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Identification of Differentially Expressed Novel Genes Against Fusarium Wilt in Chickpea Using Dot Blot Technology

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ABSTRACT

Transcriptional remodeling in immune response during vascular wilt, was studied using Dot blot technology i.e., cDNA macro-array using *Ca* EST clones of the subtracted cDNA libraries from susceptible (JG62) and resistant genotypes (WR315). Root tissue sample fromWR-315 harvested at different time intervals post *Fusarium* infection was used to evaluate the expression profile during early phase of incompatible interaction. 2112 PCR amplified clones using adapter specific primers were used and blotted onto Gene screen Nylon membrane. Total cDNA probes for differential screening was prepared from the total RNA isolated from the roots of control and pathogen infected plants for both the cultivars. Hybridized blots were scanned using Phosphor imager and 1065 clones showing more than 2.5 fold induction were further analysed. Out of these 1065 clones plasmid preps were made for 783 clones followed by automated DNA sequencing. A cut off score of 100 was put to find the best matches and the top match was used for all the downstream work. Some of the interesting genes fall in the class of regulatory proteins, signaling proteins and defense response proteins are bZIP, bHLH, Zn-finger transcriptional activators, kinases, phospahatases, G-protein coupled receptor, also the downstream proteins such as PR proteins, CHS and CHI.

Key words : Chickpea, Dotblot, EST's, Fusarium and Macro-array.

Fungal disease is responsible for most of the reduction that differentiates yield potential from harvested yield in major crop plants (Agrios, 2005). Many studies have examined the effects of fungal pathogenesis with the aim of discovering mechanisms used by infection-tolerant species and the elements that might confer tolerance to sensitive plants. Active disease resistance in plants depends on the ability of the host to recognize pathogens and initiate defense mechanisms that limit infection. A number of distinct, but interconnecting, signaling pathways are involved in disease resistance that are under both positive and negative control. These pathways operate, at least partly, through the action of small signaling molecules such as salicylate, jasmonate and ethylene (Anderson *et al.,* 1991). The interplay of different signals probably allows the plant to fine-tune defense responses in both local and systemic tissue. Until recently, the analysis of signaling processes and their interactions in plants have usually been focused on only one or a few genes at any time. Such limited studies have been unsuccessful to assess the global gene activation by different signals and pathogens in the defense response. With the advent of new technologies, it is now possible to determine the genesis of fungal pathogen tolerance to a larger scale through genome-wide expression profiling (Bevan and Waugh, 2007).

Chickpea is the third most important grain legume in the world. India is the largest chickpea producer and accounts for over 68% of the total global production (FAO, 2012). Chickpea wilt caused by *Fusarium oxysporum* f. sp. ciceri is considered to be one of the most limiting factors in chickpea production. The disease is wide-spread in appearance and on an average causes 10-20% losses, alone in India, every year (Jimenez-Diaz *et al.*, 1989 and Singh and Reddy, 1991). The existence of pathogenic races of *Fusarium oxysporum* is well established and eight races of the pathogen have been identified. With a view to incorporate the resistance genes, the genetics of resistance has also been worked out against some of the prevailing races. However, the mechanism of chickpea-*Fusarium* interaction is poorly understood. Thus, the aim of this study is to identify the differentially expressed transcript abundance in chickpea exposed to *Fusarium* infection. This may pave the way in understanding the signaling

networks involved in chickpea-*Fusarium* interaction.

MATERIAL AND METHODS

All percentage shown here are on w/v basis unless mentioned otherwise. All solutions and media were made in Milli Q and Milli RO respectively and were sterilized by autoclaving at 15 Ib/ sq inch for 20 minutes or filter sterilized by passing through a 0.22µM Millipore filter. Media or media components used were from GIBCO BRL or Difco unless mentioned otherwise. Chemicals used were of analytical grade (Glaxo or MERCK) or molecular biology (SIGMA, GIBCOBRL or USB) grade unless mentioned otherwise. Restriction enzymes and DNA modifying enzymes were obtained from NEB, MBI-Fermentas, BM, Amersham or Perkin Elmer. Buffers provided with the enzymes were used for setting up the reaction.

Biological materials and Plasmids

Cicer aritienum varieties JG 62, susceptible to wilt and WR315, resistant to wilt
Fusarium oxysporum f.sp. *ciceri* Race 1
Escherichia coli DH5á strain

Plasmid and primers used in the study

pGEM-T Easy
M13 forward primer 5' GTT TTC CCA GTC ACG ACG TTG 3'
M13 reverse primer 5' TGA GCG GAT AAC AAT TTC ACA CAG 3'
Nested primer N1 5' TCG AGC GGC CGC CCG GGC AGG T 3'
Nested primer N2R 5' AGC GTC GTC GCG GCC GAG GT 3'
Adapter primer 5' CTA ATA CGA CTC ACT ATA GGG C 3'

Media and solutions

Luria Bertani (LB) Medium (Gibco/BRL)

2x LB (for Millipore plasmid isolation)

1% Tryptone
1% NaCl
0.5% Yeast Extract

Terrific Broth

1.2% Bacto tryptone, 2.4% Yeast extract
0.4% (v/v) glycerol. Supplemented with
1/10 th volume 10X Phosphate Buffer
(2.31g K_2HPO_4, 12.54g KH_2PO_4 in 100ml H_2O).

Media with antibiotics

LB/TB with 50ìg/ml Ampicillin
LB agar with 75µg/ml Ampicillin

Potato Dextrose Broth

20% Potato infusions
2% Dextrose

EndoR(6X)

30% Ficoll 400
0.6% SDS
60mM EDTA pH8.0
0.06% Bromophenol blue.

20X SSC (1 liter)

175.3 g Sodium Chloride
88.2g Sodium Citrate

Phenol

Glaxo Laboratories Exel AR Grade.
Redistilled at 180ºC and stored at
-20°C in small aliquots.

2% Tryptone
1% NaCl
1% Yeast extract

2XL Broth

2% Bacto Tryptone
1% Yeast Extract
0.1% NaCl
0.2% Dextrose

10X TE

100mM Tris-HCl, pH 8.0
10mM EDTA, pH8.0

Potato Dextrose Agar

20% Potato infusions
2% Dextrose
1.5% Agar

50X TAE (1 Liter)

242 g Tris base
57.1 ml Glacial Acetic Acid
17.8gm EDTA (Na_3 Salt)

RNase A

10 mg/ ml
Prepared as described in
Sambrook *et al.,* (1989)

TE-Saturated Phenol

Prepared as per Ausubel *et al*., (1994)

Water Saturated Phenol
Prepared as per Ausubel *et al*., (1994)

MS medium with vitamins
Gibco BRL

GTC Buffer
4M Guanidinium-iso-thio-cyanate
42mM Sodium Citrate
0.83%LaurylSarcosine
10mM â Mercapto-ethanol

2XYT Medium
1.6% Bactotryptone
1% Bacto yeast extract
0.5% NaCl

L B Agar
LB with 1.5% Agar (Gibco/ BRL)

Phenol: Chloroform: IAA
25 parts of TE saturated phenol, 24 Parts of chloroform and 1 part Isoamylalcohol

X-Gal
20mg/ml stock in dimethylformamide
Stored in -20°C.

IPTG
0.2% stock in distilled water and filter
Stored in -20°C.

Trituration buffer (pH5.5)
100mM $CaCl_2$
70 mM $MgCl_2$
40mM NaOAc

Seed sterilization and growth of plants

Chickpea plants were raised in tubes under sterile conditions. The seeds were washed in autoclaved water for 1-2 min twice followed by 70% ethanol wash for 30 sec. Again it was washed twice with autoclaved water. The seeds were treated with 0.1% $HgCl_2$ for 3 min with vigorous shaking. Following this five washes of five min each with autoclaved water were carried out. After the last wash the bottle containing the seeds was placed in a dark place overnight.

For germination MS basal medium solidified with 0.6% agar was used. The tubes were kept in dark overnight to allow germination. Afterwards the growth conditions maintained were: 25°C temperature and 16h photo period for three weeks during growth and also during infection.

***Fusarium* culture and infection of plants**

A loop full of Fusarium culture was inoculated from the PDA slant into 100ml of potato dextrose broth in 500ml flask. The culture was incubated at 25°C under shaking conditions at 200 rpm. For infection one week old Fusarium culture was used. To collect the fungal spores, the culture was filtered twice through cheese cloth and the filtrate was collected in sterile flask in the laminar hood. To check the spore count two independent aliquots of the filtrate were taken in 1.5ml eppendorf under sterile conditions. Each of these eppendorfs were vortexed to suspend the spores evenly before dilution. The average number of spores in the filtrate was calculated from the 20 times and 100 times diluted sample in sterile water. For both the dilutions the number of spores were counted with the help of haemocytometer. The actual number of spores in the filtrate was calculated taking into account the dilution factor. The average spore count of the culture filtrate varied between 10^7 -10^8 spores/ml. For carrying out infection it was diluted with sterile water to achieve the final concentration of 10^6 spores/ml.

For infection 3 weeks old plants were uprooted from the media and swirled in autoclaved water to get rid of the agar adhering to the roots. Each plant was then placed in a tube containing 10 ml of spore suspension with a final concentration 10^6 spores/ml. For control, the plants were dipped in sterile water. The plants were held in the tubes with the help of sterile cotton and kept under the same condition as used for growth of seedlings. For harvesting, the plant material was cut at the point of seed attachment with a sterile blade and the root tissue was quick chilled by dipping in liquid Nitrogen. The harvested plant material was stored at -80°C till further use.

RNA Work

Precautions were taken to keep RNases at bay. All glassware used, were baked at 300ºC overnight. The 50ml tubes were treated with 3% H_2O_2 for few hours, rinsed with RNase-free water thrice, autoclaved for 30 min and dried before use.

All plastic wares and gel running tank, gel tray, comb were treated with 3% H_2O_2 and rinsed thrice with autoclaved water before use. Water used for RNA work was treated with 0.1% DEPC for 10-12hrs at room temperature and then autoclaved for 30min. All solutions were prepared in DEPC treated water and autoclaved for 30min.

Total RNA isolation

Total RNA from frozen plant tissue was isolated using Trizole reagent following manufacturer protocol.

Poly A+ isolation from total RNA preparation using Dynabeads

For isolating Poly A^+ representing the mRNA fraction in total RNA, Dynabeads were used. These are uniform, superparamagnetic, 2.8µm diameter, polystyrene beads with 25 nucleotide long chains of deoxythymidine covalently linked to their surface via a 5´ linker group.

Preparation of total RNA for mRNA isolation

1. The volume of total RNA (100-250 µl) was adjusted to 100 µl using DEPC treated water.
2. RNA was denatured by heating at 65ÚC for 2 min and quick chilled on ice.

mRNA isolation from total RNA

1. Total RNA (250 µg) was added to the Dynabeads oligo $(dT)_{25}$ which were already suspended in the binding buffer.
2. Thorough mixing was done by pipetting up and down. Incubation was carried out at room temperature for 5 min to allow binding of mRNA to beads.
3. The eppendorf was placed in magnetic stand for 2 min and supernatant removed.
4. The beads were washed twice with 200µl of washing buffer (10mM Tris-HCl pH 7.5, 0.15 M LiCl, 1mM EDTA) using the magnet. Care was taken to remove the traces of wash buffer completely.
5. The elution of bound mRNA was carried out by suspending the Dynabeads oligo $(dT)_{25}$ in 15µl of elution buffer (10mM Tris-HCl pH7.5) and heating to 80-90ÚC for 2min. Followed by quickly placing the tube in the magnetic stand. As the beads moved towards the magnetic side the eluate with mRNA was left at the bottom of eppendorfs. The eluate was transferred to fresh RNase free eppendorf.
6. The concentration of the eluted mRNA was estimated by measuring the absorbance A_{260} of the final preparation. From 250 µg of total RNA at least 1.5- 2µg of mRNA was isolated.

PCR Amplification of Clones

Colony PCR using adaptor specific primers

Using a sterile tip a small portion of bacterial colony from the patched plate was picked and transferred to PCR tube containing 100 µl of sterile water in 0.6 ml eppendorf tube. The lysis was carried out on thermocycler which was programmed at 100°C for 10 min. The tube was quick chilled on ice and spun. 10 µl of the lysate was transferred to fresh tube and to it 40 µl of cocktail was added. The tube was tapped, spun and subjected to PCR cycling using MJ Research thermocycler.

The PCR programme employed was

Step 1	94 °C -	1 min
Step 2	94 °C -	45sec
Step 3	68° C -	45 sec
Step 4	72° C -	1 min
Step 5	Steps 2 to 4 were cycled 35 times.	
Step 6	72° C -	10 min

The composition of cocktail used was

Component	Final concentration
10X PCR buffer	1X
25mM MgCl2	1.5 mM
10mM dNTP	200µM
Forward primer	25µM
Reverse primer	25 µM
Amplitaq	5U/8 reactions

MQ water to make final volume to 50 µl including the lysate.

Dot Blot / Slot Blot

1. The manifold was well cleaned prior to use. Genescreen membrane was cut to the exact size of the slot blot apparatus.
2. The membrane was first soaked in MQ for 5 min and then in 0.4 M Tris, pH 7.5 for 20 min.
3. The wet membrane was placed on the manifold and it was assembled.

4. 10 µl of colony PCR product was denatured by adding 10 ul of 0.5 N NaOH and 1 M NaCl to achieve final concentration of 0.25N NaOH and 0.5 M NaCl. The samples were placed at 37°C incubator for 10 min.
5. The DNA samples were diluted on ice by adding 80 µl of 0.1X SSC, 0.125 N NaOH.
6. 40µl of each sample was loaded into the manifold. The solution was allowed to remain in the well, on the membrane, for 20 min without suction.
7. Light suction was applied to the manifold until the loading buffer was drawn out of the wells. Care was taken that vacuum was not released suddenly to avoid reverse flow in the manifold.
8. 40 µl of 0.5 N NaCl, 0.5 M Tris – HCl pH 7.5 was passed through the wells and light suction was applied as mentioned earlier.
9. The membrane was fixed by UV cross linking using Stratagene UV crosslinker.

cDNA Probe Synthesis

Single stranded cDNA probe was synthesized by labeling first strand of cDNA using Superscript reverse transcriptase ;

500µg/ml oligo dT_{20} 1.5µl
mRNA 400ng (10.5 µl)
Sterile DEPC treated water to 12µl

The mixture was heated to 65ÚC for 5min, quick chilled on ice and briefly spun. The following components were added

5x first strand buffer 6µl
0.1M DTT 3µl
dNTP mix (10mM each of dATP,dTTP,dGTP and 50µM of dCTP) 1.5µl
áP^{32}dCTP 3µl

The contents of the tubes were mixed gently and incubated at 42ÚC for 2min.
2µl of Superscript was added and incubated for 1hr at 42ÚC. The reaction was terminated by heating at 70ÚC for 15 min.

Probe Purification, Prehybridization and Hybridization

The probe was purified by passing through Sephadex G50 column as described in Sambrook *et.al.,* 1989. The total count of the purified probe was determined using scintillation counter. The purified probe generally had counts more than 10^6 cpm. The blots were prehybridized at 65ÚC in Church phosphate buffer (0.5M Na_2HPO_4 / 1mM EDTA / 7% SDS) for 2-3 hrs in the hybridization incubator. The minimum time for prehybridization was 1 hr and maximum was 8 hrs. For each hybridization 10^6 cpm/ml of probe count was used. To the probe 100µg /ml sheared salmon sperm DNA was added and the mix was denatured in boiling water bath for 15min and chilled on ice for another 10 min before adding to the prehybridization buffer. Hybridization was continued for 18-20 hrs. The blots were washed twice with 2x SSC at room temperature for 10 mins each. Then they were washed in 2x SSC at 42ÚC for 10 mins. Subsequent washes were given at 42ÚC, 50ÚC, 55ÚC, 60ÚC in 1xSSC + 0.1% SDS. The blots were sealed in plastic bags and exposed on imaging plate or XO-MAT AR X-ray film and kept in -70ÚC for 3-4 days.

Plasmid DNA isolation for Sequencing

For high throughput plasmid isolation, Millipore 96 Miniprep Plasmid kit was used.

Sequencing using ABI automated sequencer.
Sequencing of clones was done using ABI automated sequencer. The isolated plasmids were sequenced using M13 reverse primer.

Sequence Analysis

For all sequences obtained, the vector sequence was removed using Vec Screen at NCBI site. The vector removed sequences were further subjected to BLASTN and BLASTX.

RESULTS AND DISCUSSION

Colony PCR was standardized to get uniform yield of PCR products followed by amplification of 2112 clones representing both libraries (1056 clones from each library) for macroarray analyses(Fig1.1). 2112 PCR amplified cDNAs were blotted onto nylon membranes in duplicates. Differential screening was performed using two different probes i.e., one from control plants and the other from plants challenged with *Fusarium*. Total cDNA probes for differential screening was prepared from the total RNA isolated from the roots of control and pathogen infected plants for both the cultivars. Hybridized blots were scanned using Phosphor imager(Fig1.2).

Out of these 1065 clones, plasmid preps were made for 783 clones (Fig1.4) followed by

Fig1.1 Agarose gel showing PCR amplified clones.

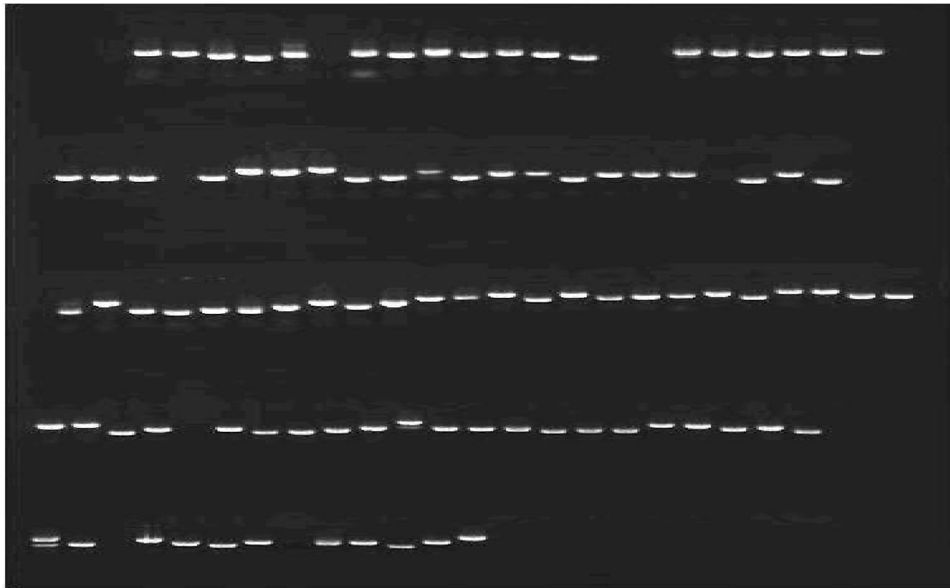


Fig1.2.Output data from Macroarray analysis(A,B PatternImages; C OverlapingImage; D ScatterPlot.

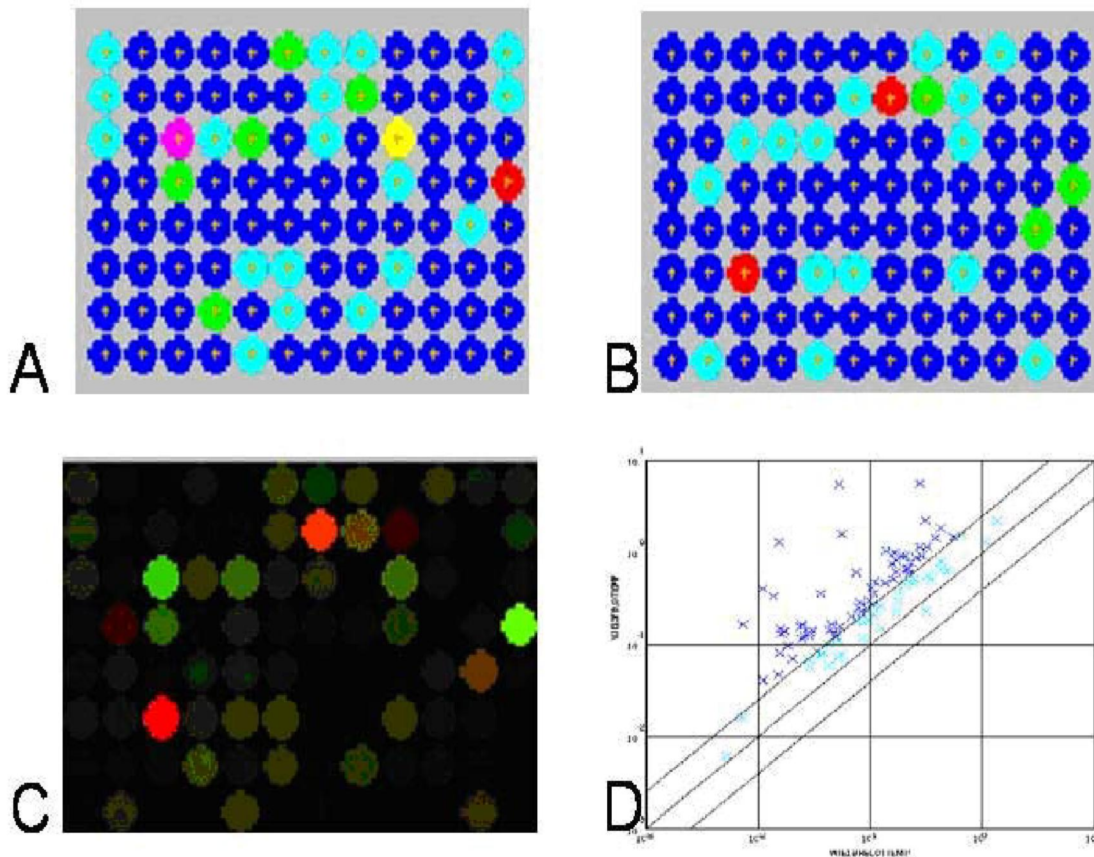


Fig1.3.Overlapping Images Showing Differentially Expressed Genes After Macroarray Analysis A-J: CompatibleLibrary;K-T: IncompatibleLibrary.

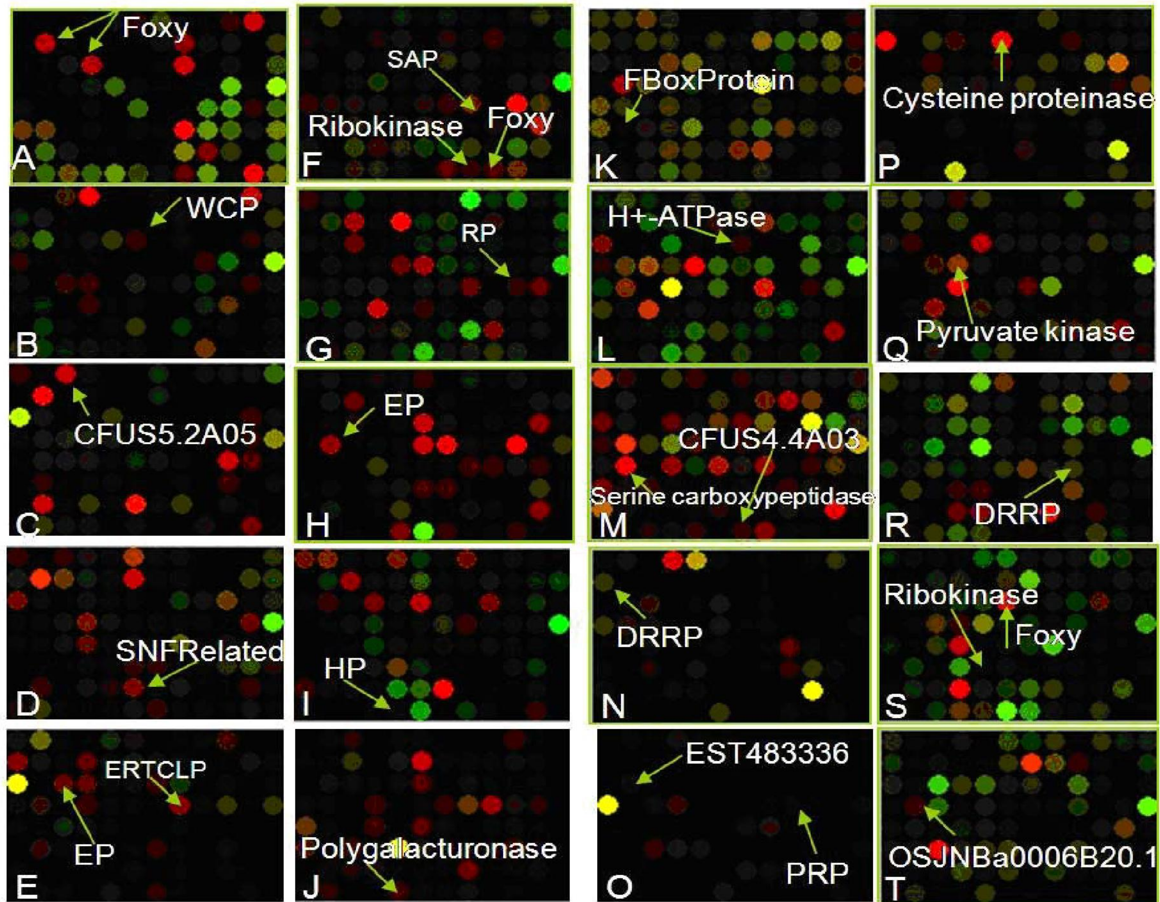
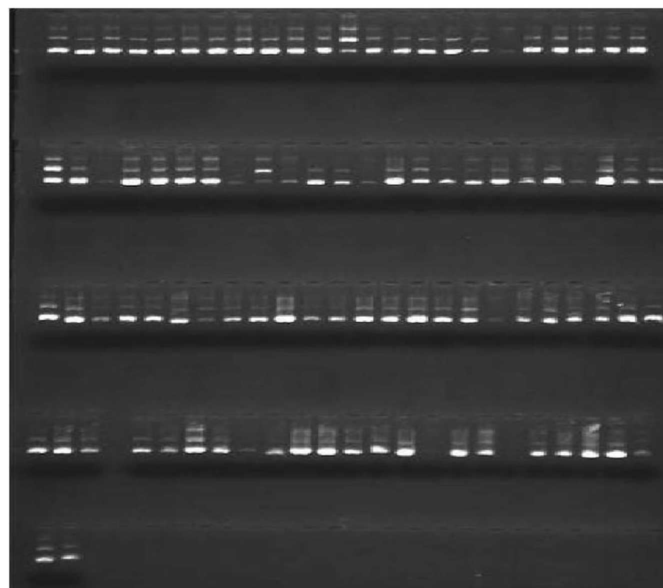


Fig.1.4Plasmid DNA isolated for automated DNA sequencing.



automated DNA sequencing. The sequences were analysed by BLAST programme after removing the vector sequence. A cut off score of 100 was put to find the best matches and the top match was used for all the downstream work. Initially all the sequences were analysed using Blast-X Programme followed by Blast-N and TBlast-x. Sequences showing no significant match after the Blast search were further analysed using Stand alone Blast to remove redundant clones. Some of the interesting genes fall in the class of regulatory proteins, signaling proteins and defense response proteins like bZIP, bHLH, Zn-finger transcriptional activators, kinases, phospahatases, G-protein coupled receptor also the downstream proteins in signal transduction pathway like PR proteins, CHS and CHI.

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