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Microarray Expression Profiling of Wilt Responsive Genes In Chickpea (*Cicer arietinum*)

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ABSTRACT

Microarray is a technology to measure and analyze the expression of thousands of genes expressed at any given time. DNA microarray technology, especially the use of Gene Chip microarrays, has become a standard tool for parallel gene expression analysis. DNA microarray uses between hundreds and hundreds of thousands of DNA probes arrayed on a solid surface to interrogate the abundance and/or binding ability of DNA or RNA target molecules. In the present study non redundant unigene set comprising 384 clones (192Clones from Incompatible library (WR315), 192 clones from compatible library (Jg62) and 72 control clones were selected for the microarray and microarray hybridization was carried out using probes prepared from resistant and susceptible plants after Fusarium infection at 0hr and 48hr in chickpea. Microarray analysis showed differences in the pattern of expression between compatible and incompatible interaction. Micro array hybridization on the array set using two different time points showed global differences in pattern of expression between compatible and incompatible interaction and the expression ratios were similar to earlier hybridization experiments for majority of genes understudy. For majority of the genes similar expression ratios were obtained using macro array and microarray.

Key words: *Chickpea,Expressed sequence tag(EST), Fusarium wilt, Genomics and Microarray.*

Plant resistance or susceptibility to disease is dictated by the genetic backgrounds of both pathogen and host. Although plants possess passive physical and chemical barriers to infection, active defence responses are only initiated after pathogen perception. Perception of both general and specific pathogen-associated molecules triggers defence responses via signal transduction cascades and transcriptional activation of numerous genes. General elicitors, including proteins, lipids and glycoproteins, trigger non-cultivar-specific defence responses (Hahlbrock *et. al*., 2003 and Montesano *et. al*., 2003),whereas specific effectors are encoded by pathogen *Avr* genes and trigger cultivar-specific defence responses through interaction with plant *R* genes according to the gene-for-gene hypothesis (Dangl and Jones, 2001). Plant R proteins possess highly conserved motifs, such as nucleotide-binding sites (NBSs), leucine-rich repeats (LRRs) and Toll/interleukin-1 receptor-like domains (Dangl and Jones, 2001), whereas pathogen *Avr* effectors lack structural similarity (Bonas and Lahaye, 2002).

Signal transduction and transcriptional activation following pathogen perception result in active defence responses, including calcium and ion fluxes, increase in reactive oxygen species (ROS) during the oxidative burst and hypersensitive cell death (hypersensitive response, HR) (Greenberg, 1997). The expression of transcription factors and protein kinases, as well as the increase in cytosolic calcium, is integral to the signalling of these defences. The expression of various defence genes also leads to the production of defensive compounds, such as pathogenesis-related (PR) proteins and phenyl propanoids (Dixon *et al*., 2002).

Chickpea (*Cicer arietinum*) is the third most important pulse crop in the world, but a major factor limiting production is a severe and destructive fungal disease known as Fusarium wilt caused by *Fusarium oxysporum* (Nene *et al* 1991). Although breeding programmes in major chickpea growing areas worldwide are focused on producing *Fusarium oxysporum* tolerant varieties, the genes and pathways of gene activation controlling effective resistance remain unknown. Hence Using microarray technology and a set of chickpea (*Cicer arietinum* L.) unigenes, expressed sequence tags (ESTs) and, the Fusarium wilt resistance response was studied in two chickpea genotypes i.e. resistant and susceptible cultivars. The expression pattern of several genes may also be used as an indicator

of the state of a cell or tissue, such as resistance or susceptibility to a disease. In this study, we constructed a cDNA microarray representing a non-redundant set of chickpea unigenes, as well as putative defence-related expressed sequence tags (ESTs) from Chickpea.

MATERIAL AND METHODS

Construction of cDNA microarray

The typical microarray experiment consisted of the following steps.

Array fabrication

PCR amplification of cDNA clones

The cDNA clones from both susceptible and resistant libraries were amplified by performing colony PCR. For template preparation, 5µl of the culture from the glycerol stocks plates was directly inoculated into 50µl of sterile water dispensed into each well of a 96-well plate. The plate was sealed and heated to 100°C for 10 minutes in a thermocycler to lyse the cells and release the plasmid DNA. In order to remove the cell debris, the plate was centrifuged at 4,000 x g for 5 minutes. 5µl of the supernatant was used as template for the PCR. The PCR reaction for each clone was carried out in 100µl volume and was set as follows:

10X PCR buffer	10.0µl
25mM $MgCl_2$	6.0µl
10mM dNTP mix	2.0µl
10µM M13 forward primer	2.0µl
10µM M13 reverse primer	2.0µl
Template	5.0µl
DNA polymerase (5 units/µl)	0.5µl
Sterile MQ water	72.5µl

PCR was performed using the following program

1. Initial denaturation	94°C for 2 min
2. Denaturation	94°C for 30 sec
3. Annealing	52°C for 30 sec
4. Primer extension	72°C for 1 min
(steps 2 to 4 cycled 30 times)	
5. Final extension	72°C for 10 min
6. Indefinite hold	4°C

Purification of PCR products

The PCR amplified products were purified using Perfectprep PCR clean up kit (Eppendorf) as per the manufacturer's protocol:

Quality check of purified PCR products

The quality of the PCR products (2µl) was checked on 1.0% agarose gels. The PCR products which showed distinct and sharp bands were further used for the array preparation. For the clones which did not amplify or which showed more than one band were excluded and again amplified as per the reaction mentioned.

Printing of cDNA clones on slides

The purified PCR products were transferred from 96-well plates to 384-well plates for long term storage. For the printing purpose, five microlitres of the PCR products from 384-well storage plates were reorganized on the fresh 384-well printing plates with 5µl of 100% DMSO. The printing was done in duplicates on the poly-L-lysine coated slides (Sigma) using a high throughput arrayer (Arrayer ESI, SDDC2) followed by UV cross linking.

Target preparation and labelling

In order to find out the differential gene expression during chickpea-*Fusarium* interaction, root tissue samples were collected from *Fusarium* infected WR-315 and JG-62 chickpea seedlings after 6h, 12h, 24h, 48h and 5d of infection. Water treated plants were taken as control. Two biological replicates were done for each time point. Total RNA was isolated from all the tissue samples using TRIzol reagent (Invitrogen, CA). 6µg of the total RNA from each sample was used for cDNA synthesis using indirect TSA labeling and detection kit Micromax (PerkinElmer). The RNA isolated from uninfected and infected tissue was labelled with flourscein and biotin, respectively.

Purification of labelled cDNAs

The labelled cDNAs were purified using microcon YM 100 columns (Millipore, Bedford, MA) as per the manufacturer's protocol :

Microarray hybridization and TSA detection

For hybridization, total 200µl each of purified flourscein and biotin labeled cDNAs were mixed and hybridized to the microarray slides in hybridization chambers (Corning) at 65ºC incubator. The slides were washed for 10 minutes in 30ml of 0.5X SSC and 0.01% SDS, 10 minutes in 30ml of

SlideNo	Cy5LabeledProbe	Cy3LabeledProbe
Cafe456-1	C-48hr post Infection	C-0hr Control
Cafe456-2	IC-48hr post Infection	IC-0hr Control
Cafe456-31	C-48hr post Infection	C-0hr Control
Cafe456-32	IC-48hr post Infection	IC-0hr Control

Figure 1.1 Labeling reactions used for the present study.

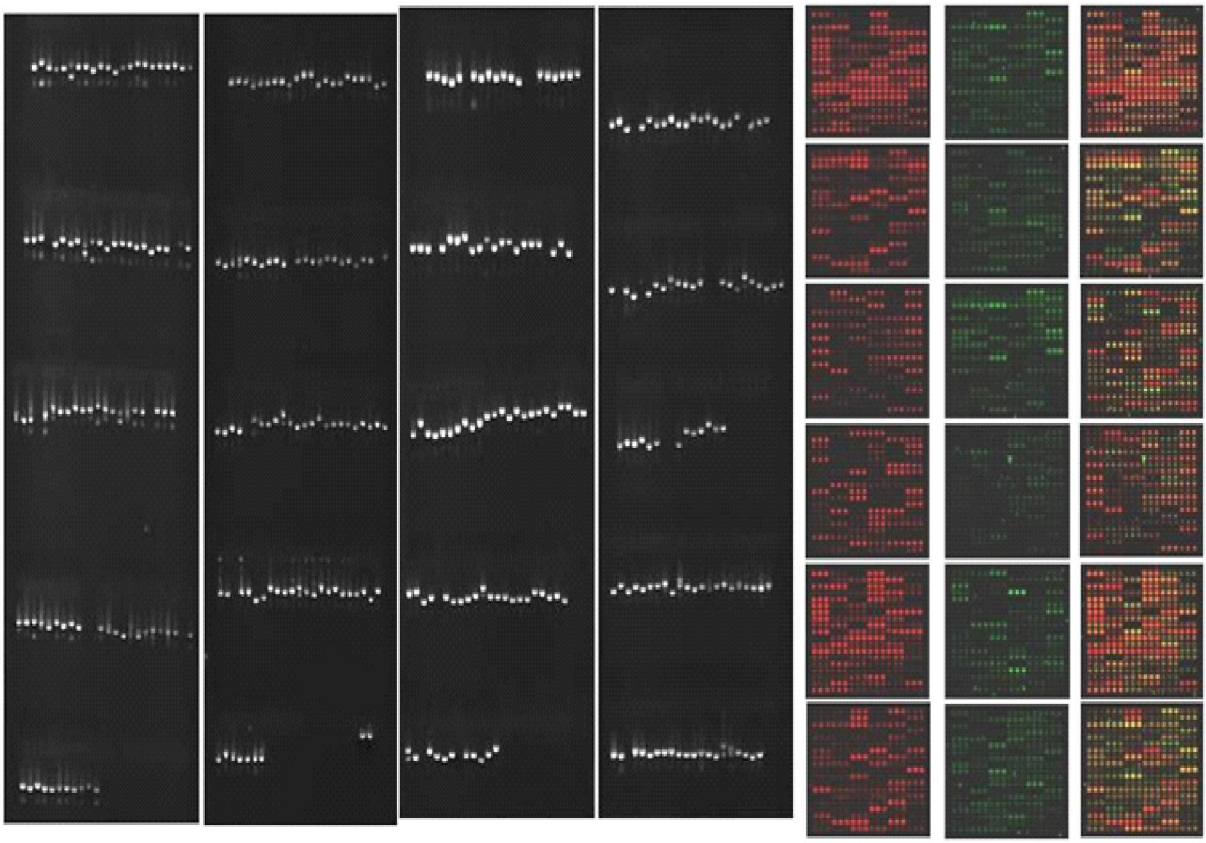

Figure 1.2. Construction of 4K Chickpea array. (A) Agarose gel showing PCR amplifiedclones used for printing on glass slides; (B)Representative microarray images obtained after scanning(Cy5); (C) Cy3; (D) Cy5/Cy3 Overlay.

0.06X SSC and 0.01%SDS and for 5 minutes in 30ml of 0.06X SSC. The slides were subsequently processed for replacement of flourscein with Cy3 and biotin with Cy5 as given below:

1. Microarray slides were incubated with 600µl of TNB-10% goat serum buffer for 10 minutes which was followed by rinsing once in 30ml TNT buffer for 1 minute with agitation.
2. The slides were then incubated with 300µl of Anti-FL-HRP conjugate solution for 10 minutes followed by rinsing of slides in 30ml of TNT buffer with agitation thrice for one minute each.
3. Slides were again incubated with 300µl of cyanine 3 tyramide solution for 10 minutes followed by rinsing in 30ml of TNT buffer three times each for five minutes.
4. The slides were incubated with 300µl of HRP inactivation solution for 10 minutes **a**nd were then rinsed, 3 x 1 minute, in 30ml of TNT buffer with agitation.
5. This was followed by incubation with 300µl streptavidin-HRP conjugate solution for 10 minutes. Then the slides were washed 3 x 1 minute, in 30ml of TNT buffer with agitation.
6. Slides were further incubated with 300µl cyanine 5 tyramide solution for 10 minutes and then washed 3 x 5 minutes, in 30ml of TNT buffer with agitation.
7. The slides were lastly rinsed for one minute in 30ml of 0.6X SSC with agitation.
8. In order to dry the slides, these were given a spin in a swinging bucket rotor at d" 3,000 x g for 2 minutes, in a 50ml conical tube.

Scanning and data analysis

Micraoarrays were scanned using Scan array 5000 scanner (PerkinElmer) to produce two separate tiff images. Spot finding and quantification of the spots were done by using scan array express software (PerkinElmer). Spots appearing bad due to poor morphology, high local background and bubbles were flagged off and were excluded from further analysis. Spots with both channel intensities less than 500 were also filtered out. Spots were quantified using an adaptive method. Avadis software (PerkinElmer) was used for further data transformation which consisted of background correction and normalization. For background correction, local background intensity of each spot was subtracted from its foreground intensity value. Due to non linearity of the data, intensity dependent Lowess normalization was applied. Cy5/Cy3 signal ratio was also calculated for each spot on the array.

Gene clustering

The clustering analysis was performed using MEV software (TIGR). The analysis included all the genes spotted on the array. Expression values for the same gene measured in each of the two genotypes were entered into analysis as independent sets of values in a single clustering analysis to detect the pattern of similarity and differences between the two cultivars. K-means clustering method was employed.

Agarose gel electrophoresis of RNA and northern blotting

Formaldehyde-denatured RNA gel electrophoresis (Lehrach et al, 1977) was performed as described by Sambrook *et al,* (1989) using formaldehyde (Glaxo) pH 3-3.5.

DNA probe preparation

All the system components were thawed on ice except Klenow which was kept in -20°C except when in use.

For a 25µl reaction 25ng of DNA fragment was taken in 16µl for random primer labeling. The DNA was denatured in boiling water bath for 5 min and quickly placed on ice for 5 min. It was spun and reaction was set up as follows:

	Volume in µl	Final Concentration
DNA	16µl	25ng
10X labeling buffer	2.5µl	1X
dNTPs(-dATP)(0.5mM each)	1 µl each	25µM each
α32PdATP	2.5µl	25uCi
Klenow DNA Polymerase (NEB) (5U/µl)	1µl	5Units

The reaction was incubated at 37°C for 2hr and then terminated by adding 2.5µl of 0.2M EDTA (pH 8.0).

To remove unincorporated label and limit high levels of radioactivity during hybridization experiments the probe was purified by gel filteration. The probe was through passed through Sephadex G-50 column as described in Sambrook et al, 1989.

Nucleic acid hybridization

Gene Screen Plus membrane was used for northern hybridizations. The blots were kept dry until probe preparation was over. The blots were wet in 2X SSC and prehybridized in (0.3 M NaCl, 50% formamide, 10%dextran sulphate and 1% SDS) at 42°C in hybridization oven. The minimum time for prehybridization was 1 hr and maximum 12 hrs. Fresh prehybridization buffer was prewarmed to 42° C and added to the hybridization bottle before adding the probe. To the probe 100µg /ml sheared Salmon sperm DNA was added and the probe was denatured in boiling water bath for 15min and chilled on ice for another 10 min before adding to the prehybridization buffer. Hybridization was carried out for 12-18 hrs at 42° C. Following hybridization blots were washed with 2X SSC at RT for 10 min. Two washes were given in 1X SSC, 0.1% SDS solution at 60°C for 10 min each. After this the blots were sealed in plastic bags and exposed to X-Omat AR X-ray film (Kodak) at -70° C for 24-36 hr.

RESULTS AND DISCUSSION

Transcript profiling of Chickpea ESTs

Schena *et. al*., 1995 first described, high-density DNA microarray methods for host pathogen interaction studies. The key unifying principle of all microarray experiments is that labelled nucleic acid molecules in solution hybridize, with high sensitivity and specificity, to complementary sequences immobilized on a solid substrate, thus facilitating parallel quantitative measurement of many different sequences in a complex mixture (Southern *et. al.,* 1999). DNA microarray techniques are particularly

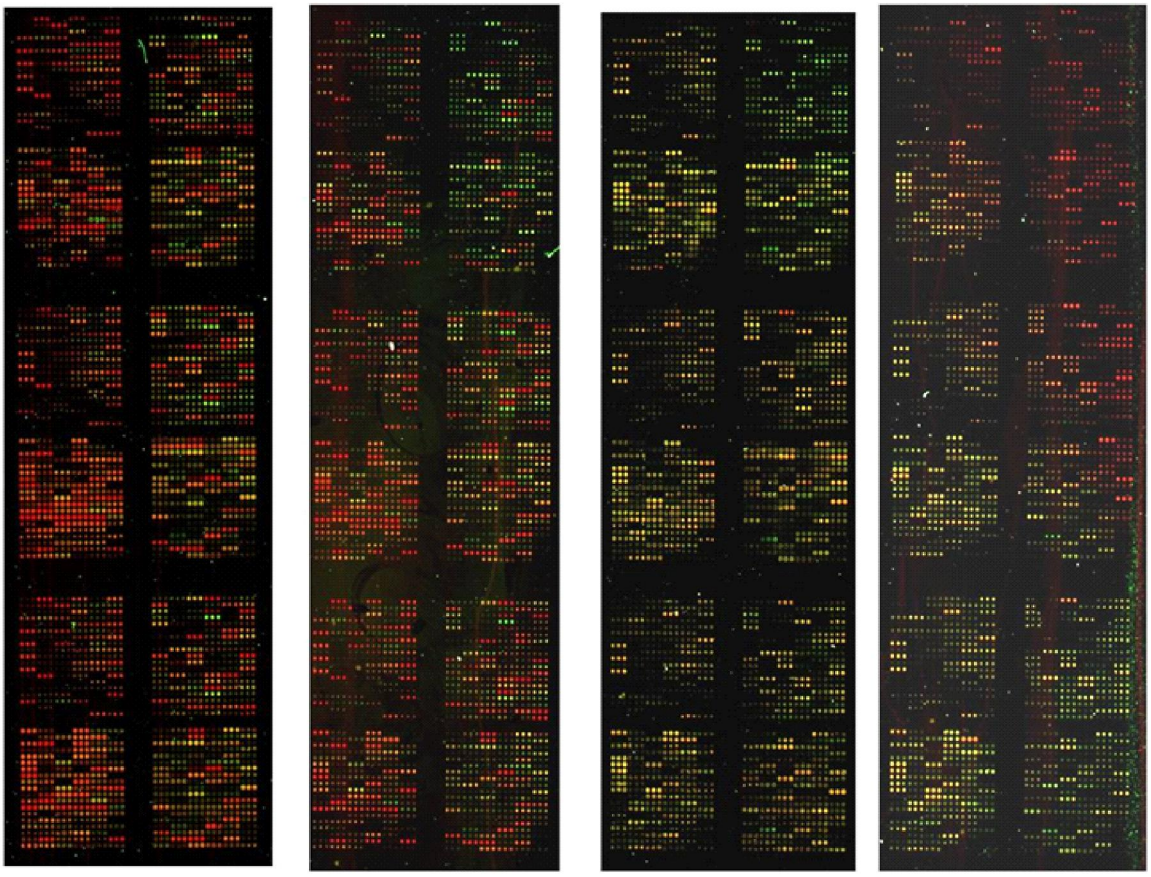

Figure1.3 Overlapping images after scanning microarray slides.

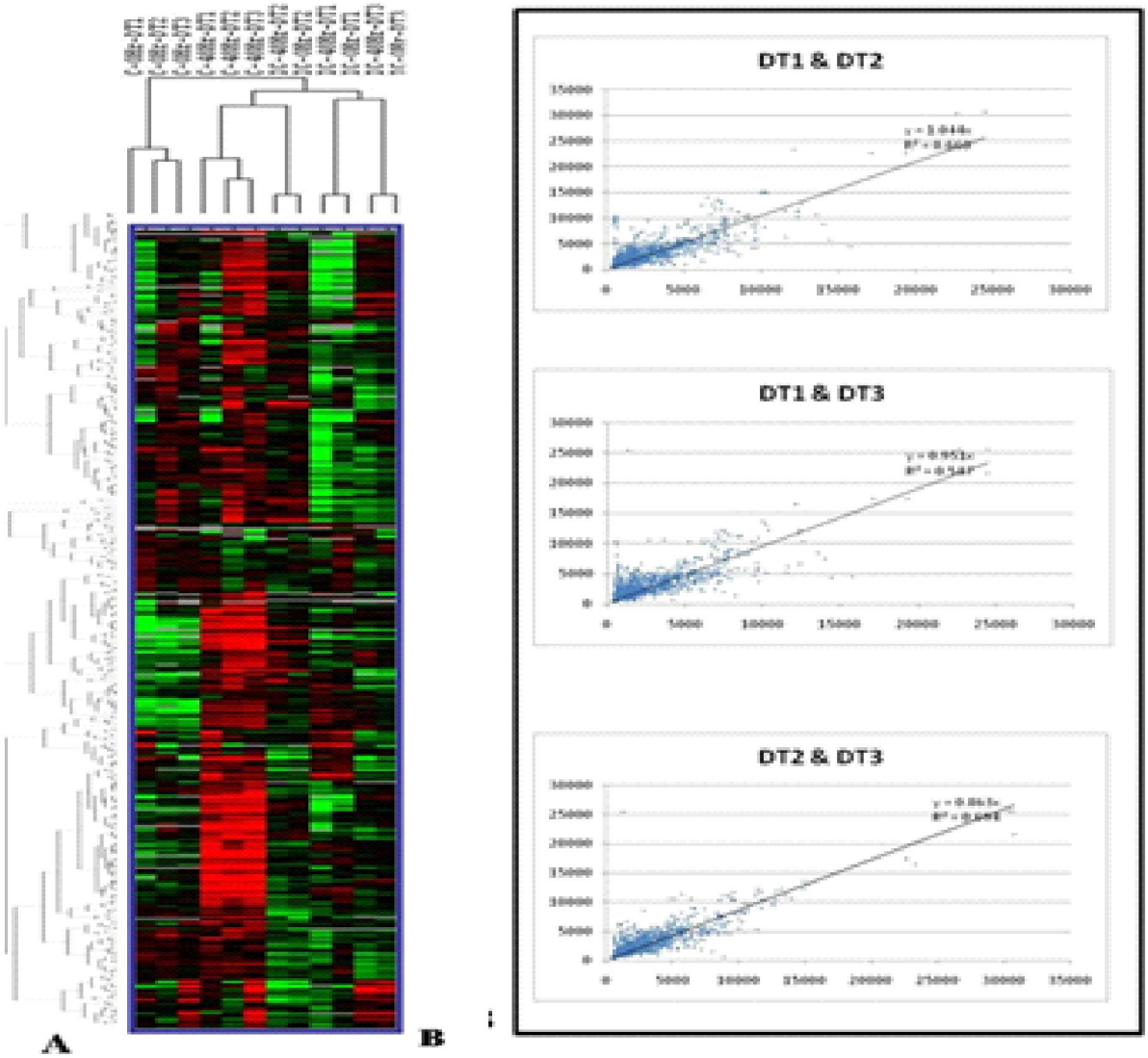

Figure 1.4. Clusterogram showing uniformity among dwell times and also differences in patterns of expression of genes between compalible and incompatible libraries. (A) Clusterogram; (B) Scaller plots.

suitable for monitoring gene expression changes in plants during plant-pathogen interactions, due to their relative simplicity, comprehensive sampling capacity and high throughput. The most attractive feature of DNA microarray techniques is that they allow researchers to examine the responses of hundreds or thousands of genes simultaneously during a given treatment. Using these expression profiles, it is possible to identify differentially present mRNA species and to hypothesize potential defense-associated function based on this differential expression. Recently, a small number of DNA microarray experiments have identified an amazing number of potential defense-related genes. Although some of these genes have previously been implicated in plant defense responses, most have not.

Microarray based on 4K array

A microarray Chip Comprising 384 clones (192 clones from Incompatible Library and 192 Clones from Compatible library falling under different classes) was prepared to standardize the microarray experimental conditions as per the layout (Figure 1.1) in terms of dwell time, humidity and selection of positive, negative and spike controls. PCR amplification (Figure 1.2) followed by purification was done to make the chipset ready for Printing on glass slide along with the controls. For Printing 5ul of purified PCR samples was put into 5ul of DMSO (100%) to get a concentration of

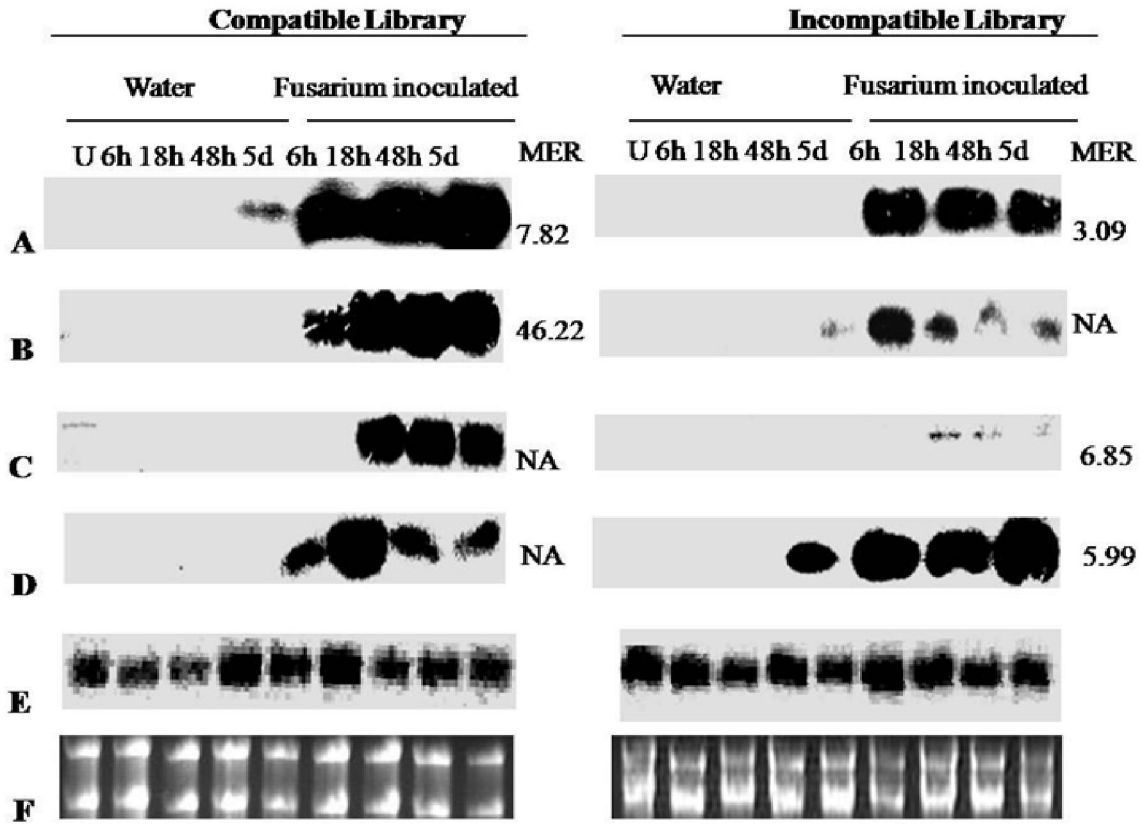

Figure 1.5. Northern blot analysis to confirm differential expression of few of the interesting clones. (A) Putative ribokinase; (B) Putative water channel protein; (C) Clone WIE_03_H_07; (D) Clone WIE_05_C_02 Note: MER (Macroarray Expression Ratio); (E) Hybridization with GAPDH; (F) EtBr stained formaldehyde gel

100 ng/ul of the sample. For the expression profiling root tissue was harvested from both susceptible and resistant cultivars following the fungal infection at 0hr and 48hr post infection. Probe labeling and microarray hybridization was carried out using TSA Kit following the manufactures instructions. After the hybridization slides were scanned using Genomic solutions Microarray scanner and the data was analysed using Genepixpro. The array was investigated to see the dwell times and humidity on the printing, picking up right controls and to standardize the hybridization process. Three dwell times viz.0.1 seconds (180 u spot size) 0.075 seconds (165 u spot size) and 0.05 seconds (150 u spot size) were investigated. The slide image and data analysis showed that the dwell time of 0.05 seconds, spot diameter (150 um) and the slide printed in presence of humidity gave better spot morphology and size uniformity (Figure 1.3) and the cluster analysis grouped the samples from different dwell times into same cluster indicating uniformity of spotting and uniform hybridization across the slide (Figure 1.4).

Identification of differentially expressed transcripts upon *Fusarium* infection

Transcripts showing more than two fold difference upon Fusarium infection were further studied. The results showed that the expression of defence related transcripts is more in resistant plants compared to susceptible plants, but the genes should be monitored at various time points and after hormone treatments before drawing any specific conclusion. Towards this goal a larger array set was constructed for analyzing global expression changes upon *Fusarium* infection in compatible and incompatible interactions based on time kinetics so that differences in either basal expression level or induction time can be correlated with the resistance/ susceptible response.

Gene expression profiles during incompatible interaction

To study the transcriptional remodeling in immune response during vascular wilt, we have developed cDNA microarray using *Ca*EST clones of the subtracted cDNA libraries from susceptible and resistant genotypes. Root tissue sample from WR-315 (resistant genotype) harvested at 24 h post *Fusarium* infection was used to evaluate the expression profile during early phase of incompatible interaction. We used indirect labeling of cDNAs following TSA protocol that incorporates flourscein and biotin labeled dUTP into the nascent cDNA from the target RNA instead of Cy3 and Cy5 modified nucleotides since it is known to negate any dye bias during the microarray experiment. A total of 257 differentially expressed unigenes were found to be associated with the early signaling pathway, of which 107 were induced and 150 repressed during incompatible interaction (Ashraf *et. al.,* 2009).

Confirmation of few of the differentially expressed genes with Northern analyses

To confirm the expression of differential genes, northern blot analysis was carried out. The genes chosen were putative ribokinase, putative water channel protein, two unidentified clones from resistant library and the results obtained were positively correlated with the earlier screening data (Figure 1.5).

LITERATURE CITED

Ashraf N, Ghai D, Barman P, Basu S, Gangisetty N, Mandal MK, Chakraborty N, Datta A and Chakraborty S 2009 Comparative analyses of genotype dependent expressed sequence tags and stress-responsive transcriptome of chickpea wilt illustrate predicted and unexpected genes and novel regulators of plant immunity. *BMC Genomics* 10:415.

Bonas U and Lahaye T 2002 Plant disease resistance triggered by pathogen-derived molecules. *Curr. Opin. Microbiol*. 5, 44–50.

Dangl J and Jones J 2001 Plant pathogens and integrated defense responses to infection. *Nature*, 411, 826–833.

Dixon R, Achnine L, Kota P, Lui C, Reddy M and Wang L 2002 The phenylpropanoid pathway and plant defence – agenomics perspective. *Mol. Plant Pathol*. 3, 371–390.

Greenberg J 1997 Programmed cell death in plant–pathogen interactions. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. 48, 525–545.

Hahlbrock K, Bednarek P, Ciolkowski I, Hamberger B, Heise A, Liedgens H, Logemann E, Nurnberger T, Schmelzer E, Somssich I and Tan J 2003 Non-self recognition, transcriptional reprogramming, and secondary metabolite accumulation during plant/pathogen interactions. *Proc. Natl. Acad. Sci. USA*, 100,14 569–14 576.

Montesano M, Brader G and Palva E 2003 Pathogen derivedelicitors: searching for receptors in plants. *Mol. Plant Pathol*. 4,73–79.

Nene Y L, Reddy MV, Haware MP, Ghaneka AM and Amin KS 1991 Field diagnosis of chickpea diseases and their control. *Information Bulletin* No. 28, ICRISAT, Hyderabad, India.

Sambrook J, Fritsch EF and Maniatis T 1989 *Molecular cloning*: *A laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Schena M, Shalon D, Davis RW and Brown PO 1995 Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270: 467-70.

Southern E, Mir K and Shchepinov M 1999 Molecular interactions on microarrays. *Nat Genet* 21(Suppl. S): 5–9.

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