Chapter 2

Differentially expressed gene transcripts in roots of resistant and susceptible chickpea plant (*Cicer arietinum* L.) upon *Fusarium oxysporum* infection

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Abstract

Differentially expressed genes in chickpea, (Cicer arietinum L.) during root infection by Fusarium oxysporum f sp ciceri Race1, were identified using cDNA-RAPD and cDNA-AFLP approaches. The former employed decamer primers on cDNA template and revealed nine differentially expressed transcripts in the resistant infected chickpea cultivar. Among the 2000 transcript-derived fragments (TDFs) screened by cDNA-AFLP, 273 were differentially expressed in chickpea roots during Fusarium infection. Only 13.65% of the TDFs were differentially regulated during pathogen challenge, while the other 86% were expressed non-differentially during the process of pathogen infection in chickpea roots. Nineteen TDFs, which expressed differentially in the resistant infected chickpea cultivar were cloned and sequenced. Two of these TDFs were similar to transcription factors like WRKY proteins and 14-3-3 proteins, while three TDFs represented the NBS-LRR type gene sequences. Two TDFs had sequence identity to genes known to have function in defense. The RAPD TDF CaFRi60 showed sequence identity to gamma-glutamyl-cysteine synthetase. Among the TDFs examined by cDNA-AFLP, 19 were confirmed by reverse northern blotting to be differentially expressed. The data confirms the effectiveness of the cDNA-AFLP technique in detecting differentially expressed genes during pathogenesis.
2.1. Introduction

Plants are constantly exposed to intimate interactions with a plethora of microbes and display a complex set of interactions, which range from symbiosis to disease. The harmful implications of some of these interactions on plant and plant-productivity lead to tremendous annual losses through reduced yields and necessitate the use of chemical fungicides. Pathogen populations dynamically change to remain diverse and stay competent in response to the constant selection pressure from changing agro-ecological conditions. As a result, crop plants are infected and parasitized by pathogens with varying degrees of specificity and severity. Considerable efforts have been directed towards understanding the molecular mechanisms underlying plant-microbe interactions (Hammond-Kosack and Jones 1996; Richter and Ronald 2000). During the initial steps of association, when a plant recognizes a potentially infectious pathogen, local defense responses aid to sequester the pathogen away from non-infected plant tissue. Events of recognition and defense by a host plant to its fungal pathogen and ability of the pathogen to overcome the plant’s defenses implies a complex, dynamic and interactive molecular network. Induction of these molecular responses necessitates up- and down-regulation of numerous but specific genes. Differential large-scale gene expression analysis in plant-pathogen interactions has resulted in identification of several defense-related transcripts (Fernandez et al., 2004; Ros et al., 2004). Direct or indirect role of these transcripts in controlling pathogen invasion to the plant tissue is also demonstrated in few cases. However, these studies are restricted to model plants and few crops such as sugarcane, tomato, coffee, cassava and rice (Durrant et al., 2000; Matsumura et al., 2003; Torres et al., 2003; Carmona et al., 2004; Fernandez et al., 2004; Zhang et al., 2004 and Kemp et al., 2005).

Chickpea (*Cicer arietinum* L.) is the third most important legume in the world and first in India. The seeds of chickpea are major source of dietary protein for human consumption especially for vegetarian population, in several parts of the world. One of the most important diseases affecting chickpeas is Fusarium wilt, caused by the fungus *Fusarium oxysporum f sp ciceri* (FOC). FOC is a soil borne root pathogen, which colonizes the xylem vessels and blocks them completely chocking nutrients/water transport that result into wilting (Bateman et al., 1996). At least four races of FOC are known to exist in India and total of 7 races throughout the world affecting all major chickpea growing areas. Worldwide chickpea yield losses from Fusarium wilt vary from 10 to 15%, however, under conditions favorable to the pathogen, wilt-disease can...
completely destroy standing crops (Halila and Strange 1996). Use of resistant cultivars is one of the most practical and cost-efficient strategies for managing chickpea wilt. However, the efficiency of resistant chickpea cultivars in wilt management is limited by various factors including, (i) pathogenic variability in the natural populations (Jiménez-Gasco et al., 2004) and location specific occurrence of races (Singh and Reddy 1991) which causes resistant cultivar to lose resistance over a period of time, (ii) susceptibility, limits exploitation of useful characters in certain varieties, e.g., double podding trait in JG-62, (iii) existence of specific races, which slows down progress in breeding program (Tekeoglu et al., 2000), (iv) breakdown of resistance, which is a consequence of directional selection for better-adapted mutants, recombinants or immigrants and also by widespread and intense deployment of R genes favored by monoculture practices.

Several studies have demonstrated that infection with *F. oxysporum* leads to various chemical and biochemical changes in chickpea. For example, positive correlation between the exudates/secondary metabolites and resistance in chickpea cultivar by production of antimicrobial microenvironment around newly emerging seedling is reported (Armero et al., 2001; Stevenson et al., 1997). Differential accumulation of chitinase, β-1, 3 glucanase and protease activities in roots of FOC resistant and susceptible chickpea cultivar upon pathogen challenge and antifungal activity of these extracts to FOC were demonstrated in earlier study from my lab (Giri et al., 1998). However, information about genetic factors that determines the outcome of interactions between *F. oxysporum* and chickpea roots are not yet identified. As an initiation towards characterization of the molecular interactions between *C. arietinum* and *Fusarium oxysporum f ssp ciceri* Race1 (FOC1), changes in the transcriptome were examined following FOC1 infection in the roots of resistant and susceptible chickpea cultivar using cDNA-AFLP approach.

The potential of the AFLP technique for generating mRNA fingerprints was first recognized by Bachem et al. (Bachem et al., 1996) for the study of differential gene expression during potato tuber formation. Since then it has been used to profile genes in a range of different systems including humans (Egert et al., 2006) animals (Fukuda et al., 1999; Vandeput et al., 2005) plants (Carmona et al., 2004; Diegoa et al., 2006; Durrant et al., 2000; Kemp et al., 2005; May et al., 1998; Simoes-Araujo et al., 2002 and Yang et al., 2003) and microbes (Decorosi et al., 2005, Dellagi et al., 2000 and Qin et al., 2000). cDNA-AFLP remains a useful technique for several reasons; it is versatile, easy, inexpensive, robust and quantitative (Reijans et al., 2003). In the present study transcript
profiles were generated and compared from three chickpea root cDNA libraries, viz., uninfected WR-315 (WR-C), WR-315 infected with FOC1 (WR-I) and JG-62 infected with FOC1 (JG-I), by subjecting them to cDNA-RAPD and cDNA-AFLP analysis. Differential expression in the identified transcripts was confirmed by reverse northern analysis of 1, 2, 4, 8 and 12 days old chickpea roots, so as to encompass early- and late-post inoculation defense responses. The three-way comparison between resistant-control (RC), resistant-infected (RI) and susceptible-infected (SI) tissues effectively negated any contaminating transcripts of pathogen origin. To the best of my knowledge this is the first demonstration that cDNA libraries can be compared by cDNA-AFLP technique.

2.2. Materials and methods

2.2.1. Chickpea growth conditions and FOC1 inoculation

*C. arietinum* seeds of cultivars Vijay (R), WR-315 (R) and JG-62 (S) were obtained from the Mahatma Phule Krishi Vidhyapeet (MPKV), Rahuri, Maharashtra, India and also from the USDA center at Washington State University, Pullman, USA. For germination, seeds were wrapped in wet sterile muslin cloth and stored at room temperature (24-26°C) in dark for 3-4 days till sprouting. While the seeds sprouted the trays and floats were made ready. The Styrofoam sheets were cut to a size that they fitted into trays, and holes were punched into the Styrofoam sheets using a cork borer in a square lattice so as to accommodate around 60 seeds. Then the sprouted seeds were transferred onto Styrofoam floats placing each sprouted seed into the holes punched earlier, and these floats were placed in the glass trays containing water and growth media and kept in controlled conditions at 22°C and 60% relative humidity under white light and normal day conditions (14 h light/10 h dark). Seedlings were grown hydroponically under sterile conditions on floats in sterile water containing macro- and micro- nutrients (half strength Hoagland’s nutrient medium, (Hoagland and Arnon 1950)).

Plants were 7 days old at the time of pathogen infection. Freshly prepared spore suspension (10 ml of 1x10⁶ spores/ml) of *Fusarium oxysporum* f.sp. *ciceri*, race 1 (FOC1) was added to the sterile hydroponic trays. After two days the water in the trays was mixed with a sterile glass rod to ensure uniform spread of the fungus. A few seeds of JG-62 (S) were sown in each tray as an indicator of infection. Seedlings grown in similar trays with no pathogen served as an uninfected plant control.

Pathogen-inoculated seedlings were removed from the floats in hydroponic trays, quickly rinsed to free the adhering fungal mycelia with sterile DEPC treated water, frozen in liquid nitrogen and stored at –80 °C till further use. Samples were collected in
duplicates for WR315, Vijay and JG-62 after 1, 2, 4, 8, 12, 16 and 20 DAI (days after infection).

2.2.2. cDNA libraries and template preparation:
Chickpea root cDNA libraries were constructed from FOC1 challenged -JG-62 (SI) and -WR315 (RI) as well as uninfected WR315 (RC). Total RNA was isolated from the root tissues of *C. arietinum* as described in section 2.2.6. Poly (A+) RNA was purified by chromatography on oligo (dT)-cellulose (Qiagen, USA) and 5 μg of the resulting mRNA was utilized to construct a cDNA library using a λ ZAP II-cDNA synthesis kit and ZAP-cDNA gigapack III gold packaging kit (Stratagene, USA) following the manufacturer’s instructions. For each library, equal amount of RNA from root tissues was pooled after 1, 2, 4, 8, 12, 16 and 20 days of infection. DNA from the phage libraries was isolated by boiling 1 ml aliquot of the library (titer of $10^8$ PFU/ml) for 5 min to denature the phages, extracted once with phenol-chloroform, precipitated with ethanol and used as template for cDNA-RAPD and cDNA-AFLP. Alternatively, cDNA inserts from these libraries were amplified using the flanking T3 and T7 promoter primers using approximately 30 ng of cDNA template. The amplification products of at least five independent PCR reactions were pooled and used for cDNA-RAPD and cDNA-AFLP.

2.2.3. RAPD primer screening with cDNA templates
A survey of differentially regulated transcripts, during pathogen infection in chickpea roots, using 200 unique deca-nucleotide RAPD primers (UBC, University of British Columbia, USA), were used to identify differentially expressed transcripts in wilt-resistant reactions. For RAPD analysis the amplified cDNA libraries from infected JG-62, infected WR315 and uninfected WR315 (control) were used as templates. Amplification was carried out in 20 μl reaction volume containing: 20 ng of chickpea root cDNA, buffer (50 mM KCl, 10 mM Tris-HCl pH-8.3, 0.1% Triton X-100), 1.5 mM MgCl$_2$, 10 mM of each dNTP, 20 pM of primer and 0.6 units of Taq DNA polymerase (Promega, Madison, WI, USA), in a Peltier Thermal Cycler DNA Engine (MJ Research, USA), programmed for 34 cycles with the following temperature profile: 30 sec at 94 °C, 1 min at 35 °C, 1.30 min at 72 °C. Cycling was concluded with a final extension at 72 °C for 5 min. PCR amplification products were electrophoresed in 1% agarose, 1X TAE (Tris-Acetate/EDTA buffer) gels, visualized by ethidium bromide staining under UV illumination.

2.2.4. cDNA-AFLP
cDNA-AFLP was performed as described by Bachem *et al.* (1996) with minor modifications. The amplified cDNA (250 ng) from the three libraries previously
described in section 2.2.2 was purified by precipitation with iso-propanol and washed with 70% ethanol. The cDNA was then digested with the restriction endonucleases EcoRI and MseI and ligated to double stranded EcoRI and MseI adapters. Pre-amplification was carried out with ‘E’ and ‘M’ primers corresponding to the EcoRI and MseI adapters with one selective base using a standard pre-amplification PCR program (30 cycles of 30s at 94°C, 30s at 52°C, and 60s at 72°C). A 1:50 (v/v) dilution of the pre-amplification product was selectively amplified with three corresponding specific base extensions at the 3’ end of the primers E and M using a standard AFLP touchdown-selective amplification program initial 12 cycles of 94°C for 30 sec; 65 – 56°C (decrease 0.7°C each cycle) for 30 sec; 72°C for 60sec; followed by 24 cycles of 94°C for 30 sec; 56°C for 30 sec; 72°C for 60 sec; (Bachem et al., 1996) and according to manufacturer’s instructions (GIBCO-BRL Life Technologies, USA). A total of sixteen such primer combinations were used for the selective amplification, the products of which were separated on a 6% denaturing polyacrylamide gel run at 1500 V and 100 W, for 3 hrs. Amplified fragments were visualized by staining the gels with silver nitrate (Sanguinetti et al., 1994). Comparison of the fingerprints obtained from duplicate PCR reactions of 8 primer-combination subsets assessed the reproducibility of the technique. In addition, to assess the reproducibility of the electrophoresis, aliquots of several amplification reactions were run on separate gels.

2.2.5. Cloning of DNA fragments and sequence analysis

**Elution and reamplification of differentially expressed bands**

DNA fragments showing differential patterns were excised from the gels and eluted in 100 μl double distilled H2O at 37°C, overnight. The eluted DNA samples were then used as templates for PCR reamplification, using 2 μl of the eluted product in a 20 μl PCR reaction containing - buffer (50 mM KCl, 10 mM Tris-HCl pH-8.3, 0.1% Triton X-100), 1.5 mM MgCl2, 10 mM of each dNTP, 20 pM of primer (the same primer set of each specific combination used in selective amplification) and 0.6 units of Taq DNA polymerase (Promega, USA), in a Peltier Thermal Cycler DNA Engine (MJ Research, USA).

The cycling conditions were same as in selective amplification but instead of touch down temperature a constant temperature of 56 °C was used. The successful reamplification of the excised DNA was verified in a 1 % (w/v) agarose gel in 1X TAE-buffer. Ethidium bromide was added to the buffer and gel to a final concentration of 0.5 mg/ml. The samples were then loaded on an agarose gel and electrophoresed for approximately 1 hr at 10 V/cm. The gels were visualized on a UV transilluminator and
the size of the bands determined by comparison to a 100 bp ladder (Bangalore Genei, India). Reamplified bands were then excised from the agarose gel and eluted using DNA-elute spin columns (Millipore, USA). Purified DNA was adenosine (A) tailed at the 3’ terminal in a reaction containing 0.5 μl PCR buffer with MgCl₂, 0.2 mM dATP and 0.6 units of Taq DNA polymerase (Promega, USA) in a final reaction volume of 5 μl and incubated at 72 °C for 30 min.

**Ligation into pGEMT**

Subsequently the DNA fragments were cloned into pGEM-T easy (Promega, USA) in an optimized insert to vector ratio of 3:1. The ligation was carried out in a 10 μl reaction volume containing 150ng DNA insert, 50 ng of linearized vector (pGEMT-easy), 3U of T4 DNA ligase in 5 μl 2X rapid ligation buffer and incubated at 4 °C overnight.

**Transformation**

Chemically (CaCl₂) competent *Escherichia coli* α-DH5 cells were prepared for transformation (Sambrook *et al*., 1989). Competent cells were mixed with plasmid DNA (100pg-100ng) and incubated on ice for 15 min. After a 2-min heat shock at 42 °C, 1 ml Luria bertani (LB) medium was added to the transformation mixture and incubated for 90 min at 37 °C. 100 μl aliquot of the mixture was plated on LBA-plates containing ampicillin 100 mg/ml, X-gal 20 mg/ml in DMF (dimethlyformamide), IPTG 0.2 g/ml in H₂O (for selection of transformed cells) and incubated for 16 h at 37 °C.

** Colony screening and plasmid isolation**

For verification of inserts single white colonies were picked with a sterile toothpick and colony PCR performed. Five independent colonies per fragment were amplified by PCR using T7 and SP6 primers and two clones per band were selected for sequencing. Plasmid DNA was isolated by the alkaline-lysis method (Sambrook *et al*., 1989). The cell pellet from 5 ml of overnight culture was suspended in 300 μl of cold solution I (100 μg/ml RNAse A, 50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) and cells were lysed by incubation with 300 μl of solution II (20 mM NaOH, 1% SDS) at room temperature for 5 min. Chromosomal DNA and cell wall components were removed by addition of 300 μl of ice-cold solution III (2.5 M potassium acetate, pH 4.8) to the lysate and centrifuged. The plasmid DNA was precipitated from the aqueous phase with 0.7 volumes of isopropanol at room temperature for 30 min and subsequently centrifuged. The DNA pellet was washed with ice-cold 70% ethanol, dried and dissolved in 30 μl TE-buffer. Typically 3-4 μg of this DNA was used for restriction analysis. Plasmid preparations were purified using the Wizard plus plasmid Preps DNA Cleanup System.
(Promega, USA) according to the manufacturer’s instructions to obtain high purity plasmids DNA (for sequencing reaction and ligation reactions).

**Sequencing**

The nucleotide sequences of the cloned fragments were determined with a MegaBACE 500 (Amersham BioSciences, USA). One μl (200 ng) of the DNA solution was used for sequencing using the DYEnamic ET Dye Terminator Sequencing Kit (Amersham Biosciences, USA) in an automated Fluorescent DNA Sequence Analyzer, MegaBACE (Amersham Biosciences, USA). The DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE is based on traditional dideoxynucleotide chain termination chemistry (Sanger *et al.*, 1977). All reactions were performed according to the manufacturer's instructions and cycle sequenced in a Peltier Thermal Cycler DNA Engine (MJ Research, USA). Primers used were T7 and SP6 Sequencing Primers. The 20mer T7 forward (5 mM) and 19mer SP6 reverse primers (5 mM) had the following sequences, respectively: Forward: 5’-TAA TAC GAC TCA CTA TAG GG-3’ Reverse: 5’-TAT TTA GGT GAC ACT ATA G-3’. Sequences were analyzed with the GenBank database using BLASTn algorithms (Altschul *et al.*, 1997).

2.2.6. Northern and reverse northern blot analysis

**RNA extraction**

Total RNA was extracted from root samples collected at different time intervals such as 1, 2, 4, 8 and 12 days after infection (DAI) using the TRIzol reagent (Invitrogen, USA) as described by the manufacturer. *C. arietinum* root tissue (100 mg) was pulverized in liquid nitrogen with autoclaved pestle and mortar, and transferred to 1 ml of TRIZol reagent. After vortexing, the lysate was stored for 5 min at room temperature and 0.2 ml chloroform was added. The mixture was shaken vigorously for 15 s and stored at room temperature for 10 min before centrifugation at 4 °C for 15 min at 12,000 g. The aqueous phase was transferred to a new tube and 0.5 ml isopropanol was added to precipitate RNA. The sample was stored at room temperature for 10 min and centrifuged at 15,000 g for 10 min at 4 °C. The RNA pellet was retained and washed with 1 ml 75 % ethanol, pelleted by centrifugation, air-dried, and dissolved in 30 μl RNase-free water. To remove contaminating DNA, the total RNA (10μg) was treated with RNase free DNaseI (0.1 U per μg RNA) at 37°C for 1 h in the presence of RNasin (0.4U) and terminated by heating at 65°C for 15 min. The RNA was precipitated with 0.1 volumes of 3 M sodium acetate buffer, pH 5.2 and 3 volumes of absolute ethanol at -70°C for 1 h. The RNA pellet was collected by centrifugation at 12,000 g for 10 min at 4°C, dried under vacuum, and
resuspended in 5 μl of DEPC-treated water. The RNA samples were quantified by spectrophotometry at 260 and 280 nm or stored at –80°C until used.

**Electrophoresis and blotting for northern**
For Northern analysis, 10 μg of total RNA from each sample; uninfected JG-62 (SC), JG-62 infected with FOC1 (SI), uninfected Vijay (RC) and Vijay infected with FOC1 (RI) at 2 and 8 DAI time intervals were subjected to electrophoresis in formaldehyde-containing 1.5% agarose gels as described by Sambrook *et al.* (1989). The samples were electrophoresed on a 1.5 % agarose gel containing 0.22 M formaldehyde and 1X MOPS buffer, pH 7.0 following standard procedures (Sambrook *et al*., 1989). The 1X MOPS buffer consists of 40 mM MOPS, pH 7.0, 10 mM sodium acetate and 1 mM EDTA. The agarose was boiled in DEPC-treated water and added to a preheated mixture of formaldehyde and 1X MOPS buffer. The solution was poured into a tray with a comb positioned appropriately under a hood, so as to prevent exposure to the dangerous formaldehyde fumes. The gel was allowed to set for 1 hr and then transferred to the electrophoresis tank containing the running buffer 1X MOPS, pH 7.0. The RNA samples (10 μg/lane) were dissolved in 0.5X MOPS, 0.22 M formaldehyde, 50% formamide, and 0.02 mg/ml ethidium bromide and denatured at 65°C for 15 min prior to loading in order to resolve the secondary structures. The samples were mixed with 0.1 volumes loading buffer (1 mM EDTA, pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol in H₂O) and loaded into the wells of the submerged gel. Electrophoresis was carried out at 100 V for 2 h at room temperature.

After the run, the entire gel was soaked sequentially in 200 ml RNase-free water for 15 min, 50 mM NaOH for 15 min and finally neutralized in10X SSPE for 30 min. Hybond N+ membrane (Amersham, USA) was cut to the size of the gel and pre-equilibrated in 1X MOPS for 15 min. RNA from the gel was transferred to the membrane under constant current (1 mA/cm²) in an electroblotting apparatus (Amersham, USA) for 2h. After the transfer, the position of the wells and the rRNA subunits were marked on the blot using a pencil. The blot was rinsed in 4x SSPE and the transferred RNA was cross-linked to the membrane under ultraviolet irradiation (70,000 μJ/cm²). After cross-linking, the membrane was baked at 80°C in a vacuum oven for 2h and stored in re-sealable polythene bags at 4°C until required (Sambrook *et al*, 1989).

**Probe preparation and hybridization**
The probe was prepared using the previously described (Section 2.2.5) DH5-α transformants carrying the TDFs. The construct was amplified by a polymerase chain
reaction using previously described T7 and SP6 oligonucleotide primers. \( \alpha P^{32}\)-dATP was incorporated in the PCR mix so as to yield a radio-labeled double stranded DNA fragment for use as a probe. Hybridization of all northern blots was carried out with four different probes thus generated using a commercial Express-Hyb solution (Clontech, USA) as per the manufacturers’ instructions; initial prehybridization, 50°C, 4h; probe hybridization, 65°C, 4h. The blots were washed thrice for 20 min in wash solution (1X SSC, 0.1% SDS at 55°C) and exposed to X-ray films (Konica, India).

**Reverse northern**

To prepare the slot blot arrays, TDFs cloned in pGEM-T easy plasmid were amplified using T7 and SP6 primers and quantified by UV spectrophotometer (Varian, USA). The HYBRI-SLOT manifold (BRL Life Technologies, Inc., USA) was arranged according to the manufacturer’s instructions, 2.5 µg DNA was denatured under 0.6M NaOH, to a volume of 20 µl and spotted on Hybond-N+ membrane crosslinked under UV illumination (UV Crosslinker, Amersham Life Sciences, USA) at 70,000 µJ/cm². Four identical filters were prepared serially, which were hybridized separately with labeled cDNA made from each of the source RNAs; uninfected JG-62 (SC), JG-62 infected with FOC1 (SI), uninfected Vijay (RC) and Vijay infected with FOC1 (RI) at different time intervals as detailed above. Alternatively, the same filter was stripped and re-hybridized with labeled cDNA from different time intervals of DAI.

**cDNA Probe preparation and hybridization**

The cDNA probes were prepared by using 5 µg of total RNA from four different samples (SC, SI, RC and RI) extracted at different time intervals. \( \alpha P^{32}\) labeled dATP was used to synthesize radiolabeled first strand cDNA using an oligo dT-18-primer and PowerScript-III reverse transcriptase (Clontech, USA) (Sambrook et al., 1989) and used to hybridize the arrays, as described in the earlier section.

**2.3. Results**

**2.3.1. Morphological changes**

Hydroponic plants on inoculation with FOC1 were observed for disease symptoms at different time intervals. The JG-62 (S) seedlings inoculated with FOC1 started developing a distinct yellow coloration at 5 DAI as compared to the uninfected healthy seedlings. At 20-30 DAI the JG-62 (S) plants showed complete wilting while the Vijay and WR-315 (R) plants along with uninfected JG-62 (S) showed normal healthy growth (Fig 2.1). In the present experiments the roots of 2 and 8 DAI were selected for analysis to isolate early and late expressing genes involved in chickpea root wilting defense. Under field conditions the root system of chickpea is robust, up to 2 m deep, with major
mass up to 60 cm. It was observed that the total root length was similar in susceptible and resistant cultivars in the uninoculated controls when observed after 20 days, which became markedly smaller and weaker in susceptible cultivar, after inoculation with FOC1 at the same time. However, in the resistant cultivar inoculation with FOC1 increased lateral root branching, which were longer and more in number (Fig 2.1). Such long lateral root branches were not observed in the susceptible inoculated plants, in which the whole root system appeared dark brown and dead.

2.3.2. cDNA-RAPD analysis

Amplification patterns of 200 RAPD primers (decamer) from UBC were studied with all the 3 cDNA libraries. Representative amplification patterns are shown in Figure 2.2. Seven TDFs, ranging in length from 260 to 650 bases were cloned, sequenced and BLASTed (Table 2.1). However, one of the seven TDFs showed none or only poor sequence similarity to any database entries hence no function could be assigned to it. It may represent a novel transcript involved in pathogen recognition, plant defense reaction and resistance. Six TDFs showed high similarity to cDNA clones from other legumes, *Medicago truncatula* and *Lotus corniculatus*. Of these, one TDF CaFRi60 showed homology to gamma-glutamyl-cystiene synthetase, which is a key enzyme in glutathione production, and known to be present in increased levels during the oxidative stress when plants are subjected to biotic or abiotic stresses (Matamoros *et al.*, 1999; May *et al.*, 1998).

2.3.3. cDNA-AFLP analysis

A total of 16 different primer combinations (25% of the total possible 64 combinations, using 3 bp extension adapters) were used on three templates to determine cDNA expression profile. The cDNA-AFLP fingerprints from three samples generated more than 2000 transcripts (averaging 130 TDFs resulting from each primer combination, Fig 2.3), which were inspected for differential expression. The TDFs were categorized into five classes as shown in Fig 2.4 and Table 2.2. A large number (86%) of transcripts were expressed in all the three samples, thus representing constitutively expressed genes in both the chickpea varieties with or without infection. The majority of differentially expressed TDFs were either up regulated or differentially expressed in resistant infected (45%), followed by TDFs up regulated or differentially expressed in susceptible infected (28%) and the lowest number of TDFs up-regulated were observed in resistant uninfected (26%) (Table 2.2). Of the sixteen primer combinations only the differentially expressed TDFs were analyzed. Several TDFs displaying an altered expression pattern in response to pathogen attack were selected for further analysis. A total of 40 differentially
accumulated TDFs from RI, ranging in length from 90 to 400 bp, were recovered from the polyacrylamide gels, reamplified and 30 of them could be successfully cloned and sequence characterized. After omitting the redundant sequences, 19 TDF sequences were submitted to NCBI as collection of ESTs and their Accession numbers are listed in Table 2.3.

2.3.4. Identification of AFLP-TDFs of known genes induced during infection:
In this study attention was focused on genes, which were differentially expressed or up regulated in the resistant infected (RI) cultivar. Among nineteen TDFs being differentially expressed during fungal infection of the resistant chickpea cultivar Vijay, ten corresponded to previously annotated protein encoding genes (Table 2.3), some of which are reported to have a potential role in defense responses, and could be grouped according to putative function (Table 2.3). CaFRi3 is differentially expressed in resistant infected (RI) with high similarity to WRKY, a well-characterized transcription factor involved in defense responses. CaFRi4 showed induced expression in RI and has homology with a 14-3-3 protein from *Pisum sativum*. 14-3-3 proteins are known to accumulate in barley leaves in response to inoculation with *Blumeria graminis* (Collinge *et al.*, 1997; Gregersen *et al.*, 1997). CaFRi9, CaFRi11 and CaFRi26 showed homology to a gene similar to NBS-LRR protein from *Ageilops tauschii*. NBS–LRR proteins are predominant class of plant defense related proteins and are known to confer resistance against many plant pathogens. A sequence encoding 60S ribosomal protein L10 was obtained in clone CaFRi42 as differentially expressed in resistant cultivar during infection. Clone CaFRi36, was similar to a mitochondrial F1 ATPase. Another clone, CaFRi51, represented a fragment of plasma intrinsic protein (pip-2 gene), which is an aquaporin located in the plasma membrane.

The homology search of the sequences of clones CaFRi12, CaFRi15 and CaFRi20 indicated their identity as transposable elements. Sequences of clones CaFRi12 and CaFRi15 were found to be similar with non-LTR retrotransposon and GAG-POL precursor gene, respectively, while clone CaFRi20 was similar to a Ty-1 copia type retroelement sequence (Table 2.3).

2.3.5. AFLP-TDFs of unknown identity induced during infection
Among the TDFs characterized, three corresponded to different ESTs reported in the databases but could not be associated with any genes described in the GenBank (Table 2.3). Two TDFs, CaFRi39 and CaFRi48 represented sequences from the wheat EST, Acc. Nos. CA681381 challenged by *E. graminis* and BJ221482, respectively, while
clone CaFRi2 was similar to a sequence in *Pinus*, which was also induced. Three other TDFs, CaFRi1, CaFRi23 and CaFRi30 did not yield any identity matches with either known gene sequences or ESTs. Though these transcripts could not be annotated, they remain positively associated to defense response of chickpea to FOC1 infection.

**2.3.6. Gene expression analysis of identified TDFs by reverse northern analysis and northern blot analysis**

Reverse northern blot hybridization is routinely employed to confirm differential gene expression of many transcripts in parallel that requires only a few micrograms of the source RNA pool. Reverse Northern blots represented in Fig. 2.5 show expression of 19 TDFs. Fig. 2.5a, shows TDFs similar to genes having established role in defense; for example, WRKY, 14-3-3 protein, NBS-LRR, chitinase and hydrolase. These genes were found to be induced in the resistant infected chickpea samples. TDFs CaFRi3, 4, 9, 11 and 33 showed a similar pattern, with increased accumulation of the transcripts at 2 DAI, followed by a substantial decrease at 8 DAI. Clones CaFRi26 and 28 were homologous to NBS-LRR and hydrolase and exhibited higher levels of transcripts even up to 8DAI. TDFs CaFRi12 and CaFRi15 both, although denote non-LTR type of retroelements, CaFRi12 was found to be expressing higher at 8 DAI as compared to CaFRi15 (Fig 2.5b). CaFRi20, a Ty-1 copia-type retrotransposon sequence, had its transcript signal decreased considerably at 8 DAI as compared to that at 2 DAI. CaFRi36 and 42 had homology to mitochondrial F1 ATPase and ribosomal protein and their transcript signals in the resistant infected cultivar at 2 DAI were higher as compared to 8 DAI (Fig 2.5c). CaFRi51 was similar to plasma intrinsic protein like sequence and showed increased transcript signal in the susceptible infected cultivar JG-62 at 2DAI; this was puzzling considering the fact that it was isolated as being up-regulated in resistant infected cultivar. TDFs CaFRi39, 2 and 48 were similar to other ESTs in the database and showed enhanced transcript signal in RI at 2 DAI (Fig 2.5d). CaFRi1, 23 and 30 with no similarities to any of the sequences in the GenBank database showed a similar expression pattern with higher transcript accumulation at 2 DAI in the resistant infected cultivar (Fig 2.5e). The expression of the seven TDFs from cDNA-RAPD was also confirmed using reverse northern (Fig 2.5f). The induced expression was observed in the resistant sample 2 days after challenge with FOC1. The transcript levels of CaFRi56 and CaFRi62 were detected even at 8 DAI though they were lower than the transcript levels at 2 DAI.
Figure 2.1: a) Chickpea seedlings hydroponically growing in growth chamber; b) JG-62 seedling showing wilting symptoms after infection with FOC1 while Vijay seedlings are healthy after infection; c) Root morphology of JG-62 and Vijay after infection; d) Difference between infected roots of Vijay covered with fungal mycelial mass and non-infected roots without any fungal mycelia.
Figure 2.2: Representative amplification profiles generated by RAPD primers. Templates were cDNA libraries, RC (lanes 1), RI (lanes 2) and SI (lanes 3). The upper number indicates the primer number used for amplification for the set of three cDNA libraries. Arrows indicate DNA fragments differentially detected in roots of resistant infected chickpea cultivar RI (WR315).

Table 2.1: Summary of the transcript-derived fragments (TDFs) clones identified by cDNA-RAPD, containing sequences induced during infection. The nucleotide-homology of the TDFs with sequences in the database using BLASTn algorithm

<table>
<thead>
<tr>
<th>Clone Id</th>
<th>GB Accession</th>
<th>Length (bp)</th>
<th>Homology</th>
<th>e-Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaFRi60</td>
<td>DR749500</td>
<td>650</td>
<td>Gamma-glutamylcysteine synthetase mRNA</td>
<td>1e-147</td>
</tr>
<tr>
<td>CaFRi58</td>
<td>DR749499</td>
<td>266</td>
<td>G max cDNA clone Gm-r1083-4905 [gi 3941321]</td>
<td>1e-20</td>
</tr>
<tr>
<td>CaFRi62</td>
<td>DR749501</td>
<td>516</td>
<td>M truncatula clone pGLSD-33B19 [gi20175779]</td>
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</tr>
<tr>
<td>CaFRi65</td>
<td>DR749502</td>
<td>312</td>
<td>M truncatula clone pHOGA-7H14 [gi 13781558]</td>
<td>1e-27</td>
</tr>
<tr>
<td>CaFRi67</td>
<td>DR749503</td>
<td>478</td>
<td>M truncatula clone MTUS-15C7 [gi 33105673]</td>
<td>4e-62</td>
</tr>
<tr>
<td>CaFRi70</td>
<td>DR749504</td>
<td>519</td>
<td>L corniculatus clone SPD012c01_f [gi 45578828]</td>
<td>8e-107</td>
</tr>
</tbody>
</table>

TDF not similar to any sequences in the GenBank
CaFRi56 | DR749498 | 379 | No match | - |
Figure 2.3: Representative amplification pattern from three cDNA libraries, resistant control (RC), resistant infected (RI) and susceptible infected (SI), displayed by cDNA-AFLP visualized on 6% polyacrylamide gel by silver staining. Templates were cDNA libraries, RC (lanes 1), RI (lanes 2) and SI (lanes 3). A) Transcripts differentially expressed in RI, B) Transcripts up-regulated in RI, C) Transcripts differentially expressed in SI, D) Transcripts up-regulated in SI and E) Transcripts differentially expressed in RC.
Figure 2.4: TDFs identified after differential display were categorized into five classes and are schematically represented as the three circles representing the three libraries under study Green- Resistant infected; Red- Susceptible infected; Blue- Resistant control, and the area under the overlap denotes the different classes detailed in Table 2.2. The area under the triangle represents the non differentially expressing TDFs. RI- resistant infected with FOC1, RC- resistant control (not infected) and SI- susceptible infected with FOC1. A, B, C, D and E are as described in Table 2.2.

Table 2.2: Differentially expressing TDFs identified after differential displays were categorized into five classes A, B, C, D and E; are described here and are schematically represented in Fig 2.4. RI- resistant infected with FOC1, RC- resistant control (not infected) and SI- susceptible infected with FOC1.

<table>
<thead>
<tr>
<th>Class</th>
<th>Origin of TDFs</th>
<th>No. of TDFs</th>
<th>Remarks/Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Bands seen only in the resistant infected (RI) sample</td>
<td>78</td>
<td>Represents genes/transcripts differentially expressed in resistant cultivar during pathogen infection.</td>
</tr>
<tr>
<td>B</td>
<td>Bands of more intensity in the RI sample</td>
<td>46</td>
<td>Represents genes/transcripts which are up regulated in resistant cultivar during pathogen infection</td>
</tr>
<tr>
<td>C</td>
<td>Bands seen only in the susceptible infected (SI) sample</td>
<td>52</td>
<td>Represents genes/transcripts differentially expressed in susceptible cultivar during pathogen infection</td>
</tr>
<tr>
<td>D</td>
<td>Bands of more intensity in the SI sample</td>
<td>25</td>
<td>Represents genes/transcripts being up regulated in susceptible cultivar during pathogen infection</td>
</tr>
<tr>
<td>E</td>
<td>Bands seen only in the resistant control (RC) sample or bands of more intensity in the RC</td>
<td>72</td>
<td>Represents genes/transcripts differentially expressed or up regulated in the resistant cultivar under no pathogen stress.</td>
</tr>
<tr>
<td><strong>Total Differentially Expressed TDFs</strong></td>
<td><strong>273</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
To validate the cDNA-AFLP and to reconfirm reverse northern expression patterns, four TDFs encoding different classes of proteins were analyzed by traditional Northern blot hybridization and kinetics of their transcript accumulation in response to pathogen challenge is shown in Fig. 2.6. The induction pattern observed in northern analysis showed that all the four TDFs tested (CaFRi4, CaFRi3, CaFRi9 and CaFRi11) were in conformity with the expression profiles observed with the cDNA-AFLP and reverse northern analysis.

2.4. Discussion

2.4.1. Application of cDNA -RAPD and -AFLP for isolation of differentially expressed transcripts in chickpea roots

The transcript profiles were compared from three cDNA libraries by cDNA-AFLP and cDNA-RAPD to successfully isolate transcripts either differentially expressed or up-regulated in resistant chickpea cultivar challenged by FOC1. The differentially expressed bands were classified depending on their origin and nature of expression into five categories as shown in Table 2.2. DNA fragments that were differentially expressed or up-regulated in resistant cultivar challenged by FOC1 (class A and B) are presumably contributing to the resistance mechanism and were preferentially cloned and sequenced.

A total of 1200 amplified fragments from cDNA-RAPD could identify 7 TDFs of class A and B. On the other hand, cDNA-AFLP experiments allowed me to survey more transcripts generated during the chickpea root infection by FOC1. A total of 273 TDFs (13.65% of all the TDFs generated), showing differential expressions, were identified from approximately 2000 TDFs generated using cDNA-AFLP. Of these 273 differentially expressed TDFs, 78 and 46 TDFs were identified as being differentially expressed (class A) and up-regulated (class B), respectively, in the roots of the resistant infected chickpea cultivar. A total of 77 TDFs (28.20% of the total differentially expressed TDFs) were identified from the susceptible cultivar, JG-62 of which 52 were differentially expressed (group C) and 25 were up-regulated (group D). The transcripts from these two groups represent the genes that are induced in the susceptible cultivar upon pathogen challenge. However, they might also represent transcripts derived from the pathogen considering intense disease progression and rampant pathogen growth. From the uninfected resistant seedlings, 72 TDFs were identified showing differential expression (TDFs in group E). The transcripts from this category would also represent genes that are either silenced or down-regulated in the resistant cultivar during pathogen challenge.
Table 2.3: Summary of the transcript-derived fragments (TDFs) clones identified by cDNA-AFLP, containing sequences induced during infection. The nucleotide-homology of the TDFs with sequences in the database using BLASTn algorithm

<table>
<thead>
<tr>
<th>Clone Id</th>
<th>GB Accession</th>
<th>Length (bp)</th>
<th>Homology</th>
<th>e-Score</th>
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<tr>
<td>CaFRi3</td>
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<td>300</td>
<td>WRKY protein</td>
<td>8e-05*</td>
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<td>14-3-3 protein</td>
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<td>CaFRi9</td>
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<td>228</td>
<td>NBS-LRR</td>
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<tr>
<td>CaFRi11</td>
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<td>286</td>
<td>NBS-LRR</td>
<td>3e-07</td>
</tr>
<tr>
<td>CaFRi26</td>
<td>DR749481</td>
<td>229</td>
<td>NBS-LRR</td>
<td>1e-33</td>
</tr>
<tr>
<td>CaFRi28</td>
<td>DR749482</td>
<td>201</td>
<td>Hydrolase alpha/beta fold family protein</td>
<td>6e-06</td>
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<tr>
<td>CaFRi33</td>
<td>DR749484</td>
<td>145</td>
<td>Class III chitinase</td>
<td>9e-08</td>
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**TDFs similar to Organelle genes**

<table>
<thead>
<tr>
<th>Clone Id</th>
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<th>Length (bp)</th>
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<tbody>
<tr>
<td>CaFRi36</td>
<td>DR749485</td>
<td>103</td>
<td>Mitochondrial F1 ATPase</td>
<td>1e-09</td>
</tr>
<tr>
<td>CaFRi42</td>
<td>DR749487</td>
<td>155</td>
<td>60S ribosomal protien L10</td>
<td>2e-09</td>
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<tr>
<td>CaFRi51</td>
<td>DR749489</td>
<td>88</td>
<td>Plasma Intrinsic protein [pip-2 gene]</td>
<td>3e-06</td>
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</table>

**TDFs similar to Retroelements**

<table>
<thead>
<tr>
<th>Clone Id</th>
<th>GB Accession</th>
<th>Length (bp)</th>
<th>Homology</th>
<th>e-Score</th>
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</thead>
<tbody>
<tr>
<td>CaFRi12</td>
<td>DR749493</td>
<td>400</td>
<td>Non-LTR retroelement</td>
<td>2e-05</td>
</tr>
<tr>
<td>CaFRi15</td>
<td>DR749496</td>
<td>206</td>
<td>GAG-POL precursor gene</td>
<td>8e-11</td>
</tr>
<tr>
<td>CaFRi20</td>
<td>DR749479</td>
<td>170</td>
<td>Ty-1 copia retrotransposon</td>
<td>1e-76</td>
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</table>

**TDFs similar to known ESTs**

<table>
<thead>
<tr>
<th>Clone Id</th>
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<th>Length (bp)</th>
<th>Homology</th>
<th>e-Score</th>
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<tbody>
<tr>
<td>CaFRi39</td>
<td>DR749486</td>
<td>273</td>
<td>cDNA clone wlm24.pk0020.e10</td>
<td>1e-70</td>
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<tr>
<td>CaFRi2</td>
<td>DR749490</td>
<td>305</td>
<td>pinus induced compression wood</td>
<td>2e-04</td>
</tr>
<tr>
<td>CaFRi48</td>
<td>DR749488</td>
<td>284</td>
<td>cDNA clone wh25g01</td>
<td>1e-41</td>
</tr>
</tbody>
</table>

**TDFs not similar to any sequences in the GenBank**

<table>
<thead>
<tr>
<th>Clone Id</th>
<th>GB Accession</th>
<th>Length (bp)</th>
<th>Homology</th>
<th>e-Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaFRi1</td>
<td>DR749497</td>
<td>199</td>
<td>No match</td>
<td>-</td>
</tr>
<tr>
<td>CaFRi23</td>
<td>DR749480</td>
<td>137</td>
<td>No match</td>
<td>-</td>
</tr>
<tr>
<td>CaFRi30</td>
<td>DR749483</td>
<td>90</td>
<td>No match</td>
<td>-</td>
</tr>
</tbody>
</table>

* Short sequence protein BLASTx
When the total up-regulated (45.43%) and down-regulated (54.57%) transcripts were compared, the latter slightly outnumbered the former though the difference was not significant (P=0.1301) when compared using one sample t-test. This indicated that the genes, which are down-regulated, might also play an equally important role as genes, which are up-regulated during the course of pathogen infection.

Despite the recent development of high-throughput full-genome expression systems like microarray, which rely on comparison of two samples and prior knowledge of gene sequences, cDNA-AFLP would remain a useful technique since several transcript pools can be compared in the same experiment.

In the present study three tissue samples, resistant-uninoculated control, resistant challenged and susceptible challenged were compared, which could choose transcripts expressed only in resistant-infected roots offering an advantage to effectively negate the pathogen-derived transcripts. Another important feature of this study is comparison of transcripts from cDNA libraries. Libraries of cDNA are in use since long, mainly for cloning specific genes, and recently for generating ESTs. A novel approach of cDNA-AFLP was exploited with libraries to demonstrate successful isolation of differentially expressed transcripts. This has several advantages, viz., 1) it requires a simple PCR with flanking vector primers to rescue the cDNAs in the library, 2) based on the TDF sequence, full-length cDNA can be easily isolated from the library either by PCR with primers designed from the TDF or by screening the library with the TDF. One disadvantage in the PCR amplification before AFLP may be the reduced sensitivity to differences between the transcript levels that may lead to failure in discriminating the marginally differing transcripts.

2.4.2. Identification of transcription factors induced during FOC1 infection
From the cDNA-AFLP experiments, 19 differentially expressing TDFs were isolated, of which 10 showed homology with known genes (Table 2.3). Interestingly, 7 TDFs belonged to genes known to be associated with defense response. CaFRi3 corresponded to a WRKY protein and was up regulated specifically in roots of the resistant chickpea cultivar upon FOC1 infection. These results were consistent with the early expression of WRKY transcript in coffee two days after challenge by rust fungi *Hemileia vastatrix*, detected by RT-PCR technique (Fernandez *et al.*, 2004). Plant WRKY DNA binding proteins recognize a TGAC core sequence in various W-box elements that are present in promoters of several defense-related genes (Rushton and Somssich 1998).
Figure 2.5: Reverse northern analysis of TDFs identified by cDNA-AFLP and cDNA-RAPD in chickpea roots. 19 cDNA-AFLP TDFs and seven cDNA-RAPD TDFs were hybridized with 5 μg of total RNA from JG-62 susceptible control (SC), JG-62 susceptible infected (SI), Vijay resistant control (RC) and Vijay resistant infected (RI) at 2 DAI and 8 DAI during the FOC1 infection. The following groups of TDFs were analyzed;

**Figure 2.5 (a):** TDFs similar to defense related genes.
Figure 2.5 (b): TDFs similar to retroelement like sequences

Figure 2.5 (c): TDFs similar to genes encoded by mitochondria and ribosome
Figure 2.5 (d): TDFs similar to other EST sequences in the database.

Figure 2.5 (e): TDFs showing no homology to any sequences in the GenBank database.
Figure 2.5 (f): TDFs identified by cDNA-RAPD in chickpea roots; CaFRi 60 corresponds to gamma-glutamyl-cystiene synthatase.

Figure 2.6: Induction of transcript accumulation in chickpea roots after challenge with FOC1 analyzed by Northern blot. Expression levels for clones CaFRi4 (14-3-3 like protein), CaFRi3 (WRKY like protein), CaFRi9 and CaFRi11 (NBS-LRR) at 2 DAI in susceptible control (SC), susceptible infected (SI), resistant control (RC) and resistant infected (RI) are shown. 10 μg total RNA from roots was examined. Ribosomal RNAs were stained with ethidium bromide (Et. Br.).
It is evident from recent reports that WRKY transcription factors are implicated in the rapid responses of plants to wounding, pathogens or inducers of disease resistance (Chen and Chen 2000; Durrant et al., 2000; Fernandez et al., 2004; Hara et al., 2000; Wang et al., 1998 and Yang et al., 1999). The pathogen-induced WRKY DNA-binding proteins may serve as common transcriptional activators that regulate the expression of a large set of pathogen-responsive genes throughout the plant kingdom (Du and Chen 2000). Direct evidence of the involvement of WRKY proteins in defense process remained limited until recently, when a common regulatory component that mediated cross-talk between the antagonistic salicylic acid- and jasmonic acid-dependent defense signaling pathways, was identified as Arabidopsis WRKY70 (Li et al., 2004). Tobacco chitinase gene CHN50 (Fukuda et al., 1999) was reported to contain the GTAC core sequence in W box element of its promoter region to which WRKY protein binds to induce its expression. Interestingly the CaFRi33 that was observed to be induced is similar to class III chitinase transcript. The transcript CaFRi4 showed induced expression only in the resistant infected chickpea (Fig 2.5a) with homology to a 14-3-3 protein from Pisum sativum. Reverse northern blot analysis revealed that, CaFRi4 showed maximum expression at 2 DAI, which decreased at later stages of infection. Lower level of expression of this transcript was seen in roots of the uninfected susceptible and resistant varieties as well as the susceptible infected. 14-3-3 proteins are phosphoserine/threonine-binding proteins - they bind to a range of transcription factors and other signaling proteins, and play important roles in the regulation of plant development and stress responses (Richter and Ronald 2000). These proteins participate in the defense reaction by regulating the proton pump (H+-ATPase) to initiate the hypersensitive response to fusicoccin, a fungal toxin produced by Fusarium sp (Roberts 2003). Fusicoccin, known as activator of H⁺ATPase is also reported to stabilize interaction between 14-3-3 and regulatory domain of H⁺ATPase protein (Bunney et al., 2002; Roberts 2003). It, therefore, falls into place to find 14-3-3 induced by FOC1 infection, moreover TDF CaFRi36 represents transcript encoding ATPase like protein. The transcripts for 14-3-3 encoding protein were also reported to accumulate in barley leaves in response to inoculation with Blumeria graminis (Collinge et al., 1997; Gregersen et al., 1997). These proteins appear to be more strongly induced in the resistant cultivar than in the susceptible cultivar and the highest induction of this transcript has been reported in the 2-week-old resistant potato cultivar at 72 hrs after infection with Phytophthora (Ros et al., 2004).
2.4.3. Resistant gene TDFs induced during biotic stress in chickpea

CaFRi9, CaFRi11 and CaFRi26 showed homology to NBS-LRR like sequence from *Ageilops tauschii*. Reverse northern blot analysis of these TDFs showed differential expression in resistant infected roots when compared to the controls of both, the resistant and susceptible, chickpea roots (Fig 2.5a). The levels of CaFRi9 and 11 in the susceptible cultivar upon FOC1 infection were lower than that of constitutive signals in resistant cultivar. However, higher accumulation of CaFRi26 transcript was observed in the resistant infected as compared to other transcripts even after the 8 DAI (Fig 2.5a), though its level was lower as compared to 2 DAI. The proportion of CaFRi26 was higher than that of the other two transcripts, CaFRi9 and CaFRi11, at 8 DAI. NBS–LRR protein is a predominant class of plant R proteins that confers resistance to many plant pathogens. Although it is not yet clearly understood how these proteins function, experimental evidences indicate that the pathogen recognition is primarily determined by the highly sequence-variable LRR modules (Deslandes *et al*., 2002). Members of this gene family are involved in conferring resistance to tomato vascular wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* race 2 (12C), which show structural similarity to resistance genes that contain a NBS-LRR motif (Ori *et al*., 1997). CaFRi28 and CaFRi30 having sequence similarity to hydrolase and chitinase respectively were isolated being differentially expressed in resistant infected seedling and the same was also confirmed by reverse northern (Fig 2.5a). Antifungal hydrolases are reported to be induced in conjunction with other pathogenesis-related proteins in typical systemic acquired resistance responses (Bol *et al*., 1990; Ryals *et al*., 1996). Hydrolases have been implicated in induced resistance response against FOC race 0 in chickpea root (Cachinero *et al*., 2002). Differential induction of chitinase activity in the resistant chickpea cultivar, Vijay and its association with resistance reaction was shown in earlier study from my laboratory (Giri *et al*., 1998). Of the seven RAPD-TDFs only one TDF, CaFRi60 showed sequence identity with a known gene glutamyl cystiene synthatase, which is a key enzyme in glutathione production, and known to have increased levels during the oxidative stress when plants are subjected to biotic or abiotic stress conditions (Matamoros *et al*., 1999; May *et al*., 1998).

Four TDFs were selected for RNA gel blot analysis to validate the cDNA-AFLP and reverse northern expression patterns. The induction pattern observed in northern analysis showed that the four TDFs tested (CaFRi4, CaFRi3, CaFRi9 and CaFRi11) largely confirmed the expression profiles observed with the cDNA-AFLP analysis as well as the reverse northern (Fig 2.6). Northern analysis showed that the 14-3-3 like
protein (CaFRi4), WRKY like protein (CaFRi3) and NBS-LRR like gene transcripts (CaFRi9 and CaFRi11), are mainly induced in the resistant chickpea cultivar at 2 DAI with FOC1 and that their transcription decreased with longer periods of challenge. Nevertheless, the cDNA-AFLP technique allowed the isolation of differentially expressed genes under the conditions tested.

2.4.4. Retrotransposons induced during pathogen challenge

Two transcripts CaFRi12 and CaFRi15 showed homology to non-LTR sequences while CaFRi20 was similar to Ty copia-like element from *C. arietinum*. These transcripts were differentially regulated in roots of the resistant infected cultivar, Vijay (Fig 2.5b), though the level of expression in the uninfected resistant cultivar was higher than the expression level in the roots of uninfected as well as infected susceptible chickpea cultivars. The transcript level of CaFRi12 was higher in resistant infected seedling even at 8 DAI as compared to the other two TDFs, CaFRi15 and CaFRi20, all representing the Transposable Element (TE) like sequences. Retrotransposons have been proposed to capture the inducible promoters of defense genes or in corollary; they could have provided their inducible promoters to some plant defense genes (Grandbastien et al., 1997; Takeda et al., 1999). Many transposons (Tnt1A, Tnt1B, Tnt1C, BARE-1 and Tto1) are also reported to be induced during biotic and abiotic stress (Casacuberta and Santiago, 2003). Retroelements are known to be found in resistant gene clusters like the Fusarium wilt resistance locus in melon (Fom-2) that contains two retroelement-like sequences and three sequences with similarity to DNA transposons (Joobeur et al., 2004). Resistance gene clusters in plants conferring race-specific resistance are often large tandem repeats of highly polymorphic genes. The rice *Xa21* gene family has been shown to contain a high number of transposable elements including LTR-retrotransposons and MITEs inserted within the different genes (Song et al., 1998). In a largely accepted view, the high variability needed to evolve new resistance specificities in host plant is generated by the insertions of transposable elements (Reijans et al., 2003).

2.4.5. TDFs identified in compatible interactions: High expression in susceptible cultivar

Unlike other TDFs CaFRi51 was a unique clone that showed higher expression in susceptible-infected root tissue as compared to the control and resistant infected cultivar (Fig 2.5c). The BLAST search identified this TDF to be a Plasma Intrinsic Protein (PIP-2 gene), which is an ‘aquaporin’ (the closest hit is from pea gi|5139538; E score = 3e-06). Aquaporins represent a fairly large family of genes having role in nutrient uptake and
phloem loading/unloading of water and nutrients (Luu and Maurel 2005). They are also
known to be expressed in high amount under drought conditions (Jang et al., 2004). The
FOC infection causes wilt by plugging the conducting strands, which creates virtual
drought like conditions in root tissue, which is likely to trigger expression of aquaporins.
It is expected that this condition be pronounced in roots of susceptible cultivar as the
pathogen infects several parts of the root. The disease responsive TDF, CaFRi51
(although isolated as differentially expressed from roots of resistant cultivar) having
homology with aquaporins might have produced higher signal in infected susceptible
roots in the reverse northern experiment. Nevertheless this TDF needs more
experimentation to explain its anomalous behavior, before classifying it as ‘leakage’ in
cDNA-AFLP.

The cDNA-AFLP technique was thus a very useful tool in the global survey of
the genes expressed in chickpea during the infection with F. oxysporum. The technique
could identify many transcripts involved in the host-pathogen interactions. The study
shows that the chickpea defense response exhibits similarities to that of earlier known
defense responses in different plant species. The data generated from such studies would
provide the initial clues for guiding further functional studies of resistance in chickpea
Fusarium interactions.

2.4.6. Correlation of genes and genetics of wilt resistance in chickpea
The obligatory self-pollination and thousands of years of repeated selection might have
streamlined the genome/gene pool of chickpea and as a result it has become extensively
uniform. The lack of diversity in cultivated chickpea has been well reviewed by Abbo et
al. (2003). RFLP was of less use in detecting polymorphism in chickpea, because of the
homogeneous genome of Cicer (Simon and Muehlbauer 1997), RAPD also revealed low
polymorphism in chickpea germplasm (Sant et al., 1999). The amount of genetic
variation detected within C. arietinum using AFLP was less than that detected within
almost all of the wild Cicer species indicating that most of the cultivated Cicer
accessions are genetically similar (Nguyen et al., 2004). Three independent loci for
resistance to race 1 have been reported by Upadhyaya et al. (1983a; 1983b) and Singh et
al. (1987) and designated as h1, h2, and h3. Their studies have indicated that dominant
alleles at the first two loci (H1H1H2H2h3h3) give early-wilting (wilt susceptible), but
recessive allele at both the loci (h1h1h2h2h3h3) confers complete resistance (wilt
resistant). Further, the recessive allele in homozygous form at any one of these two loci
(h1h1H2H2; H1H1h2h2) is reported to give rise to the intermediate or late-wilting
phenotype (Brindha and Ravikumar, 2005; Singh et al., 1987). The genotypes of the
susceptible (JG-62) and resistant (WR-315 and Vijay) varieties chosen in the present study probably differ only at the two loci, H1 and H2. The resistant lines were designated to be homozygous recessive at all the three loci (h1h1h2h2h3h3) while the susceptible line, JG-62, was characterized as having the genotype H1H1H2H2h3h3. As the two resistant varieties WR-315 and Vijay have similar genotype for wilt resistance, the TDFs isolated from cultivar WR-315 by cDNA-AFLP could be validated in Vijay; both, the cDNA-AFLP and reverse northern profiles of these TDFs agreed to a large extent. Mapping and tagging of Fusarium resistance using molecular markers was attempted by several workers and these efforts identified QTL clusters of resistant genes (Ratnaparkhe et al., 1998; Santra et al., 2000; Sharma et al., 2004, Spielmeyer et al., 1998; Winter et al., 2000). Identification of resistance genes in chickpea was also attempted using resistance gene analogues (RGA), or known resistance genes, which revealed marginal differences among the resistant and susceptible chickpea cultivars (Flandez-Galvez et al., 2003; Rajesh et al., 2002; Tekeoglu et al., 2002). The efforts by me to employ RGAs for screening these libraries did not reveal significant differences (data not shown). It is understood that the difference between resistance and susceptibility depends on early detection of pathogen and prompt induction of defense responses (Hammond-Kosack and Jones 1996). The transcription factors 14-3-3, WRKY and the NBS-LRR identified in this study represent key factors governing this detection and activation thus differentiating between susceptibility and resistance in chickpea. It would, therefore, be interesting to map them to check if they are associated with the previously identified loci that have resistance gene clusters (Huettel et al., 2002; Tekeoglu et al., 2000).

In conclusion, TDFs accumulating in resistant cultivar roots challenged by FOC1 were successfully identified by employing transcript profiling techniques. cDNA-AFLP is a robust and useful technique to compare more than two cDNA libraries. Transcription factors 14-3-3, WRKY and NBS-LRR were induced in early responses in chickpea roots with FOC1 infection. While structural genes like hydrolase, chitinase, gamma-glutamyl-cystiene synthatase and aquaporin also mark the chickpea defense response.