicates for each sample type were averaged prior to clustering.

In Silico isolation of the promoter region

The selected peroxidase class III gene EST was used to found corresponding gene sequence from the gene bank in National center of biotechnology information (NCBI) using basic alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/sites/gquery) based on their

best match obtained by BLAST searches (Altschul *et al.*, 1997). The corresponding gene was used to find the 5` upstream genomic UTR region of this gene, supposedly harboring different promoter regulatory elements, from soybean genome at http://www.phytozome.net/soybean. The extracted upstream fragment was tested for plant Cis-acting regulatory DNA elements using PLACE database http://www.dna.affrc.go.jp/PLACE/signalscan.html (Higo *et al.*, 1999; Prestridge, 1991).

Forward and reverse primers were designed to isolates -1440 bp upstream of the ATG transcription start site using Primer3 (v. 0.4.0), http://frodo.wi.mit.edu/primer3/(Rozen and Skaletsky 2000) (Table 2). The primer quality and compatibility was tested *In Silico* using FastPCR software (FastPCR© 1999-2010, Institute of biotechnology, University of Helsinki, Finland). Finally, Insilco PCR was conducted to examine primer efficiency and the expected amplicon length using *in silico* PCR (http://insilico.ehu.es/user_seqs/ PCR/).

Genomic DNA extraction

Mortar and pistol were used to grind 0.1 g fresh 10 day old leaves from Soy bean (William82 cv.) in liquid nitrogen until it became fine powder. Genomic DNA was extracted from samples using the Qiagen DNeasy Plant Mini Kit following the manufacturer's instructions. Extraction was followed by treatment with RNase-A (Sigma, St. Louis, MO; R-4875) for 30 min at 37°C according to (Mansour *et al.*, 2008). The quality and quantity of extracted DNA was measured by a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Delaware, and USA). DNA samples were adjusted to 50 ng/µl with ddH_2O before PCR amplification.

High fidelity PCR amplification

Specific Polymerase chain reaction (PCR) was used to isolate upstream genomic regions from Peroxidase class III gene promoter region that were then sequenced to confirm harboring desired genomic region. For the accurate amplification of the gene promoter region, Platinum® Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) was used with specific primers to isolate the 5 upstream region of the gene following manufacturer instruction. Amplification reactions were performed according to Williams *et al.* (1990) in 50 µl volumes. Briefly, each reaction mixture containing 1 X of 10 X High Fidelity PCR Buffer (600 mM Tris-SO_4 (pH 8.9), 180 mM Ammonium Sulfate), 0.2 mM of 10 µM dNTPs mix (each dATP, dCTP, dGTP and TTP) (Pharmacia), 2 mM from (50 mM $MgSO_4$), 0.2-0.4 µM of each primer, 50 ng of genomic DNA, and 1.0 unit of Platinum® Taq DNA Polymerase High Fidelity and autoclaved ddH_2O up till 50 ul. The PCR program was used as suggested by manufacturer as follows; Initial denaturation: 94°C for 30 seconds to 2 minutes followed by 25-35 cycles of: Denature: 94°C for 15-30 se-

conds, Anneal: 55°C for 15-30 seconds, Extend: 68°C for 1 minute per kb of PCR product). Finally, final extension for 10 minutes step at 68°C.

Gel electrophoresis

Ten µl of PCR products were separated on agarose (1.2%) gel electrophoresis, stained and loaded in 2 µl EZ-VISION™ ONE (Amresco, USA), at 100 Volts in 1 X SB (10 mM NaOH solution with boric acid pH is 8.5) and photographed on a UV transilluminator (Pharmacia) by a Canon S5 digital camera with a UV filter adaptor. A negative control which contained all the necessary PCR components except template DNA was included in PCR runs.

Purification of PCR products

DNA fragments containing Peroxidase class III gene promoter region were purified from PCR products using MinElute PCR Purification Kit (Qiagen, Santa Clarita, CA) following the manufacturer's instructions. Mixed lengths fragments were separated by E.gel DNA Purification system Using E-Gel® CloneWell™ Agarose Gels (Invitrogen, Carlsbad, CA)

Cloning into E. coli pENTR vector

Purified peroxidase class III gene promoter region DNA fragments with expected size, 1440 pb, were inserted into pENTR vector (pENTR™/TEV/D-TOPO® Cloning Kit., Invitrogen.) for cloning in Escherichia coli (One Shot® Mach1™-T1R Chemically Competent) (Invitrogen, Carlsbad, CA). Transformed cells were grown overnight on LB plates median supplemented with 50 ug/ul Kanamycin.

PCR amplification for confirming positive clones

Individual colonies were selected and grown overnight in 5 ml of LB media supplemented with tetracycline (10 mg L^{-1}) for promoter fragment: *pJan25T*. Cells from overnight cultures were collected by centrifugation and the plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen, Santa Clarita, CA). Isolated plasmids from each culture were used as template in a 20 µl PCR reaction to confirm the presence and orientation of the promoter using Recombinant Taq Polymerase following manufacturer instructions (Invitrogen, Carlsbad, CA). PCR was started with an initial denaturation at 94°C for 5 min followed by 25 cycles of denaturation at 94°C for 35 s, annealing at the primer T_m (Table 1) for 35 s, and extension at 72°C for 2 min per kb of PCR product. Final extension of the amplification was for 10 min at 72°C. All PCR reactions were performed using a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad, USA).

Preparing samples for sequencing

Plasmids (*pENTR*) harboring the Purified Peroxidase class III gene promoter region were isolated from overnight culture using the QIAprep miniprep system (Qiagen, Santa Clarita, CA) and prepared for sequencing. The DNA inserts

were sequenced using the ABI Big Dye Terminator Cycle Sequencing Kit and run on the ABI Prism 3100 Genetic Analyzer (Perkin-Elmer, Applied Biosystems, and Foster City, CA). Both clones were completely sequenced in both directions and found to be identical except for few nucleotides.

Insilco sequence analysis

Nucleotide sequences of the isolated the Peroxidase class III gene promoter fragment was analyzed Insilco and compared with the original expected sequence using the DNASTAR analysis software (DNASTAR, Inc., Madison, WI). The overlapping identical sequences were assembled into tentative consensus sequences referred to here as conting which was queried manually through Gen-Bank using blastn, blastx, tblastn, tblastx, blastp, rpsblast (Altschul *et al.*, 1997) and against Soy bean genomic data base at http://www. phytozome.net/soybean. Unigenes affiliated with regulatory region were queried manually through the Gene Ontology Database (The Gene Ontology Consortium, 2004). Sequence alignments were conducted using the ClustalOmega program within MEGA v.6.0 (Tamura *et al.*, 2013) and showed by Jalview v.2.8.1 (Waterhouse *et al.*, 2009). The phylogenetic analyses were conducted using MEGA v.6.0. Our sequences was compared with other case studies by using BioEdit Sequence Alignment Editor (North Carolina State University, USA) (Hall, 1999), MULTALIN (Combet *et al.*, 2000), CLUSTAL W (Thompson *et al.*, 1994), and FastPCR (Kalendar *et al.*, 2009) after data mining at the servers of NCBI (Altschul *et al.*, 1997) and GGB (Grape Genome Browser) http://www.genoscope.cns.fr), following ML phylogram editions (Maximum Likelihood, Hillis *et al.*, 1994) by MEGA4 program (Tamura *et al.*, 2013).

Transfer of the peroxidase class III gene promoter from the pENTR plasmid to the pJan25 series

To test the new vectors and ensure that the selection markers and Peroxidase class III gene promoter site worked properly together, the promoter fragment, 1440 bp from the transcription start site, was transferred from a *pENTR* clone to the pJan25T plasmids using the Gateway® LR Clonase™ II Enzyme Mix kit following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Plasmids harboring the promoter region were used to transform *E. coli* (One Shot® Mach1™-T1R chemically competent cells, Invitrogen, Carlsbad, CA). The transformed cells were grown overnight at 37°C on LB selective media containing 10 mg L-1 tetracycline. Plasmids were isolated from overnight cultures using the QiaPrep Spin Miniprep Kit (Qiagen, Santa Clarita, CA) and used to transform onion cells via the biolistic gene transfer method (Sanford, 1990) as a raid test for their expression ability.

Microprojectile bombardment of onion cells

Microprojectile bombardment was conducted using the Biolistic Particle Delivery System (PDS-1000/He, BioRad, Hercules, CA) according to the manufacturer's instructions. Gold microprojectiles coated with 6 µl of *pJan25T* construct (1 µg/µl) were bombarded into onion cells at 1100 PSI. The *pJan25T* harboring construct the peroxidase class III gene promoter fragment was delivered into three replicates.

Fluorescence was monitored using a Nikon SMZ 1500 stereomicroscope with an eGFP filter (Nikon Corporation; Tokyo, Japan). Stereomicroscope images were captured with an Optronics MagnaFire model S99802 CCD camera (Optronics; Goleta, CA). The functionality of the Peroxidase class III gene promoter fragment promoter was tested by driving expression of the *GUS* reporter gene. Bombarded onion tissue was incubated with *GUS* staining solution to determine β-glucuronidase activity after 24 h. *GUS* staining solution consisted of 2 mM 5-bromo-4-chloro-3-indolyl glucuronide, 100 mM potassium phosphate buffer pH 7.0, 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% Triton X-100 (Jefferson *et al*., 1987). The colorimetric reaction proceeded by immersion in *GUS* stain and subsequent vacuum infiltration with 500 µl of *GUS* stain for 10 min. Tissue was subsequently incubated at 37°C overnight to promote development of the *GUS* stain.

Heat shock transformation of chemically competent Agrobacterium rhizogenes (strain K599)

The *pJan25T* plasmids construct containing the Peroxidase class III gene promoter fragment were isolated from overnight cultures using QIAprep Spin Miniprep Kit (Qiagen, Santa Clarita, CA). About 2-4 ul of the isolated plasmids were used for transformation of *Agrobacterium rhizogenes* (strain K599) using heat shock transformation method. Briefly, after a short incubation in ice, a mixture of 100 ul of chemically competent bacteria and 2-4 ul (50-150 ug/ul) of plasmids is placed at 42°C for 45 seconds (heat shock) and then placed back in ice immediately. 250 ul of SOC media is added and the transformed cells are incubated at 37°C for 1 hour with agitation. To be assured of isolating colonies irrespective of transformation efficiency, two quantities of transformed bacteria were plated. Transformed *Agrobacterium rhizogenes* (strain K599) cells were grown for 2-3 day on LB plates containing 5 ug/ml tetracycline.

Soybean root transformation with Agrobacterium rhizogenes

Seedlings, 7 to 9 days old, were used for transformation. Generally, 120 seedlings were used for each the construct which planted in hydrated Promix potting media. 2 days old *Agrobacterium rhizogenes* cultures (strain *K599*), grown in TB (Terrific Broth) media supplemented with 5ug/ml Tetracycline at 26°C, were

re-suspended in 3% sucrose with 500 uM acetosyringone pH 5.7 media and used for transformation at OD 0.8 measured by spectrophotometer wave length 600 nm. Plants were cut at the soil line and transferred to 50 ml beaker (15 to 20 cut plants/beaker) filled with 30 ml of resusbeded *Agrobacterium rhizogenes* cells in MS solution. The Co-cultivation solution was vacuum infiltrate into the plants for 30 minutes. The seedlings were cultivated into the same solution over night for about 15 hours inside of a clear plastic tub, partially covered with its lid, on a rotating platform, moving at 65 rpm at 26°C. The plantlets washed thereafter with reverse osmosis water and transferred in the small beakers for up to 4 days after the post cocultivation wash. The post-cocultivation-washed plants were then cultivated in vermiculite to grow.

Nematode and plant preparation

Nematode culture was performed according to Alkharouf and Matthew (2004). Nematode infection was determined by acid fuchsin staining (Byrd *et al.*, 1983) at the end of the experiment. Eggs from SCN, H. glycines population NL1-RHp, which infects as Race 3 in deferential screening, were incubated in sterile water at room temperature on a rotary shaker at 25 rpm to promote hatching. After 2 days the Juveniles were collected and concentrated by centrifugation to approximately 3,000 J2/ml. Using a method similar to Hermsmeier *et al.* (1998), 20 radicals were excised and placed in a pinwheel formation with the root tips pointing inward on a circle of sterile Whatman 3 mm paper on top of Gamborg's B5 media (GIBCOBRL, Gaithersburg, MD, USA) in Petri dishes. Replicate experiments using different isolations of J2 nematodes were conducted. Petri dishes, each containing 20 roots, were set up for the experiments. Each set of 20 roots was inoculated with 3,000 J2s in sterile water. The control replicates received the same volume of sterile water. After 3, 6, and 9 days per infection (dpi) of exposure to the nematodes, the roots of both mock-treated and infected roots were rinsed in deionized water to remove any debris and J2s that had not infected the roots. Stereomicroscopy was performed with a Nikon SMZ 1500 stereomicroscope (Nikon Corporation, Tokyo, Japan). Images were captured with an Optronics Magna Fire model S99802 CCD camera (Optronics, Goleta, CA).

Gene ontology analyses

Sequences data for this article have been deposited with the EMBL/GenBank data libraries.

RESULTS AND DISCUSSION

Mathematical modeling of peroxidase class III gene expression in response to nematode infection:

The first week of nematodes life cycle after infection is very essential for it to control and damage the plant (Fig. 1). The prompt and continuous expression of resistant genes in the site of infection dur-

ing this period should decrease the risk factor by increasing the plant resistance. The continuous expression of peroxidase class III gene in the first 9 days of nematodes life cycle indicates that the gene promoter can drive and over-expresses resistant genes efficiently to encounter nematodes infection in the first week of infection.

Microarray analysis of isolated tissues from the nematodes site of infection using Laser capture Micro-dissection LCM showed continues over-expression of Peroxidase class III gene (Klink *et al.*, 2009). The gene expression fold of the gene was changed over the time course of the infection from (526.877) times, (584.6685) times and (219.324) times after 3, 6 and 9 days respectively (Table 2).

As the result indicates, the maximum level of expression was reached after 6 days and started to decline to half this level after 9 days and expected to decline gradually. By fitting the data mathematically, we modeled the expression pattern of this gene (Fig. 2). The mathematical modeling of the gene expression indicates its prompt response and predicts its declining continuous expression until ten days. Thus, this result indicates that the expression of any resistance gene drive by this promoter may also decline after 10 days of infection.

Isolation and cloning of peroxidase class III promoter

The peroxidase class III gene promoter was isolated using a custom designed primers based on the genomic available sequences in the *Phytozome* website as described in Material and methods section (Table 1). The isolated fragments were purified, cloned and sequenced in forward and reverse direction multiple times (Fig. 3).

Bioinformatics analysis of peroxidase class III promoter

The resulted sequences were used to build consensus sequence using *Cap3* software (a DNA sequence assembly program online (Huang and Madan, 1999). The program uses forward–reverse constraints to correct assembly errors and link Coting by using BioEdit v. 7.2.5 (Hall, 1999) unresolved ‘noisy’ nucleotide sites at both ends of the sequence removed. NCBI Pair-wise alignment between the sequences of isolated fragments and the published online sequence should high similarity with few SNPs that has been detected as described in Material and methods section.

Testing expression of peroxidase class III promoter for eGFP and GUS

The 1440 bp upstream from ATG transcription start site of the peroxidase class III gene were isolated, supposedly harboring different promoter regulatory elements were transferred to a collection of Soy bean plants inside a promoter-less custom made plasmid *pJan25T* designed in our Laboratory for promoter testing

applications (Alzohairy *et al.*, 2013) (Fig. 4).

The transformed cells should transient expression of both eGFP (Fig. 4a) and *GUS* (Fig. 4b). This preliminary result indicates that the construct is working in plant cell and the peroxidase classes III promoter can drive genes for expression.

Transformation of peroxidase promoter: pJan25T construct to collections of soybean roots

Transgenic plants roots were screened and monitored for GFP and *GUS* activity after infection with the root-parasitic nematode (*H. glycin*) after 3, 6 and 9 days.

The induction of eGFP and GUS expression by nematode infection

Transformed roots were challenged by nematodes infection. The expression of *GUS* in the root was observed around infection site. This results shows that the peroxidase class III gene has stress responsive promoter against nematodes infection in plants. This promoter will be useful for expressing genes and gene products at the site of nematode feeding that may inhibit the life cycle cyst nematode (SCN) (*H. glycines*) (Fig. 6).

SUMMARY

Successful engineering of transgenic plants for resistance to nematodes requires promoters that over-express resistant genes in the proper cells at the proper time. Gene promoter region controls the temporal and spatial expression of genes. In previous results, Time-course Microarray experiments exhibit the activation of responsive gene in different time points. The data at feeding site showed that the peroxidase class III gene has a stress responsive promoter for nematodes infection and activated upon nematode infection. The transcriptional activation fold change of this gene was tested after 3, 6 and 9 days post nematode infection showing highly responsive and continuous active promoter at the site of infection. The mathematical modeling of its expression is indicated that its expression is prompt and predicts it's declining after ten days. The Pfam domain analysis of peroxidase class III gene showed that this protein is responsible to catalyze a number of oxidative reactions which responsive to many other stresses too. In this investigation we have identified and isolated the promoter region of the peroxidase class III gene which activated in *Glycine max* during nematode infection as well as infection and propagation of *Phytophthora sojae* fungus. The promoter region, -1440 bp upstream of the ATG transcription start site were isolated, cloned, sequenced and transferred to a collection of soybean plants roots inside a custom made vector, *pJan25T*. The *Agrobacterium rhizogenes* root transformation system was used so that whole plants will not need to be regenerated. The transformed plant roots were screened by infection of the root-parasitic nematode *Heterodera glycin* using eGFP as visual marker gene for trans-

formation and *Gus* as a reporter gene for promoter activity. This active and responsive promoter could be used in the future experiments to over-express genes and gene fragments that may inhibit the life cycle of the nematode at the feeding site.

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Table (1): PCR primer pairs composition used for promoter isolation peroxidase class III gene.

Primer	Length	GC%	Expected Size	TM
F 5` GGAAGAAGTACACGACGGAGA 3`	21	52.38	2797	59.34
F 5` TATGACTGCCCCAGACCCTA 3`	20	55.00	1440	60.47
R 5` GTGTTTGCTTGAGAGGGTGT 3`	20	50.00	-	57.77

Table (2): Expression fold change (FC) of peroxidase class III gene responsive promoter after 3, 6 and 9 days.

Gene accession		FC after 3 days	FC after 6 days	FC after 9 days	Functional Category	Gene code
GenBank ID	Similar in *Arabidopsis* T.					
CF809087	AT5G05340	526.877	584.669	219.324	Class_III_ peroxidas	K

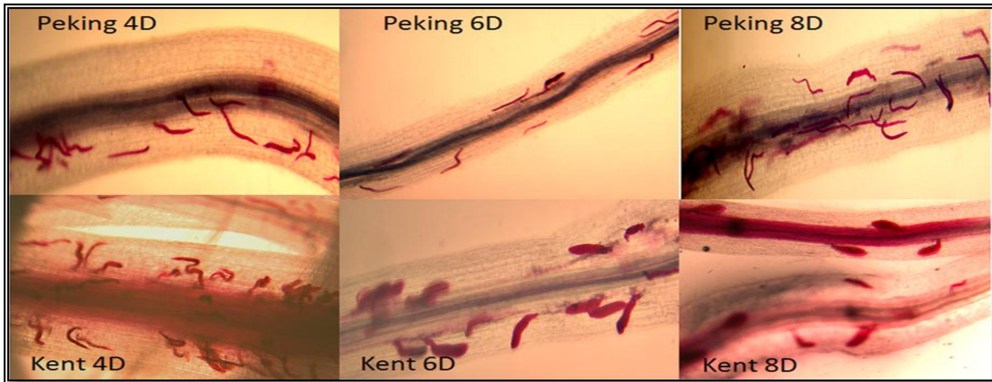

Fig. (1): Timeline of SCN at 4, 6, 8 days after infection in a Resistant (Peking, upper panel) and susceptible (Kent, lower panel) soybean cultivar.

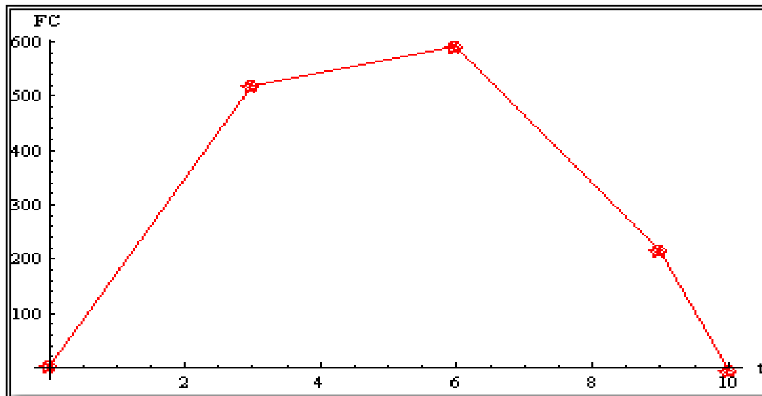

Fig. (2): The expression data from our experiments were fitted using mathematical program.

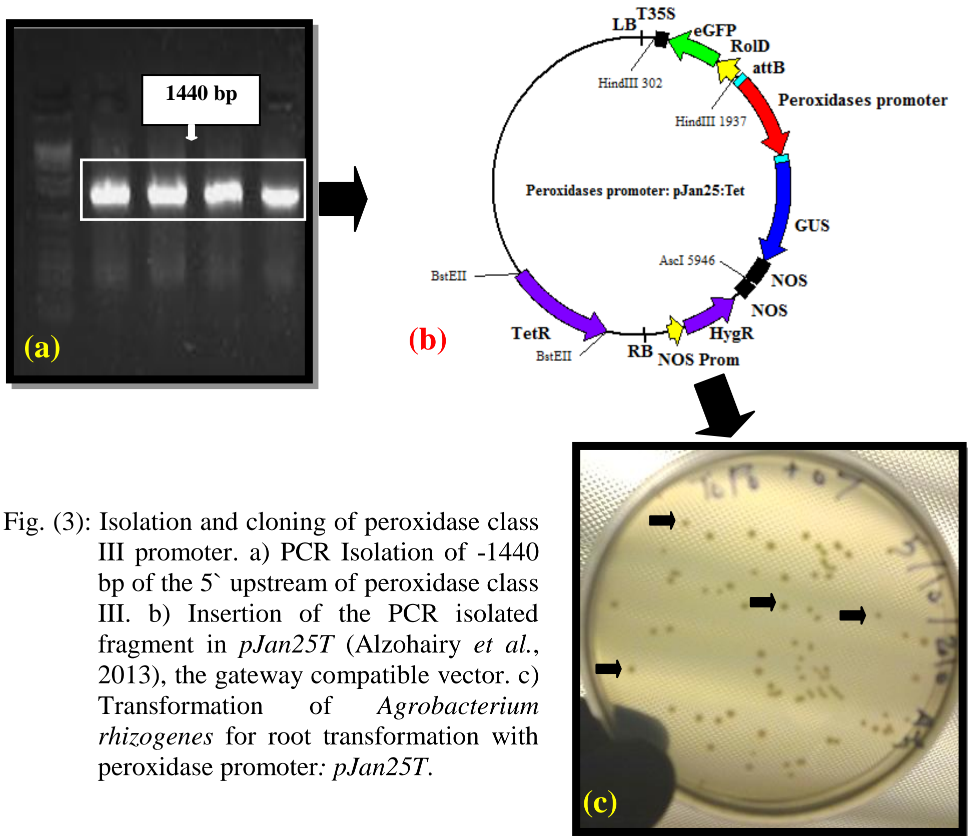

Fig. (3): Isolation and cloning of peroxidase class III promoter. a) PCR Isolation of -1440 bp of the 5` upstream of peroxidase class III. b) Insertion of the PCR isolated fragment in *pJan25T* (Alzohairy *et al.*, 2013), the gateway compatible vector. c) Transformation of *Agrobacterium rhizogenes* for root transformation with peroxidase promoter*: pJan25T*.

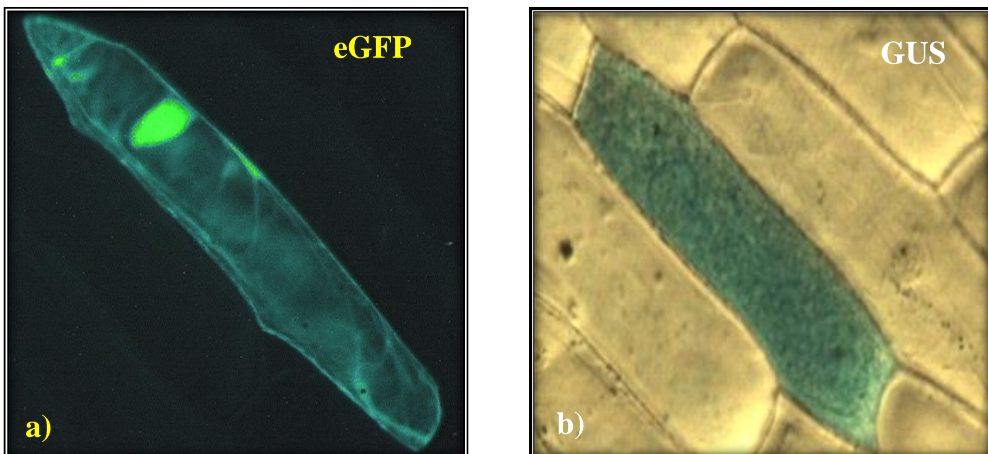

Fig. (4): Isolation, cloning and expression of active Peroxidase class III gene stress responsive promoter in Onion cells after Biolistic transformation with the plasmid peroxidase promoter: *pJan25T*. a) The transient expression of eGFP in onion cells. b) The transient expression of *GUS* in onion cells.

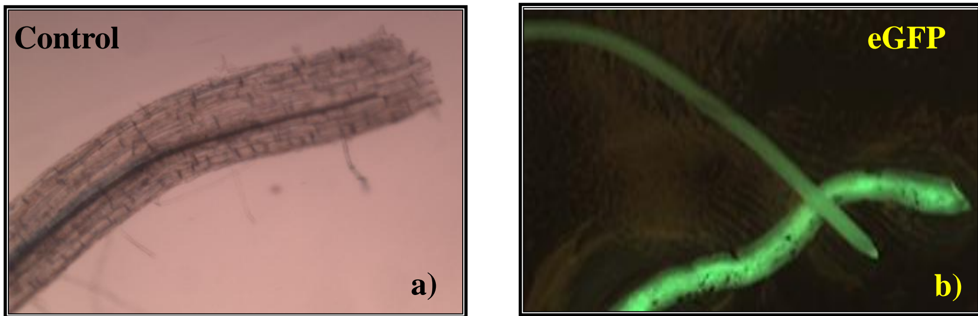

Fig. (5): Transformation of peroxidase promoter*: pJan25T* to *soy*bean roots using *Agrobacterium rhizogenes*. a) The non-transformed roots show no expression of eGFP, b) The transient expression of eGFP in soybean roots.

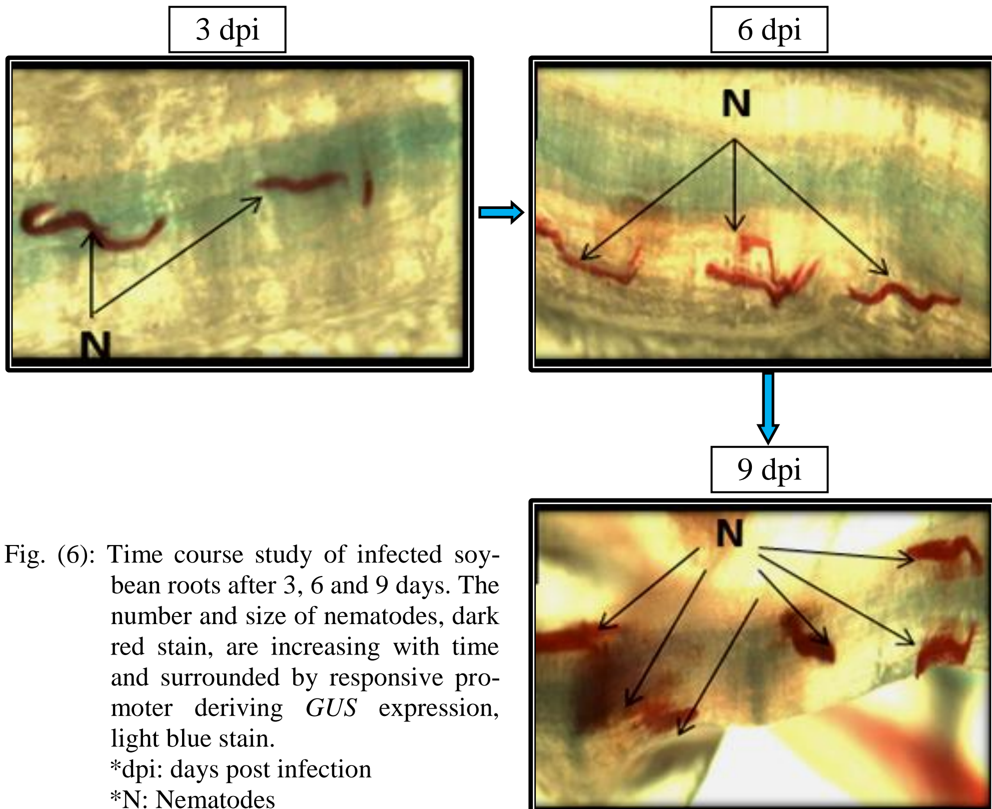

Fig. (6): Time course study of infected soybean roots after 3, 6 and 9 days. The number and size of nematodes, dark red stain, are increasing with time and surrounded by responsive promoter deriving *GUS* expression, light blue stain.
*dpi: days post infection
*N: Nematodes