Host-Pathogen interaction with reference to Chickpea and *Fusarium oxysporum*

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CERTIFICATE

Certified that the work incorporated in the thesis entitled "**Host-Pathogen interactions with reference to Chickpea and *Fusarium oxysporum***" submitted by Mr. Suhas B Nimbalkar was carried out under my supervision. The material obtained from other sources has been duly acknowledged in the thesis.

Date: 18th July 2007

Dr. Vidya S. Gupta
(Research Guide)

DECLARATION

I hereby declare that the thesis entitled "**Host-Pathogen interactions with reference to Chickpea and *Fusarium oxysporum*.**" submitted for the degree of Ph.D. at the University of Pune has not been submitted by me for a degree at any other university.

Date: 18th July 2007

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List of abbreviations

ABA - abscissic acid
ACC - aminocyclopropane carboxylic acid
AFLP - amplified fragment length polymorphism
avr - gene for avirulence
°C - degree Celsius
Ca1433 - *Cicer arietinum* 14-3-3 gene
CC - coiled coil
cDNA - complementary deoxyribonucleic acid
cDNA-AFLP - complementary deoxyribonucleic acid-amplified fragment length polymorphism
CDPK - calcium dependent protein kinase
cM - centimorgan
CTAB - hexadecyl-trimethyl-ammonium bromide
DAI - days after infection
DEPC - diethyl pyrocarbonate
DDRT - differential display reverse transcription
DTT - dithiothreitol
EDTA - ethylenediamine tetra acetate
EST - expressed sequence tag
FOC - *Fusarium oxysporum* f. sp. *ciceri*
FOC1 - *Fusarium oxysporum* f. sp. *ciceri* race1
g, mg, μg, ng - gram, milligram, microgram, nanogram
GRP - glycine rich proteins
h - hour
HCl - hydrochloric acid
HMGR - 3-hydroxy-3-methylglutaryl CoA reductase
HR - hypersensitive response
IAA - iso-amyl alcohol
IAA - indole acetic acid
IFR - isoflavone oxidoreductase
IPTG - isopropyl-thio-galactoside
ISSR - inter simple sequence repeat
JA - jasmonic acid, jasmonate
L, mL, μL - liter, milliliter, microliter
LB - luria-bertani
LG - linkage group
LOX - lipoxygenases
LRR - leucine rich repeat
LTR - long terminal repeat(s)
LZP - leucine zipper containing protein
λ260, λ280 - absorption at 260nm, 280nm
M, mM, μM - molar, millimolar, micromolar
MAS - marker assisted selection
MAPK - mitogen activated protein kinase
min - minute
MITE - miniature inverted tandem elements
mmole, μmole - millimole, micromole
MeJ - methyl jasmonate
NaCl - sodium chloride

NaOH - sodium hydroxide
NBS - nucleotide binding site
NIPs - nodulin-like intrinsic proteins
nm - nanometer
ORF - open reading frame
PAGE - polyacrylamide gel electrophoresis
PAL - phenyl ammonia lyase
PAMP - pathogen associated molecular pattern
PIPs - plasma membrane intrinsic proteins
PRP - proline rich protein
QTL - quantitative trait loci
QRT-PCR - quantitative RT-PCR
R genes - resistance genes
RAPD - random amplified polymorphic DNA
PCR - polymerase chain reaction
RFLP - restriction fragment length polymorphism
RGA - resistance gene analog
RIL - recombinant inbred line
RNAi - ribose nucleic acid interference
ROS - reactive oxygen species
rpm - revolutions per minute
RT-PCR – reverse transcriptase-polymerase chain reaction
SA - salicylic acid, salicylate
SAGE - serial analysis gene expression
SAR - systemic acquired resistance
SDS - sodium dodecyl sulphate
sec - second
SIPs - small basic intrinsic proteins
SNAP - single nucleotide amplification polymorphism
SNP - single nucleotide polymorphism
SSC - sodium chloride sodium citrate
SSH - subtractive suppression hybridization
SSR - simple sequence repeat
STMS - sequence tagged microsatellite site
TAE - Tris-acetate EDTA
TBE - Tris-borate-EDTA
TDF - transcript derived fragment
TE - Tris-EDTA
TEs - Transposable elements
TRIS - tris-hydroxymethyl aminomethane
TIGR - The Institute For Genome Research
TLP - thaumatin like protein
Tn - transposon
TIPs - tonoplast intrinsic proteins
TIR - toll-interleukin receptor
VIGS - viral induced gene silencing
U - unit
X-Gal - 2-bromo-3-chloro-4-indolyl-galacto-pyranoside

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Chapter 1

Introduction and Review of Literature

Abstract

Plants are constantly exposed to intimate interactions with a plethora of microbes and display a complex set of interactions, which range from symbiosis to diseases. The harmful interactions detrimental to plant and plant-productivity lead to multi billion annual losses impelling the use of chemical fungicides. Pathogen populations stay competent and dynamically change to remain diverse in response to the constant selection pressure from changing agro-ecological conditions. The crop plants are thus, infected and parasitized with varying degrees of specificity and severity. Considerable efforts have thus been directed towards understanding the molecular mechanisms underlying plant-microbe interactions. When a plant recognizes potentially infectious pathogens, the local defense responses are activated to sequester the pathogen away from non-infected tissue. The recognition and defense by a host plant to its fungal pathogen and the ability of the pathogen to overcome the plant's defenses, constitutes a complex and dynamic interactive molecular network. Induction of these molecular responses necessitates up- and down-regulation of numerous but very specific genes. Differential large-scale gene expression analysis in plant-pathogen interactions has resulted in identification of several defense related transcripts (Ros *et al*., 2004; Fernandez *et al*., 2004). Direct or indirect role of these genes in controlling pathogen invasion to the plant tissue is also demonstrated in a few cases. However, these studies are mostly restricted to model plants and some of the crop plants such as cassava, sugarcane, tomato, rice, coffee etc (Durrant *et al*., 2000; Matsumura *et al*., 2003; Torres *et al*., 2003; Carmona *et al*., 2004; Zang *et al*., 2004; Feranadez *et al*., 2005; Kemp *et al*., 2005).

One of the most important diseases affecting chickpea is Fusarium wilt, caused by the soil borne fungus *Fusarium oxysporum* f sp *ciceri* (FOC), a root pathogen, which colonizes the xylem vessels and blocks them completely leading to wilt (Bateman *et al*., 1996). Several studies have demonstrated that infection with *F. oxysporum* preceeds various chemical and biochemical changes in chickpea (Stevenson *et al*., 1997; Armero *et al*., 2001). However, information about genetic factors that determines the outcome of interactions between *F. oxysporum* and chickpea roots needs a further detailed study.

1.1. *Cicer arietinum* L., the host plant

Chickpea are the third most important legume crop with a worldwide production of about 9.2 Mt (Million tones) (Fig. 1.1). In India chickpeas are the most important legume crop as revealed by the India's contribution (accounts for ~60%) towards the world's total production as well as area under cultivation (6700 thousand hectares) (Fig. 1.1) (http://faostat.fao.org). Though, chickpeas are grown and locally consumed, India is also the world's largest importer of chickpeas accounting for about 20% of global imports (Source: http://faostat.fao.org). These figures reflect on the growing demand for chickpea as well as the immense strain on crop production and yield.

1.1.1. Morphology

Chickpea (*Cicer arietinum* L.) has a deep tap root system, which enhances its capacity to withstand drought conditions; it is well adapted to areas having relatively cooler climatic conditions and a low level of rainfall. The aerial portion is profusely branched, erect or spreading, reaching a height of 0.2-1 m, appearing glandular pubescent, olive, dark green or bluish green in color. Leaves are imparipinnate, glandular-pubescent with 3-8 pairs of leaflets with rachis ending in a terminal leaflet. Leaflets are ovate to elliptic, 0.6-2.0 cm long, 0.3-1.4 cm wide; margin serrate, apex acuminate to aristate, base cuneate; stipules 2-5 toothed or absent. The inflorescence consists of solitary flowers, sometimes 2 per inflorescence and borne on 0.6-3 cm long peduncles, 7-10 mm long calyx; while the bracts are triangular or tripartite; the corolla is 0.8-1.2 cm long and varies from white, pink, purplish (fading to blue), or blue. The staminal column is diadelphous (9-1) with a sessile, inflated and pubescent ovary (Duke, 1981; Cubero, 1987; van der Maesen, 1987). The seeds (1-2 or maximum 3) are contained in a pod which is rhomboid ellipsoid, inflated and glandular-pubescent. The seed color varies from cream, yellow, brown, black, or green. Seeds may be rounded to angular with a smooth or wrinkled, or tuberculate seed coat, which is laterally compressed with a median groove around two-thirds of the seed forming a beak at the anterior end; during the cryptocotylar germination cotyledon tips remain in the seed coat in intimate contact with the endosperm (Duke, 1981; Cubero, 1987 van der Maesen, 1987).

1.1.2. Center of origin and distribution

Chickpea is an ancient crop that marks its origin well before 10,000 B.C. when it was used by the 'hunter-gatherer' for sustaining their communities. The regions of Turkey and the ancient city of Jericho domesticated this crop around 7,500 B.C., which is considered as its centre of origin by Ladizinsky, (1975) while van der Maesen (1987)

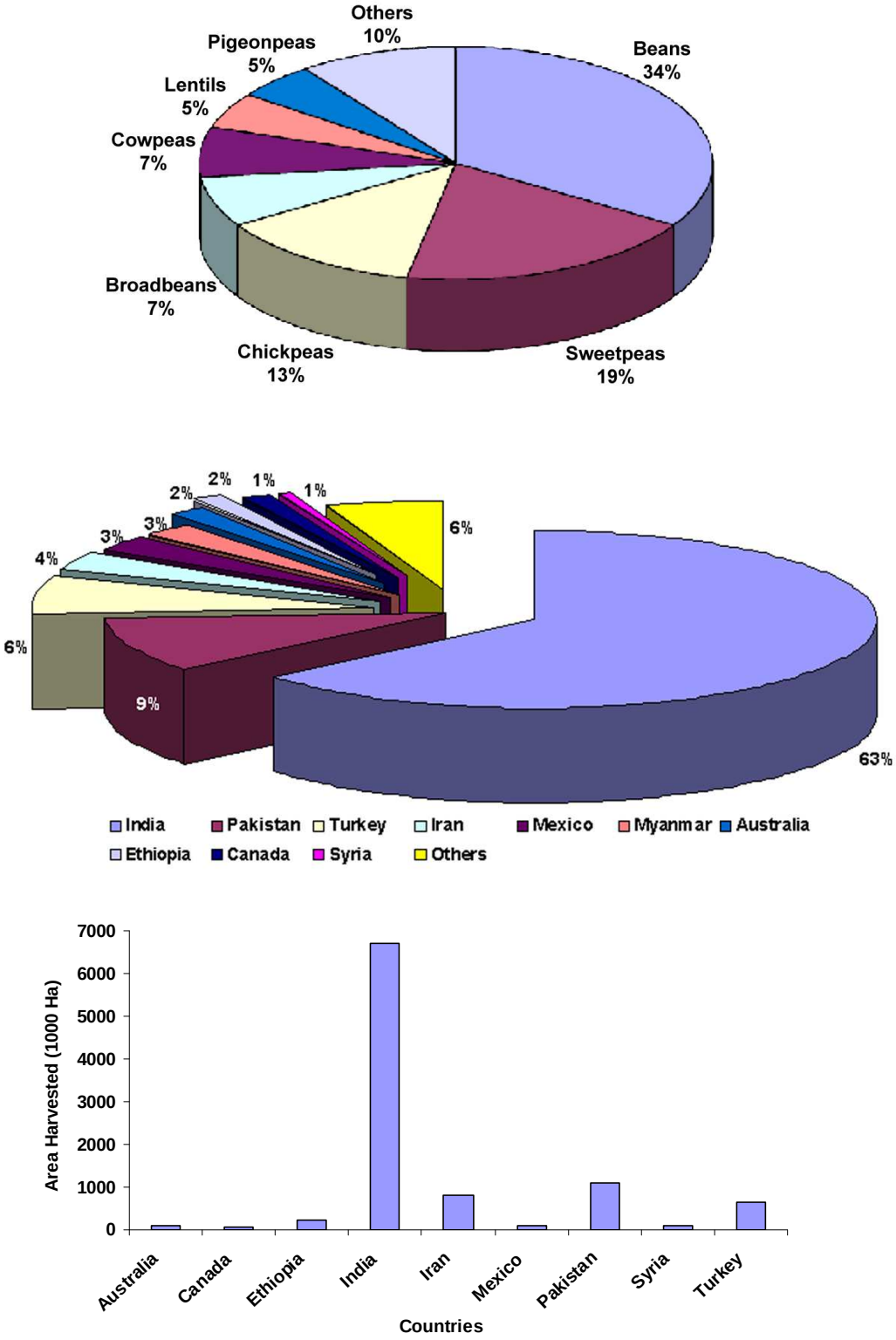


Figure 1.1: Contribution of Indian agriculture to global production of chickpea. a) Global production of chickpea in comparison with other legume crops. b) India is the world's largest producer of chickpeas, contributing to >60% of the total global produce. c) Area under cultivation of chickpea. Source: FAO Data (http://faostat.fao.org).

recognized the southeastern part of Turkey adjoining Syria as the possible center of origin of chickpea based on the presence of the closely related annual species, *C. reticulatum* Ladizinsky and *C. echinospermum* P.H. Davis. *C. reticulatum* is regarded as the progenitor of cultivated chickpea, *C. arietinum*, which although morphologically resemble each other (Ladizinsky, 1975).

Botanical and archeological evidence shows that chickpeas were first domesticated in the Middle East and were widely cultivated in India, Mediterranean area, the Middle East, and Ethiopia since antiquity. In the New World, it is important in Mexico, Argentina, Chile, Peru and the U.S. and also in Australia. Wild species are most abundant in Turkey, Iran, Afghanistan, and Central Asia (Duke, 1981). Chickpea was brought to the Western Europe and was known in many areas in the Bronze Age, most popularly, Italy and Greece. People in these regions consumed chickpea in various forms like roasted as snacks, raw, carbonized or in broth. Many past writings have also been found telling the uses and importance of chickpeas both medical and as a food crop. With time, many other varieties of chickpea were developed as it spreads in many areas of Asia and Australia. During the First World War, Germany cultivated chickpea as a coffee substitute.

Chickpea has majority of its cultivation in dry areas of the Indian subcontinent (Saxena, 1990) and India is the principal chickpea producing country with a share of 90% in this region. Presently, the most important chickpea producing countries are India (63%), Pakistan (9%), Turkey (6%), Iran (4%), Mexico (3%), Myanmar (3%), Ethiopia (2%), Australia (2%), and Canada (1%).

1.1.3. Season

The yields from chickpea is maximum when grown on sandy, loam soils having an appropriate drainage system as this crop is very sensitive to excess water. The production of chickpea or ‘chana’ is also affected in excessive cold conditions. Chickpea is sown in the months of September to November in India and is considered as a rabi crop. The desi type chickpea reaches physiological maturity by 95-105 days and Kabuli type by 100-110 days. The plant is harvested when its leaves start drying and shedding and can be done manually or with the help of a harvester. In India, it is harvested in February, March and April. This crop is often cultivated as a sole crop but sometimes it is also grown rotationally with other crops such as sorghum, pear millet, wheat and coriander. Pale yellow, dark brown or reddish chickpea are some of the varieties that are grown today.

1.1.4. Nutrition

Chickpea (*Cicer arietinum* L.) belongs to the family leguminosae and is commonly known as Chana, Bengal gram or garbanzo. It is a self-pollinating plant with a diploid genetic content of 2n=16, with a C value of 1C=931Mbp. Chickpea is mainly employed for human consumption and also a small proportion forms the part of animal and poultry feed. Chickpea has one of the highest nutritional compositions of any dry edible legume and is not reported to contain any specific major anti-nutritional factors (Williams & Singh, 1987). On an average, chickpea seed contains 23% protein, 64% total carbohydrates, 47% starch, 5% fat, 6% crude fiber, 6% soluble sugar and 3% ash. The mineral component is high in phosphorus (340 mg/100 g), calcium (190 mg/100 g), magnesium (140 mg/100g), iron (7 mg/100 g) and zinc (3 mg/100 g). Chickpea protein has the highest digestibility when compared to other dry edible legumes. The lipid fraction is high in unsaturated fatty acids, primarily linoleic and oleic acids. They are also a good source of calcium, magnesium, potassium, phosphorus, iron, zinc and manganese (Ibrikci *et al*., 2003). Chickpeas do not contain as high amounts of isoflavones as soybeans do (USDA-ARS, 2004), but provide more beneficial carotenoids such as β-carotene than genetically engineered "Golden Rice". Thus, chickpea is considered a functional food or nutraceutical (Agharkar, 1991; McIntosh & Topping, 2000; Charles *et al*., 2002). While it is a cheap source of protein and energy in the developing world, it is also an important food to the affluent populations to alleviate major food-related health problems. However, more research is necessary to elucidate and extend the food and nutraceutical benefit of this important food legume through breeding.

Having a capacity to stand in drought conditions, this crop doesn't have the requirement of being fed with nitrogen fertilizers. Chickpea through its BNF (Biological Nitrogen Fixing) capability meets 80% of its nitrogen requirement and can fix up to 140 kg N/ha from air. It leaves substantial amount of residual nitrogen behind for subsequent crops and adds much needed organic matter to maintain and improve soil health, long-term fertility and sustainability of the agro-ecosystems.

Commercially, the species is grouped into desi (small, colored seed coat) and kabuli (large, white or cream seed coat) types. To a certain extent this classification overlaps with the macrosperma and microsperma races proposed by Moreno and Cubero (1978). Classification also reflects utilization: whereas kabulis are usually utilized as whole grains, desis as whole seeds, de-hulled splits (dhal) or flour. Seeds are ground to flour and used in confectionery. Young shoots or green pods, shelled for the peas and

eaten as a snack or vegetable. Chickpea is also known for its use in herbal medicine and cosmetics. An acrid liquid from the glandular hairs of the plant is collected by spreading a cloth over the crop at night, which absorbs the exudation with the dew. The exudate contains about 94% malic acid and 6% oxalic acid and is used medicinally.

Kabuli × desi crosses are used in many breeding programs to combine genes for cold tolerance, bold seededness, resistance to Ascochyta blight and long vegetative growth more frequently found in kabuli types, while genes for heat and drought tolerance, resistance to Fusarium wilt and early flowering contributed by the desi types (Singh, 1987).

1.1.5. Yield and losses

The potential seed yield of about 5 t/ha has been reported in chickpea. However, the realized seed yield hovers around 850 kg/ha (world average ≈ 0.8 t/ha, FAOSTAT, 2005) which has stagnated over the years (Fig 1.2). Series of biotic and abiotic stresses reduce the yield and yield stability leaving room for only marginal improvements. This affects development of widely adapted cultivars and susceptibility to several biotic and abiotic stresses. Generally the crop produces excessive vegetative growth under high input conditions and is unable to translate the biomass into high seed yields. The major abiotic constraints to productivity include drought, heat, cold and salinity and the key biotic constraints are Ascochyta blight (*Ascochyta rabii*), Fusarium wilt (*Fusarium oxysporum*), Dry root rot (*Rhizoctonia bataticola*), Botrytis grey mould (*Botrytis cinerea*), Collar rot (*Sclerotium rolfsii*), Root-knot nematode (*Meloydogyne incognita* and *M. javanica*), Stunt-virus, Pod borer (*Helicoverpa armigera*), and Cutworm (*Agrotis ipsilon*).

Amongst the causal agents of biotic stresses, about 67 fungi, 3 bacteria, 22 viruses and 80 nematodes have been reported on chickpea (Nene *et al.*, 1996) but only few of these cause economically important diseases (Haware, 1998). There has been an increase in different chickpea pathogens like fungi, bacteria and viruses over a period of past 17 years. The maximum number of pathogens has been reported from India alone with the number rising to 89 pathogens in 1995 from 35 in 1978 (Nene *et al.*, 1996). The insect *Helicoverpa armigera* which feeds on foliage, flowers and developing seeds, is the most important pest of chickpea, while stunt is the most important and prevalent viral disease in most chickpea growing regions of the world.

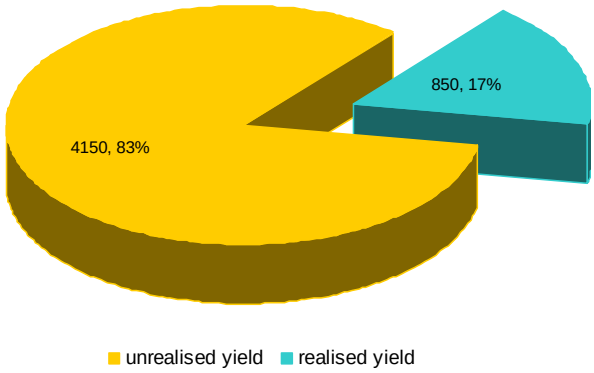


Figure 1.2: The potential seed yield of about 5 t/ha has been reported in chickpea. However, the realized seed yield (productivity) hovers around 850 kg/ha (world average ≈ 0.8 t/ha, FAOSTAT, 2005) which has stagnated over the years. The huge unrealized potential yield loss is due to various biotic and abiotic factors.

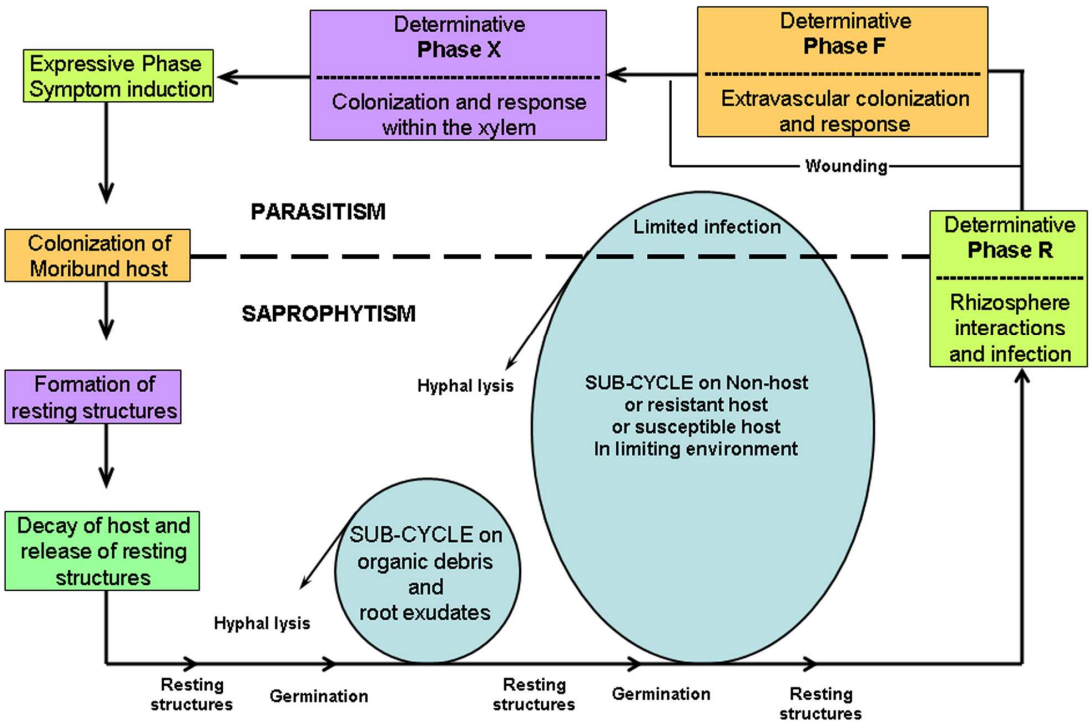


Figure 1.3: Schematic representation of the life cycle of wilt causing soil borne fungi, depicting saprophytic and parasitic growth and successive phases of colonization and pathogenesis (Beckman and Roberts, 1995)

1.1.6. Diseases

Among the economically important fungal diseases of chickpea are root diseases like Fusarium wilt and root rots caused by a complex of soil borne fungi, foliar diseases like Ascochyta blight and Botrytis gray mould, of which wilt and blight are the most devastating diseases affecting chickpea in tropical and temperate regions, respectively. Especially Ascochyta blight and Fusarium wilt, pod borer, drought and cold are major constraints to yield improvement and adoption of the crop by farmers. Therefore, improving resistance to biotic and tolerance to abiotic stresses as well as a general increase in dry matter are major aims of chickpea breeders around the world.

1.2. *Fusarium oxysporum* f. sp. *ciceri*- the chickpea wilt pathogen

1.2.1. Classification:

Classification and identification schemes for *Fusarium* are traditionally based exclusively on a morphological species concept derived from cultural characteristics, shared morphological trait of the anamorph, host range, and to a lesser extent, teleomorph micromorphology (Booth, 1971). The systematics of *Fusarium* remains controversial and confusing (Gams and Nirenberg, 1989), due to the conflicting morphological species concepts employed in taxonomic treatments of this genus (Booth, 1971; Gerlach and Nirenberg, 1982; Nelson *et al.* 1983). Gerlach and Nirenberg's system (1982) is the most differentiated, including 73 species and 26 varieties; while 44 species and 7 varieties have been recognized by Booth (1971) and, 30 species by Nelson *et al.* (1983). On the other hand, in more recent times molecular systematics, based on discrete DNA sequence data, offers an objective, phylogenetically based system of classification for *Fusarium* and its teleomorphs (Bruns *et al.* 1991). Previous investigations employing cladistic analysis of DNA sequences from multiple unlinked loci in *Fusarium* species have revealed the utility of gene phylogenies inferred from mitochondrial small subunit (mtSSU) rDNA, nuclear 28S rDNA, β-tubulin gene and nuclear translation elongation factor 1α (O'Donnell *et al.* 1998; Baayen *et al.*, 2000), however, nuclear rDNA ITS gene tree was found to be composed of non-orthologous sequences (O'Donnell and Cijelnik, 1997).

1.2.2. Habitat and host range:

Fusarium is a large cosmopolitan genus of pleoanamorphic hyphomycetes whose members are responsible for a wide range of plant diseases (Farr *et al.*, 1989), mycotoxicoses and mycotic infections of humans and other animals (Nelson *et al.*, 1994). The species *Fusarium oxysporum* is well represented among the soil borne fungi, in every type of soil, all over the world (Burgess, 1981) and is considered to be a normal

constituent of the rhizosphere of plants (Appel and Gordon, 1994). Some strains of *Fusarium oxysporum* are pathogenic to different plant species; they operate by penetrating into the roots and causing either root rots or tracheomycosis by invasion of the vascular system, causing wilt and sudden death disease leading to severe economic damages to many crop species. Typically the vascular wilt causing *Fusarium oxysporum* species invade only living root tissues, tend to be specialized or host specific, and suppressed by saprophytes (Hillocks, 2001). Depending on the plant species and plant cultivars infected, *Fusarium oxysporum* is classified into more than 120 *forma speciales* (Armstrong and Armstrong, 1981) and further subdivisions into races are often made based on their virulence to a set of differential host cultivars (Cornell, 1991). However, the genetic basis of host specificity (forma speciales) and cultivar specificity (pathogen races) of *F. oxysporum* is not understood (Baayen *et al*., 2000). The presently accepted classification for the Fusarium wilt pathogen *Fusarium oxysporum* f. sp. *ciceri* is: Form-class: Fungi Imperfecti, Form-order: Moniliales, Form-family: Tuberculariaceae, Form-genus: *Fusarium*, Form-species: *oxysporum*, forma specialis *ciceri*. *Fusarium oxysporum f.sp. ciceri* is reported from most of the chickpea growing areas.

1.2.3. Fusarium life cycle

Insight into the life cycle of wilt pathogens is important to understand their survival, causation of disease in a spatial framework and interactions at the host-parasite interface leading to disease resistance or susceptibility. Beckman and Roberts (1995) have addressed these topics and proposed a model explaining the interactions between vascular wilt causing pathogens and their host plants, wherein, the pathogens have distinct saprophytic and parasitic phases in their life cycles. The life cycle of soil-borne, wilt causing fungi including their saprophytic and parasitic growth and successive phases of colonization and pathogenesis is represented in Figure 1.3. There are three distinct phases in the pathogen lifecycle **i)** Determinative Phase, **ii)** Expressive Phase and **iii)** Saprophytic Phase. In the determinative phase the extent of colonization of the host vascular system is determined, while in the expressive phase mainly disease symptoms are developed, and the saprophytic phase is characterized by the survival of the pathogen by formation of long-lived resting structures. During disease congenial conditions, the pathogen after invasion of the root tissue, acquires significant cortical colonization, then it enters the second phase of vascular invasion and spreads along with the transpiration pull. The spread and colonization of the xylem vessels by the pathogen plugs the conducting vessels leading to disruption of water uptake by the plants and thus causes wilting in the susceptible plants.

1.3. Wilt: The disease

1.3.1. A chronological over view

Wilt in chickpea was first reported by Butler in 1918. McKerral (1923) who considered the disease to be soil borne and the putative causal organism *Fusarium* spp. were isolated from the soil samples analyzed. An association of *Fusarium* spp. and *Macrophomina phaseolina* (Tassi) Goid, with wilted plants was reported by Narsimhan (1929) and Dastur (1935). However, the latter could not prove pathogenicity of the isolated *Fusarium* spp. and concluded that wilt was due to abiotic factors (Dastur, 1935). Later 300 *Fusarium* isolates were identified, isolated and classified into non-pathogenic types, wilt causing types and seed rotting types by Prasad and Padwick (1939) who also identified *Fusarium* spp. to be the causal agent of chickpea wilt.

1.3.2 Disease management

Fusarium wilt, caused by *Fusarium oxysporum* f.sp. *ciceris* (FOC), is a major constraint to chickpea production worldwide (Jalali & Chand, 1992). Annual chickpea yield losses due to Fusarium wilt vary from 10-15% (Trapero-Casas & Jimenez-Diaz, 1985; Jalali & Chand, 1992), and at times under specific conditions is capable of completely destroying the crop (Halila & Strange, 1996).

1.3.3. Cultural practices

As chickpea wilt pathogen is monocyclic the FOC population in soil persists due to the longevity of reproductive units and thus makes it difficult to eliminat them from wilt sick fields (Haware *et al.*, 1986; 1996). Chickpea wilt has been reported to increase with increased levels of soil inoculum. Occurance of wilt disease, its severity and disease progression is directly proportional to the density of the pathogen population. Presence of high levels of FOC propagules leads to 100% wilting much earlier than lower initial levels of FOC propagules (Bhatti and Kraft, 1992). Thus it may be possible to early forecast the severity of diseases induced by soil-borne pathogens by assessing the initial pathogen population (Fry, 1982).

Avoidance of planting in heavily infested fields is advised to minimize the effects of wilt disease; however, availability of land is a limiting factor in Indian conditions. Moreover, as the pathogen can survive in soil for longer periods (Haware *et al.*, 1996) crop rotation, is not an effective practice for reducing wilt incidence. On the other hand, cultural practices like deep ploughing during summer and removal of host debris from the field can considerably reduce inoculum levels. Solarization (covering the soil with transparent polythene for 6-8 weeks during summer months) is known to effectively control wilt in chickpea (Chauhan *et al.*, 1988). However, it is not a practical option in

India as the poor farmer is already strapped for resources. Control of seed transmission of wilt can be achieved by using disease free seed, obtained from plants grown in disease free areas. The seed-borne inoculum can also be controlled by seed dressing with fungicides like Benlate-T (benomyl 30%+thiram 30%) at 0.25% rate (Haware *et al.*, 1978).

1.3.4. Biocontrol

The most effective and practical way to manage wilt is to use resistant cultivars (Jimenez-Diaz *et al*., 1991; Jalali & Chand, 1992; Kraft *et al*., 1994; Jimenez-Gasco, *et al*., 2004). However, occurrence of pathogenic races in FOC curtails the effectiveness of host resistance. Seven FOC races (0-6) have been identified (Haware & Nene, 1982; Jimenez-Diaz *et al*., 1993). Races 1-4 were first described in India (Haware & Nene, 1982). Later, race 0 was reported in California (USA), Israel, Lebanon, Spain, Syria, Tunisia and Turkey; and races 1 and 6 were identified in California, Israel, Morocco and Spain. Cultivation of varieties possessing resistance to specific races of the pathogen prevalent in a region or locality is the most economical disease management strategy (Jalali and Chand 1992). Genetic analysis has indicated that resistance to wilt race 1 is governed by (a) single gene (Kumar and Haware 1982), (b) two genes (Upadhyaya *et al*., 1983a; 1983b) and (c) three genes (Singh *et al*., 1987). Partially recessive alleles in homozygous form at either of the first two loci and the dominant allele at the third locus delay wilting (Table 1.1), but any two of these alleles together confer complete resistance (Singh *et al*., 1987).

The use of biological control using either bacterial or fungal antagonists may enhance the effectiveness of resistant cultivars for management of Fusarium wilt in chickpea. Biological control by non-host *F. oxysporum* isolates (Ogawa & Komada, 1985; Paulitz *et al*., 1987; Mandeel & Baker, 1991; Alabouvette *et al*., 1993; Hervas *et al*., 1995; Larkin *et al*., 1996; Fuchs *et al*., 1997; Hervás *et al*., 1997) and incompatible races of the same *forma specialis* (Biles & Martyn, 1989; Martyn *et al*., 1991; Hervas *et al*., 1995) is seen as a promising strategy for management of Fusarium wilt diseases. Hervas *et al*. (1995) showed that prior inoculation of germinated chickpea seeds with either incompatible FOC races or non-host *F. oxysporum* isolates can suppress Fusarium wilt caused by the highly virulent FOC race 5. Further studies (Hervas *et al*., 1997; 1998) supported the potential of the non-host *F. oxysporum* isolate Fo90105 as a biocontrol agent against Fusarium wilt of chickpea.

Various mechanisms are involved in the biological control of Fusarium wilt diseases by non-host *F. oxysporum* isolates, these include saprophytic competition for nutrients; parasitic competition for infection sites; and enhanced resistance due to rapid induction of defense responses within the host (Schneider, 1984; Alabouvette, 1986; Matta, 1989; Mandeel & Baker, 1991; Fuchs *et al*., 1997).

These mechanisms may function in parallel and not necessarily exclusive of one another, and several other mechanisms are speculated to be responsible for disease suppression by many biocontrol agents (Mandeel & Baker, 1991). In previous studies, it was shown that certain plant defense responses, namely phytoalexin synthesis and accumulation of chitinase and β-1,3-glucanase activities, may be involved in the non-host resistance of chickpea against non-host *F. oxysporum* isolates (Armero *et al*., 1993; Cabello, 1994; Armero, 1996). Later, Stevenson *et al*. (1997) concluded that chickpea phytoalexins (the pterocarpans maackiain and medicarpin) are fundamental components of the resistance mechanism of this plant to Fusarium wilt.

1.4. Host responses to pathogen

1.4.1. Resistance mechanisms

Several different kinds of resistance mechanisms are exhibited by the plants, which are more or less regulated via different genetic frameworks. Additionally, there are several different definitions of the forms of resistance, which have been changed over a period. The four categories i) escape, ii) tolerance, iii) resistance and iv) immunity, described by Chahal and Gosal (2002) are fairly descriptive of the various mechanisms that influence the occurrence and severity of disease from a crop yield perspective.

***Escape*:** The mechanism relies on avoidance of contact with the disease causal agent. Abscission of diseased leaves or growth and flowering early in the season are examples of escape mechanisms. The escape strategy can also be utilized to some extent by agronomical practice, like early or late planting and the use of fertilizers (Barbetti *et al*., 1975; Chahal and Gosal, 2002). Deployment of early maturing varieties is one of the regular practices in several crops.

***Tolerance*:** Here although the plant may show some visible disease symptoms, it does not suffer any adverse effects from infection, while the pathogen also is able to reproduce. A variant of tolerance is recovery, where a diseased plant is restored to healthy status by various *in planta* mechanisms. Examples include the woody plants, which form new xylem tissue around *Verticillium*-infected tissues (Hiemstra, 1998).

Table 1.1: The genetic constitution and Fusarium wilt to race 1 reactions of chickpea cultivars

Cultivar	ICC #	Genetic constitution	Wilt reaction
JG-62	4951	$H_1\ H_1\ H_2\ H_2\ h_3\ h_3$	Early-wilting
K 850	5003	$h_1\ h_1\ H_2\ H_2\ h_3\ h_3$	Late-wilting
C 104	4928	$H_1\ H_1\ h_2\ h_2\ h_3\ h_3$	Late-wilting
H 208	4954	$H_1\ H_1\ H_2\ H_2\ H_3\ H_3$	Late-wilting
WR 315	8933	$h_1\ h_1\ h_2\ h_2\ h_3\ h_3$	Resistant
CPS 1	10130	$h_1\ h_1\ h_2\ h_2\ h_3\ h_3$	Resistant
P 436-2	554	$h_1\ h_1\ h_2\ h_2\ h_3\ h_3$	Resistant
BG 212	11088	$h_1\ h_1\ h_2\ h_2\ h_3\ h_3$	Resistant
JG-74	6098	$h_1\ h_1\ h_2\ h_2\ h_3\ h_3$	Resistant

(http://www.icrisat.org/ChickPea/Pedigree/Chickpeaintro.htm accessed on 29-07-07)

***Resistance*:** It is a hereditary capability to limit pathogen growth. Resistance does not necessarily imply complete abolishment of pathogen activity. The common distinction of different forms of resistance is the vertical and horizontal resistance (Parlevliet and Zadoks, 1977; Vanderplank, 1984), effective against different pathogens, depending on their life style and reproductive strategies (McDonald and Linde, 2002). In vertical resistance the plant has ability to completely block the pathogen growth, the determinant of virulence of the pathogen. Vertical resistance is further sub-divided into race-specific resistance, where the resistance is active against some genotypes (races) of the pathogen, but not all races; while race non-specific resistance is the ability to block all known isolates of a pathogen, but where some plant genotypes show susceptible phenotype (Hammond-Kosack and Parker, 2003). Vertical resistance can be due to the presence of a resistance (*R*) gene according to the gene-for-gene resistance model (Flor, 1947) where the plant *R* gene recognizes a pathogen avirulence (*Avr*) gene, leading to a rapid response and resistance.

Horizontal resistance limits the disease progression of a wide range of pathogen genotypes, the determinant of aggressiveness of the pathogen. Horizontal resistance is often inherited as quantitative trait loci (QTLs). This type of resistance can be governed by multiple factors, and is in some cases referred to as 'basal resistance' (Hammond-Kosack and Parker, 2003), which can be confusing since induced resistance due to recognition of non-specific pathogen components like chitin or flagellin often is referred to as 'basal resistance' (de Torres *et al*., 2006). The horizontal ("basal") resistances can also be governed through non-induced components like physical characteristics of the plant, toxin resistance and its chemical composition (i.e. the chemical structure of its antimicrobial secondary metabolites, like glucosinolates, phytoalexins, oxylipins etc.). Horizontal resistance does not breakdown like gene-for-gene type resistance, but may erode over time.

***Immunity or non-host resistance*:** As all pathogens are not able to attack all plants, the events where all interactions between all genotypes of a pathogen and all genotypes of a plant are incompatible (= no disease develops) are designated as immunity or non-host resistance. There have been many hypotheses about the mechanisms of non-host resistance – **i)** the pathogen fails to recognize the plant as a potential host, **ii)** the plant contains multiple "*R* genes" or "*R* genes" targeting indispensable structures of the pathogen, which makes it virtually impossible for the pathogen to break the induced

resistance of the plant (Hammond-Kosack and Parker, 2003; Holub and Cooper, 2004), **iii)** the pathogen lacks the appropriate virulence factors and is thus unable to overcome the basal resistances of the non-host (Holub and Cooper, 2004).

1.4.2 Defense responses

Active plant defense against invading pathogens is achieved by recognition of pathogen followed by changes in structural and biochemical components that are differentially regulated depending on the incoming stress. Perception of both general and specific pathogen-associated molecules triggers defense responses via signal transduction cascades and transcriptional activation of numerous genes. Active defense responses are being elucidated in various plants, which include calcium and ion fluxes, increase of reactive oxygen species (ROS) during the oxidative burst (Lamb and Dixon 1997) and hypersensitive cell death (HR) (Greenberg 1997). The expression of transcription factors and protein kinases, as well as elevation in cytosolic calcium, is integral to the signalling of these defenses (Grant and Mansfield 1999). The expression of various defense genes also leads to the production of antimicrobial compounds such as pathogenesis-related (PR) proteins (Van Loon and Van Strien 1999) and phenylpropanoids (Dixon *et al*. 2002).

The numerous defense responses vary in their timing ranging from rapid responses, such as HR and callose depositions, followed by induced defenses like the salicilic acid (SA)- or methyl jasmonate (MeJ)-induced antimicrobial peptides. One of the rapid responses against the pathogen is deposition of callose that work as a barrier against pathogens that try to penetrate the cell and limits nutrient leakage from the cell, thus being efficient against both necrotrophs and biotrophs (Flors *et al*., 2005). However, callose deposition is reported to negatively influence SA accumulation which leads to the counter-intuitive result that loss of callose synthase can result in enhanced resistance against some biotrophic pathogens (Vogel and Somerville, 2000). Other modulations of the physical barriers against the pathogen are also known, such as lignification and thickening of the cell wall.

A long lasting resistance is then achieved by the plant, such as systemic acquired resistance (SAR), which in essence keeps the plant on alert to defend itself from future attacks (Grant and Lamb, 2006). Grafting studies have shown that SAR requires SA locally. The mobile signal still remains elusive, but is dependent on a lipid transfer protein (Maldolando *et al*., 2002). Another induced resistance requires ET, JA and (cytosolic) NPR1 is referred to as induced systemic resistance (ISR), a long lasting

response triggered by non-pathogenic rhizobacteria, which is not associated to elevated levels of pathogenesis-related (PR) proteins (Pieterse *et al*., 2001). ISR is, in many respects, to be regarded as a priming of defenses (Verhagen *et al*., 2004), similar to BABA (β-amino-butyric acid)-induced resistance (BABA-IR). BABA-IR is, however, dependent on the SAR or an ABA-dependent signalling, depending on pathogen (Ton and Mauch-Mani, 2004).

The main function of many of the classes of pathogenesis related (PR) proteins (van Loon and van Strien, 1999) is to weaken the cell wall of the pathogen, such as glucanases, chitinases, osmotin (Narasimhan *et al*., 2003), cyclotides (Kamimori *et al*., 2005), defensins (Thomma *et al*., 2002) and thionins (Carrasco *et al*., 1981), or to inhibit their ability to degrade plant tissue via enzyme inhibitors such as proteinase inhibitor (cyclotides) or amylase inhibitor (some defensins) activity. The defensins show target specificity to different types of cell walls and appear to interact with them using electrostatic interactions. The subsequent membrane disruption may, however, not be the only mode of action of this group of proteins, but rather includes disruption of RNA, DNA or protein synthesis (Thomma *et al*., 2002). Over expression analyses of a pea defensin and a pea pathogen-responsive dirigent family (lignan/lignin biosynthesis) protein in *B. napus* background displayed enhanced resistance to *L. maculans*, illustrating the functional role of these classes of pathogen-responsive proteins in resistance (Wang *et al*., 1999). Some of the PR proteins may also act via direct induction of cell death (Narasimhan *et al*., 2001), possibly also as an effect of severe ion leakage over the cell wall. Despite confirmed antimicrobial activities *in vitro*, most PR proteins only give a moderate effect on resistance when over expressed (van Loon *et al*., 2006).

The intracellular receptors are characterized by nucleotide-binding and leucine-rich repeat domains [so-called NB-LRR ("Nibbler") proteins] (Dangl, 2007). NB-LRR proteins are structurally analogous to the animal NOD/NLR/CATERPILLAR superfamily of intracellular proteins and also act as microbial sensors. Plant NB-LRR proteins are activated upon delivery of a virulence factor into the cell's interior by viral, bacterial, fungal, oomycete, or insect pathogens (Jones and Dangl, 2006). Activation of NB-LRRs leads to a faster and stronger response that can include rapid cell death at and surrounding the infection site. Improper activation of this output response could be dangerous leading to cell death, stunting of plant growth, and resulting in loss of seed yield (Zhang *et al*., 2003; Heidel *et al*., 2004). Hence, the plant immune system must differentiate between a harmless or helpful microbe and a pathogenic one, and translate

that recognition into an appropriately graded output. The output is controlled in the nucleus by plant-specific transcriptional regulators, many of which are members of the WRKY family (Euglem, 2005). NB-LRR proteins inhabit a variety of subcellular locations, from the inner face of the cell membrane to the cytosol (Jones and Dangl, 2006). In tomato, the resistance genes *I*, *I-1*, *I-2* and *I-3* have been described (Huang and Lindhout, 1997). One of these, *I-2* , has been cloned and belongs to a large group of *R* genes that encode intracellular proteins with nucleotide binding site (NBS) and leucine-rich repeat (LRR) domains (Simons *et al*., 1998). This *I-2* gene is specifically expressed in tissue surrounding xylem vessels (Mes *et al*., 2000), suggesting that resistance to xylem colonizing *F. oxysporum* is (mainly) mediated by xylem contact cells, which is in accordance with earlier histological observations (Beckman and Roberts, 1995). These contact cells are likely to respond to avirulence factors that are secreted by the fungus in xylem sap. More recently, the Xa27 a novel R protein was cloned from rice (Gu *et al*., 2005) that does not share homology with other R proteins. Interestingly, expression of the resistant *Xa27* allele occurs only in the vicinity of tissue infected by *Xanthomonas oryzae* pv. *oryzae*. The identification of Xa27 marks the first example of a differentially expressed R protein whose induction specificity dictates resistance.

1.4.3. Defense responses in chickpea

In chickpea too, there have been attempts in studying the transcriptional profiling of certain potential defense-related genes after *A. rabiei* inoculation, SA treatment and MeJ treatment (Cho and Muehlbauer 2004; Cho *et al*. 2005). These efforts studied the transcript levels by RNA gel blots and reverse transcription-polymerase chain reaction (RT-PCR) and showed that, although differential expression was observed for all treatments among the transcripts studied, resistance to *A. rabiei* did not correlate with SA- and MeJ-mediated regulation of the defense-related transcripts. Further, many putative *A. rabiei* defense-related genes were identified by Coram and Pang (2006) by employing microarrays constructed using a representative non-redundant set of chickpea unigenes, as well as putative defense-related cDNAs from grass pea (*Lathyrus sativus* L.). Gene expression changes were investigated in three genotypes with ranging levels of resistance/susceptibility to *A. rabiei* over two time-points after treatment with SA, MeJ and the immediate ethylene precursor aminocyclopropane carboxylic acid (ACC). These studies showed active defense mechanisms in chickpea and identified putative genes potentially involved in these responses, including the rapid synthesis of PR proteins, presence of an oxidative burst and the synthesis of putative cell-wall strengthening proteins, antimicrobial proteins, and numerous proteins of unknown identity. The specific transcripts that were reported as potentially predictive of *A. rabiei* resistance

included PR proteins, β-1,3-glucanase, SNAKIN2 antimicrobial peptide, hypothetical proline-rich protein (PRP), disease resistance response protein DRRG49-C, leucine-zipper containing protein (LZP), environmental stress-inducible protein (ESP), polymorphic antigen membrane protein (PAMP), Ca-binding protein and several unknown or unclear proteins (Coram and Pang 2006). Rapid expression of PR proteins in resistant genotypes (IC and FL) was considered important for *A. rabiei* resistance, and these proteins were induced by one or more signalling compounds (SA) in resistant genotype. Interestingly, elevation of cytosolic Ca^{2+} was not induced by the signalling compounds used, which may be attributed to the elevation of Ca^{2+} being a defense-activating signal in itself (Reddy 2001), possibly requiring pathogen perception to be triggered. However, two calmodulin-like proteins (DY396411 and DY396364) were up-regulated in resistant genotype, after SA treatment, which also represent Ca-binding proteins.

Full-length sequences for chalcone synthase and phenylalanine ammonia-lyase (PAL) cDNA, both important enzymes in defense response, have been reported in chickpea (Hanselle *et al*., 1999; Hein *et al*., 2000). The elicitation of chickpea cultured cells led to several rapid responses including an oxidative burst, extracellular alkalinization followed by intracellular acidification, transient K^+ efflux, and activation of defense-related genes, all within 2 h (Mackenbrock *et al*., 1993). Rapidly and transiently expressed genes encoded the first soluble enzyme in the pterocarpan biosynthesis part of the medicarpin and maackiain malonylglucoside phytoalexin pathway (Mackenbrock *et al*., 1993), a NADPH: isoflavone oxidoreductase (IFR; Tiemann *et al*., 1991) and at least eight members of the cytochrome P450 protein family also involved in isoflavone synthesis. This suggests that isoflavone metabolism is of considerable importance for resistance to *A. rabiei* (Barz & Mackenbrock, 1994; Overkamp *et al*., 2000; Cho *et al*., 2005). Increased expression was also found for mRNAs of rab and rac type small GTP binding proteins (Ichinose *et al*., 1999), and for genes encoding two glycine-rich proteins (GRPs), which displayed maximum expression 5 days post infection and are probably involved in fortification of cell walls by oxidative cross-linking of cell wall components (Cornels *et al*., 2000). In *planta*, a pathogenesis-related thaumatin-like protein (TLP) gene, PR-5a, and a second cDNA coding for a slightly larger TLP (PR-5b), presumably located in the vacuole, were elicited much faster in an *A. rabiei*-resistant chickpea cultivar than in a susceptible cultivar.

1.4.4. Chickpea defense responses to *Fusarium*

The host in response to pathogen invasion, presents defenses, mainly at two levels (**i**) Structural: in the vascular tissue, where the upward movement of the pathogen is arrested by compartmentalization of the pathogen through the formation of callose, gelgum and

tyloses, which are mainly the derivatives of celluloses and hemi-celluloses and progressive suberinization and lignin deposition (**ii**) Biochemical: the endodermis and xylem parenchyma, where the invading pathogen is restricted by infusion of phenolic compounds, and by hydrolytic enzymes like chitinases and glucanases. Fungal elicitors are known to induce the production of phenyl ammonia lyase (PAL) and peroxidase which are involved in the synthesis and depolymerization of lignin precursors. The rapid increase and higher levels of PAL and peroxidases activity was found in resistant cultivars as compared to the susceptible cultivars (Aguilar *et al*., 2000). Phenolics may function as either phytoalexins or be incorporated into structural barriers such as phenol-conjugated, lignified or suberised cell walls of appositions (Aist, 1983). Phytoalexins have been implicated as fundamental components of chickpea resistance mechanism to Fusarium wilt (Stevenson *et al*., 1997).

However, the genetics of Fusarium resistance is complex, since at least for resistance to race 1, a minimum of two out of three detected resistance genes are required (van Rheenen, 1992). Several studies employing inter- and intra-specific recombinant inbred line (RIL) populations have demonstrated the organization of resistance genes for Fusarium wilt races 1, 3, 4 and 5 (*foc*1, *foc*3, *foc*4 and *foc*5; Mayer *et al*., 1997; Ratnaparkhe *et al*., 1998; Tullu *et al*., 1998; Winter *et al*., 2000; Sharma *et al*., 2004) in two adjacent resistance gene clusters on linkage group (LG) 2 flanked by STMS markers GA16 and TA96 (*foc1–foc4* cluster) and TA96 and TA27 (*foc3–foc5* cluster), respectively (Fig 1.4). Apart from the resistance genes *per se*, other sequences coding for proteins putatively involved in the chickpea's defense reaction were localized in close vicinity to the Fusarium resistance gene clusters, like the sequence of one of the markers tightly linked to the *foc4* and *foc5* loci is similar to a PR-5 thaumatin-like protein gene and another is homologous to the gene for anthranilate *N*-hydroxy cinnamoyl-benzoyltransferase, a regulator of the phytoalexin pathway, both important components of the plant's defense against pathogens (Fig 1.4). Huttel *et al*., (2002) isolated a series of RGAs from both *C. arietinum* and *C. reticulatum* using two degenerate primer pairs targeting sequences in the NBS domain. A total of 48 different RGAs were grouped into 9 different sequence classes, and were members of the Toll-interleukin receptor (TIR)-NBS-LRR and coiled coil (CC)-NBS-LRR groups. Thirty of these RGAs were mapped on the reference genetic map of chickpea (Winter *et al*., 2000), where they could be located on principally five linkage groups, some of them as clusters on LGs 2 and 5, respectively (Fig 1.4). While, Flandez-Galvez *et al*. (2003) mapped 12 RGA markers that clustered on three LGs.

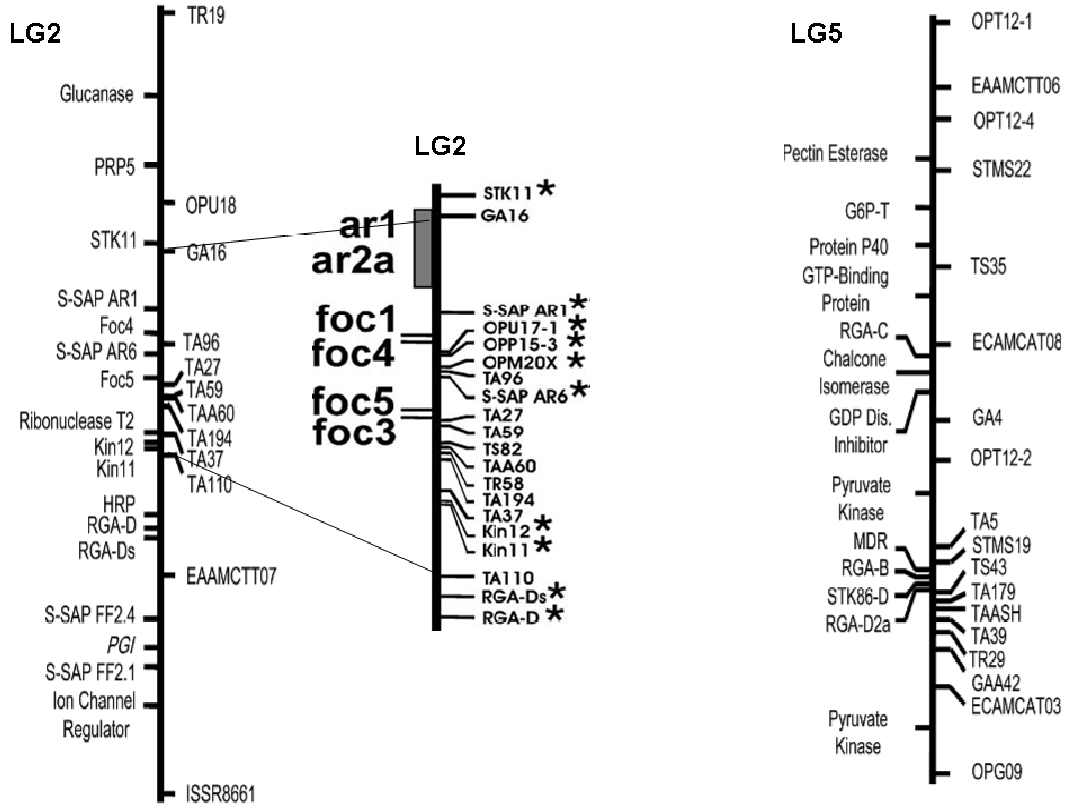


Figure 1.4: LG2 and LG5 from the integrated genetic map of chickpea generated by Millan *et al*. (2006) with data from Winter *et al*. (2000), Huttel *et al*. (2002) and Pfaff & Kahl (2003). Abbreviations for the different genetic markers are given in these papers. Markers on the left of the vertical bar are derived from genes and those on the right are STMS or dominant framework markers. Only a few markers necessary for demonstrating the context within the linkage groups are shown. Detailed map of LG2 is in the centre depicting the vicinity of the Fusarium resistance gene clusters including Fusarium resistance (foc) genes and QTL for Ascochyta blight resistance (ar1, ar2a, indicated by the shaded box) on the left side of the vertical bar. Markers on the right are STMS and DAF markers from Benko-Iseppon *et al*., (2003) and RGA markers from Huttel *et al*., (2002). Loci marked with an asterisk are potentially involved in pathogenesis, either encoding RGAs or pathogenesis-related proteins. (See Millan *et al*., 2006 for more detail).

It is usually accepted that the difference in resistant and susceptible cultivars lies in the speed with which they can activate the defense mechanisms and accumulate substances like callose to restrict the growth and spread of the pathogen. However, there is still a debate about the role of fungal toxins in vascular wilt diseases. *Fusarium oxysporum* is known to produce the toxin 'fusaric acid' in culture filtrates, but most of the disease symptoms are postulated to be caused by the plant response to infection.

Previous genetic studies directed towards functional understanding of genomic information in chickpea and Fusarium interaction seem incomplete due to unavailability of the wide range of genomic information or gene expression data. Most of the functional genomic approaches to identify genes for traits of interest were attempted by direct cloning of defense genes (Ichinose *et al*., 1999) or resistance gene homologues (Huttel *et al*., 2002) based on the information obtained from other model species. Although, directly applying the methods or genomic information from model plant species to chickpea may not always resolve the important issues. Getting a global perspective of the interaction transcriptome is thought to be as important as generation of one dimensional sequence data.

1.5. Genomics

The goal for the post-genomic era of plant biology is to assign functions for every gene in the plant genome. Functional genomics uses large scale methods in order to describe functions and interactions of genes by studying genomic sequences, transcripts, proteins, metabolites and phenotypes (Bouchez and Höfte, 1998; Somerville and Somerville, 1999; Colebatch *et al*., 2002a, 2002b; Holtorf *et al*., 2002; Kennedy and Wilson, 2004; Steinmetz and Davis, 2004). High throughput technologies of genomics, transcriptomics, proteomics, metabolomics and phenomics have shifted the focus from single gene research towards a holistic understanding of gene function. None of the methods used in isolation provide enough information to infer function for an unknown gene, instead, combined data from different functional genomics tools brings us close to this goal. Most of the tools used in functional genomics are based on traditional methods which have been adjusted for high throughput systems. At the genome level (DNA), gene function is studied using sequencers and bioinformatics while several methods have been developed in order to analyze the transcriptome (RNA) of an organism. Microarrays are the most widely and routinely used tools to study transcriptome activity, but also gel based (Differential display, cDNA-AFLP) and sequence based (ESTs, SAGE, MPSS) methods are available. The proteome can be studied with 2Dgels, 2D Liquid Chromatography (2DLC), Mass Spectrometry (MS) and Biomolecular Interaction Analysis Mass

Spectrometry (BIAMS) and the metabolome with, for example, Gas-Chromatography Mass-Spectrometry (GCMS). For phenotypic analysis, several different methods for mutant screens have been developed, although mostly in Arabidopsis.

Determination of transcript patterns in plants by qualitative and quantitative means is of importance to plant molecular biology. The quantitative and qualitative comparison of individual mRNA concentrations present in samples originating from different genotypes, developmental stages, growth conditions, different stimulus (inducers, pathogens pests or other stress) will enable us to identify genes that are differentially expressed and hence may have specific metabolic, morphogenetic, stress alleviatory or defensive functions. The sequence information has experienced a rapid growth, with concomitant increase in the number of putative proteins with unknown functions. The many genome projects, which focus on the genome sequencing, identify only approx. 30-40% of its coding sequences corresponding to known proteins. Also, there are more than 30-40% sequences which are orphans sharing no sequence homology to any known gene.

Due to this gap in understanding, currently the functions of about half of the many-sequenced genome's open reading frames are still not known. The value of transcript pattern analysis in assessing roles of novel sequences in the given organism cannot be ignored, since the similarity of expression patterns of sequences of unknown function with those of known genes would at least indicate functional homology. The potential of global transcript analysis can be seen by the four results that are returned: identify new markers for research, reveal *cis*-acting elements and *trans*-acting factors that cluster with a pattern of regulation, identify novel patterns of regulation, and find clusters of co-regulated genes with functional convergence that suggests the physiological relevance of their regulation (Kuhn, 2001). Further, when the transcript patterns are compared with the proteome data, it would enable to determine the preferential regulation of the intracellular concentration of specific proteins occurs at the level of transcription or by post-transcriptional mechanisms such as mRNA translation efficiency or partitioning between subcellular compartments (Kuhn, 2001).

Initially isolation of genes for which products and mutants were not known was accomplished by differential screening of cDNA libraries. Liang and Pardee (1992) developed the *in vitro* technique for the determination of transcript patterns known as differential display reverse transcription PCR (DDRT-PCR). For the first time it was possible to determine simultaneously, a large part of the transcripts present in a

eukaryotic cell within a single experiment with high sensitivity. The technique found wide applicability, and for few years no other method was available by which comprehensive transcript patterns of eukaryotic cells could be obtained. The use of DDRT-PCR, has been reviewed in both eukaryotes (Matz and Lukanyov, 1998) and prokaryotes (Fislage, 1998). Briefly, after cDNA synthesis using reverse transcriptase and an oligo-dT primer that anneals to the 3' poly-A tail of mRNA, subsets of cDNA populations for comparison are PCR amplified with short, non-specific oligonucleotide primers, in combination with oligo-dT primers, and visualized on polyacrylamide gels. Fischer *et al*. (1995) used DDRT-PCR in conjunction with amplified fragment length polymorphism (AFLP). AFLP, till then was a method used for the characterization of genomic DNA developed by Vos *et al*. (1995). The new technique, termed restriction fragment length polymorphism-coupled domain directed differential display (RC4D), which provided a useful tool to detect differentially expressed members of individual gene families.

SAGE (serial analysis of gene expression) is another technique, developed by Velculescu *et al*. (1995), where short fragments termed as tags are extracted from cDNA molecules, concatenated, cloned and sequenced. The abundance of a tag in the sequence reflects the abundance of the corresponding mRNA in the tissue from which the cDNA was prepared. The cDNA-AFLP technique (Bachem *et al*., 1996) is based on the selective PCR amplification of adapter-ligated restriction fragments derived from cDNA. cDNA-amplified fragment length polymorphism (cDNA-AFLP), which belongs to the category of Differential Display (DD) methods, is based on the discrimination of the cDNAs obtained by PCR, corresponding to mRNAs derived from different samples. Currently it's the era of microarray hybridization technology, which has seen rapid growth and popularity since its emergence with the simultaneous quantitative determination of mRNA concentrations of a small set of Arabidopsis genes by a cDNA microarray (Schena *et al*., 1995). The technology has now developed to a level where the state of expression of complete genomes can be recorded with high accuracy on a single chip. Yet this technique has not stagnated the development of other quantitative large scale screening methods. The throughput of the most advanced AFLP and SAGE protocols is comparable to microarrays. In contrast to other DD methods, cDNA-AFLP allows systematic study of transcriptome through the use of selective fragment amplification (Vos *et al*, 1995; Bachem *et al*, 1998) and, considering its good reproducibility, sensitivity and correlation with northern analysis, it has become the most frequently applied DD technique.

1.5.1. A Method to study interaction transcriptome

The fundamental issue of any diseases is to globally and integratively understand the interactions between pathogens and their hosts by using fast and effective techniques. Most defense responses are accompanied by rapid transcriptional activation of many genes (>1% of the genome), often with unknown function (Durrant *et al.* 2000; Maleck *et al.* 2000; Schenk *et al.* 2000). The advent of DNA microarray technology has revolutionized the study of plant-pathogen interactions and has already provided novel insight into the involved pathways and their interactions with one another. However, in plant species, for which it is difficult to generate microarrays, cDNA-AFLP technique lends itself to gene discovery. The technique has meager requirements with no need of prior sequence information. While being very sensitive, it allows the detection of low abundance transcripts. Additionally, the set-up cost for a laboratory to use this technique is lower than that for microarrays as no special equipment is required, making it a method of choice for a large number of researchers (Ramonell and Somerville, 2002).

In this study, cDNA-AFLP (Fig 1.5) and cDNA-RAPD, both gel based tools for the analysis of the chickpea transcriptome, have been utilized. Transcriptional analysis is a powerful tool to discover novel genes and to get information on the expression of unknown genes in different tissues of plants under different external stimuli. Though in the recent years, functional analysis of the transcriptome using microarrays has become the most prominent tool to study and understand gene function mainly because of its throughput and extensiveness. The gel and sequence based transcriptional analysis methods still hold their place due to inherent numerous advantages that they offer.

1.5.2. cDNA-AFLP for differential gene expression profiling

In this section the cDNA-AFLP techniques available to determine transcript patterns and to identify differentially expressed genes in plants have been summarized. A major challenge in analyzing plant-microorganism interactions is often the small amount of biological material available. This limitation has been overcome using PCR based methods, initially developed for DNA fingerprinting, which allows comparing profiles of gene expression (following conversion of mRNA to cDNA). A step by step schematic representation of the cDNA-AFLP protocol detailing the steps and different components involved has been made in Fig 1.5.

Isolation of differentially expressed genes from both host and pathogen or symbiont during their interaction might be dependent on the method of cDNA synthesis.

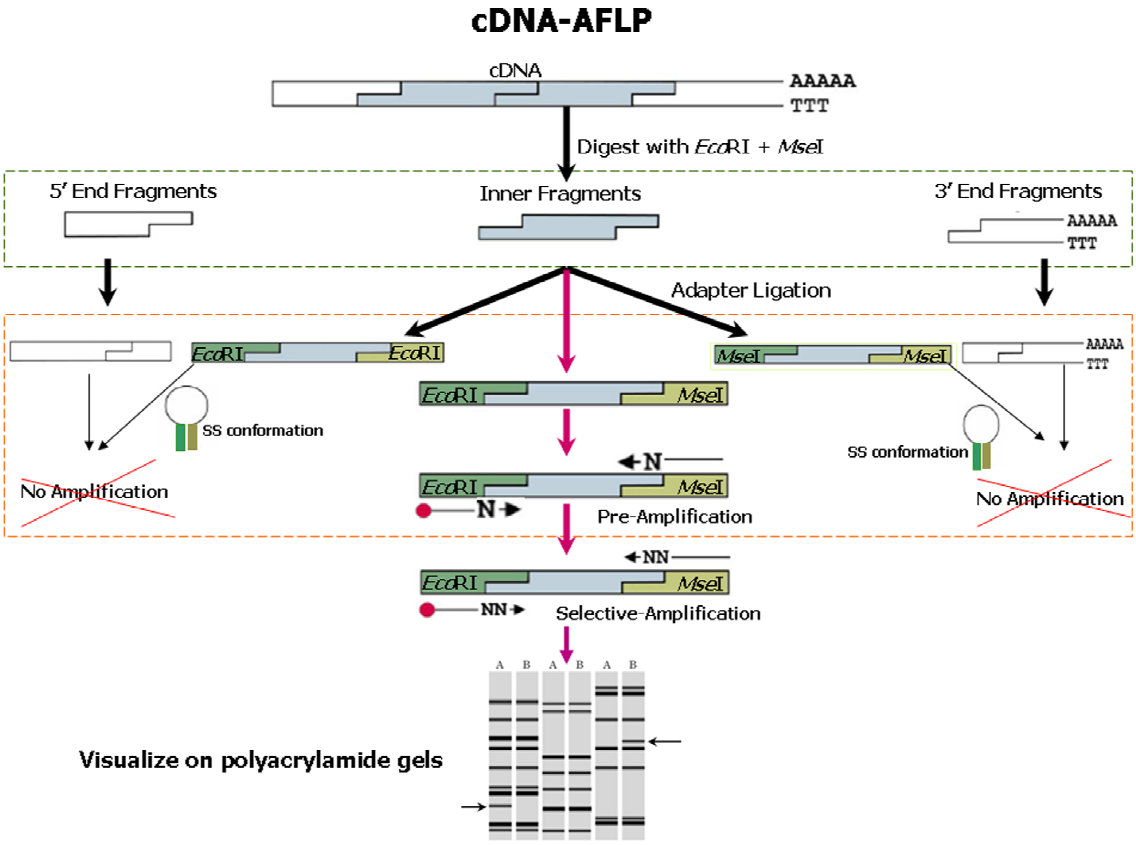


Figure 1.5: The scheme of classical cDNA-AFLP. Double stranded cDNA were synthesized from total RNA or mRNA (cDNAs rescued from λ-Zap phase library as in present case) and were digested with *Mse*I and *Eco*RI enzymes, which recognize four and six bp, respectively. A complete digest of plant cDNA with these enzymes produces five different types of fragments: *Mse*I/*Mse*I, *Mse*I/*Eco*RI, *Eco*RI/*Eco*RI, *Mse*I/poly(A) and *Eco*RI/poly(A) fragments. Following digestion, double-stranded adapters were ligated to the restriction fragments to generate templates for amplification. PCR amplification is carried out in two steps (i) pre-amplification and (ii) selective-amplification. In the pre-amplification step, around 15 cycles of amplification were carried out using primers without extensions. The products of this reaction were then subjected to a second round of PCR amplification using primers bearing one, two or three additional nucleotides extensions at their 3' end, allowing only a set of subpopulation to be amplified. The amplicons were separated on a polyacrylamide gel and visualized by silver staining/autoradiography. RNA probes from different sources (A, B) produce different cDNA-AFLP banding pattern, which allowed differentially expressed cDNAs to be identified (arrows). (Bachem *et al*., 1996; Kuhn, 2001).

Of the studies referred to above (Benito, *et al.* 1996; Seehaus and Tenhaken, 1998; Geri, *et al.* 1999; Lapopin, *et al.* 1999; Timmusk, and Wagener, 1999; Hermsmeier, *et al.* 2000), only two (Lapopin, *et al.* 1999; Hermsmeier, *et al.* 2000) have reported identification of both host and microorganism cDNAs. Both these reports involved eukaryote-eukaryote interactions in which both host and microorganism mRNAs possess 3' poly-A tails and were thus converted to cDNA using an oligo-dT primer. However, prokaryotic organisms do not generate mRNAs with 3' poly-A tails, and thus synthesis of bacterial cDNA cannot be performed using an oligo-dT primer. Recently, using a mixture of 11-mer primers designed to anneal to conserved regions in the 3' ends of enterobacterial genes, representative cDNAs have been synthesized from the bacterial plant pathogen *E. carotovora*. Differential gene expression in *E. carotovora* grown in different media was profiled using cDNA-AFLP (Dellagi, *et al.* 2000). But the situation is different when studying fungal pathogen wherein mRNA with 3' polyA tail is expected. In such situations the software program 'Eclat' developed by Friedel *et al*., (2005) can be used which checks the origin of the EST/TDF generated as to fungal or plant (http://mips.gsf.de/proj/est/). Currently application of 'Eclat' is optimized for *Blumeria*/Barley system. Making this program broad based to accommodate other systems would greatly enhance its utility. Like DDRT-PCR, cDNA-AFLP is derived from a DNA fingerprinting method and again involves selective PCR amplification of sub-sets of cDNA populations for comparison on polyacrylamide gels. The work of Dellagi *et al.* (2000) offers the possibility of distinguishing between differentially expressed bacterial and plant genes during the *E. carotovora*-potato interaction by using different strategies for cDNA synthesis. Synthesis of cDNA from the interaction using an oligo-dT primer should produce cDNA specifically derived from eukaryotic mRNA (i.e. from the plant), whereas priming with the 11-mer oligonucleotides should efficiently synthesize cDNA from the bacterium. The two cDNA populations can then be compared by profiling them using cDNA-AFLP. The cDNA-AFLP technique has also been effectively used to identify tomato cDNAs that are up-regulated in the resistance response to *Cladosporium fulvum*, when R protein Cf-9 is activated by the Avr9 protein from the pathogen (Durrant, *et al.* 2000). Several plant genes that were rapidly induced by Avr9 elicitation were also up-regulated by wounding, again indicating that common pathways might be activated in both, the defense and the stress. The authors did not report clear identification of pathogen cDNAs in this analysis. The gene expression profiling approach of cDNA-AFLP allows all components of the interaction transcriptome (genes that are up- and down-regulated or constitutively expressed) to be

identified simultaneously. Nevertheless, in spite of their documented success, these techniques do not allow rapid, high-throughput generation of cDNA sequence data, because large numbers of PCR primer combinations are required to profile all transcripts within an infected eukaryotic cell.

Ditt *et al*., (2001), made an attempt to systematically explore the host gene expression response to *Agrobacterium.* In addition to identifying factors that might be relevant for transformation, such a study of changes in gene expression helps elucidate the general response of the plant to *Agrobacterium* infection. This information could be compared with the responses of plants to other pathogens and symbionts. The use of a differential screen, the cDNA–AFLP, helped to examine the initial response of gene expression in plant cells exposed to *Agrobacterium*. It was shown that a number of plant transcripts have altered expression at 24 and 48 h after interaction with *Agrobacterium*, and the proteins encoded by these genes have a putative role in plant signal transduction and in defense response. Many plant-pathogen interactions are governed by specific interactions between pathogen avirulence (Avr) genes and corresponding plant resistance (R) genes. An interaction where a corresponding pair of R gene and Avr gene is present and expressed, results in incompatibility and the plant is resistant. When one of the two is inactive or absent, the interaction is compatible and the plant is susceptible. By cDNA-AFLP analyses, transcripts were identified that specifically accumulate after pathogen infection in potato. Using RNA from potato leaves infiltrated with *P. syringae* pv. *maculicola* for 3 and 12 h, several bands were detected that correspond to genes which show increased expression in response to bacterial infiltration (Petters *et al*., 2002).

Carmona *et al*., (2004) determined through cDNA-AFLP molecular events associated with the sugarcane resistance after infection with *P. melanocephala* by isolating cDNA sequences that are induced in a highly resistant somaclone. Somaclonal variation has been widely employed for plant genetic improvement (Karp 1991). In sugarcane, this source of variability has been used in the selection of clones resistant to biotic and abiotic stress (Ramos *et al*. 1996). Kemp *et al*., (2005) identified and characterized some of the genes induced in cassava leaves in response to the incompatible pathogen *Pseudomonas syringae* pv. tomato (*Pst*). This tomato pathogen induces the HR in cassava leaves; the interaction was chosen because of the slow and incomplete expression of ‘resistant’ or ‘tolerant’ cultivars of cassava to *Xam*. In addition, leaf invasion is the typical initial means of infection; the resulting water-soaked angular lesions often extend to a systemic vascular phase.

Table 1.2: Use of cDNA-AFLP in combination with other techniques aids in generating more specific information.

cDNA-AFLP in combination with-	Targeted goals	Reference
Imaging techniques to probe the location of specific molecules; screening for variation in architecture using IR spectroscopy	Transcription and translation of the genes; the assembly of the components.	McCann *et al*., 2001
In silico AFLP	Suitability of Restriction enzymes for cDNA-AFLP	Breyne *et al*., 2003
BSA (Bulk Segregant Analysis)	May be effective for detecting expressed sequence tags. Cloning candidate genes for a given trait	Barcaccia *et al*., 2001; Cho *et al*., 2005; Guo *et al*., 2006
'**Gene Swinger**' program of affymetrix	Analyze for variant expression of ESTs with no known match or function	Kemp *et al*., 2005
Metabolic profiling	Compares differential expression of the genes and respective metabolites	Goosens *et al*., 2003; Rischer *et al*., 2006
Publicly available marker genotype data and application of classical linkage analysis	Identifies numerous transcriptional regulatory loci explaining the variation in gene expression phenotypes.	Vuylsteke *et al*., 2006
'**E*clat***' program	To find the origin of ESTs as to plant or fungal.	Friedel *et al*., 2005

Cho *et al*., (2005) have attempted to **(1)** screen for genes, showing constitutively different expression levels between resistant and susceptible recombinant inbred lines (RILs) using cDNA-AFLP and **(2)** elucidate their genetic significance in conferring resistance to Ascochyta blight. They identified a flavanone 3-hydroxylase (F3H) like TDF and found it to be lower in RILs susceptible to pathotypes I and II of *A. rabiei* than in RILs resistant to both pathotypes. F3H was mapped to linkage group 5. Santaella *et al*., (2004), used cDNA-AFLP to isolate genes from cassava that are expressed during infection by *Xam*. The expression pattern of a selected set of transcript-derived fragments (TDFs) was confirmed by quantitative reverse transcription-PCR (QRT-PCR).

Escalettes *et al*., (2006) have applied the cDNA-AFLP technique on the partially resistant apricot cultivar 'Goldrich' infected with PPV to characterize any functional or structural genes whose expression is modified during virus infection. As it is expected these candidates might be linked to the compatible or incompatible interaction of PPV with its woody host plant (*Prunus armeniaca* L.). Genes that show enhanced or repressed expression during virus infection may be involved either in the host defense mechanism or in the infection process. Van de Velde *et al*., (2006), presented a comprehensive transcriptomics dataset that demonstrates that nodule senescence is a complex and regulated process. Transcript-profiling analysis of developmental nodule senescence was performed in the legume *M. truncatula* through a modified cDNA-AFLP protocol (Breyne *et al*., 2003). A very specific sampling method coupled to cluster analysis allowed distinct stages of the nodule senescence process to be identified.

cDNA-AFLP is a robust, selective and sensitive expression profiling technique which can also be effectively combined with other methods to generate more specific data depending on the aim of the experiments as highlighted in Table 1.2. The standard hybridization based techniques like microarrays are close ended, wherein certain strain-specific genes will not be assessed and important information will not be revealed. Relying solely on closed expression systems limits applicability and progress will be hampered since the scope of these methods is restricted only to already known genes. Open expression systems, which do not require prior knowledge of sequences, are more widely applicable and inherently have the advantage of identifying and assessing new genes (Reijans *et al*., 2003). PCR-based techniques have shown to be more sensitive than hybridization-based techniques (Hoheisel and Vingron 2000), and are more suited to identify new, low-expressed genes. cDNA-AFLP is a PCR-based, open transcript profiling technology and combines both the feature of high-throughput analysis and detection of rare expressed transcripts.

1.6. Objectives of the thesis work

The importance of the chickpea crop in Indian perspective and the devastating damage caused by its pathogen *Fusarium oxysporum* f.sp. *ciceri* necessitated the work, which was carried out keeping in mind the following objectives

1) To identify differentially expressed genes in chickpea during FOC1 pathogen challenge by cDNA-RAPD and cDNA-AFLP.
2) To compare the differentially expressed transcripts from Fusarium wilt-susceptible and -resistant chickpea varieties.
3) To isolate and clone the differentially expressed gene transcripts from the roots of resistant cultivar infected with FOC1.
4) To confirm the expression patterns of the TDFs by reverse northern and northern analysis.
5) To isolate, the cDNAs encoding complete ORFs of 14-3-3 genes from *C. arietinum*, to clone and sequence them.
6) To study the expression pattern of the *Ca1433* genes in roots of wilt-susceptible and -resistant varieties of *C. arietinum* during FOC1 pathogen challenge.
7) To survey the database for transposable elements in TC and EST sequences.
8) To analyze the abundance to the transposable element sequences in different crops, tissue and under different conditions (stress and unstressed).

1.7. Organization of the thesis

After setting up of the objectives for the study the execution of the experiments was done in a phase wise manner which led to the formation of the thesis. In this thesis I have made an attempt to take a step further in the realm of information currently available on plant fungal interactions based on a study of the genes expressed during the interaction between chickpea and *Fusarium*. The thesis is organized into five chapters, the first being the introduction to the plant and the pathogen under the study and a detailed review of the techniques currently available to study the interaction transcriptome. Each chapter starts with its own individual abstract, introduction, materials and methods, results and discussion, relevant to the topic covered therein. Second chapter describes the differentially expressed genes identified during chickpea and Fusarium interaction. The third chapter describes the isolation, cloning and characterization of 14-3-3 genes from chickpea which were identified using cDNA-AFLP in the second chapter. The fourth chapter describes the presence and abundance of transposable elements in the EST database since significant representation of transposable elements were identified using cDNA-AFLP. In the fifth and final chapter I have summarized the entire gist of the work.


Chapter 2

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


The research work described in this chapter has been published as a full-length paper in PMPP (2006); 68: 176-188.

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 sp *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^{o}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^{o}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 1x10^{6}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 ^{0}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 ^oC, 1 min at 35 ^oC, 1.30 min at 72 ^oC. Cycling was concluded with a final extension at 72 ^oC 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 *Eco*RI and *Mse*I and ligated to double stranded *Eco*RI and *Mse*I adapters. Pre-amplification was carried out with 'E' and 'M' primers corresponding to the *Eco*RI and *Mse*I 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 94ºC for 30 sec; 65 – 56ºC (decrease 0.7ºC each cycle) for 30 sec; 72ºC for 60sec; followed by 24 cycles of 94ºC for 30 sec; 56ºC for 30 sec; 72ºC 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 H_2O at 37^{o}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 $MgCl_2$, 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 ^{o}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 $MgCl_2$, 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 ^{o}C overnight.

Transformation

Chemically ($CaCl_2$) 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 ^{o}C, 1 ml Luria bertani (LB) medium was added to the transformation mixture and incubated for 90 min at 37 ^{o}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 H_2O (for selection of transformed cells) and incubated for 16 h at 37 ^{o}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 (AmershamBiosciences, 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 ^{o}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 ^{o}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^{o}C for 1 h in the presence of RNasin (0.4U) and terminated by heating at 65^{o}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^{o}C for 1 h. The RNA pellet was collected by centrifugation at 12,000 g for 10 min at 4^{o}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^{o}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 H_2O) 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/cm^2) 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/cm^2). 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. αP^{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^{o}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 70,000µJ/cm^2. 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. αP^{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 synthatase, 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.

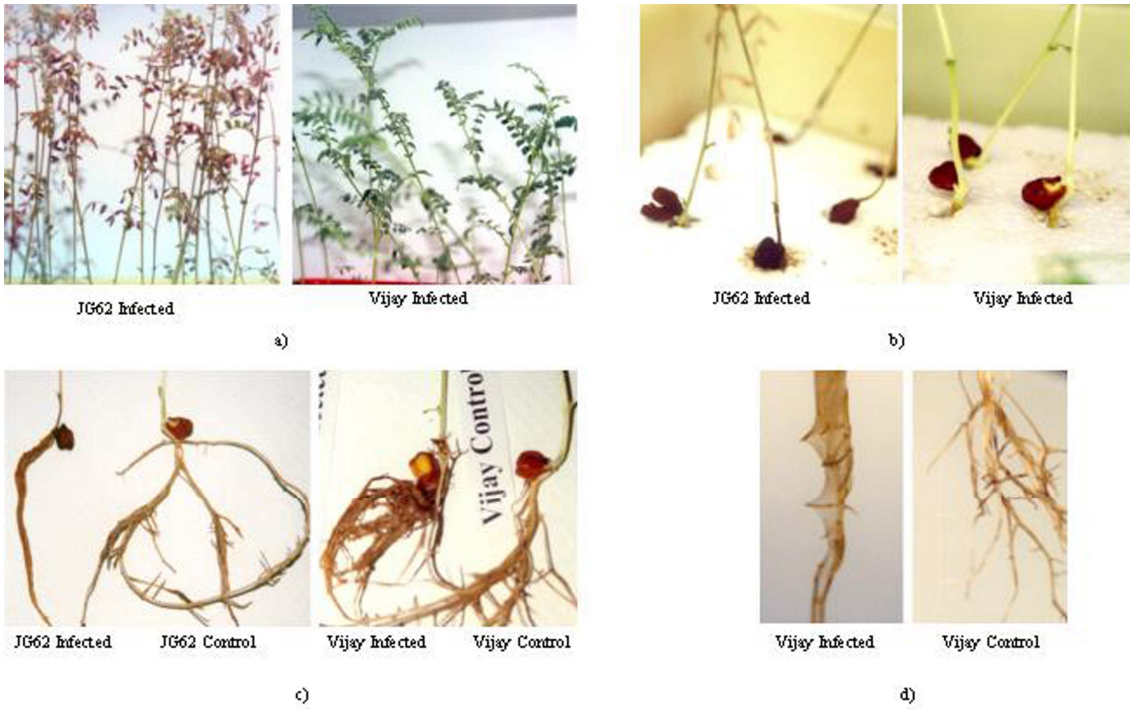


Figure 2.1: a) Chickpea seedlings hydroponically growing in growth chamber; b) JG-62 seedling showing wilting symptoms after infection with FOC1 while Vijay seedlings are healthy after infection; c) Root morphology of JG-62 and Vijay after infection; d) Difference between infected roots of Vijay covered with fungal mycelial mass and non-infected roots without any fungal mycelia.

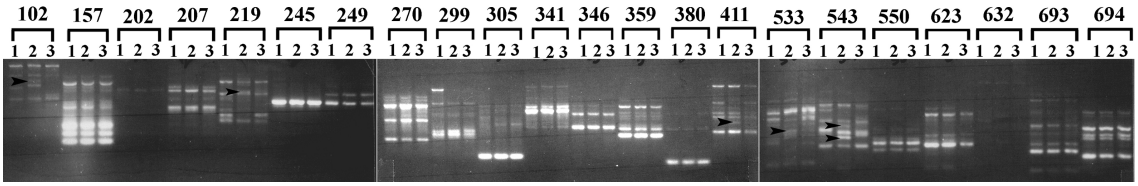


Figure 2.2: Representative amplification profiles generated by RAPD primers. Templates were cDNA libraries, RC (lanes 1), RI (lanes 2) and SI (lanes 3). The upper number indicates the primer number used for amplification for the set of three cDNA libraries. Arrows indicate DNA fragments differentially 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

Clone Id	GB Accession	Length (bp)	Homology	e-Score
TDF similar to known genes				
CaFRi60	DR749500	650	Gamma-glutamylcysteine synthetase mRNA	1e-147
TDF similar to known ESTs				
CaFRi58	DR749499	266	*G max* cDNA clone Gm-r1083-4905 [gi 3941321]	1e-20
CaFRi62	DR749501	516	*M truncatula* clone pGLSD-33B19 [gi20175779]	7e-138
CaFRi65	DR749502	312	*M truncatula* clone pHOGA-7H14 [gi 13781558]	1e-27
CaFRi67	DR749503	478	*M truncatula* clone MTUS-15C7 [gi 33105673]	4e-62
CaFRi70	DR749504	519	*L corniculatus* clone SPD012c01_f [gi 45578828]	8e-107
TDF not similar to any sequences in the GenBank				
CaFRi56	DR749498	379	No match	-

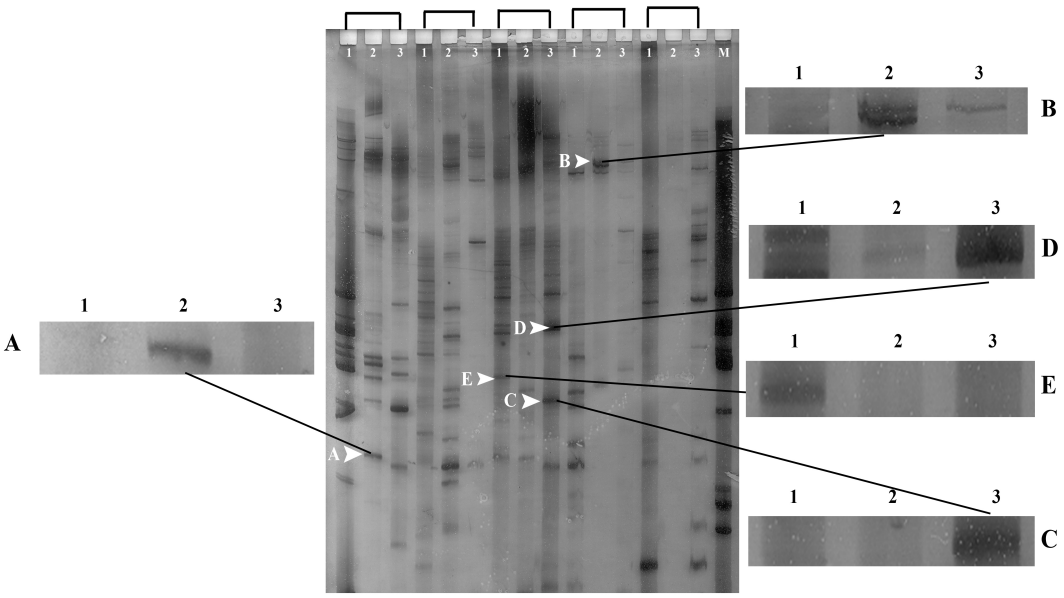


Figure 2.3: Representative amplification pattern from three cDNA libraries, resistant control (RC), resistant infected (RI) and susceptible infected (SI), displayed by cDNA-AFLP visualized on 6% polyacrylamide gel by silver staining. Templates were cDNA libraries, RC (lanes 1), RI (lanes 2) and SI (lanes 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.

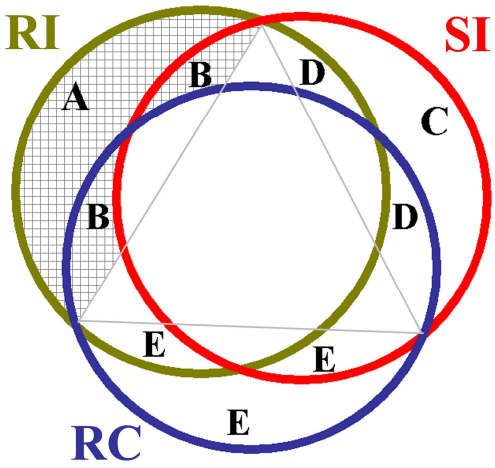


Figure 2.4: TDFs identified after differential display were categorized into five classes and are schematically represented as the three circles representing the three libraries under study Green- Resistant infected; Red- Susceptible infected; Blue- Resistant control, and the area under the overlap denotes 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.

Class	Origin of TDFs	No. of TDFs	Remarks/Significance
A	Bands seen only in the resistant infected (RI) sample	78	Represents genes/transcripts differentially expressed in resistant cultivar during pathogen infection.
B	Bands of more intensity in the RI sample	46	Represents genes/transcripts which are up regulated in resistant cultivar during pathogen infection
C	Bands seen only in the susceptible infected (SI) sample	52	Represents genes/transcripts differentially expressed in susceptible cultivar during pathogen infection
D	Bands of more intensity in the SI sample	25	Represents genes/transcripts being up regulated in susceptible cultivar during pathogen infection
E	Bands seen only in the resistant control (RC) sample or bands of more intensity in the RC	72	Represents genes/transcripts differentially expressed or up regulated in the resistant cultivar under no pathogen stress.
	Total Differentially Expressed TDFs	**273**	

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

Clone Id	GB Accession	Length (bp)	Homology	e-Score
TDFs similar to genes having role in defense				
CaFRi3	DR749491	300	WRKY protein	8e-05*
CaFRi4	DR749492	262	14-3-3 protein	2e-23
CaFRi9	DR749494	228	NBS-LRR	2e-22
CaFRi11	DR749495	286	NBS-LRR	3e-07
CaFRi26	DR749481	229	NBS-LRR	1e-33
CaFRi28	DR749482	201	Hydrolase alpha/beta fold family protein	6e-06
CaFRi33	DR749484	145	Class III chitinase	9e-08
TDFs similar to Organelle genes				
CaFRi36	DR749485	103	Mitochondrial F1 ATPase	1e-09
CaFRi42	DR749487	155	60S ribosomal protien L10	2e-09
CaFRi51	DR749489	88	Plasma Intrinsic protein [pip-2 gene]	3e-06
TDFs similar to Retroelements				
CaFRi12	DR749493	400	Non-LTR retroelement	2e-05
CaFRi15	DR749496	206	GAG-POL precursor gene	8e-11
CaFRi20	DR749479	170	Ty-1 copia retrotransposon	1e-76
TDFs similar to known ESTs				
CaFRi39	DR749486	273	cDNA clone wlm24.pk0020.e10	1e-70
CaFRi2	DR749490	305	pinus induced compression wood	2e-04
CaFRi48	DR749488	284	cDNA clone wh25g01	1e-41
TDFs not similar to any sequences in the GenBank				
CaFRi1	DR749497	199	No match	-
CaFRi23	DR749480	137	No match	-
CaFRi30	DR749483	90	No match	-

* 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).

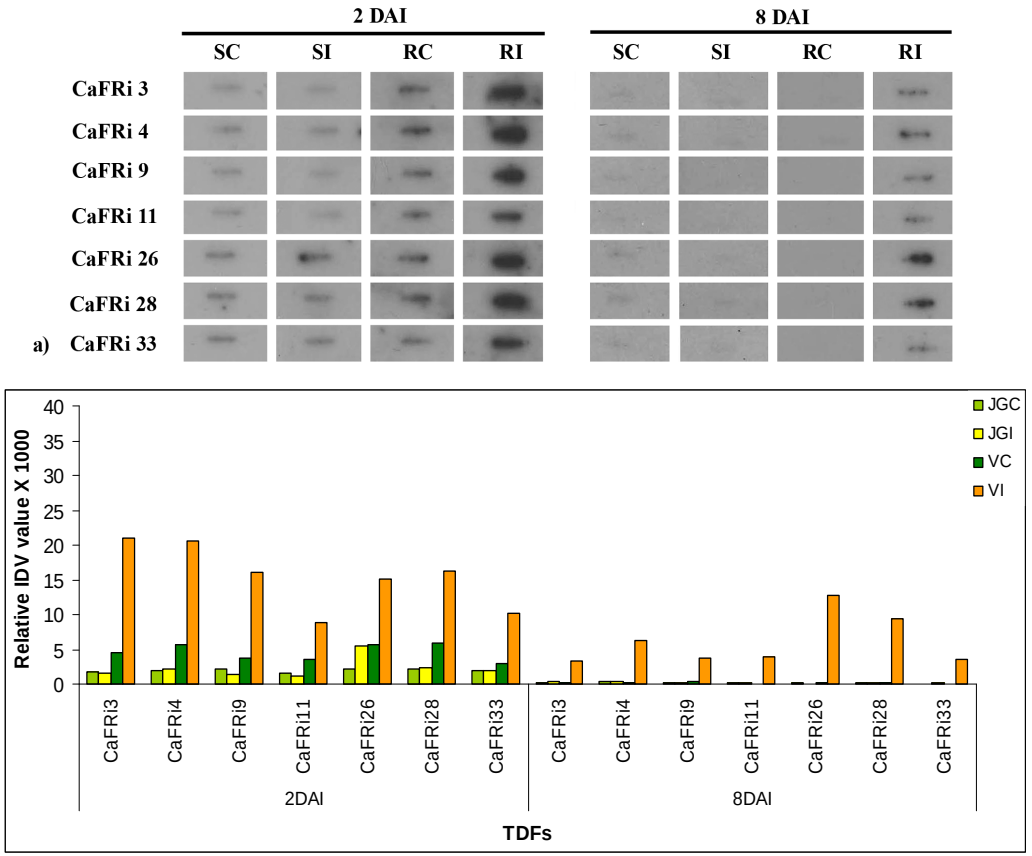


Figure 2.5: Reverse northern analysis of TDFs identified by cDNA-AFLP and cDNA-RAPD in chickpea roots. 19 cDNA-AFLP TDFs and seven cDNA-RAPD TDFs were hybridized with 5 µg of total RNA from JG-62 susceptible control (SC), JG-62 susceptible infected (SI), Vijay resistant control (RC) and Vijay resistant 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.

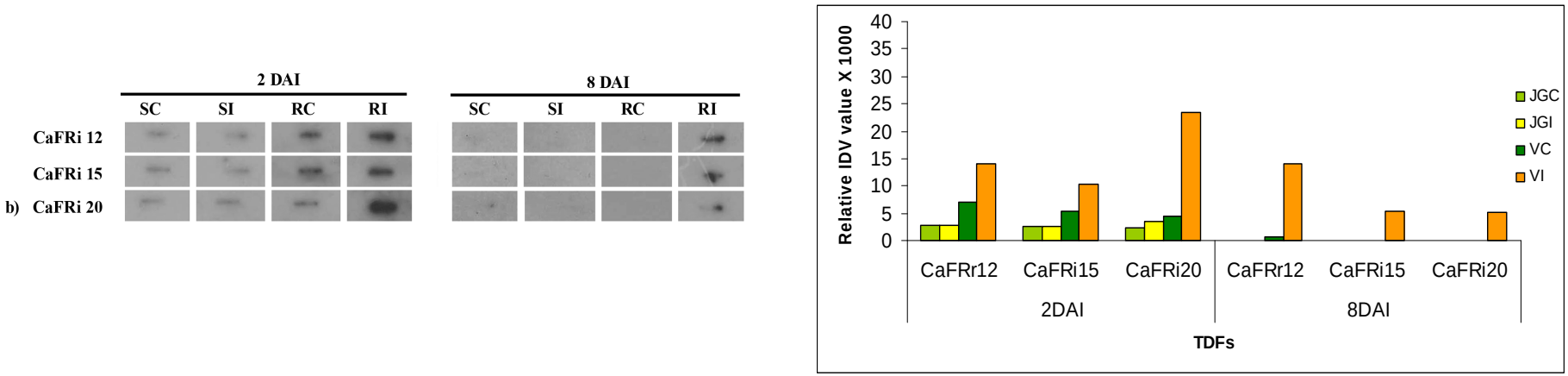


Figure 2.5 (b): TDFs similar to retroelement like sequences

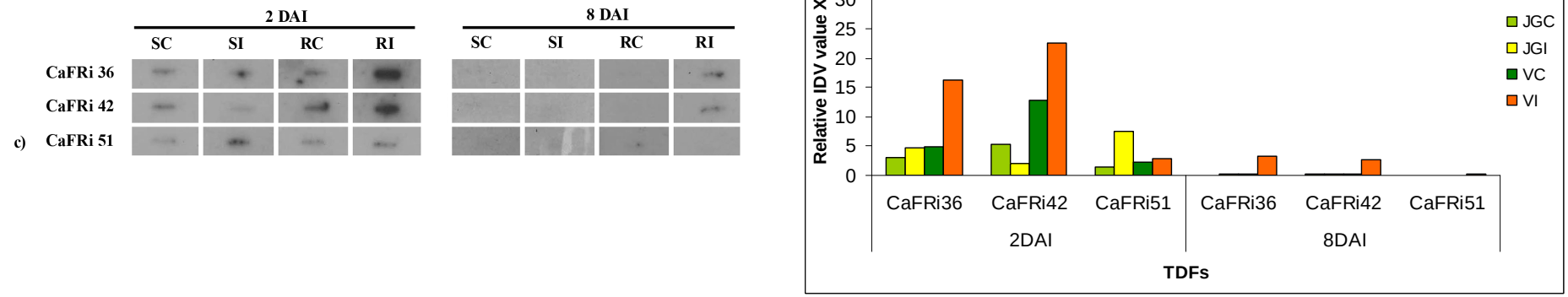


Figure 2.5 (c): TDFs similar to genes encoded by mitochondria and ribosome

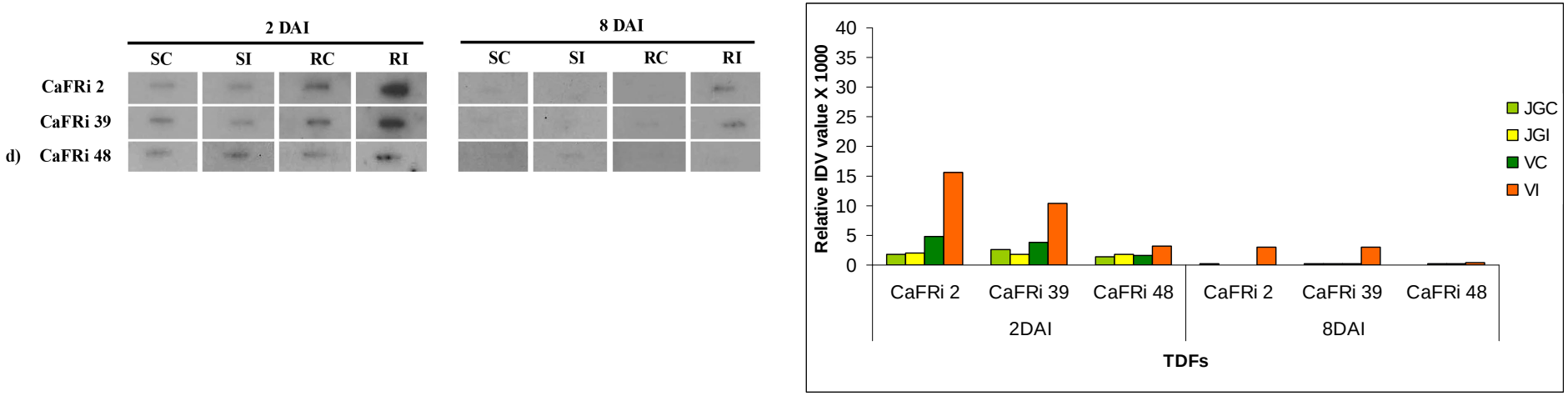


Figure 2.5 (d): TDFs similar to other EST sequences in the database.

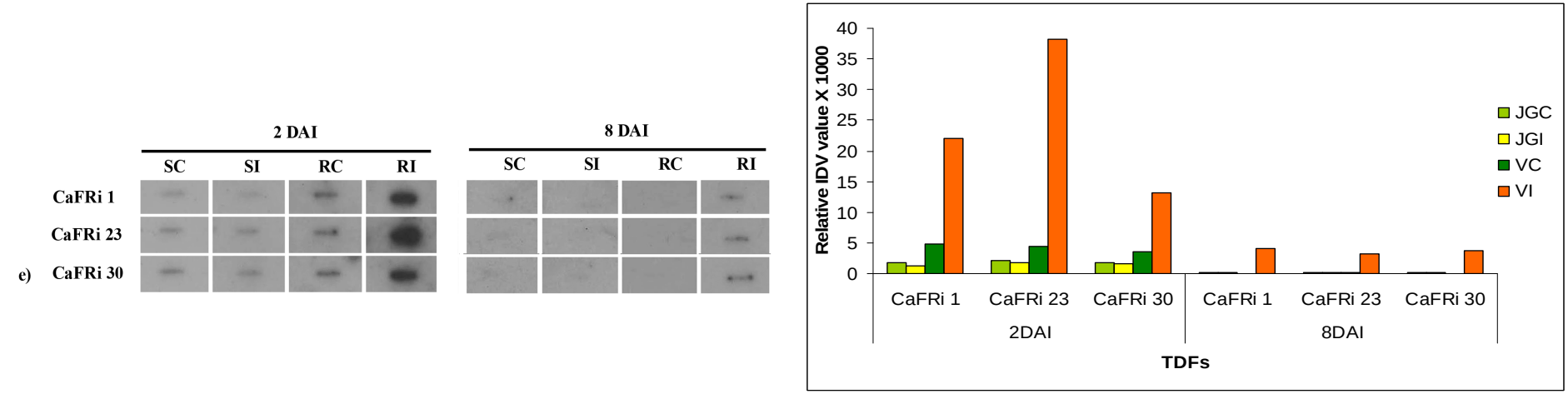


Figure 2.5 (e): TDFs showing no homology to any sequences in the GenBank database.

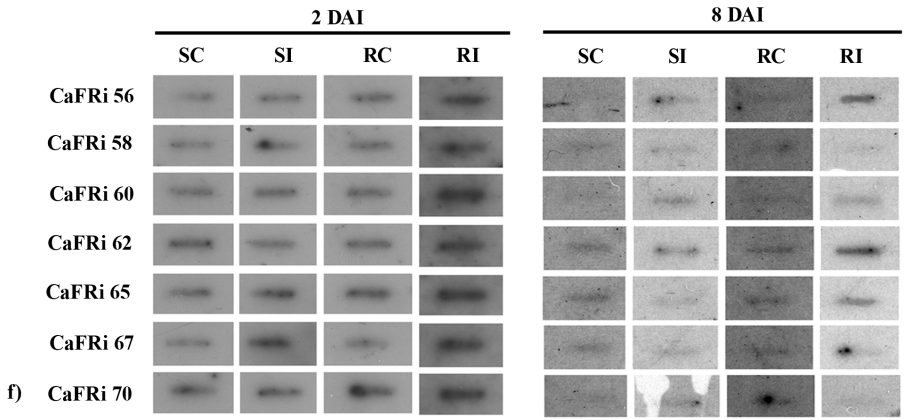


Figure 2.5 (f): TDFs identified by cDNA-RAPD in chickpea roots; CaFRi 60 corresponds to gamma-glutamyl-cystiene synthatase.

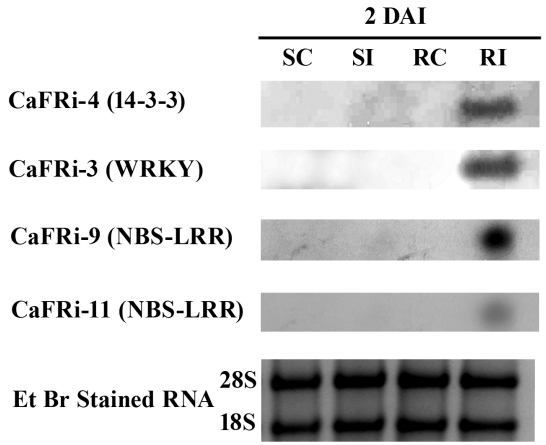


Figure 2.6: Induction of transcript accumulation in chickpea roots after challenge with FOC1 analyzed by Northern blot. Expression levels for clones CaFRi4 (14-3-3 like protein), CaFRi3 (WRKY like protein), CaFRi9 and CaFRi11 (NBS-LRR) at 2 DAI in susceptible control (SC), susceptible infected (SI), 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 *Phytopthora* (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 h_1, h_2, and h_3. 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 ($h_1h_1h_2h_2h_3h_3$) while the susceptible line, JG-62, was characterized as having the genotype $H_1H_1H_2H_2h_3h_3$. 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.

Chapter 3

Molecular cloning of 14-3-3 genes from chickpea (*Cicer arietinum*) and their characterization.

The research work described in this chapter has been communicated as a full-length paper to Plant Science

Abstract

Fusarium oxysporum is a ubiquitous soil-borne fungus, existing as both, pathogenic and nonpathogenic variants which colonizes root cortex. The pathogenic strain of the fungus invades the plants' vascular system and plugs the conducting strands leading to wilt. However, the molecular events that lead to onset of the disease or resistance in host plants are poorly understood. In earlier chapter (2), a TDF corresponding to 14-3-3 has been isolated as one of the stress responses in chickpea challenged by *Fusarium oxysporum* f.sp. *ciceri* race1 (FOC1). In the present chapter I have investigated expression of two 14-3-3 isoforms in chickpea roots in response to the pathogen challenge. Semi quantitative RT–PCR using gene specific primers revealed constant basal-levels of 14-3-3 transcripts in chickpea roots under normal growth conditions, and diverse transcription patterns in response to FOC1 pathogen attack in the roots of FOC1 resistant (Vijay) and susceptible (JG-62) varieties of chickpea. Pathogen responsive gene expression patterns of 14-3-3s indicated an expression-specificity of the isoforms in the 14-3-3 gene family. Phylogenetic analysis of full-length cDNA sequences and deduced amino acid sequences showed that these two 14-3-3 isoforms were clustered in two different groups that are presumably involved in the signaling pathways in response to *Fusarium* infection.

3.1. Introduction

The 14-3-3 proteins were characterized initially during a survey-and-catalogue project of proteins that appeared to be specific to mammalian brain tissue (Moore and Perez, 1967). Operational designations were given to proteins based on chromatography elution and starch-gel electrophoresis profiles. Several three-numbered proteins appeared to be brain specific, including 15-4-1, 4-4-2, and 14-3-3. For the 14-3-3 proteins, subsequently this three-numbered designation has become their *de facto* name, even though it conveys no functional information. Some proteins from this initial catalogue were renamed once a function was determined, but for the 14-3-3 proteins the three numbered designation nomenclature has persisted.

In the recent times the Greek letter designations are used for the members of the *Arabidopsis* 14-3-3 family of proteins. This nomenclature is in keeping with the early 14-3-3 literature, which originally differentiated the 14-3-3 protein variants as isoforms that were eluted differentially during column chromatography of brain extracts. However, the current list of 14-3-3 isoforms from *Arabidopsis* is based on gene sequences rather than biochemical differentiation. In addition, the three-letter gene name for *Arabidopsis* 14-3-3s is GRF (general regulatory factor) (Rooney and Ferl, 1995), and *Arabidopsis* 14-3-3 gene and isoform designations often include the name GF14 (G-box factor 14-3-3 homolog) preceding the Greek letter (Wu *et al*., 1997). In the time since their initial discovery, 14-3-3s have been found in every eukaryotic organism tested, yet remain absent from the genomes of prokaryotes (Aitken *et al*., 1992; Ferl *et al*., 1994; Ferl, 1996; Wang and Shakes, 1996; Rosenquist *et al*., 2000). Hence, the 14-3-3s appear to be a wide-spread, but essentially eukaryotic family of proteins, and are now considered an integral part of signal transduction.

In plants, a number of 14-3-3 genes have been identified, and additional functional roles of their products, have begun to be identified, apart from their interactions with the H^+-ATPase. For example, initially the 14-3-3 proteins from *Arabidopsis* and maize were found as part of a transcription factor complex (de Vetten *et al*., 1992; Lu *et al*., 1992). Later it was identified that 14-3-3 proteins complex with the maize transcription factors EmBP1 and VP1 and may function as adapter molecules to establish a complex between the two factors (Schultz *et al*., 1998). A different role for 14-3-3 proteins could be assigned due to their involvement in the regulation of nitrate reductase (NR) (Bachmann *et al*., 1996; Moorhead *et al*., 1996). NR is regulated by

phosphorylation, and its activity is inhibited when 14-3-3 proteins are bound specifically to the phosphorylated form of the protein.

One of the notable components of the plant defense network is the 14-3-3 proteins which have been previously shown to be upregulated during pathogen attack (Roberts and Bowles, 1999). These phosphoserine-binding proteins regulate the activities of a wide array of targets via direct protein-protein interactions. 14-3-3 proteins are also known to be involved in transcription by interacting with the G-box binding complexes (de Vetten *et al*., 1992; Lu *et al*., 1992) and TATA box binding proteins (Pan *et al*., 1999). The 14-3-3 family is found across several organisms and usually consists of multiple genes as well as protein isoforms. The diversity arising from multiple isoforms and functions, derived from large number of organisms, has led to ambiguity regarding nomenclature and function of 14-3-3s. However, several evidences have strongly linked 14-3-3 proteins to the metabolic and signaling pathways for plant growth regulation and responses to environmental stress (Chen *et al*., 2006). The most prominent paradigm involving function(s) of 14-3-3s in plants includes regulation of plasma membrane H^+-ATPase, nitrate reductase (NR) and sucrose phosphate synthase (SPS) (Chung *et al*., 1999; Sehnke *et al*., 2002). The 14-3-3 proteins play key functional roles in many critical physiological pathways that are regulated by phosphorylation and complete the signal transduction process by binding to the phosphorylated targets (phosphoserine/threonine) or effect structural changes that regulate or modulate activity of the target proteins. This functional trait is deeply engrained in the highly conserved structural core of the 14-3-3 dimer, which provides grooves for binding two specifically phosphorylated peptides. The primary diversity among 14-3-3 isoforms lies in the N and C termini, with the C-terminal region forming a flexible hinge guarding access to the central core region (Sehnke and Ferl, 2000).

Chickpea, a globally important food legume crop, suffers immense damages due to the wilt disease, caused by the soil-borne fungus *Fusarium oxysporum*. In previous chapter (2), using cDNA-AFLP I could isolate a TDF from FOC1 challenged chickpea roots that revealed homology to the reported 14-3-3 sequences. In this study we have isolated and cloned 2 full-length 14-3-3 isoforms and have studied their differential expression pattern in roots when challenged with the pathogen. The 14-3-3 gene expression was compared between chickpea varieties that were either susceptible (JG-62) or resistant (Vijay) to FOC1. Kinetics of expression of two 14-3-3 genes derived from

resistant chickpea root cDNA was studied by semiquantitative RT-PCR at 1, 2, 4 and 8 days after infection (DAI) with FOC1.

3.2. Materials and Methods

3.2.1. Plant material, growth conditions and stress treatment

Chickpea plants of wilt-susceptible (JG-62) and -resistant (Vijay) cultivar were grown hydroponically under sterile conditions on floats in trays containing 3L of half strength Hoagland's medium (Hoagland and Arnon, 1950). Fifty plants were grown in each tray in a controlled environmental growth conditions at 22^{o}C and 60% relative humidity under white light and normal day length (14 h light/10 h dark). Seedlings were infected with the pathogen (FOC1) after 7 days of growth of the seedlings. Freshly prepared spore suspension (10 mL of 10^{6}spores/mL) of FOC1 was added to the sterile hydroponic trays. Stirring the water in the trays with sterile glass rod at regular intervals ensured uniform spread of fungus and infection. Five seeds of JG-62 (S) were sown in each tray as an indicator of successful infection and wilting. Seedlings grown in similar trays with no pathogen inoculation served as an uninfected plant control. At harvest, the seedlings were removed from the floats in hydroponic trays and the infected roots were briefly rinsed with sterile DEPC treated water, to free off the adhering fungal mycelia, quickly frozen in liquid nitrogen and stored at -80 ^{0}C till further use. Samples were collected in duplicates for Vijay and JG-62 after 1, 2, 4 and 8 DAI.

3.2.2. RNA extraction and cDNA preparation

Total RNA was extracted from root samples collected at different time intervals such as 1, 2, 4 and 8 DAI with FOC1 using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Total RNA was treated with RNase free DNaseI (0.1 U per µg RNA) at 37^{o}C for 1 h in the presence of RNasin (0.4U) and terminated by heating at 65^{o}C for 15 min to remove the contaminating genomic DNA. The RNA was precipitated with 3M sodium acetate (pH 5.2) and absolute ethanol at -80^{o}C for 1 h, pellet collected by centrifugation at 12,000 g for 10 min at 4^{o}C and resuspended in 10 µl of DEPC-treated water. Five microgram of total RNA thus obtained was used to synthesize first strand-cDNA by incubating at 70^{o}C for 10 min in the presence of 100 µM oligo d$(T)_{18}$ primer and snap chilled on ice. Reverse transcription was carried out in a 20 µl reaction containing 1µl (10U/µl) Powerscript RT III (Clontech, USA), 2 µl (10X) first strand synthesis buffer (250 mM Tris-HCl, pH 8.3, 250 mM KCl, 20 mM MgCl2), 1 µl DTT (50 mM), 1 µl RNasin (10U) and 2 µl dNTPs (0.5 mM) at 42^{o}C for 1.5 h, and terminated by heating at 70^{o}C for 10 min. Three independent replicates of the reaction were pooled and used as template for PCR amplification.

3.2.3. Primer design, cloning and sequencing of 14-3-3 isoforms from chickpea roots

Among the differentially expressed fragments previously (chapter 2) studied in wilt resistant chickpea cultivar Vijay, a specific 262 bp TDF (DR749492) had been identified with significant similarity to 14-3-3 like protein. Based on its homology to 14-3-3 gene sequences from other plants (*Medicago truncatula*, *Vicia faba* and *Pisum sativum*), two sets of primers were designed using Fast PCR software (Kalendar, 2007) as in Table 3.1 (Sigma Aldrich, USA). The first set of primers (F1-R1 and F2-R2) were designed based on *Glycine max* (U70535) and *Vicia faba* (AB050953) sequences while the second set (F3-R3 and F4-R4) was designed using the sequences from *Cicer arietinum* (DR749492), *Pisum sativum* (AJ238682) and *Vicia faba* (Z48504), wherein the 262 bp (DR749492) sequence matched with AJ238682 and Z48504.

The primers generated an amplicon of ~790 bps, from all the four samples viz- susceptible control (SC), susceptible infected (SI), resistant control (RC) and resistant infected (RI) chickpea cultivar roots. cDNA derived as explained above was used as templates in a PCR reaction of 20 µl volume consisting of: 1.5 mM $MgCl_2$, 50 mM KCl, 2.5 mM each of dATP, dCTP, dGTP and TTP (AmershamBiosciences, USA), 10 µM primer and 0.6U *Taq* DNA polymerase (Promega, USA). Amplification was performed in a PTC-200 (MJ Research Inc., USA) programmed for: 94°C for 1 min followed by 35 cycles each comprising 30 sec at 94°C, 1 min at 55°C and 1.30 min at 72°C with a final extension of 5 min at 72°C. Amplification products were resolved on 1.0% agarose in 0.5X TAE buffer (pH 8.0) and visualized by ethidium bromide staining. All the reactions were repeated at least thrice to ensure reproducibility.

The fragment(s) thus amplified were ligated into pGEMT-Easy vector (Promega, USA) and transformed into competent *E. coli* α-DH5 cells. Plasmid was isolated from positive transformants (white colonies against blue colonies) by the alkaline lysis method (Sambrook *et al.* 1989) and purified using the Wizard Plus Miniprep DNA purification system (Promega, USA). The insert was sequenced bi-directionally from four representative clones in two independent replicates in an automated fluorescent sequence analyzer using DYEnamic terminator chemistry (MegaBace, AmershamBiosciences, USA).

3.2.4. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Following reverse transcription, PCR amplification was carried out in a final reaction volume of 20 µl using 1 µl of 1:20 diluted cDNA (derived from the root mRNA preparation, as described above). A typical cycle consisted of a denaturation step at 94°C for 1 min, followed by primer annealing at 56°C for 30 sec (for 14-3-3), 50°C (for 18S

RNA) and extension at 72°C for 1 min. The PCR reaction consisted of 25 such cycles and a final step of incubation at 72°C for 5 min. The PCR products thus obtained were analyzed on a 1.0% agarose gel. PCRs were carried out in three independent replicates. The dilution and number of cycles were chosen such that the PCR reaction was still in logarithmic phase. RT-PCR of 18S RNA was used as a control for integrity and normalization of quantity of all the test RNA samples. Identities of the amplified PCR products (of expected size) were confirmed by sequencing and analysis of the amplicons in all cases.

3.2.5. Sequence analysis of 14-3-3 deduced protein

The BLAST 2.0 program (Altschul, *et al*., 1997) was used for nucleotide and (translated) polypeptide homology search of the sequences of 780 and 786 bp amplicons against the reported sequences in the GenBank and EMBL databases and alignment was performed. The amino acid sequence was deduced from 14-3-3 gene sequence from chickpea and the primary and secondary structures of the protein were analyzed by using GenBank tools, CPH-model (Lund, *et al*., 2002) and Bioedit (Hall, 1999) software for Molecular Biology. 14-3-3 amino acid sequences retrieved after BLASTx were aligned using Clustal-X (1.8) software (Thompson *et al*., 1997). 14-3-3 sequences from following species were used for sequence comparison and phylogenetic analysis: *Medicago truncatula* (ABE79090), *Vicia faba* (BAB17822, P42653, P42654), *Pisum sativum* (CAB42546, CAB42547), *Vigna angularis* (BAB47119), *Lycopersicon esculentum* (T07383, T07387, T07389, CAB65693), *Gossypium hirsutum* (ABD63905), *Maackia amurensis* (AAC15418) and *Populus canescens* (AAD27823).

The nucleotide/polypeptide sequences of the two isoforms of chickpea 14-3-3 were also compared with 14-3-3 protein family of *Arabidopsis* using the following sequences from genbank database (nucleotide accession numbers, protein accession numbers, greek letter isoform name): GRF9 (U60444, AAB49334, μ), GRF10 (U36446, AAA79699, ε), GRF8 (U36447, AAA79700, κ), GRF6 (U68545, AAB08482, λ), GRF4 (L09111, AAB06231, ϕ), GRF1 (L09112, AAA96323, χ), GRF2 (M96855, AAA32798, ω), GRF3 (L09110, AAA32799, ψ), GRF5 (L09109, AAB06585, υ) and GRF7 (U60445, AAB49335, ν). The phylogenetic tree was constructed using ClustalX, Treeview (Page, 1996)/ PhyloDraw software (http://pearl.cs.pusan.ac.kr/phylodraw/). Further the cDNA sequences and polypeptide sequences were also blasted using the CVit BLAST tool at www.medicago.org site. This BLAST tool aids in the positioning of the blasted sequences on to the *Medicago* physical map.

Table 3.1: Summary of primers used for isolation and semi quantitative RT-PCR analysis of the two 14-3-3 gene isoforms and their differential expression in susceptible infected and resistant infected sample of chickpea roots.

Acc. No. used for primer design	Primer (5'- 3')	Gene (Acc. Number)
	[F]: CCG GTC CGC CTA TGG TGT GCA CCG G	18S RNA
	[R]: CCT CTG ACT ATG AAA TAC GAA TGC CCC	
		Ca1433-1
U70535, AB050953		(EF565383; EF643372)
Degenerate	[F1]: AGA TC[S] [K]C[S] ATG GC[H] TCC [W]CC AA	
	[R1] AAC AT[S] [S][W]A A[K][S] G[W]A AAC [Y]A[Y] CA	
Specific	[F2]: TCC ATG GCA TCC TCC ATC GAA ACC TTC G	
	[R2]: TTA CTC TGC ATT ATC TCC TAC AGG	
DR749492, AJ238682, Z48504		*Ca1433*-2 (EF565384; EF643373)
Degenerate	[F3]: ATG GCC ACC GCA CCA AC[W] CC[K] CG[K] G	
	[R3]: GGT GCT GAT GAA AT[Y] AAA GAA GC	
Specific	[F4]: CCG ATG GCC ACC GCA CCA ACA CCG C	
	[R4]: CTG TGG TTC ATC ATT GCC TTT AGG TGC	

[K]: (T/G); [S]: (C/G); [W]: (T/A); [Y]: (T/C)

3.3. Results

3.3.1. Isolation and cloning of two isoforms of 14-3-3 from chickpea

A TDF (DR749492) annotated to be a 14-3-3 like transcript has been earlier isolated from chickpea roots. This TDF spans a part of 5' UTR region and extends into the ORF. This led to the isolation and characterization of the full-length 14-3-3 sequence. Based on the 14-3-3 gene sequences reported for legumes in the GenBank two sets of gene specific primers for N and C terminal regions of the 14-3-3 sequence were designed (Table 3.1) such that they included the start and stop codons, respectively. The amplicon from the first set of primers (F1-R1 and F2-R2) encoded a 780 bp ORF and was designated as *Ca1433*-1 (Fig 3.1), while the amplicon from the second set (F3-R3 and F4-R4) encoded a 786 bp ORF which was designated as *Ca1433*-2 (Table 3.1 and Fig 3.1). The identity of the amplicons was confirmed by bi-directional sequencing of multiple clones in independent replicates. The sequences thus obtained, encoded the entire ORF of the 14-3-3 proteins. Sequence analysis revealed that both the fragments showed significant identity to the reported 14-3-3 proteins from many plants. Further the analysis of the *Ca1433*-1 sequence indicated that the first 'ATG' of the sequence was the valid beginning codon and the longest open reading frame (ORF) encoded a putative polypeptide of 259 amino acids while *Ca1433*-2 similarly encoded a putative polypeptide of 261 amino acids with the first 'ATG' as the valid start codon of the longest open reading frame. The predicted proteins had molecular weights of 29.26 kDa and 29.33 kDa, with theoretical pI of 4.72 and 4.71, respectively.

3.3.2. Comparison of 14-3-3s from chickpea and other plants:

Figure 3.2 shows a sequence alignment (Clustal-X) of the two isoforms of chickpea 14-3-3 (*Ca1433*-1 and *Ca1433*-2), with 14-3-3 proteins from *Vicia faba, Medicago truncatula, Pisum sativum, Vigna angularis, Lycopersicon esculentum, Nicotiana tabaccum, Goyssipium hirsutum, Maackia amurensis* and *Populus canescens*. The alignment illustrated that the 14-3-3 gene is conserved in these plants. All the isoforms share a conserved core region, with the N- and C- termini being the most divergent. The two chickpea 14-3-3 genes (*Ca1433*-1 and *Ca1433*-2) share a 69% identity between them with highly variable C terminal end (Fig 3.2).

A dendrogram constructed based on these sequences shows that, the chickpea 14-3-3 sequences form two distinct groups where *Ca1433*-1 is grouped along with 14-3-3 D isoform from *Vicia faba* while *Ca1433*-2 is closer to 14-3-3 A isoform from *Vicia faba* (Fig 3.3).

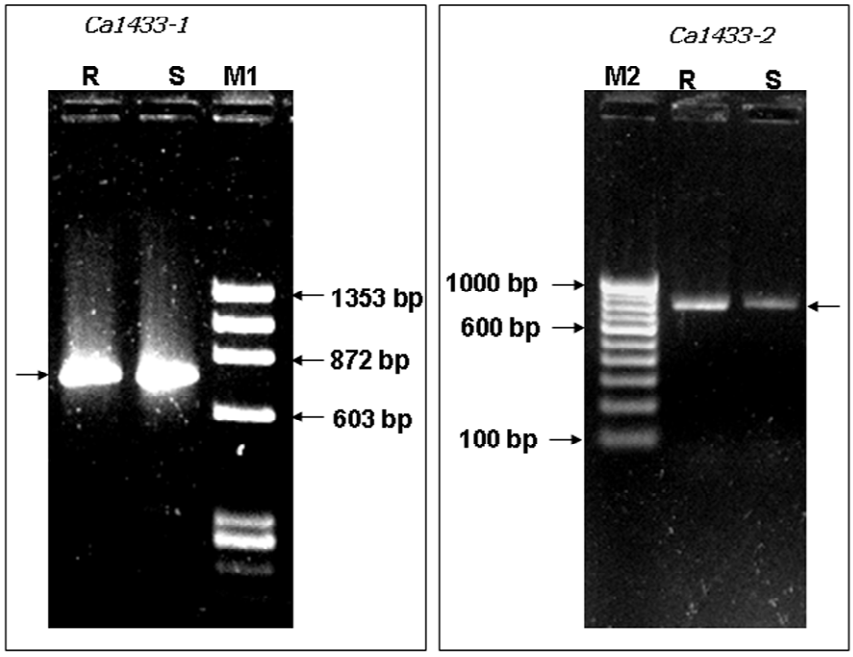


Figure 3.1: RT-PCR amplification of the two 14-3-3 genes from *Cicer arietinum* root cDNA. The amplified products were cloned into pGEMT-E vector and sequenced using MegaBace DNA Analyzer (AmershamBiosciences, USA). Lanes marked R- Resistant cultivar; S- susceptible cultivar; M1- φX174 *Hind*III digested marker and M2- 1000 bp ladder.

3.3.3. Comparing *Ca1433*-1 and *Ca1433*-2 with *Arabidopsis* 14-3-3

The 14-3-3 family in *Arabidopsis* has been extensively studied at the gene, mRNA and protein level. The chickpea 14-3-3 deduced protein sequences were especially aligned separately with thirteen isoforms from *Arabidopisis*. The *Arabidopsis* isoforms range in length from 241 to 268 amino acids and the family members break into two major evolutionary branches, the Epsilon (ε) group and the Non-Epsilon (Non-ε) group. An alignment of the two chickpea 14-3-3 isoforms with the *Arabidopsis* sequences reveals some interesting information (Fig 3.4). The chickpea 1433-1 isoform groups with the epsilon types and the 1433-2 isoform into the non-epsilon type (Fig 3.4).

The *Ca1433*-1 and *Ca1433*-2 were localized on to the linkage groups of *Medicago* physical map. The *Ca1433*-1 was localized on the linkage group 2, 3, 4 and 5 while *Ca1433*-2 was positioned at two different places on linkage group 3 (Fig 3.5)

3.3.4. Comparison of *Ca1433*-1 and *Ca1433*-2 between SC, SI, RC and RI

The *Ca1433*-1 gene transcripts expressed in the susceptible (JG-62) and the resistant (Vijay) chickpea varieties infected with FOC1 and control were cloned and sequenced separately from all the four samples studied (SC, SI, RC and RI). The sequence analysis of cDNA as well as the amino acids revealed no variation in the *Ca1433*-1 sequence between the susceptible (EF565383) and resistant (EF643372) varieties with or without infection. The *Ca1433*-2 gene was also isolated from the susceptible (EF565384) and the resistant (EF643373) chickpea varieties in all the four samples, sequenced, and found no sequence variation between these four samples. Thus, for both the chickpea 14-3-3 genes studied there was no sequence variation between the two varieties, under both, normal or pathogen challenged conditions.

3.3.5. Semi-quantitative RT-PCR

Transcription profiling of the two different 14-3-3 genes was undertaken by SQ-RT–PCR, and the results are summarized in Fig 3.6 and Fig 3.7. Under normal growth conditions (control) expression of *Ca1433*-1 appeared to be higher in the roots of susceptible cultivar (SC) as compared to the resistant cultivar (RC) or equal in both. When challenged, (infected with FOC1) the expression of *Ca1433*-1 increased in both the RI and SI samples, though the level of transcripts was higher in the SI samples (Fig 3.6).

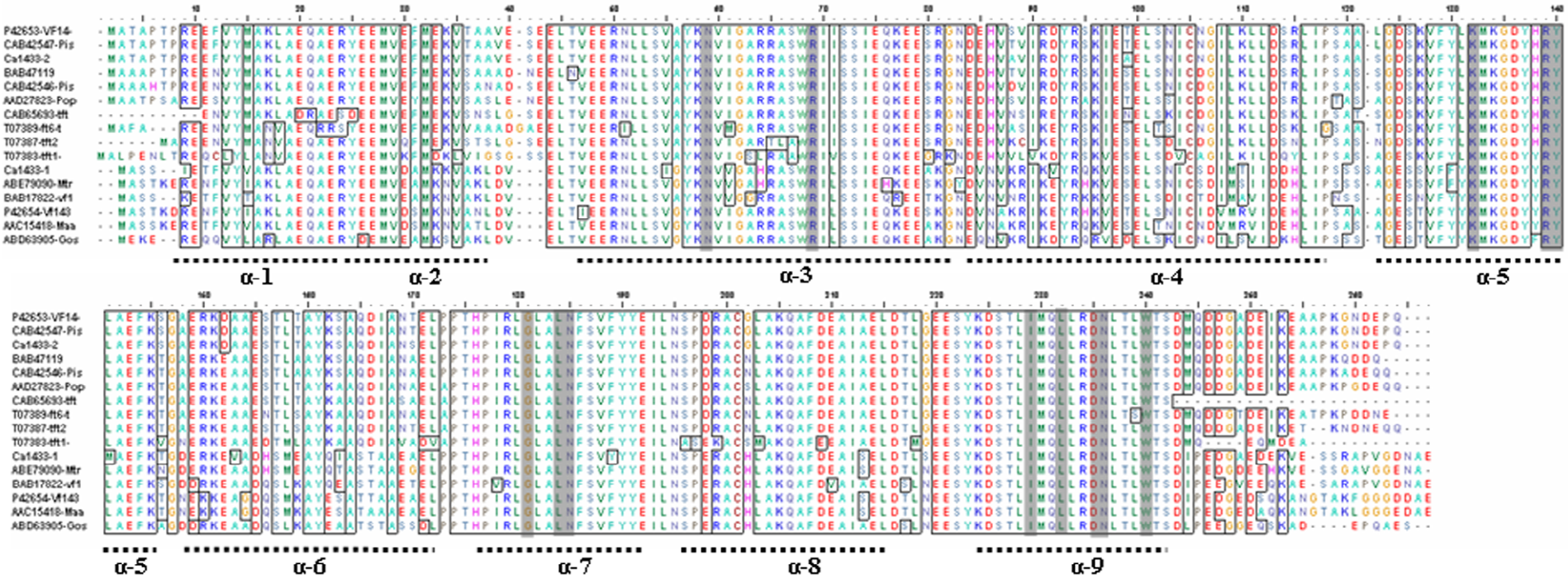


Figure 3.2: Deduced amino acid sequence alignment of the two chickpea 14-3-3 isoforms with 14-3-3 protein sequences from *Medicago* (ABE79090), *V. faba* (BAB17822, P42653, P42654), *P. sativum* (CAB42546, CAB42547), *V. angularis* (BAB47119), *L. esculentum* (T07387, T07389, T07383, CAB65693), *G. hirsutum* (ABD63905), *M. amurensis* (AAC15418), *P. canescens* (AAD27823), retrieved using BLASTx from GenBank. Outlined boxes represent the identical regions in the alignment. Shaded boxes represent residues which form contact with the target proteins and form the conserved motifs among all sequences across species barrier. The dotted line indicates the α-helices as predicted by CPH modeling program.

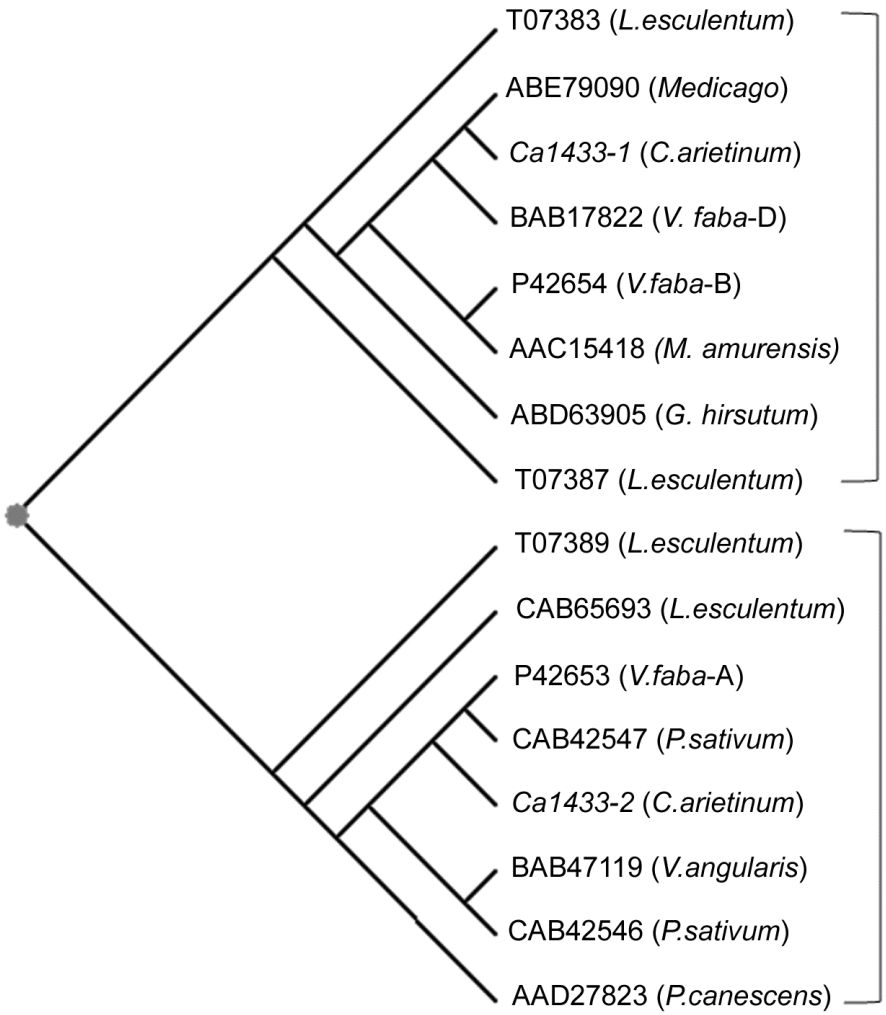


Figure 3.3: The phylogenetic tree generated using deduced amino acid sequences of the two chickpea 14-3-3 gene isoforms (*Ca1433*-1 and *Ca1433*-2) and the protein sequences retrieved from the GenBank database by each of the isoform using BLASTp. The 14-3-3 proteins are placed within two major groups. Note that the two chickpea 14-3-3s are classified into different group. Alignment made using Bioedit/ Clustal W and neighbor joining (NJ) tree generated using PhyloDraw.

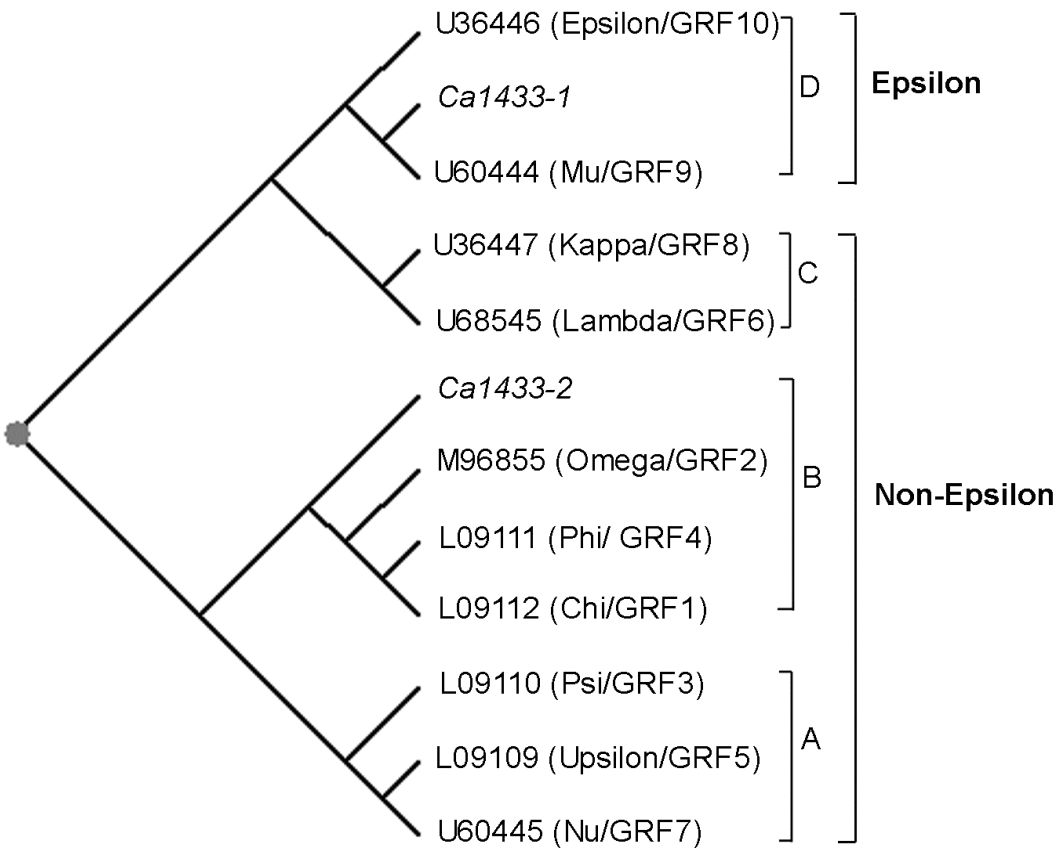


Figure 3.4: A phylogenetic tree shows the topology of the chickpea (*Ca1433*-1 and *Ca1433*-2) and *Arabidopsis* (GRF) 14-3-3 families separated into four major groups, based on cDNA sequences. A, B, and C groups are in the *Arabidopsis* non-ε-group and group D belongs to ε-group. Similar groupings were observed when amino acid sequences were compared. Note that the two chickpea 14-3-3s are classified into different group *Ca1433*-1 in ε-group and *Ca1433*-2 in non-ε-group. Alignment made using Bioedit/ Clustal W and neighbor joining (NJ) tree generated using PhyloDraw.

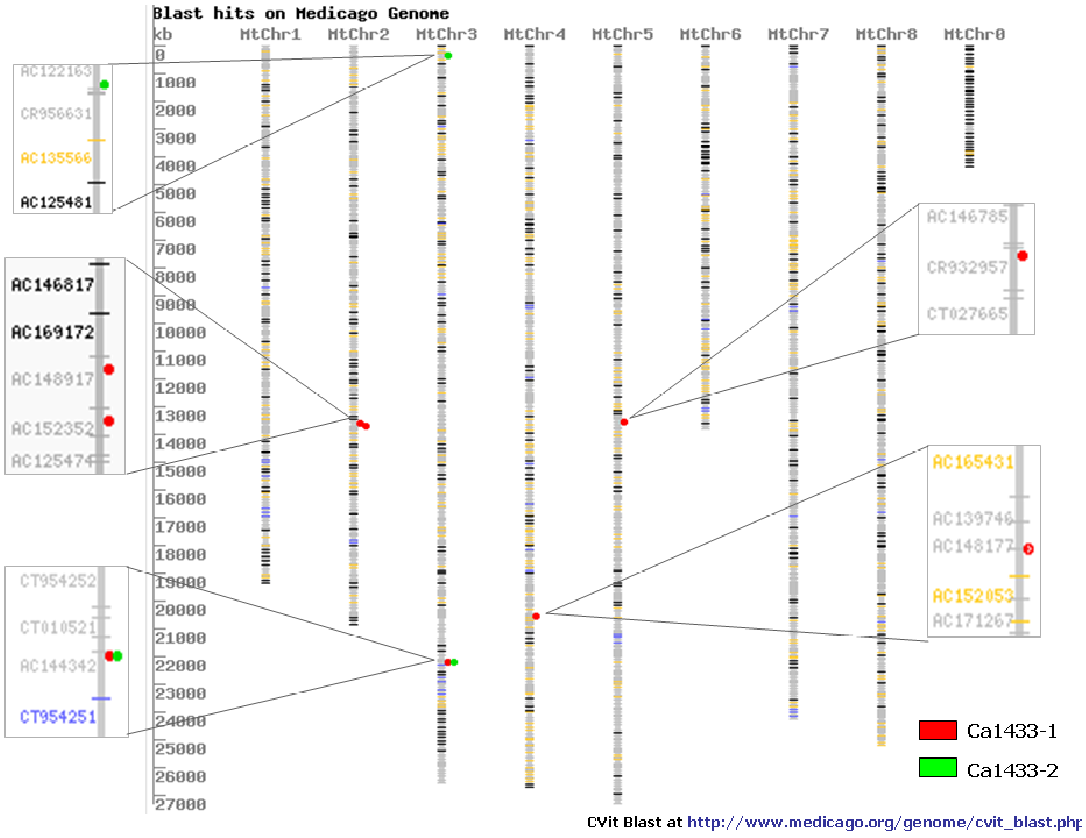


Figure 3.5: The *Ca1433*-1 and *Ca1433*-2 genes from *C. arietinum* were blasted using the CVit BLAST tool to place them on the *Medicago* physical map. *Ca1433*-1 gave hits at five positions on chromosomes 2, 3, 4 and 5. *Ca1433*-2 gene gave hits at two positions on chromosome 2 only.

In the susceptible cultivar the *Ca1433*-1 transcripts were induced by FOC1 infection at 1 DAI, their levels peaked at 2 DAI and increased accumulation continued till 4 DAI. In the resistant cultivar the *Ca1433*-1 transcripts levels were lower during FOC1 infection. The expression pattern in 8 DAI was similar to 1 DAI (Fig 3.6).

Under normal growth conditions expression of *Ca1433*-2 appeared to be at least 2 fold higher in the roots of resistant cultivar (RC) as compared to the susceptible cultivar (SC) at 1, 2 and 4 days. Under the challenged conditions the expression of *Ca1433*-2 increased in both the RI and SI samples, though the level of transcripts was higher in the RI (Fig 3.7). The *Ca1433*-2 transcripts were induced at 1 DAI; their levels peaked at 2 DAI and finally dropped at 4 DAI in SI and RI as well. The expression pattern in 8 DAI was similar to 1 DAI.

3.4. Discussion

The presence of 14-3-3 proteins in several diverse organisms has been well documented (Ferl, 1996). 14-3-3 proteins are a large family of around 30 kDa acidic proteins that exist as homo- and hetero-dimers within all eukaryotic cells. These seemingly ubiquitous proteins are employed in wide variety of cellular functions. 14-3-3 proteins are phosphoserine/threonine-binding proteins that bind to a range of transcription factors and other signaling proteins, and play important roles in the regulation of plant development and stress responses (Roberts, 2003). Two optimal phosphoserine/threonine-containing motifs, RSXpSXP and RXXXpSXP are recognized by all 14-3-3 isotypes in the mammalian systems (Yaffe, 2002). A high degree of similarity has been found between the amino acid sequences of nematode, yeast, plant and human 14-3-3 proteins (Lapointe *et al*., 2001). However, the amino acids towards the N-terminus are conserved to a degree of only 14% and there is very little amino acid conservation towards the C-terminus (Chung *et al*., 1999).

3.4.1. Structure-function considerations of 14-3-3 protein

Protein sequences of 14-3-3s are highly conserved across the evolutionary lineages; moreover, extreme conservation of the core region of 14-3-3s makes the animal structure a very likely fit to the general features of plant 14-3-3s (Fig 3.2) (Ferl *et al*., 1994; Ferl, 1996). The crystal structure has been solved for two mammalian 14-3-3s (Liu *et al*., 1995; Xiao *et al*., 1995) yet both fail to resolve the N- and C- termini, which are highly divergent among isoforms. Thus, it is possible to consider the paradigm regulatory features of 14-3-3s to be associated with the central conserved core while recognizing that the divergent termini might contribute to specific regulatory functions.

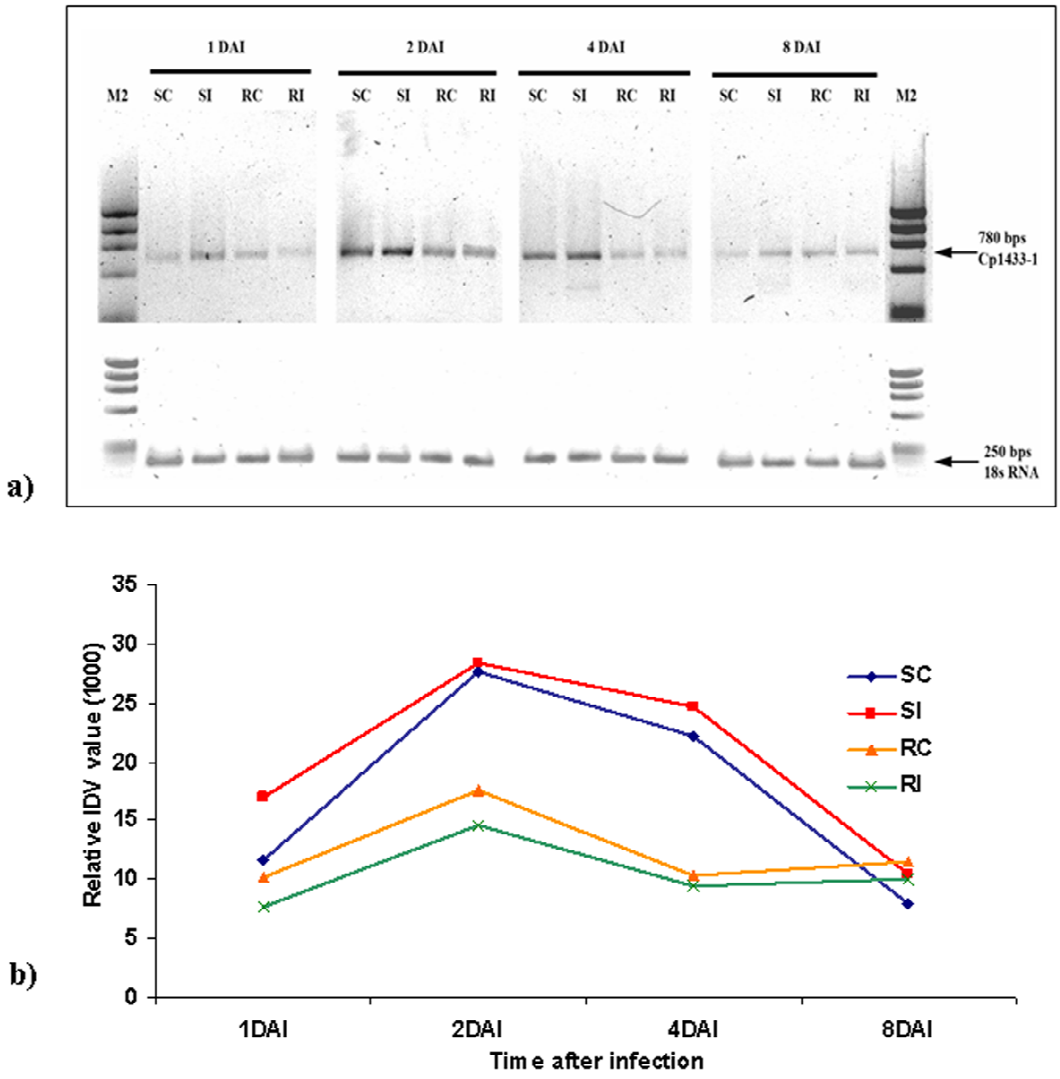


Figure 3.6: Semi-quantitative RT-PCR of *Ca1433*-1. **a)** Reverse transcription (RT)–PCR analysis of *Ca1433*-1 gene expression patterns in the incompatible and compatible chickpea-FOC1 interactions in a time course of 1-8 DAI (Days after Inoculation). Susceptible control (SC), susceptible infected (SI), resistant control (RC) and resistant infected (RI) chickpea root samples; Lanes M2 is φX *Hind*III digest. Independent experiments were carried out with similar results. The transcripts of *Ca1433*-1 showed increased levels in SI. RT-PCR of 18S RNA was used to check the uniform quantity of the cDNA concentration used in RT-PCR. **b)** Abundance of the transcripts was quantitated by AlphaImager (Alpha Innotech, USA). Expression levels at different time points as compared to the control were plotted.

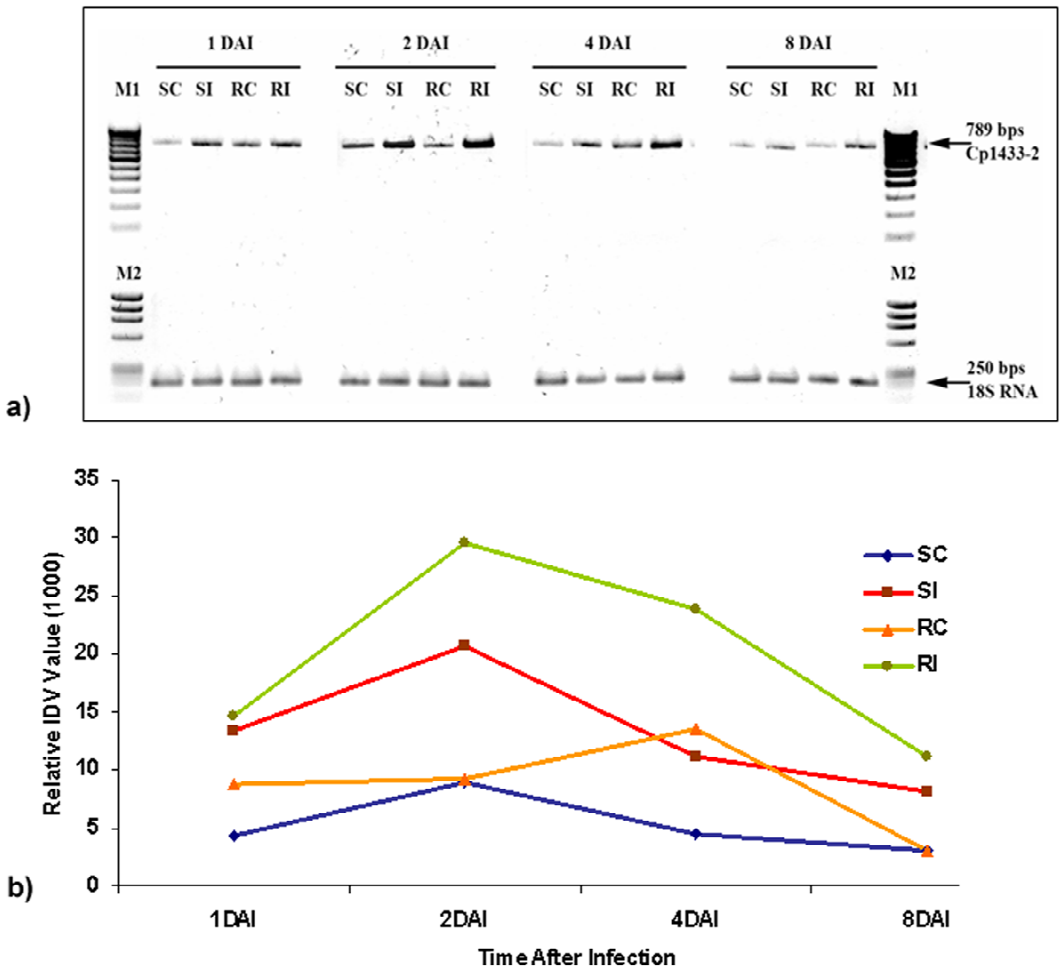


Figure 3.7: Semi-quantitative RT-PCR of *Ca1433*-2. **a)** Reverse transcription (RT)–PCR analysis of *Ca1433*-2 gene expression patterns in the incompatible and compatible chickpea-FOC1 interactions in a time course of 1-8 DAI (Days after Inoculation). Susceptible control (SC), susceptible infected (SI), resistant control (RC) and resistant infected (RI) chickpea root samples; Lanes M1 1000bp ladder, Lanes M2 is φX *Hind*III digest. Independent experiments were carried out with similar results. The *Ca1433*-2 transcript showed increased induction in the RI sample. RT-PCR of 18S RNA was used to check the uniform quantity of the cDNA concentration used in RT-PCR reactions. **b)** Abundance of the transcripts was quantitated by AlphaImager (Alpha Innotech, USA). Expression levels at different time points as compared to the control were plotted.

Both chickpea 14-3-3 genes without exception, showed a highly variable N and C terminal region with a conserved core region. The main 14-3-3 structural feature is a double-barreled, W-shaped clamp formed from the essentially antiparallel helices of the dimer pair (Fig 3.8). Each monomer produces a channel that accommodates interaction with a phosphorylated peptide from the target protein (Yaffe *et al*., 1997; Petosa *et al*., 1998).

The amino acid sequences of *Ca1433* genes were deduced and the primary and secondary structures of the protein were analyzed by using a combination of various stand-alone and online programs. A region in 14-3-3 protein, which uses primary forces for binding to H^+-ATPase is formed by residues Lys56, Arg63 and Arg136, Tyr137, Lys129, Asn182, Asn233, Trp237 and Glu189 (Wurtele, *et al*., 2003) while residues Gly178, Leu181, Ile226, Leu229, Asp232, and Val185 are involved in binding by forming the secondary forces (Van der Waals), these residues are the signature features of 14-3-3s and found to be conserved across species as well as in the two *Ca1433*s.

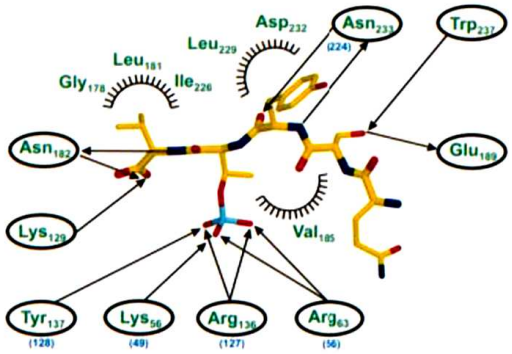
The Ca1433 proteins were predicted to contain nine α-helices, which are essential for its function (Fig 3.9). In 14-3-3 dimers, the α-helices form a putative palisade around an amphipathic groove, which constitutes the binding site for target proteins. The residues lining the groove are highly conserved between species. The invariant residues were conserved in both the isoforms of chickpea 14-3-3 which include the hydrophobic Leu182, Leu231 and Leu237 as well as the basic Lys57, Arg64, and Arg68 (Fig 3.9). The helix α-5 has polar groups belonging to the conserved amino acids Lys130, Asp134, Arg137, and Tyr138 (Fig 3.9). 14-3-3 binding to phosphoserine is probably mediated by the cluster of basic amino acids Arg64, Arg68, Lys130, and Arg134 on the basic face of the groove (Fig 3.9).

3.4.2. Two 14-3-3 isoforms in chickpea

The 14-3-3 genes are known to be present as a large family in many plants studied hitherto viz. *Arabidopsis* (15), Rice (8), Tomato (10), Poplar (6), Potato (6) and *Vicia faba* (4). The presence of a large and diverse 14-3-3 family in plants is justified by the requirement of a battery of regulators and corresponding responses to deal with complex environmental and developmental changes. At least two isoforms of 14-3-3 were observed in chickpea, though there is a strong possibility of presence of more. The 14-3-3 gene sequences from chickpea, when aligned with other plant 14-3-3s, they tend to get segregated into two distinct groups. While *Ca1433*-1 is grouped along with the D form, the *Ca1433-2* is closer to A isoform of *Vicia faba* (Fig 3.3).

a)

b)



c)

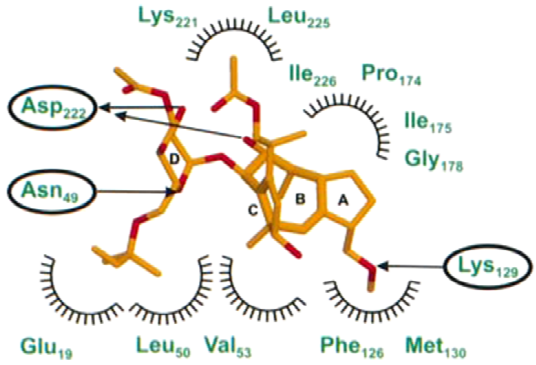


Figure 3.8: Phosphopeptide binding to plant 14-3-3. (a) Ribbon plot of two different orientations of the dimeric tobacco 14-3-3c protein (green) bound to the peptide Gln-Ser-Tyr-pThr-Val (yellow), which constitutes the C-terminal end of PMA2, an H^+-ATPase isoform from *Nicotiana plumbaginifolia*. (b) Scheme of the interaction between peptide and protein, where half circles indicate residues forming van der Waals interactions, and arrows denote hydrophilic interactions between the indicated residues and the corresponding atoms of the peptide. The numbers of some conserved amino acids in human 14-3-3ζ are indicated in parentheses (blue). (c) Contacts between the toxin and the 14-3-3, with symbols as in B; carbon and oxygen atoms are in orange and red, respectively. (Wurtele *et al*., 2003)

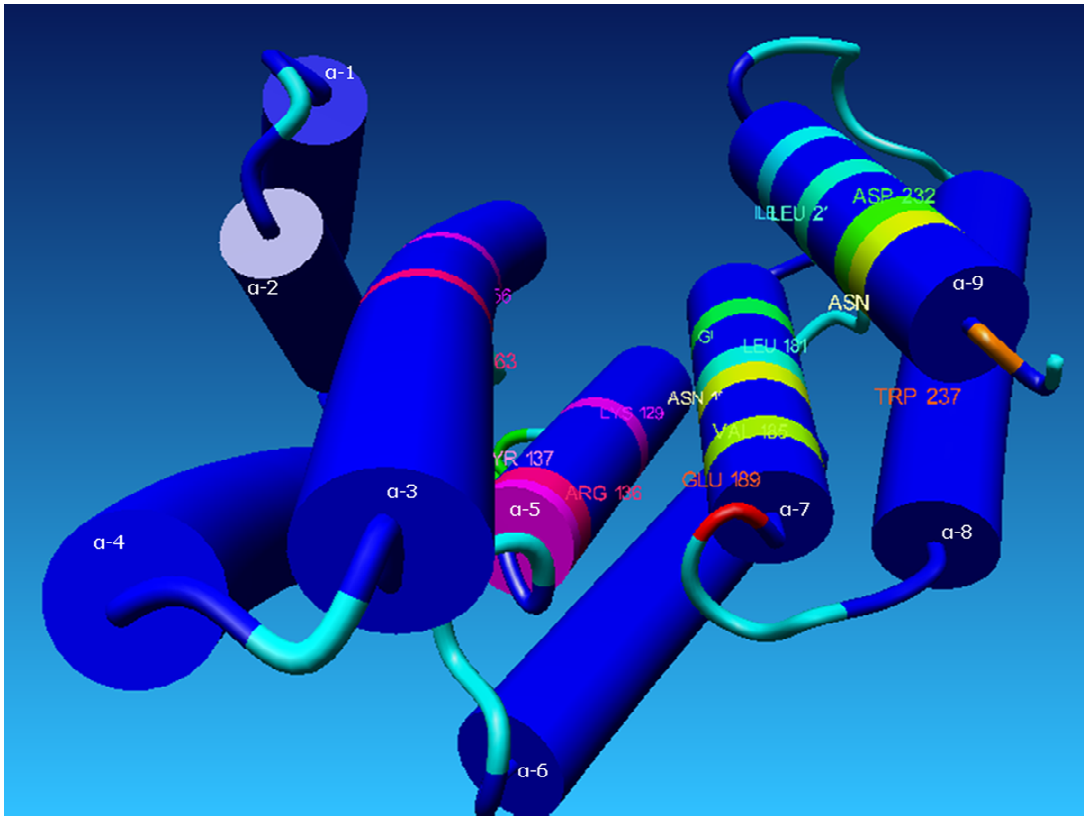
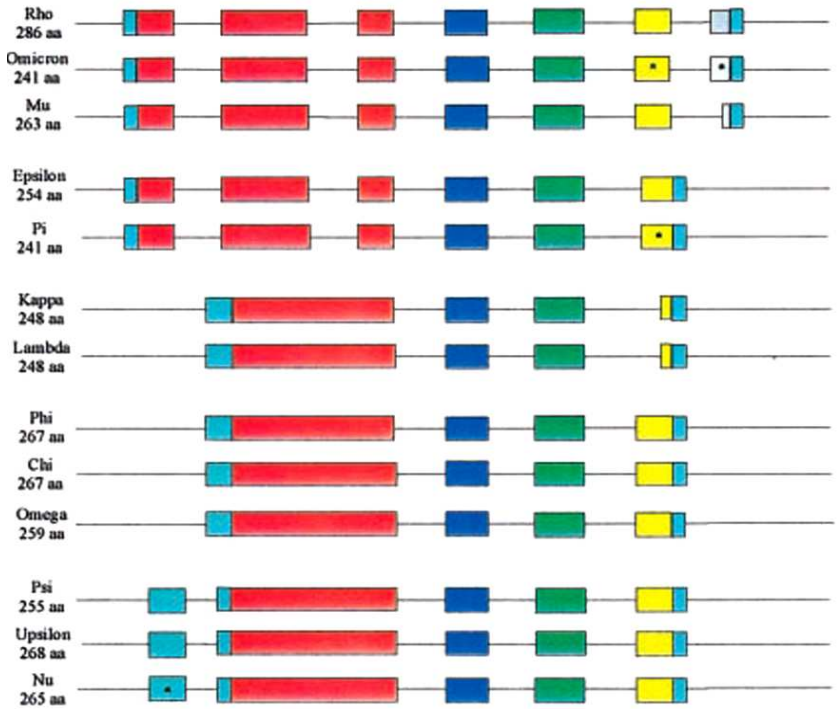


Figure 3.9: A 3D model for Ca1433-2 was built using deduced amino acid sequences which were loaded into the CPH-modeling server to generate the PDB file based on homology. The picture was generated using YASARA software (www.yasara.org). On one side of the groove - helices 3 and 5 - cluster of charged and polar residues. On the other side - helices 7 and 9 - patch of hydrophobic residues. Residues lining the concave surface of the groove are mostly conserved among different isoforms of the 14-3-3 family.

3.4.3. Structural and functional divergence in 14-3-3 family

The 14-3-3s exhibit significant diversity in eukaryotes; with yeasts have only two, while *Arabidopsis* has 15 different 14-3-3 genes including two pseudo genes (DeLille *et al*., 2001; Rosenquist *et al*., 2001; Sehnke *et al*., 2002). Though, 14-3-3 proteins are homologous and similar across genera, the variations within the *Arabidopsis* 14-3-3 s is a fair representation of its degree and location in most of the plant species. However, in the 14-3-3 families, the functional diversity seems largely unresolved (Ferl, 2004). The potential for specific functions among isoforms is supported by the unique C- and N-termini. The *Arabidopsis* 14-3-3 isoforms, based on sequence alignment of the central core region and the gene structure considerations, are divided into two distinct groups, the ε- and non-ε-groups having distinct evolutionary lineages prior to the separation of animals and plants (Ferl *et al*., 1994) (Fig. 3.4 and Fig 3.10). The ε-group comprises ε, π, ρ, ο, and μ isoforms and the non-ε-group isoforms, comprises κ, λ, ω, χ, φ, ν, υ and ψ. The ε members possess two additional N-terminal introns and also appear to have additional C-terminal introns. All the non-ε members all exhibit a well conserved exon (4) and intron (3) structure (Fig 3.10). The ν and υ are the only two non-isoforms present in chloroplasts, indicating specificity among non-ε isoforms for their differential localization and suggesting that chloroplast-specific functions might be limited to ν and υ (Sehnke *et al*., 2000). The non- ε group members contain the EF hand-like divalent cation-binding motif (Lu *et al*., 1994) which is a conserved mechanism of plant 14-3-3s binding to the target proteins. The *Ca1433*-1 when aligned with these characterized isoforms grouped with the ε-group and *Ca1433*-2 with the non-ε-group suggests a similar architecture for the 14-3-3s from chickpea (Fig 3.4). The *Ca1433*-1 is grouped in this second sub-branch of the ε group and is closer to μ isoform (Fig 3.4). Though, *Ca1433*-2 is grouped in the second sub-branch of the ε group along with ω, χ, and φ, it remains separated from them. However, the extreme divergence of the C-terminal regions prohibits intron identification based solely on sequence data.

Distinct grouping of the ε-isoforms in animals suggests an early divergence from the other isoforms (Wang and Shakes, 1996). The *Ca1433*-1 isoform groups with the ε-types and is closer to μ, thus suggesting an ancient origin (Fig 3.4). The ε-isoforms are more similar to yeast and plant isoforms than other animal isoforms at numerous residues, and thus may have retained functional characteristics of the ancestral protein. The known invertebrate 14-3-3 proteins group with the non-ε mammalian isoforms.



Greek Name	Greek Letter	GRF Gene	GRF Accession #	Genbank Accession # for cDNA	BAC Accession #	Original Publication
Rho	ρ				AC013427	
Omicron	ο				AC007894	Rosenquist 2000
Mu	μ	GRF 9	AF145301	U60444	AC007087	Wu et al. 1997
Epsilon	ε	GRF 10	AF145302	U36446	AC068562	Wu et al. 1997
Pi	π				AC012680	
Kappa	κ	GRF 8	AF145300	U36447	AB011479	Wu et al. 1997
Lambda	λ	GRF 6	AF145298	U68545	AL353995	Jarillo et al. 1994
Phi	ϕ	GRF 4	AF001414	L09111	AC079605	Lu et al. 1994
Chi	χ	GRF 1	U09377	L09112	AL161513	Lu et al. 1994
Omega	ω	GRF 2	U09376	M96855	AC013430	Lu et al. 1992, 1994
Psi	ψ	GRF 3	U09375	L09110	AB005231	Lu et al. 1994
Upsilon	υ	GRF 5	AF001415	L09109	AL391145	Lu et al. 1994
Nu	ν	GRF 7	AF145299	U60445	AC021640	Wu et al. 1997

Figure 3.10: The information about Arabidopsis 14-3-3 gene structures. Colored boxes represent exons, and are color coded as to their similarity between genes. The thin lines represent introns, which are shown to denote position only. The light-blue boxes represent 59 leader and 39 trailer sequences. The asterisks identify putative exons, cases where cDNA sequences are not yet available. Information available is outlined to the right of each 14-3-3 gene. Rho, omicron, and pi are the least well characterized of the Arabidopsis 14-3-3s; thus, very little information is available. (DeLille *et al*., 2001).

Most of the current 14-3-3 isoform diversity is attributed to the independent duplication events after the divergence of the major eukaryotic kingdoms. *Ca1433*-2 isoform grouped with the non-ε types and possibly could be a more evolved form of 14-3-3 in chickpea (Fig 3.4).

The ε and non-ε groups have a different structure in certain region of the molecule and exhibit variation in target binding preferences (Sehnke *et al*., 2006). The relatively conserved core region is the basis for conservation of a general theme, subtle changes in the core and the divergent termini serve to define isoform-specific function(s). The domain within the extreme amino-terminal 26 residues is essential for dimerization of 14-3-3 proteins (Aitken, 2002). The variability in the eight to twenty one residues at N-terminal of 14-3-3 isoforms may limit the possible homo- or hetero-dimer combinations. Specificity of function of 14-3-3 proteins arises due to such a restriction of combination.

3.4.4. Differential expression of *Ca1433*-1 and *Ca1433*-2

The constitutive levels of both *Ca1433-1* and *Ca1433-2* are comparable in the susceptible and resistant cultivar roots. There is some upregulation of both the transcripts at 2 DAI in the absence of challenge which tapers further at 4 and 8 DAI. Upon induction by FOC1, *Ca1433-1* transcript was significantly upregulated in susceptible roots at 4 and 8 DAI as compared to the resistant cultivar roots (Fig 3.6). This response diminished further at 8 DAI. On the other hand, transcript *Ca1433-2* showed greater amplitude of induction in resistant roots which begins as early as 1 DAI and peaks at 2 and 4 DAI (Fig 3.7). It was found reduced slightly at 8 DAI but showed significantly higher levels of transcription as compared to the susceptible tissue. Thus *Ca1433*-2 was found to respond positively and more strongly to pathogen while *Ca1433*-1 responded negatively to the pathogen challenge. The lower level of *Ca1433*-2 induction in the susceptible infected sample compared to both, the resistant infected as well as the resistant control ruled out the ontogenetic control of this isoform. Similar observations were made where the 14-3-3 transcripts were undetectable in roots of susceptible wheat infected by the 'take-all' fungus *Gaeumannomyces graminis* (Guilleroux and Osbourn 2004). At least four rice 14-3-3 genes (GF14), GF14b, GF14c, GF14e and Gf14f were differentially regulated during interactions with *Magnaporthe grisea* and *Xanthomonas oryzae* pv. *oryzae*, - the incompatible interactions showed a stronger induction of the genes than the compatible interactions (Chen *et al*., 2006). The chemical inducers like benzothiadiazole, methyl jasmonate, ethephon and hydrogen peroxide have shown to

induce these GF14 genes. 14-3-3 protein involved in signaling appear to be more strongly induced in 2-week-old potato resistant variety than in the susceptible variety and the highest induction of this transcript was observed in the 2-week-old Bettina plants at 2 DAI (Ros *et al*, 2004).

3.4.5. Functional variability of *Ca1433*-1 and *Ca1433*-2

14-3-3 proteins interact with a wide range of target proteins involved in stress responses, and regulate their functions such as signaling or transcription activation or defense (Roberts *et al*., 2002), *e.g.*, the *Arabidopsis* 14-3-3 genes, RCI1/RCI1A and RCI2/RCI1B, are regulated during cold acclimation (Jarillo *et al*., 1994); 14-3-3 isoforms in *Arabidopsis* activate a stress-responsive calcium-dependent protein kinase, CPK-1 (Abarca *et al*., 1999; Camoni *et al*., 1998b); the tobacco T14-3-3 is induced in the perception of the salt stress (Chen *et al*., 1994). 14-3-3s are involved in responses to light/dark transitions, anoxia and changes in nutrient supply (Bunney *et al*., 2001).

Ca1433-2 was observed to be closer to 'A' isoform in *V. faba*, interestingly this A form has been shown to have a high affinity towards the H^+ ATPase than the other isoforms. This *Ca1433-2* thus could play an important role by activating the H^+ proton pump as an early event in sensing and signaling the pathogen attack. 14-3-3s have been found involved in plant defense responses mainly by regulating the proton pump (H^+-ATPase) to initiate the hypersensitive response to fusicoccin, a fungal toxin (Roberts *et al*., 2002). The effect of fusiccocin on a pathogen-resistance response and fusicoccin-induced expression of several genes, including 14-3-3, has already been shown in tomato (Roberts and Bowles, 1999). 14-3-3 transcripts were over expressed in rice during infection with *Magnaporthe grisea* (Manandhar *et al*., 1999) and in barley as non-host hypersensitive response to inoculation with *Blumeria graminis* (Brandt *et al*., 1992; Gregersen *et al*, 1997). In white spruce and hybrid poplar 14-3-3s were up-regulated by elicitors of wounding or defense (Lapointe *et al*., 2001).

The ε types of 14-3-3s are localized in plastids and reported to finely coordinate the complex starch metabolism, especially by binding to starch synthase III. Antisense transcripts of 14-3-3 led to over accumulation of starch in *Arabidopsis* leaves (Sehnke *et al*., 2001). Moreover, 14-3-3s are shown to modify the way starch branch and accordingly produce amylase, amylopectin or glucan. The ε type *Ca1433*-1 down regulation as seen in roots of resistant cultivar, may be involved in production of callose, successfully compartmentalizing the pathogen and restricting its growth. Diurnal conversion of starch to glucose is also known to operate stomatal opening and closing,

thereby controlling the transpiration and water uptake. Since the pathogen factor is expected to be pronounced in susceptible cultivar wilt like situation is more severe and wider in the root tissue. *Ca1433*-1 could possibly control stomatal opening, therefore found expressed in susceptible cultivar. Since the pathogen factor is largely under control in the resistant cultivar, its expression terminates sooner. On the other hand *Ca1433*-2 is disease responsive and could be involved in signal transduction pathway, triggering the cascade of molecular responses leading to resistance. Similar feature was reported in earlier work from my laboratory, where the chitinase, glucanase and proteinase activities were reduced in resistant cultivar as soon as the pathogen activities are controlled (Giri *et al*., 1998) while higher expression of aquaporins is seen in susceptible cultivar (Nimbalkar *et al*., 2006).

3.4.6. Promoter effect on 14-3-3 expression?

The nucleotide and deduced amino acid sequences of *Ca1433*s (both *Ca1433*-1 and *Ca1433*-2) from the SC, SI, RC and RI samples was compared. The *Ca1433*-1 sequences showed no variation between these different samples. Comparing the nucleotide and deduced amino acid sequences of *Ca1433*-2 from the SC, SI, RC and RI samples also revealed no sequence variation between these samples. However, both the genes *Ca1433-1* and *Ca1433-2*, exhibited a differential expression pattern upon challenge with FOC1, hinting towards the role of regulatory elements (promoter domain composition) in governing the expression of 14-3-3 isoforms. The promoters are known to contain mostly the same motifs; however, there are also several sequences that differentiate the activity of the genes (Aksamit *et al*., 2005), *e.g.*, a unique motif activated by salicylic acid and virus infection is present in the potato 14-3-3 16R isoform but not in the 20R isoform, which however, contains unique sequences responsive to ethylene and metal ions. This suggests that unique elements within the promoters distinctly regulate each isoform. In contrast to the conserved coding sequence of 14-3-3 isoforms, their promoters show several important differences (Aksamit *et al*., 2005), *e.g.*, the promoter 16R contains two unique sequences TTGACC (–430) an elicitor responsive element (ElRE), required for plant defense signaling (Chen and Chen 2000), and TTWTWTTWTT (–259) a T-box found in a SAR/MAR sequence. The E1RE sequence in 16R promoter might suggest the involvement of the 14-3-3 proteins in plant defense mechanisms upon pathogen infection and salicylic acid induction. Since, the sequence data from chickpea cDNA from the present experiments showed no variation in coding sequences and while the expression analysis of mRNA strongly suggests the differential expression of 14-3-3 isoforms, it is possible that the difference lies in their promoter region containing specific inducible

domains. The need for the precise regulation of the response to various stimuli could explain existence of several isoforms with promoters dedicated to a given set of stimuli. These findings could be helpful in further deciphering the regulation of metabolic pathways by 14-3-3 proteins.

3.4.7. Fusicoccin and 14-3-3 interaction

The phytotoxic fusicoccin (FC) profoundly alters plasma membrane traffic by stimulating apoplast acidification, hyperpolarizing the membrane potential and consequently increasing proton-motive force (Oecking *et al*., 1994). FC binds to a single "receptor" in higher plants, and polypeptides isolated as FC-binding proteins from three different plant species have been identified as products of the 14-3-3 gene family (Kourthout and de Boer, 1994; Marra *et al*., 1994; Oecking *et al*., 1994). It has been demonstrated that a functional FC-binding site is formed from a complex between the C-terminal regulatory domain of the H^+-ATPase and 14-3-3 proteins and that both proteins are required for FC binding (Baunsgaard *et al*., 1998). 14-3-3 proteins are a family of regulatory proteins that have attracted much attention in recent years because of the identification of interactions between various mammalian 14-3-3 isoforms and proteins involved in signal transduction, particularly protein kinases and phosphatases (for review, see Aitken, 1996).

In plants it is found that the Fusicoccin receptor is an oligomer composed of 14-3-3 protein homologs (Oecking *et al*, 1994), the fusicoccin receptor are distinguishable from other cellular 14-3-3 proteins by their tight association with the plasma membrane. The FC-binding activity in epidermal microsomal fraction increased upon the pathogen attack, suggesting that 14-3-3s are involved in an epidermis specific response to the fungus probably through activating the proton pump (H^+-ATPase) to stimulate the HR (Finni *et al*., 2002). In white spruce and hybrid poplar it is shown that 14-3-3s are up-regulated by wounding or wounding elicitors, or by chitosan and jasmonic acid, two defensive elicitors, and H^+-ATPase is a potential target for the 14-3-3-mediated regulation during stress (Lapointe *et al*., 2001). Caffeic acid/5-hydroxyferulic acid O-methyltransferase (OMT1) and ascorbate peroxidase implicated in plant defense or oxidative stress are identified to interact with 14-3-3s by yeast two-hybrid screening (Zhang *et al*., 1997a and 1997b). Similar to FC, other two inducers of programmed cell death, tunicamycin and brefeldin A, also induce the accumulation of 14-3-3 proteins (Malerba *et al*., 2004). Interestingly, a 14-3-3-interactor, AKR2, is an ankyrin-repeat containing protein and negatively regulates transcription factors that mediate defense responses (Yan *et al*., 2002; Kuhlmann *et al*., 2003). The AKR2-antisense plants

developed HR-like lesions with increased H_2O_2 generation and exhibited increased resistance to a bacterial pathogen (Yan *et al*., 2002).

3.4.8. Fusaric acid and 14-3-3 interactions

It has been shown that fusaric acid induces an early hyperpolarisation followed by a marked depolarization of membrane potential in the roots of *Ricinus* (Pavlovkin *et al*., 2004), tomato (D'Alton and Etherton, 1984), corn (Pavlovkin, 1998) and *Egeria densa* leaves (Marrè *et al*., 1993). It's proposed by D'Alton and Etherton (1984) that the initial hyperpolarisation could be due to an early stimulation of the proton pump by the acidification of the cytosol consequent on the entry in to the cell of the undissociated form of the fusaric acid. The immediate effect of fusaric acid along with fusicoccin on the ATPase of *Ricinus* root cells was studied and showed that the functional activity of ATPase is directly influenced by fusaric acid, because fusaric acid directly influenced fusicoccin caused hyper polarization of membrane potential (Pavlovkin *et al*., 2004). This response may point to dependent sites or modes of action of an alternation of the ATPase activity by fusaric acid and fusicoccin. With prolonged exposure to fusaric acid, the membrane continued to depolarize and this depolarization became irreversible. The probable explanation, for the irreversible depolarization is, that fusaric acid cause reduction in ATP levels (Köhler and Bentrup, 1983; D'Alton and Etherton, 1984) that are necessary for electrogenic extrusion of H^+ and hence maintenance of the membrane potential. Other investigators have found that fusaric acid reduces respiratory rates in tomato and *Egeria* plants (Naeff-Rooth and Reusser, 1954; Marre *et al*., 1993).

In addition, the H^+-ATPase has been considered a ''switch'' participating in signal transduction pathways in response to pathogens (Schaller and Oecking, 1999). Therefore, it will be relevant to reveal if the action of 14-3-3s on the ATPase activity forms part of the plant responses to the pathogen interaction in chickpea Fusarium wilt. It remains to be established whether this could be one, hitherto undescribed, but possible event during the interactions of chickpea with *Fusarium*. The natural openings in the plants are shut by an invading pathogen, to impede its pursuit by potential antagonists inside the plant host. However, fusicoccin produced by mycotoxigenic *Fusarium* opens plant stomata (Marre, 1979) and can reverse stomatal closure, the effect of syringomycin (Mott and Takemoto, 1989).

3.4.9. Comparative genome analysis among legumes

The *Medicago truncatula* sequencing project was initiated in 2003, with funding from the National Science Foundation and the European Union's Sixth Framework Program to

complete sequencing of the remaining euchromatic genespace. This initiative was started with a view that the map-anchored, high-quality sequence would provide an extremely valuable basis for genomic comparisons with other plant genomes, and as a foundation for improving many crop and forage legumes. Their site www.medicago.org has many tools, of which CVit- BLAST (chromosome visualization tool) helps to place a given sequence (Protein/Nucleotide) on to the *Medicago* physical map. The 14-3-3 genes (*Ca1433*-1 and *Ca1433*-2) isolated during present investigation were BLAST searched against *Medicago* genome sequences using the CVit- BLAST tool, to study their distribution on different chromosomes and to find their precise mapping positions in terms of anchored BAC clones on the physical map. *Ca1433*-1 was found to be distributed on chromosomes 2, 3, 4 and 5 while *Ca1433*-2 was placed on chromosome 3 only but at two different places (Fig 3.5). The analysis of sequences flanking the *Ca1433*-2 gene on chromosome 3 of *Medicago* revealed the region to be gene rich. The region is clustered with RGA like sequences. These regions would provide good sequence information to study the chromosomal region flanking the 14-3-3 genes in chickpea.

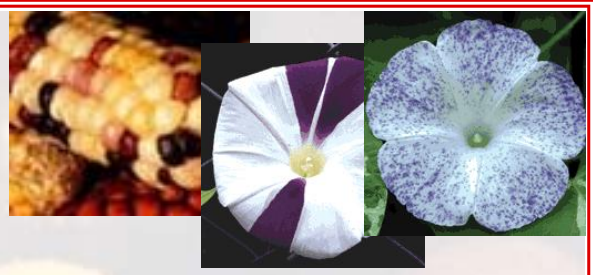
The chromosomal position of a gene affects its regulation and level of expression. Location on a genetic map is an important technical factor for correlating gene effects with phenotype, and forms the basis of quantitative trait analysis (Sasaki and Sederoff 2003). Comparative studies will also provide more information on the evolution of intergenic regions. Studies of intergenic regions in maize (Bennetzen and Ma 2003) first showed the dynamic nature of the intergenic regions and the role of retrotransposons and other elements. These findings provided support for the idea that genome size depends more on the number and activity of mobile elements than on the number of functional genes.

During the evolution of angiosperms there is occurrence of only a finite number of chromosomal rearrangements. Thus it is expected that a significant block/s of genetic material, may be syntenic among genomes of related species (Michelmore 2000). In legumes three species *viz*. *Glycine max* (soybean), *Lotus japonicus* and *Medicago truncatula* have been in focus for the comparative genomic efforts out of which the latter two are considered as model species. Agricultural traits, physiology, and genetics of these genera have been extensively studied. Among cool season food legumes, it has already been shown that nearly 40% of the lentil map arrangements can be found in pea while this number may not be as high in chickpea (Weeden *et al*., 1992). Comparing the

maps of pea, chickpea and lentil has revealed at least five genomic regions that resemble each other (Simon and Muehlbauer 1997). With the information of using conserved portions of the genome of pea, which is much better defined than that of its relatives such as chickpea, genomic arrangements can be predicted for other genomes. It is through comparative genomics that researchers will deduce the mechanisms and pathways by which plant genes and genomes have diverged to give the diversity of form, function and adaptation that now characterize the world's flora (Ku *et al*., 2000) In summary, the grain legumes exhibit an enormous amount of variation and this variation is silently awaiting commercial exploitation. Recent advances in yield increase of wheat, rice, and maize have raised hopes that similar results may be possible with the grain legumes by merging classical plant breeding techniques and newer genetic engineering approaches.

Earlier studies on macrosynteny, have shown that legumes resemble other plant families - including Gramineae, Solanaceae, and Brassicaceae - in terms of within-family genome conservation (Young *et al*., 2003). Microsynteny appears to be widespread within legumes. On the basis of the 1000 anchored soybean BAC-end sequences, Yan *et al*., (2002) found that more than half of soybean BAC contig groups exhibit microsynteny with *M. truncatula*. Of these, more than 80% showed extensive microsynteny. Microsynteny among legumes was the basis for two highly significant success stories resulting in the cloning of extracellular receptor kinases like genes that have crucial roles in Nod-factor perception. Legume microsynteny was essential in the cloning of NN1 from *Medicago sativa* and SYM2 from *Lotus japonicus*, along with their respective orthologues DMI2 in *Medicago truncatula* and Sym19 in pea (Endre *et al*., 2002; Stracke *et al*., 2002).

These 14-3-3 genes have been mapped and analyzed in different crops, *e.g.* analysis of the tan spot-resistance QTL in wheat by Faris *et al*. (1999), showed that 14-3-3 gene is more significantly associated with disease resistance and contributes more towards phenotypic variation for two important diseases of wheat; tan spot and leaf rust. 14-3-3 gene along with other defense responsive genes is shown to have an additive effect on blast resistance in rice (Liu *et al*., 2004). Finally, it would thus be interesting to see how these two *Ca1433* genes behave in the chickpea populations since earlier studies in different crops have revealed a positive association of 14-3-3 genes to the disease resistance.



Chapter 4

Transposable elements induced by biotic stress as revealed by cDNA-AFLP and the survey of database.

The research work described in this chapter has been communicated as a full-length paper to Functional and Integrative Genomics

Abstract

The life cycle of active transposable elements (TEs) essentially includes transcription, translation, and integration of daughter copies of the DNA sequences. Environmental factors, biotic and abiotic stress conditions are known to increase TE activity. Using cDNA-AFLP I detected Ty-*copia* like TEs in chickpea root cDNA library differentially activated by wilt pathogen, *Fusarium oxysporum* f. sp. *ciceri* Race 1 (FOC1). Expression of these TEs was confirmed by reverse northern analysis. Interestingly, the retrotransposons were observed to be highly upregulated in the incompatible interaction (resistant cultivar) but not in the compatible interaction (susceptible cultivar) of chickpea with FOC1. Analysis and comparison among different groups of crops using publicly available 490209 unique ESTs and tentative clusters (TCs) from different tissues and growing conditions from cereals (rice, wheat and maize); solanaceae members (potato, tobacco and tomato) and legumes (*Medicago*, soybean and lotus) were carried out. The TEs in cereals were significantly higher than those present in legumes or solanaceae members. A total of 557,359 ESTs from *G. max* and *M. truncatula* arising from 119 and 61 different libraries, respectively were analyzed to estimate the frequency and distribution of active transposable elements in the legumes during stressed and unstressed conditions. The percentage of ESTs similar to retrotransposons in legumes was significantly higher ($P < 0.05$) in cDNA libraries from tissues during different stress conditions than in cDNA libraries from tissues, which were unstressed. Furthermore, total of 169,610 ESTs from legume roots was studied. The percentage of ESTs similar to retrotransposons in legume roots was significantly higher ($P < 0.05$) in root cDNA libraries during different stress conditions than in root cDNA libraries that were unstressed. Thus, identification of retrotransposons within the available EST database provides an indirect estimation of the patterns of transcriptional activity of these repetitive elements.

4.1. Introduction

Retrotransposons are the mobile genetic elements ubiquitous to plant genomes. They are present in high copy number, comprising bulk of the genome amounting to more than 50% of genomic DNA in some plants. Despite the popular perception they being the parasitic elements, retrotransposons benefit their host plants in several ways, they are thought to increase mutation rate thereby providing new regulatory properties for genes (Wesseler *et al.,* 1995) or believed to contribute to DNA repair (Moore and Haber 1996) or to the centromere function (Miller *et al.,* 1998) and towards host's responses to pathogen. Such positive contribution of the retrotransposons is crucial for their multiplication and survival. Transposable elements (TEs) are genetic elements that are able to mobilize/transpose themselves into another location within the genome.

TEs have been classified in two groups according to their mode of transposition (Fig 4.1): class I elements or retroelements include long-terminal repeat (LTR) retrotransposons, such as Ty1-*copia* and Ty3-*gypsy*-like elements, non-LTR (LINEs and SINEs) and retroviruses (Jakowitsch *et al.,* 1999; Kumar and Bennetzen 1999; Schmidt, 1999), they replicate and reinsert at multiple sites in a complex process involving DNA dependent RNA transcription, translation of the RNA into functional proteins, RNA-dependent DNA synthesis (reverse transcription), and reintegration of newly generated retroelement copies into the genome (Table 4.1). The replicative mode of transposition of retrotransposons can rapidly increase their copy number and thus increase plant genome size, for example they represent half or more of the nuclear DNA in wheat (Echenique *et al.,* 2002) and maize (SanMiguel and Bennetzen, 1998). The class II elements or DNA transposable elements, like *Ac*, *Tam1*, *En/Spm*, and also elements such as miniature inverted tandem elements (MITEs) (Wessler *et al.,* 1995; Casacuberta *et al.,* 1998), transpose by an excision/repair mechanism and involve only DNA. The class II TEs are usually present in a low copy number, probably as a consequence of their 'cut and paste' mechanism of transposition and they usually do not greatly influence plant genome size (Kunze *et al.,* 1997; Casacuberta and Santiago, 2003.)

Transposition is not a random process; it is regulated at 1) transcriptional initiation, 2) post-transcriptional and 3) at transpositional level. Many retroelements studied were observed to be activated following biotic and abiotic stress. Various biotic factors such as viral, bacterial and fungal inoculation (Pouteau *et al.,* 1994; Mhiri *et al.,* 1999) elicitors like fungal extracts (Pouteau *et al.,* 1994; Takeda *et al.,* 1999) are reported to activate the retrotransposons. Besides, tobacco Tto5 and Tto1 are found to be induced by chemical inducers like salicylic acid and methyl jasmonate.

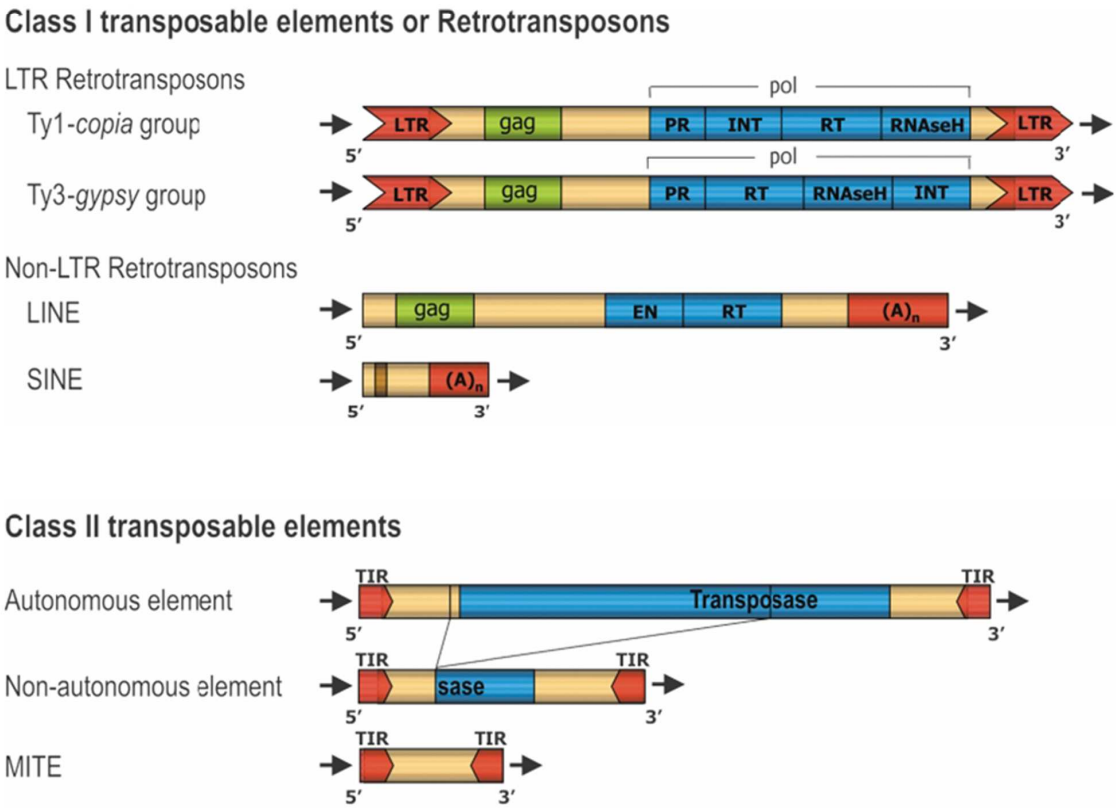


Figure 4.1: Transposable elements classified as class I and class II types based on the presence and arrangement of genes. (Casacuberta and Santiago, 2003)

Table 4.1: General classification of transposable elements based on their mode of transposition and gene arrangement

Retrotransposons (**Class I**)	Transposons (**Class II**)	**MITEs**
Transpose through an RNA intermediate.	Transpose directly via a DNA intermediate.	TEs with characteristics of both class I and class II elements.
Rapid increase in copy number due to replicative mode of transposition, can be extremely high in eukaryote genomes.	As a consequence of their 'cut and paste' mechanism of transposition, usually present in a low copy number.	Structural characteristics similar to defective class II elements; high copy number high sequence and size conservation of subfamilies suggest that they can be amplified from a very limited number of progenitors a characteristic of class I elements.
Divided into		
Long Terminal Repeat **(LTR)**	(**non-LTR**)	
Further subdivided into the Ty1-copia and the Ty3-gypsy, based on the arrangement of Gag and Pol (PR, INT, RT and RNAseH) genes.	Subdivided into long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs).	
LTR retrotransposons have long terminal repeats (LTRs) of variable length (from 100 bp to several Kb)	Lack LTRs and are transcribed from an internal promoter.	

Both, biotic and abiotic stress are known to stimulate the expression or the transposition of mobile elements in yeast (Rofte *et al*., 1986, Bradshaw and McEntee, 1989), *Drosophilla* (Junakovic, 1988), animal (Chinnadurai, 1991) and plant systems (Osterman, 1991; Pouteau, *et al.,* 1991; Walbot, 1992). One of the best-characterized plant retrotransposons is the Tnt1 (*Nicotiana tabaccum* Ty1-*copia* like) element from *Nicotiana tabacum* (Grandbastien *et al.*, 1989). Tnt1 is expressed only in roots and not in other healthy tobacco tissues (Pouteau *et al.,* 1991). Expression of Tnt1 is induced strongly by stress such as pathogen attacks and inducers of microbial origin, and a close correlation has been shown between Tnt1 transcription and plant defense responses (Pouteau *et al.,* 1994; Mhiri *et al.,* 1996; Grandbastien *et al.,* 1997; Mhiri *et al.,* 1997, 1999). Recent studies have shown that Tnt1 transcription is followed by transposition, and that fungal extracts efficiently activate Tnt1 mobility. The key step in controlling Tnt1 amplification appears to be transcription, as newly transposed Tnt1 copies show high sequence similarities to the sub-populations of transcribed Tnt1 elements (Melayah, *et al*., 2001).

Host cells and the retroelements must have evolved mechanisms to minimize the negative effects of TE activity. It has been proposed that DNA methylation limits spread of TE. In maize, analysis of repetitive DNAs has routinely shown that they are cytosine methylated. These methylated DNAs have been shown to be mostly LTR retotransposons (SanMiguel *et al.,* 1996). In fact, *de novo* methylation was first detected during inactivation of DNA transposable elements of the mutator and AC/DS families (Bennetzen *et al.,* 1993: Kunze *et al.,* 1997). In maize, retros that make up to 50-80% of the genomic DNA constitute only 10% or less of the transcripts in most tissues indicating good control over the transcription activity. Many retros show unique patterns of developmental and /or environmental regulation. Several others show organ specific expression, eg. Tn1 of tobacco, BARE of barley and PREM 2 in maize have been detected primarily in roots, leaves and young microspores, respectively (Poteau *et al.,* 1991; Suoniemi *et al.,* 1996a, b; Turcich *et al.,* 1996)

Ty1-*copia* like retrotransposon element was identified in chickpea and their potential for diversity assessment among wild and cultivated species of *Cicer* was studied (Sant *et al.,* 2000) as there is sequence heterogeneity among Ty1-*copia* elements in chickpea. The copy number is higher in the cultivated *Cicer arietinum* compared with *Cicer reticulatum* (Sant *et al.,* 2000).

In the present chapter I have identified one Ty1-copia like sequence (a Class I type TE) and two non-LTR retrotransposon like sequences, which were transcriptionally active when the chickpea plants were challenged with the pathogen *Fusarium oxysporum* f.sp *ciceri* race1 (FOC1). I here survey the available EST database to analyze the expression pattern of the TEs primarily in legumes and in other crops. The frequency of retrotransposons and transposons in the gene indices of The Institute For Genome Research (TIGR) database and in the National Center For Biotechnology Information (NCBI) database was estimated using keyword driven searches. Occurrence of TEs in libraries representing tissue, stages of development, response to biotic and abiotic stimulus etc, serves as a good indicator of temporal and spatial expression patterns of TE. In light of this, it is planned to validate the observation of transposon activation in chickpea roots challenged by FOC1 and to survey patterns of transcriptional activity in legumes among the different classes of TEs ie. retrotransposons and transposons in the different plant cDNA libraries (ESTs).

4.2. Materials and methods

4.2.1. Plant materials and pathogen infection

The seeds of chickpea varieties Vijay (Resistant), WR315 (resistant) and JG-62 (susceptible), were obtained from the Mahatma Phule Krishi Vidyapeeth (MPKV) at Rahuri, Maharashtra, India. Sprouted seeds were transferred on to the Styrofoam floats in the glass trays filled with sterile water (half strength Hoagland's medium; Hoagland and Arnon, 1950) and placed in a growth chamber at 22^{o}C and 60% relative humidity under white light and normal day conditions (14 h light/10 h dark) as detailed in previous chapters.

The plants growing in hydroponic trays containing sterile water were challenged with freshly prepared spore suspension of FOC1 after 7 days. To ensure uniform spread of the fungus, the water in the trays was mixed with a sterile glass rod after two days. Uninoculated trays served as a control.

4.2.2. cDNA-AFLP

cDNA-AFLP was performed as described in chapter-2 in which the amplified cDNA library was used as the DNA templates. The root cDNA libraries were amplified using T3 and T7 primers flanking the cDNA insert in the library. At least five amplifications were carried out separately and pooled. These amplified libraries served as the starting template for the cDNA-AFLP. Selective amplification products were separated on a 6% polyacrylamide gel run at 1500 V, 100 W, for 3 hrs. Gels were stained using the standard silver staining technique (Sanguinetti *et al.*, 1994).

4.2.3. TDF isolation and sequence analysis and reverse northern

TDFs that were differentially expressed in the resistant-infected cultivar (WR315) were eluted from the gels, cloned into pGEM-T Easy Vector (Promega, USA) and transformed in *Escherichia coli* α-DH5 competent cells prepared by $CaCl_2$ method as described in chapter-2. Plasmid DNA (200 ng) was used for sequencing using the DYEnamic ET Dye Terminator Cycle Sequencing Kit (AmershamBiosciences, USA) in MegaBace DNA Sequence Analyzer (AmershamBiosciences, USA). The sequences were compared with the GenBank database using Bastn algorithms (Altschul *et al.*, 1997).

Total RNA was extracted from chickpea root tissues collected at 1, 2, 4, 8 and 12 DAI intervals using TRIzol (Invitrogen, USA) method according to manufacturer's instructions. Total RNA (5 µg) was used in the reverse transcription reaction containing oligo dT(18) primer, PowerScript reverse transcriptase (Invitrogen, USA) and αP^{32} dATP according to standard techniques (Sambrook, *et al.,* 1989), to generate the first strand cDNA probes. Reverse northern blot hybridizations were accomplished using these αP^{32} radio-labeled cDNA probes generated by RT-PCR labeling as described in chapter-2.

4.2.4. Database survey

Medicago truncatula and *Glycine max* EST sequences were obtained from TIGR (The Institute for Genome Research; http://www.tigr.org). Database searches were carried out using the keywords search tool option in the TIGR and NCBI public database. The data retrieved using keywords "copia", "gypsy", "gag pol", "integrase", "reverse transcriptase", "retrotransposon" or "retroelements" and "non-LTR" were grouped as retrotransposons and the data retrieved using "transposons" or "transposable element" were grouped as transposons. Use of these keywords could retrieve sequences of the entire TEs excluding only some ESTs, which were not clustered or annotated, that might have certain similarity to retrotransposons. The retrieved sequences were edited to eliminate repetitions if any. Telomerase types of reverse transcriptase sequences were also eliminated.

All the unique sequences and tentative clusters (TCs) (TIGR database) from nine crops species and all sequences, genomic and cDNA from *Cicer* (1437 sequences in NCBI database) were included in this survey which encompassed a total of 490209 unique ESTs. TCs from various tissues and growing conditions were included in this study. A total of 180 libraries from both *Glycine max* and *Medicago truncatula* were screened, of which 330,436 ESTs in 119 libraries from *Glycine max* and 226,923 ESTs

in 61 libraries from *Medicago truncatula* were used in this study to find the distribution of the TEs in these legumes. I also segregated these sequences according to distribution in different plant parts as well as during stress (biotic or abiotic) and unstressed conditions. The TEs in the root tissue from these two legumes were also studied, to generate a picture of the activity of TEs in roots in response to both abiotic and biotic stress including pathogenic and symbiotic interactions. A total of 169,610 ESTs from legume roots were studied. Comparison of the frequencies of TEs in different tissues and stress conditions was performed using their percentages and 2-sample *t*-test was performed on them to test their significance levels.

4.3. Results and Discussion

Like several other plant genomes, chickpea shows abundant dispersal of retrotransposons (SanMiguel *et al*., 1996; Heslop-Harrison, *et al*., 1997; Sant *et al*., 2000), which seem to be conserved within the genus *Cicer*. *Ty1-copia* sequences have been detected in closely related *Vicia* species (Pearce, *et al.*, 1996), while the chromosomal localization of a Ty1-*copia* family was determined in chickpea (Brandes *et al.*, 1997). Sequence heterogeneity between two Ty1-*copia* sequences CA1 and CR10 from *Cicer arietinum* and *Cicer reticulatum* has also been reported (Sant *et al.*, 2000) and used for detection of genetic diversity in *Cicer*. The total number of Ty1-*copia* elements estimated was approximately 600 copies and 10 copies per diploid genome of *C. arietinum* and *C. reticulatum*, respectively (Sant *et al.*, 2000). Two groups of retrotransposon-like sequences of a Ty3-*gypsy* element, *CaRep1* and *CaRep2* were isolated from chickpea and their structure, genomic organization and distribution among the wild species of the genus *Cicer*, were studied (Staginnus *et al.*, 1999). The present chapter contributes information on the expression pattern of the repetitive elements of chickpea. RNA transcripts from retroelements have the potential to be included in cDNA libraries constructed from these mRNA populations as they have a poly-A tail. The abundance of these elements in the RNA population included in the cDNA libraries is expected to represent the transcriptional activity in chickpea genome.

4.3.1. Expression of retroelements during biotic stress

A number of transcripts were identified by cDNA-AFLP to be differentially expressed in resistant infected chickpea cultivar which were cloned and analyzed as reported earlier chapters. Of these, 3 corresponded with transposable elements (Table 4.2). Chickpea plants were subjected to conditions of pathogen challenge simulating field conditions and the RNA was isolated from two individual genotypes at specific time points that were challenged or not challenged with the fungal pathogen FOC1.

Table 4.2: Summary of the transcript-derived fragments (TDFs) identified by cDNA-AFLP, containing sequences induced during infection. The nucleotide-homology of the TDFs with sequences in the database using BLASTn algorithm is detailed below.

Clone Id	GB Accession	Length	Homology	e-Score
CaFRi12	DR749493	400	Non-LTR retroelement	2e-05
CaFRi15	DR749496	206	GAG-POL precursor gene	8e-11
CaFRi20	DR749479	170	Ty-1 copia retrotransposon	1e-76

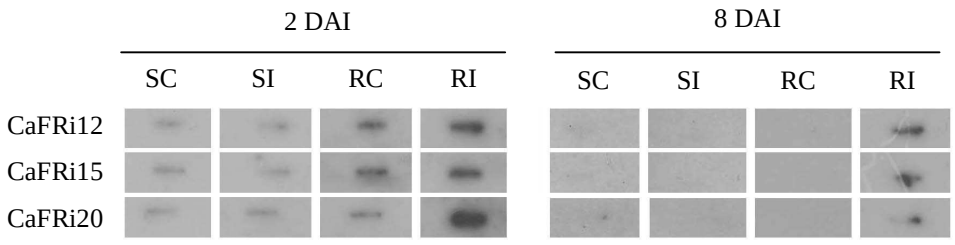


Figure 4.2: Reverse northern of TDFs similar to retroelements identified by cDNA-AFLP. CaFRi12 (DR749493) and CaFRi15 (DR749496) were similar to non-LTR like retrotransposon and CaFRi20 (DR749479) was similar to Ty1-copia like sequence.

It was observed that the retrotransposon transcript levels in roots correlated with the exposure of the plants to pathogen challenge in the resistant genotype Vijay (Fig 4.2). The maximal transcript abundance was detected in roots exposed to the pathogen at 2 DAI.

Time-course RNA reverse northern blot analysis revealed more abundance of retrotransposon transcripts at 2 DAI during pathogen attack (Fig 4.2). The TDFs CaFRi12 and CaFRi15 showing homology with non-LTR like retrotransposon showed to have increased transcript levels in the resistant infected cultivar even at 8 DAI interval though the levels of the transcript reduced when compared to the 2 DAI stage. The transcript levels of the TDF clone CaFRi20 having homology with a Ty1-copia like element from *Cicer* reduced considerably at 8 DAI interval in the resistant infected cultivar. No signals were detected in samples from the control and challenged plants of susceptible cultivar JG-62 at 8 DAI.

Interestingly, the retrotransposons were observed to be highly upregulated in the incompatible interaction (resistant cultivar) and not in the compatible interaction (susceptible cultivar). The data suggests that the retroelements are highly sensitive to the biotic stimuli in the resistant chickpea cultivar, as was seen with the Ty1-*copia* type element OARE-1 from oat which was also scarcely activated in the susceptible phenotype (Kimura *et al.,* 2001). This may not be true in all the cases, as was observed for the expression of Tnt1 retrotransposon, which was induced equally in both, the resistant as well as the susceptible phenotypes (Grandbastein *et al.,* 1998).

4.3.2. Database survey

To generate a global picture of retrotransposon activity, publicly available expressed sequence tags (ESTs) were analyzed for TEs in the TIGR database for graminae (wheat, rice and maize), fabaceae (*Medicago truncatula*, *Lotus japonicus* and *Glycine max*) and solanaceae (potato, tomato and tobacco) in the present study. A total of 490,209 unique sequences from the gene indices of the TIGR database were surveyed for the presence of TEs. The gene indices include both TC and singleton EST sequences, TC (Tentative consensus) sequences were analyzed because they were more likely to represent full-length transcripts and singletons because they represent unique sequences. Fifty five percent of these sequences were from graminae, 26.30% from fabaceae, and 18.60% from solanaceae (Table 4.3).

Most copies of retroelements are inactive as seen by their representation in the mRNA population which is smaller than in the genomic DNA. For example, 50–80% of the genomic DNA in maize is made up by the retrotransposons but constitutes less than

10% of the mRNA in most tissues (Kumar and Bennetzen 1999). This percentage is similar to those observed in *Drosophila* and yeast. In Drosophila the levels of transcripts homologous to Ty1-copia-type retrotransposons range between 0.5 to 3% of the total RNA and in yeast 5 to 10% of the polyadenylated RNA (Singer and Berg 1991). Previous studies have shown that some of these retroelements are also transcribed in different legume species (Garber *et al.,* 1999; Madsen *et al.,* 2005) though no quantification of their abundance is available. Translation products of Ty1-copia-type retrotransposons have been recently detected immunologically in barley (Jaaskelainen *et al.,* 1999).

The accumulation of 557,359 ESTs from different cDNA libraries of *Medicago truncatula* and *Glycine max* from different tissues, developmental stages, and environmental conditions provides a unique opportunity to estimate the relative proportion of these retroelements in these cDNA libraries. Previous studies based on database surveys have analyzed ESTs from various plant species (Rossi *et al.,* 2001; Vicient *et al.,* 2001; Khul *et al.,* 2004). The EST data used in these analyses contained ESTs, unique sequences or tentative consensus (TCs) sequences, but was restricted to only one crop like sugarcane or onion or wheat or genes specific to one group of plants like legume specific genes (Graham *et al.,* 2004). To provide a better estimate of the frequency, type and the distribution of retroelements in plant ESTs, I analyzed the ESTs from the TCs of different crops into putative groups of different transposable elements, thereby reducing the redundancy in the data sets and analyzed retroelements in the coding regions from graminae, fabaceae and solanaceae in the TIGR database. Sampling of these ESTs in the database for plant transposable element (TE) sequences revealed that 0.27% (1,340 of 490,209) unique sequences studied were annotated as TEs (Table 4.3). Of these, 15.14% (203 of 1,340) were homologous with class I DNA elements (Ac/Ds or En/Spm), and the remaining were homologous with class II RNA elements [*copia, gypsy,* or non–long terminal repeat (LTR) retrotransposons].

I also have analyzed the sequences, both genomic as well as the ESTs from *Cicer* in the NCBI database as the TIGR database did not include the *Cicer* sequences in their gene indices. In the NCBI database we searched for *Cicer arietinum* mRNA sequences annotated as TEs. The survey revealed that of the total 1,437 mRNA sequences 132 could be retrieved as TE sequences (both expressed and non-expressed) of these 132 sequences 17 sequences were reported as ESTs or mRNA transcripts which indicate them to be transcriptionally active.

Table 4.3: Total number of unique sequences (TIGR database) from nine crops and the number of unique sequences annotated as different TEs.

			Class I	Class II	
		Total Unique Seq	**Retrotransposons**	**Transposons**	**Total TE**
			Total		
Legumes	*Medicago truncatula*	36878	68	17	85
	Lotus japonicus	28460	18	4	22
	Glycine max	63676	73	15	88
		129014	**159[0.123]**	**36[0.027]**	**195 [0.151]**
Cereals	*Oryza sativa*	89147	532	98	630
	Triticum aeistivum	122282	120	10	130
	Zea mays	58582	167	41	208
		270011	**819[0.303]**	**149[0.055]**	**968 [0.358]**
Solanaceae	*Solanum tuberosum*	38239	42	2	44
	Nicotiana tabaccum	21107	75	10	85
	Lycopersicon esculentum	31838	42	6	48
		91184	**159[0.174]**	**18[0.019]**	**177 [0.194]**
Total		**490209**	**1137**	**203**	**1340**

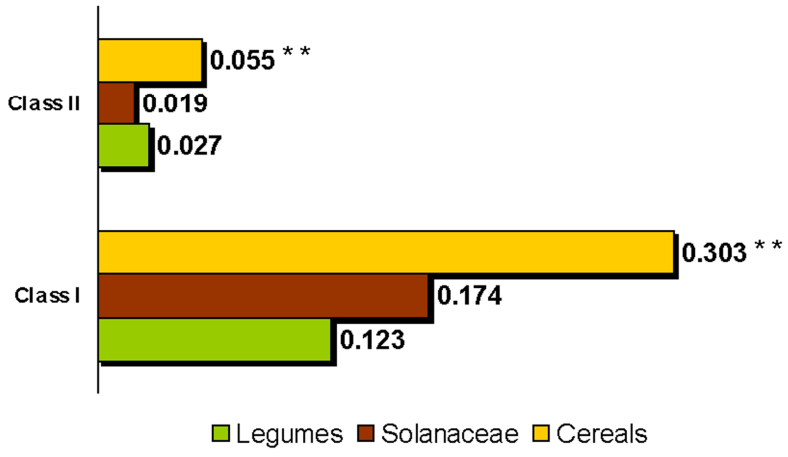


Figure 4.3: The frequency of TCs from different crops annotated as transposable elements in the TIGR database is represented as percentages. ** indicates that the percentage of TEs was significantly higher (P≤ 0.05) as calculated using one sample *t*-test.

The 17 TE sequences comprised transcripts identified during unstressed conditions (5); biotic stress (3); and abiotic stress (9). Among the TE like transcripts the total expressed transcripts during stressed conditions were higher than during unstressed conditions

To estimate the level of transcription of different retrotransposons during different biotic and abiotic stress conditions is important because transposition of these retroelements seems to be regulated mainly at the transcriptional level. For the tobacco *Tto1* and rice *Tos17* Ty1-*copia*-type retrotransposons a correlation between transcription and transposition has been demonstrated (Hirochika 1993; Hirochika *et al*. 1996). Therefore, control mechanisms of the transcription may be crucial to minimize deleterious effects of retrotransposon transposition on the host. The plant retrotransposons have shown different levels of transcription under stress conditions. Therefore, unique sequences and the TC sequences of the legumes included in this study were separated into ESTs that were obtained from plants under biotic stress (virus, fungal infection or mychorrizal), abiotic stress (cold, heat, drought, tissue culture, wounding and chemical agents), simultaneous biotic and abiotic stress (including libraries from etiolated seedlings), or no stress (Table 4.4).

Tentative clusters were selected in all nine plants for analyzing the presence of class I and class II TEs. Further, TEs from individual EST clones from the legumes were analyzed during stress and non-stress conditions. Of the nine crops selected, *Medicago truncatula*, *Lotus japonicus* and *Glycine max* were grouped into fabaceae. Rice, wheat and maize were grouped as graminae, while potato, tobacco and tomato formed the solanaceae group. The total unique sequences annotated as TEs (includes both retroelements and DNA transposons) in the different groups were; 0.151% in fabaceae, 0.358% in graminae and 0.194% in the solanaceae group (Table 4.3). Graminae had significantly the highest number of total TEs followed by solanaceae and then fabaceae, though there was no significant difference between the fabaceae and solanaceae group for the total TEs as revealed by the two sample *t*-test. Again the total numbers of retrotransposons in the different groups were analyzed and the data suggests that retrotransposons (class I) are generally more transcriptionally active in the grasses than in other groups of plants, although transcription occurs in all groups. It was observed that graminae had the highest number of retroelements or retrotransposons (class I) among the selected groups with 0.303% and was highly significant when compared to solanaceae [0.174%] and fabaceae [0.123%]. When I analyzed the DNA transposons (class II) in these different classes, graminae had a significantly higher number of transposons [0.055%] than the other two groups (Table 4.3 & Fig 4.3).

Table 4.4: Transposable elements during different stress conditions in *M. truncatula* and *G. max*, represented as the percentages which are calculated using the individual total of the ESTs in different conditions.

			Class I		Class II		Total TEs		Total ESTs	
Unstress			0.053 [106]		0.030 [60]		0.083 [166]		199225	
Stress	Abiotic		0.066 [104]		0.030 [48]		0.096 [152]		157371	
	Biotic	Pathogenic	0.048 [35]	0.083 [126]	0.011 [8]	0.010 [16]	0.059 [43]	0.094 [142]	72718	150045
		Symbiosis	0.117 [91]		0.010 [8]		0.128 [99]		77327	
	Total Stress		0.074 [230]		0.020 [64]		0.095 [294]		307416	
	Total stress/unstress		0.066 [336]		0.024 [124]		0.090 [460]		506641	
Mixed			0.084 [43]		0.021 [11]		0.106 [54]		50718	
Grand total			0.067 [379]		0.024 [135]		0.092 [514]		557359	

Note: The figures in parenthesis represent the actual number of ESTs similar to TEs. (On 30-06-07 the number of ESTs was unchanged)

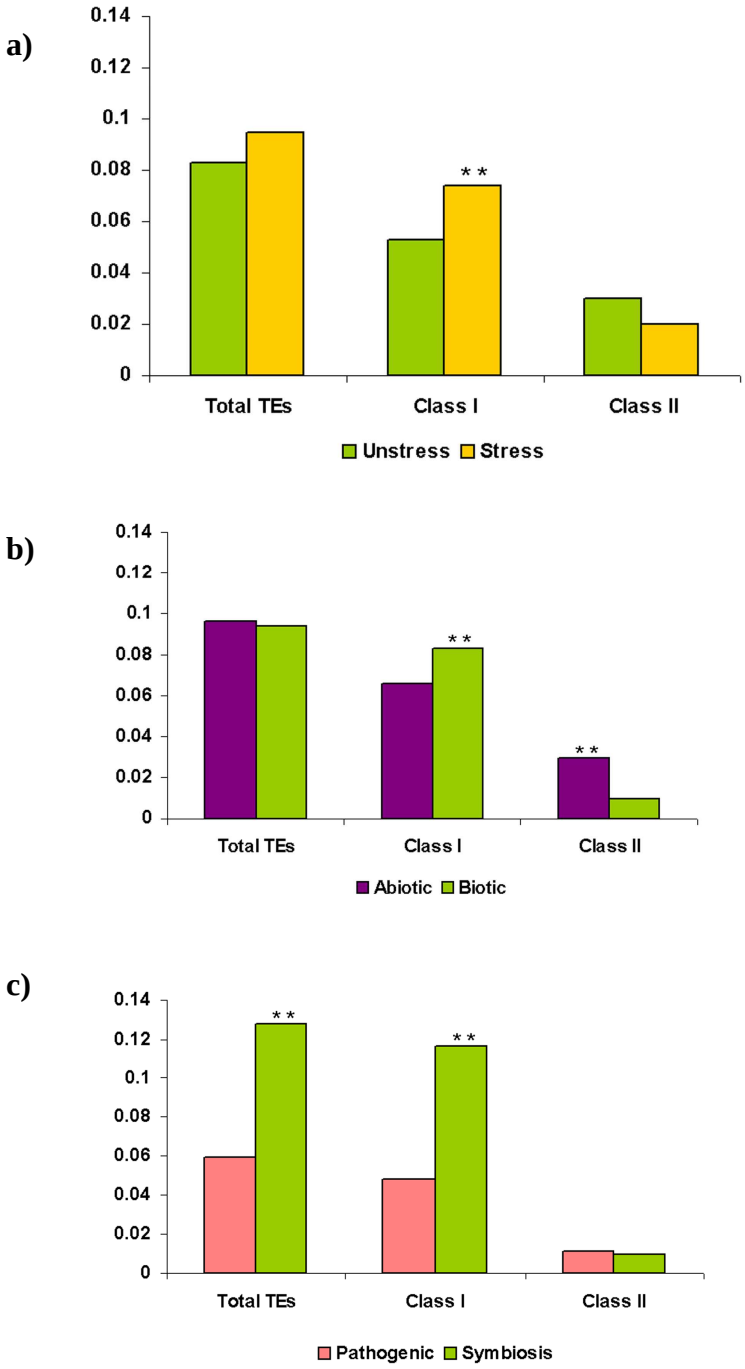


Figure 4.4: Frequency of transposable element like ESTs in the TIGR database represented as percentages from *Medicago truncatula* and *Glycine max.* a) Frequency of TEs during stress (biotic and abiotic) and unstress conditions. b) Frequency of TEs during biotic and abiotic stress conditions. c) Frequency of TEs during biotic stress i.e. pathogenic or symbiotic interactions. ** indicates that the percentage of TEs was significantly higher ($P \leq 0.05$) as calculated using *t*-test.

There was also no significant difference in the number of DNA transposons (class II) between the solanaceae [0.019%] and the fabaceae [0.027%]. Caution should be taken in this type of analysis as the ESTs in the databases are derived from a mixture of cDNA construction and sequencing methods, and display length variations combined with the inherently partial nature of EST sequences, may cause accessions for retrotransposon transcripts to be missed and cause hindrance in the annotation of ESTs in general.

4.3.3. Transposable elements during different stimuli in legumes

Two legume species in particular have been the focus of large EST projects over the last few years - *Glycine max* (soybean), *M. truncatula*, and more recently *L. japonicus*. Thus, a large fraction of all the genes in these species are represented in the current EST collections. As the EST collections represent cDNA libraries derived from specific organs harvested at defined developmental stages, or plants subjected to certain biotic and abiotic stresses, it is possible to use bioinformatics to infer something about the relative expression of thousands of different genes under a variety of conditions.

The total TEs (both class I and class II) during stress [0.094%] were more but the difference was not significant compared to the TEs during unstressed conditions [0.083%]. The correlation between the EST frequencies observed during the survey of the retrotransposons and transposons (Table 4.4 and Fig 4.4a) suggests that these frequencies reflect a particular transcriptional pattern of the transposable elements screened in this study. The increased frequency of ESTs of transposable elements in the cDNA libraries from stressed plants correlates well with the experimental evidence (Kumar and Bennetzen 1999; Vicient et al. 1999) and database survey (Vicient *et al.*, 2001; Echenique *et al.*, 2002) suggesting that the observed frequencies are the result of a particular transcription pattern rather than an artifact produced by genomic contamination of the cDNA libraries.

Total TEs (both class I and class II) during the abiotic stress [0.096%] were marginally more than the TEs during biotic stress [0.094%] and not significant (Fig 4.4b). In the biotic stress TEs during symbiosis [0.128%] were more than TEs during pathogenic stress [0.059%]. The retrotransposons (class I) were also significantly [P=0.0046] more during stress conditions [0.073%] than in unstressed conditions [0.054]. However, retrotransposons were significantly [P=0.0006] more during the biotic stress [0.083%] than in the abiotic stress [0.066%] (Fig 4.4b). Within the biotic conditions the retrotransposons were more during symbiosis [0.112%] than during pathogenic stress (Fig 4.4c). The DNA transposons (class II) were slightly more during the unstressed conditions [0.03%] than during stress conditions [0.01%].

Table 4.5: Transposable elements during different stress conditions in the root tissue of *M truncatula* and *G max*, represented as the percentages which are calculated using the individual total of the ESTs in different conditions.

			Class I		Class II		Total TEs		Total ESTs	
Unstress			0.061 [24]		0.012 [5]		0.074 [29]		39115	
Stress	Abiotic		0.091 [26]		0.014 [4]		0.105 [30]		28499	
	Biotic	Pathogenic	0.070 [15]	0.107 [106]	0.014 [3]	0.011 [11]	0.084 [18]	0.115 [117]	21405	98723
		Symbiosis	0.117 [91]		0.010 [8]		0.128 [99]		77318	
		Total Stress	0.103 [132]		0.011 [15]		0.115 [147]		127222	
	Total stress/unstress		0.076 [156]		0.009 [20]		0.086 [176]		202714	
Mixed			0.030 [1]		0		0.030 [1]		3273	
Grand total			0.092 [157]		0.011 [20]		0.104 [177]		169610	

Note: The figures in parenthesis represent the actual number of ESTs similar to TEs. (On 30-06-07 the number of ESTs was unchanged)

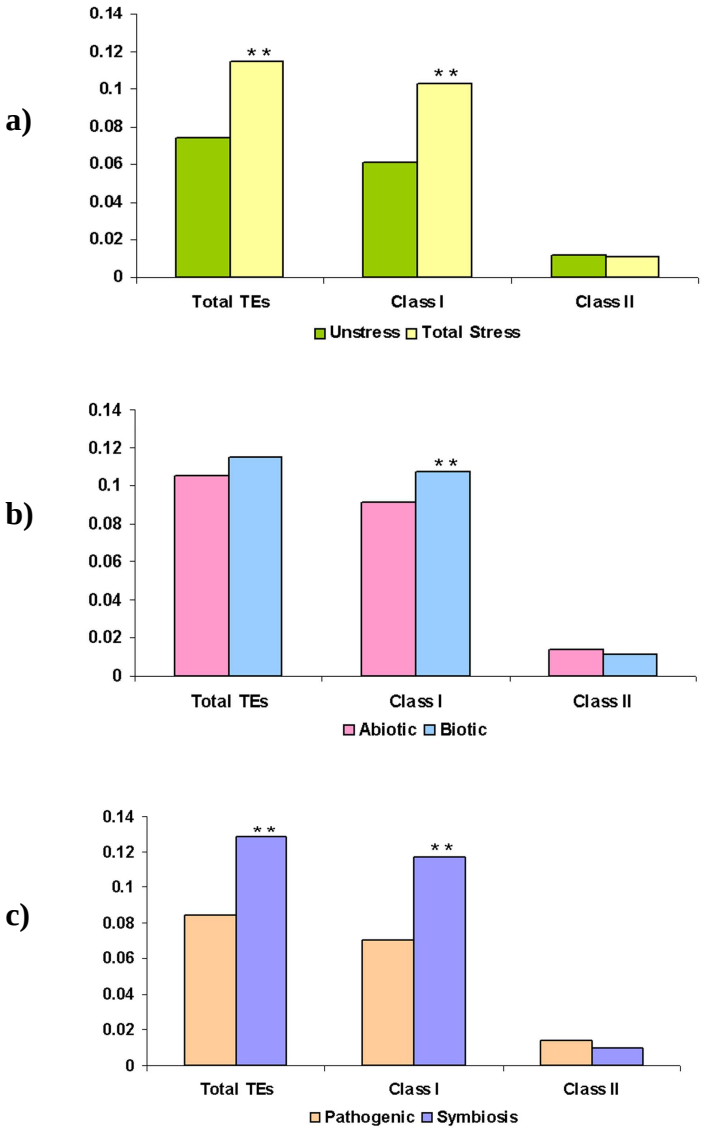


Figure 4.5: Frequency of transposable element like ESTs in the TIGR database represented as percentages from the roots of *Medicago truncatula* and *Glycine max* during different stress conditions. a) Frequency of TEs in the roots during total stress (biotic and abiotic) and unstress conditions. b) Frequency of TEs in the roots during biotic and abiotic stress conditions. c) Frequency of TEs in the roots during biotic stress i.e. pathogenic or symbiotic interactions. ** indicates that the percentage of TEs was significantly higher ($P \leq 0.05$) as calculated using *t*-test.

Within the stress conditions the transposons (class II) were significantly [P=0.0001] more during the abiotic stress [0.03%] than in biotic conditions [0.01%] (Fig 4.4a). In the biotic stress conditions the transposons were more during pathogenic stress [0.011%] than during symbiosis [0.009%] but were not significant (Fig 4.4c). Under both conditions stressed and unstressed the retrotransposons (class I) were significantly more than the DNA transposons (class II).

4.3.4. Transposable elements during stress in legume roots

Legume roots offer a unique site in study of plant-microbe interaction, comprising both symbiotic as well as pathogenic associations. Study of the expression of transposable elements in the legume roots helps to understand their activity during these different associations. In roots, the transposable elements showed similar pattern, where the total TEs (both class I and class II) were significantly [P=0.0291] more in the stress conditions [0.115%] than in unstressed conditions [0.074%] (Table 4.5 & Fig 4.5a), and the TEs in biotic stress [0.118%] were slightly higher when compared to the TEs during the abiotic stress [0.105%] but not significant (Table 4.5b). The total TEs during symbiosis [0.128%] were not significantly higher than the TEs during pathogenic stress [0.084%]. The retrotransposons (class I) in roots were significantly [P=0.0174] higher during the stress conditions [0.103%] than in unstressed conditions [0.061%]. In the stress conditions the biotic stress [0.107%] had more retrotransposons than in abiotic stress [0.091%] and in biotic stress the retrotransposons were not significantly higher during symbiosis [0.113%] than in pathogenic stress [0.070] (Fig 4.5c). The DNA transposons (class II) in roots did not show any significant difference between the two groups i.e. stressed and unstressed conditions.

4.3.5. Transposable elements in different tissues of legumes

Fabaceae, the third largest family of plants and the source of many crops, has been the focus of many genomic studies. The legumes provide a unique system of symbiotic and pathogenic interactions found, and thus provide numerous targets for functional genomics research. Symbiosis with soil microbes to obtain fixed nitrogen is unique to legumes, while mycorrhizal association is also found in majority of higher plants. There are nearly 129,014 unique sequences (TCs and ETs) representing the fabaceae available from 'The Institute for Genome Research' (TIGR browser, http://www.tigr.org/, November, 2005). *Lotus japonicus* was not include in the study as many plant parts and different stress conditions as compared to *Medicago* and *G max* are not available.

Table 4.6: Transposable elements in different plant tissues in *M truncatula* and *G max*, represented as the percentages which are calculated using the individual total of the ESTs in different tissue types.

	Class I	**Class II**	**Total TEs**	**Total ESTs**
Roots	0.092 [157]	0.011 [20]	0.104 [177]	169610
Shoots	0.064 [31]	0.027 [13]	0.091 [44]	47900
Leaves	0.050 [41]	0.011 [9]	0.061 [50]	81024
Seedlings	0.039 [30]	0.039 [30]	0.079 [60]	75794
Seeds	0.066 [51]	0.018 [26]	0.084 [65]	77080
Flowers	0.043 [14]	0.081 [26]	0.125 [40]	31966
Cell culture	0.050 [15]	0.040 [12]	0.090 [27]	29973
Total TE	0.067 [339]	0.024 [124]	0.090 [463]	513347
Mixed	0.090 [40]	0.024 [24]	0.115 [51]	44012
Grand Total	0.067 [379]	0.024 [135]	0.092 [514]	557359

Note: The figures in parenthesis represent the actual number of ESTs similar to TEs. (On 30-06-07 the number of ESTs was unchanged).

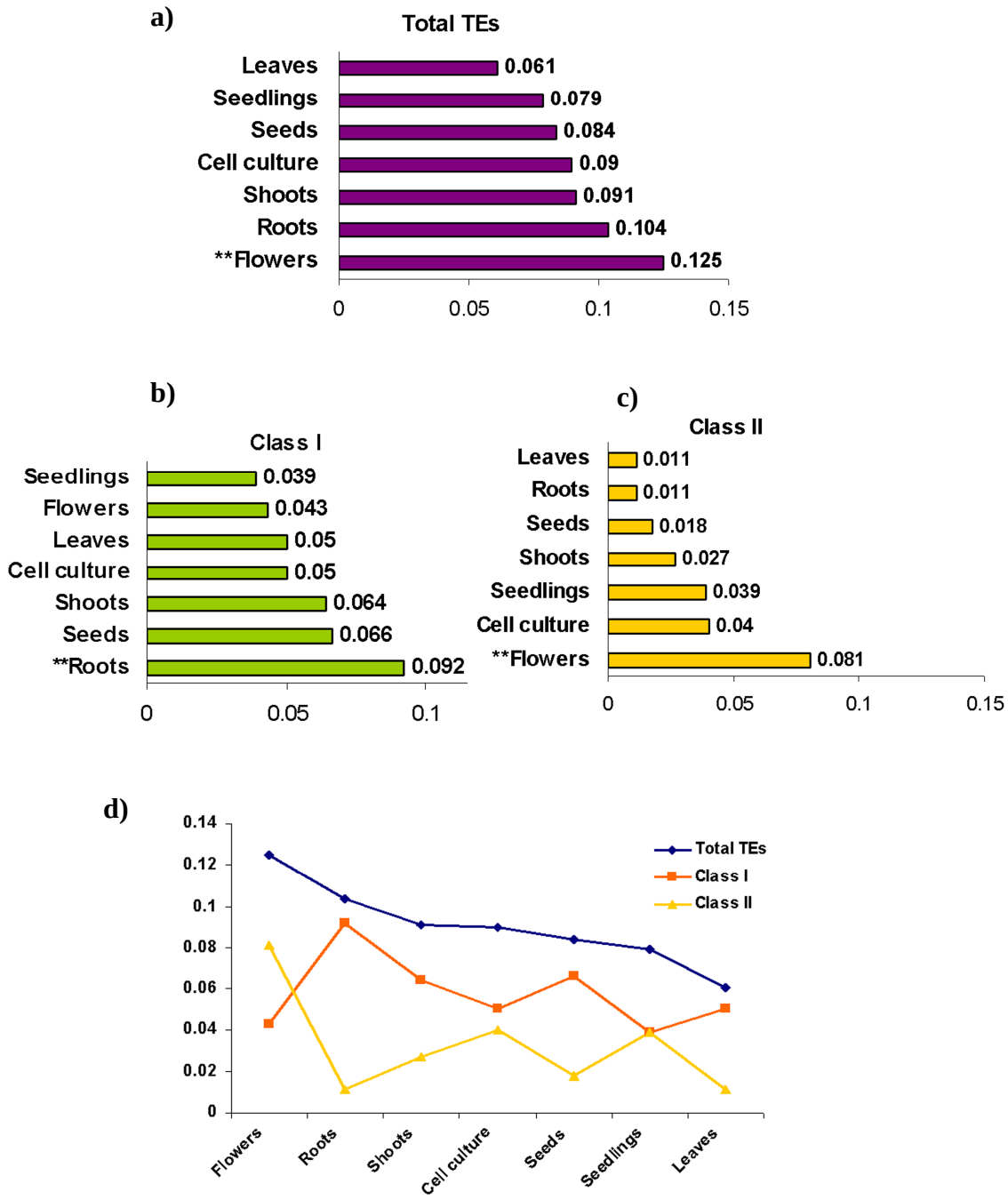


Figure 4.6: Number of ESTs in the TIGR database annotated as TEs from different plant parts of *Medicago truncatula* and *Glycine max* represented as percent. a) Total TEs from both plants and their distribution in different parts of the plant. b) Class I TEs and their distribution in different parts of the plant. c) Class II TEs and their distribution in different plant parts. d) Combined graph depicting the presence of TEs in different plant parts. ** indicates that the percentage of TEs was significantly higher ($P \leq 0.05$) as calculated using *t*-test.

The ESTs were assorted in seven different groups with respect to plant parts; roots (included nodules, root hair and hypocotyls), shoots (included meristametic tissue, epicotyl, vegetative buds and stems), leaves, seedlings (included plantlets and whole seedlings), seeds (included pods, seed coat and cotyledons), flower (included all floral parts) and cell culture (all type of tissue grown as cell culture), as these were the common plant tissue for the two legume crops under study. Only the plant tissue those were common to the two crops were included in this study and those, which weren't common, were left out.

Total percentage of TEs was highest from flower [0.125%] and roots [0.104%] followed by shoots [0.091%] and cell culture [0.090] then seed [0.084%] and seedling [0.079%], the total TEs in leaves [0.061%] were the lowest and highly significant when compared to the TEs in flower and root (Table 4.6 & Fig 4.6a). When retrotransposons from different plant parts were compared it was observed that roots had the highest number of retrotransposons [0.092%] followed by seeds [0.066%] though these two were not significantly different from each other (Fig 4.6b). Seedling [0.039%] and flower [0.043%] had the least number of retrotransposons than in other groups the difference was highly significant when compared to the roots. In all the five groups except the flowers and seedling the retrotransposons were higher than the transposons but was significantly more only in roots and seeds. In seedling there was no difference between the retrotransposons and transposons while in the flowers the number of transposons was more than the retrotransposons (Fig 4.6c). However, it should be noted that only transcription and ultimate integration in tissues giving rise to gametes is heritable; the ESTs here are derived from many types of tissues (Table 4.6). Therefore, the active transposable elements in floral or seed tissue would account for the heritable changes. An interesting trend shown by the class I and class II TEs was observed, wherein the frequencies of both class of TEs showed an inverse trend (Fig 4.6d).

Among the 490,209 unique ESTs searched in this report, retrotransposons represent about 0.23% of transcripts, the frequency being higher among graminae than in solanaceae or fabaceae. The frequency would be several-fold higher where the database was searched using BLASTx with a complete retrotransposons sequence. Secondly, I have shown that the transposable elements are differentially up-regulated during the pathogen challenge in chickpea. In plants, the copy number for Ty3-*gypsy* elements is generally high, up to 20,000 with the exceptions of the Athila and RIRE elements of Arabidopsis and rice which are represented by 30 copies. Athila and RIRE and other

Ty3-*gypsy* group elements have not been found among retrotransposons that are active in plants Madsen *et al*. (2005). Similar to this when the database was searched for Ty3-*gypsy*, the search retrieved very few sequences as compared to Ty1-*copia*.

In conclusion although role of TE in plant defense against invading pathogen is ambiguous, the present study provides clear evidence of increased activation in resistant cultivar roots challenged with wilt pathogen FOC1. In legume roots TE activity found to be significantly higher during both symbiotic and pathogenic interactions with microbes.

Chapter 5

General discussion and future directions

5.1 Summary

In the present work, differential expression of chickpea genes was studied from resistant and susceptible varieties in response to *Fusarium oxysporum* f.sp.*ciceri* race1 infection. Many gene transcripts that were upregulated in the resistant cultivar upon FOC1 infection were identified. The transcripts identified in this study along with the other known genes possibly involved in host-pathogen interactions have been schematically represented in Fig 5.1. Of the genes upregulated in resistant cultivar during pathogen attack, WRKY and 14-3-3 were identified for the first time in chickpea, though their role in defense is described and understood in many other plant species (Roberts and Bowles, 1999; Chen *et al*., 2006). Previously described defense genes in host-pathogen interactions like NBS-LRR, chitinase, hydrolase, ATPase, and gamma glutamyl-cysteine synthetase were observed to be upregulated in resistant cultivar upon FOC1 infection. Interestingly, LTR and non-LTR types of retrotransposons were seen to be upregulated in chickpea roots during pathogenic stress. The host-pathogen interaction was marked by few genes whose identity could not be established, either due to the smaller size of the obtained sequence or they could be new genes hitherto undescribed.

For the first time, full-length 14-3-3 genes from chickpea were isolated and cloned. 14-3-3 genes are present as a fairly large family in eukaryotes especially in plants. Two isoforms of the 14-3-3 genes in chickpea (*Ca1433*-1 and *Ca1433*-2) were identified and hypothesized for presence of more isoforms. The *Ca1433*-1 gene was of epsilon type suggesting it to be of primitive origin, while *Ca1433*-2 was of non-epsilon type, which is known to be more evolved. Of the two isoforms *Ca1433*-2 appeared to be positively associated (upregulated upon FOC1 infection) with the resistant cultivar during FOC1 infection while the *Ca1433*-1 was observed to be positively associated with the susceptible cultivar during FOC1 infection. In the susceptible cultivar the pathogen infects the roots and grows rapidly, the mycelial mass thus plugs the xylem vessels disrupting upward flow of water leading to pronounced wilt. In parts of the root tissue the disrupted water flow creates virtual drought like condition. Usual response of the plant to the drought like conditions is to increase transpiration in an attempt to draw more water into the xylem. This is facilitated by opening of the stomata and also through the increase in aquaporins in the root tissue. Thus, *Ca1433*-1 could possibly be involved in stomatal opening and the TDF, CaFri51, an aquaporin, is therefore, found expressed at higher levels in susceptible cultivar.

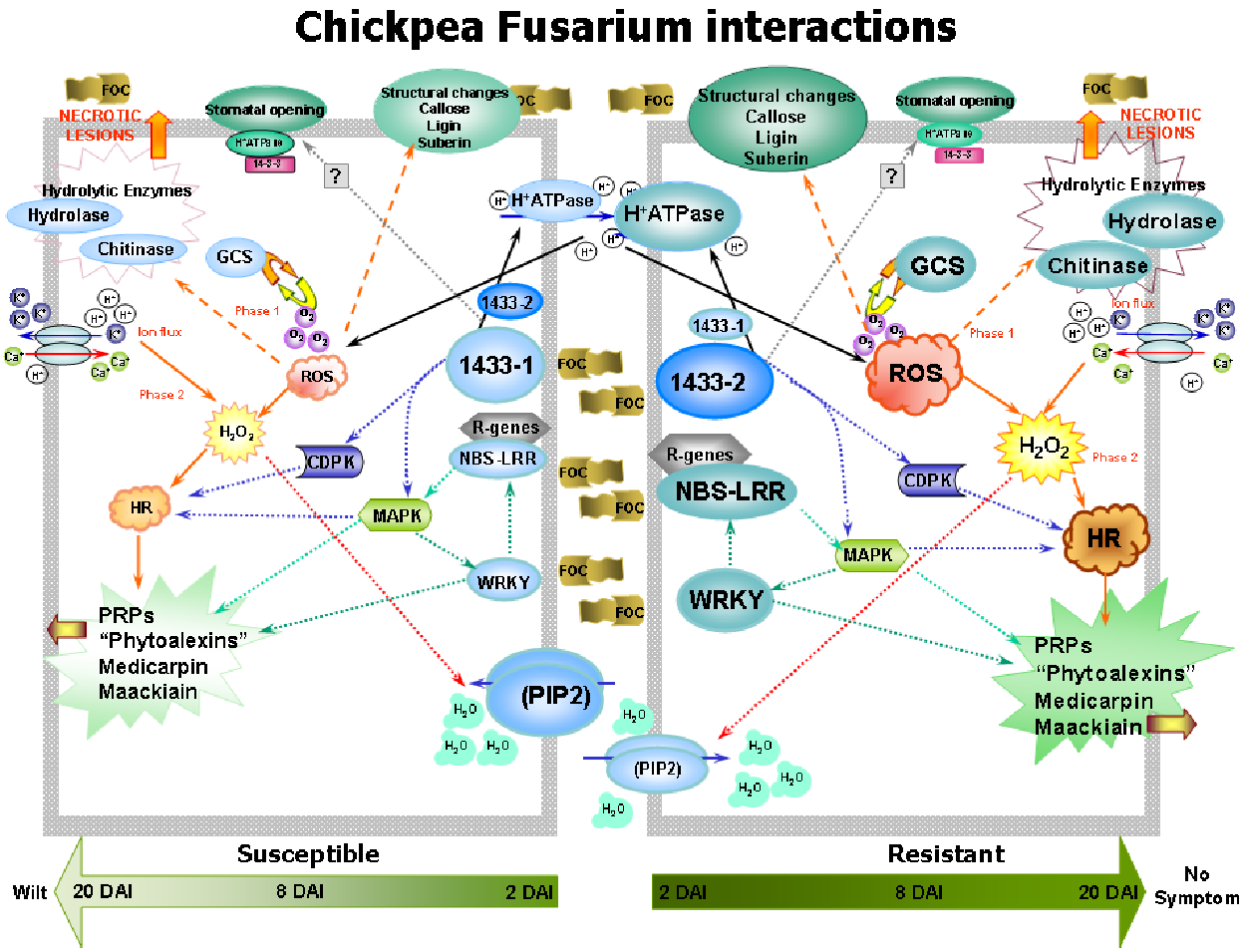


Figure 5.1: Schematic representation of different genes that are induced during the host-pathogen interaction in the resistant and susceptible varieties.

On the other hand *Ca1433*-2 is disease responsive and involved in signal transduction pathway, triggering the cascade of molecular responses leading to resistance. *Ca1433*-2 could possibly act via ATPase activation through the accumulation of reactive oxygen species (ROS), which is supported by the fact that gamma glutamyl-cysteine synthetase, a key enzyme in ROS scavenging is also upregulated during pathogen attack. Interestingly, a TDF (CaFRi36) encoding ATPase like protein was identified, which was upregulated during pathogenic stress.

Most resistance proteins are receptor-like protein kinases of the nucleotide-binding site-leucin-rich-repeat (NBS-LRR) class and composed of different combinations of conserved elements. In this study, I could identify at least three NBS-LRR like transcripts. Huttel *et al*. (2002) isolated a series of RGAs from both *C. arietinum* and *C. reticulatum*. A total of 48 different RGAs fell into 9 different sequence classes, and were members of the Toll-interleukin receptor (TIR)-NBS-LRR and coiled coil (CC)-NBS-LRR groups. Of these, 30 were mapped to five linkage groups on the reference genetic map of chickpea (Winter *et al*., 2000), some of them as clusters on LGs 2 and 5, respectively. Besides this, a direct approach towards isolation of resistance genes from chickpea resulted in mapping 12 RGA markers that clustered on three LGs (Flandez-Galvez *et al*., 2003). However, efforts to directly clone a Fusarium or Ascochyta resistance gene via the candidate gene approach have not yet been successful, which could be attributed to the low level of polymorphism in the chickpea genome in addition to the highly conserved NBS-coding region used for designing the primers that probably eluded the mapping of many RGAs. Targeting at the more variable LRR coding region of the genes in accordance with new methods to detect polymorphisms such as EcoTILLING (Comai *et al*., 2004) may be more successful.

The other three transcripts identified were CaFRi12, CaFRi15 and CaFRi20 which showed sequence identity to retrotransposon sequences. These transcripts were infection responsive being differentially regulated in roots of the resistant infected cultivar. Previous studies have proposed that retrotransposons have captured 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 reported to be induced during biotic and abiotic stress (Casacuberta *et al*., 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). It is opined that the high variability needed to evolve new resistance specificities in host plant is generated by the insertion of transposable elements (Reijans *et al*., 2003).

To generate a global picture of retrotransposon activity with special reference to pathogen infection, the publicly available expressed sequence tags (ESTs) for TEs in the TIGR database were analyzed for graminae (wheat, rice and maize), fabaceae (*Medicago truncatula*, *Lotus japonicus* and *Glycine max*) and solanaceae (potato, tomato and tobacco). A total of 490,209 unique sequences from the gene indices of the TIGR database were surveyed for the presence of TEs. Graminae had significantly the highest number of total TEs followed by solanaceae and then fabaceae. Among the TEs found in Graminae the retrotransposons of class I with 0.303% had significantly high number when compared to solanaceae [0.174%] and fabaceae [0.123%]. The DNA transposons (class II) were also significantly higher in graminae [0.055%] than the other two families.

Within fabaceae the retrotransposons (class I) were significantly [P=0.0046] expressed more during stress [0.073%] than in unstressed conditions [0.054]. However, retrotransposons were significantly [P=0.0006] more during the biotic stress [0.083%] than in the abiotic stress [0.066%], while within the biotic conditions they were more during symbiosis [0.112%] than during pathogenic stress. Similar trends were observed in the roots of fabaceae.

5.2. Updating Beckmans's model of vascular wilt

In the case of vascular wilts xylem tissues provide a convenient and effective system for study of host-parasite interactions. Xylem vessels, by virtue of their design and function, become a dangerous zone for extensive and rapid distribution of parasites in case there is a breakdown in the system. Perforation plates at the ends of vessels are the sites serving as check points that screen fungal spores from the transpiration stream (Beckman, 2000). However, organisms (like the vascular pathogens) that can quickly germinate, penetrate these end-walls and sporulate in the next xylem element, present a serious threat to the plant. Nevertheless, it takes 2-3 days for a successful fungal vascular parasite to germinate, grow through vessel end-walls and produce mature spores that can abscise and be carried further upward in the transpiration stream in the next vessel element. Thus, the plant has a period of 2-3 days, to seal off the infection sites (Beckman and Roberts, 1995) as represented in a model of the defense system in xylem tissue (Fig 5.2).

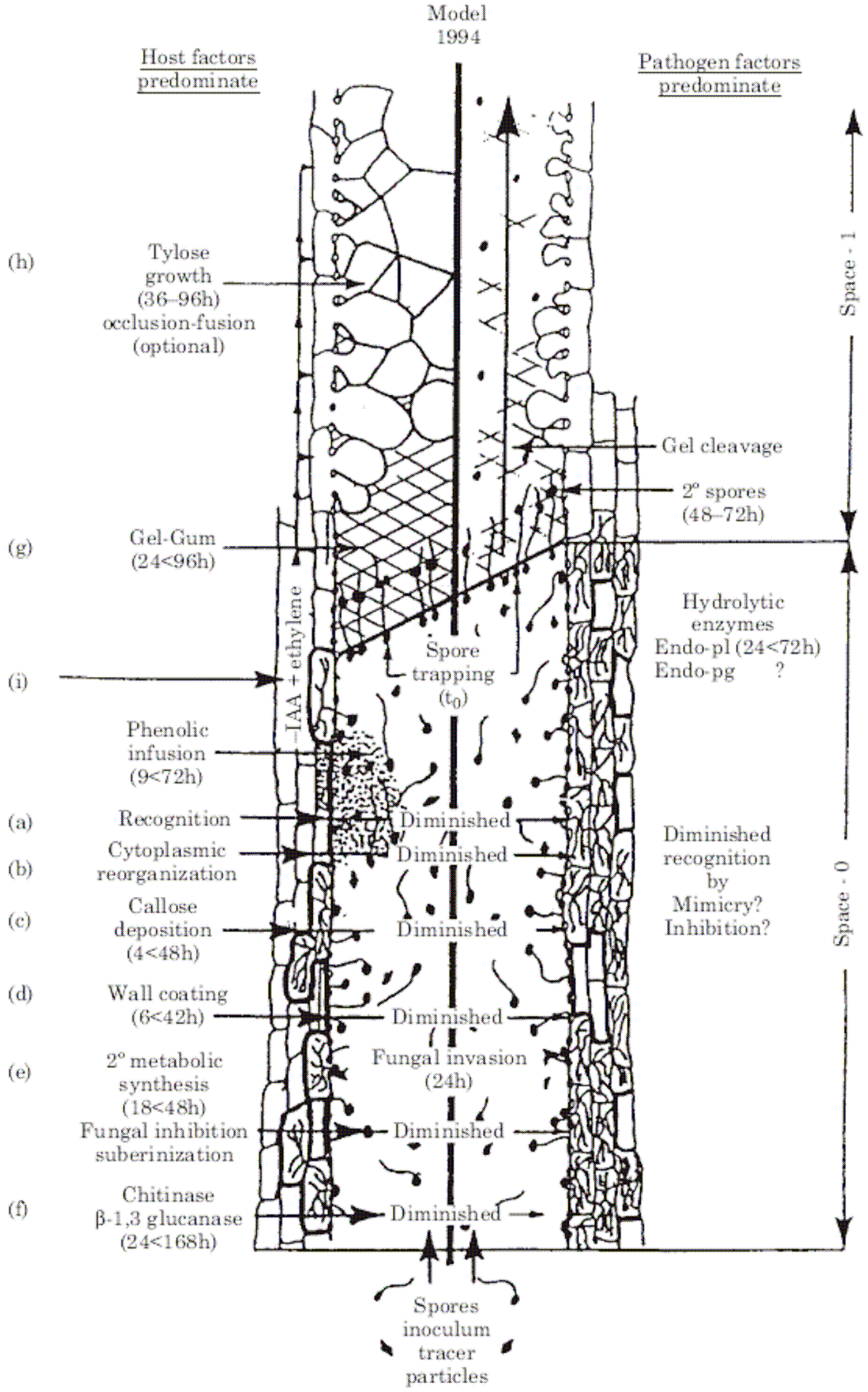

Model
1994
Host factors
predominate
Pathogen factors
predominate
(h)
Tylose
growth
(36–96h)
occlusion-fusion
(optional)
Gel cleavage
2° spores
(48–72h)
Space - 1
(g)
Gel-Gum
(24<96h)
Hydrolytic
enzymes
Endo-pl (24<72h)
Endo-pg ?
(i)
+IAA + ethylene
Spore
trapping
(t_0)
Phenolic
infusion
(9<72h)
(a)
Recognition
Diminished
(b)
Cytoplasmic
reorganization
Diminished
Diminished
recognition
by
Mimicry?
Inhibition?
(c)
Callose
deposition
(4<48h)
Diminished
Space - 0
(d)
Wall coating
(6<42h)
Diminished
(e)
2° metabolic
synthesis
(18<48h)
Fungal invasion
(24h)
Fungal inhibition
suberinization
Diminished
(f)
Chitinase
β-1,3 glucanase
(24<168h)
Diminished
Spores
inoculum
tracer
particles

Figure 5.2: A time-space model of longitudinal and lateral host-parasite interactions that occurs within Space-0 (the initially inoculated vessel element below a vessel ending, and the surrounding vascular parenchyma tissue) and in Space-1 (the next vessel above and its surrounding parenchyma tissue). The left side of the model shows the various defense processes of the host [(a)-(i) in chronological order] and the times of their occurrence in Space-0 and Space-1 that, when they predominate, serve to inhibit a vascular parasite and to localize the infection. The right side of the model shows the processes of the pathogen and the times of their occurrence in Space-0 and Space-1 that, when they predominate, enable the parasite to escape Space-0 and traverse Space-1. Inoculum is initially introduced through severed vessels (bottom of model) and drawn upward through Space-0 by transpirational pull within 10 min (Time-0). Note that phenolic infusion from a phenolic-storing cell into the vessel is clearly visible by 9 h after inoculation. The movement of IAA and ethylene upward from the point of phenolic release and oxidation (i) to initiate gel extrusion and tylose growth is hypothetical, but many-fold increases in the concentrations of IAA and ethylene in infected vascular tissues have been documented. (Reproduced from Beckman and Roberts, 1995).

The plant responses to the pathogen can be categorized into longitudinal and lateral defenses. The build up of indole acetic acid (IAA), oxidative burst and activation of H^+ pumps, provide for a longitudinal defense in the xylem tissues. While the lateral defense within xylem parenchyma tissues wherein the cells surrounding infected vessels react either by means of hypersensitivity or the deposition of callose which then becomes lignified and/or suberised (Beckman *et al*., 1989). Cells responding with the HR were never found to be invaded. The relative success or failure of the callose response to limit tissue invasion varied with the genetic complement of the host and parasite (Beckman 2000). The longitudinal and lateral components of defense and the approximate times of their occurrence, together with the actions of a potential pathogen are presented in the model (Fig. 5.2).

Defense mechanisms of plants against pathogens include *inter alia* the production of reactive oxygen species, synthesis of antimicrobial phytoalexins, induction of hydrolytic enzymes (*e.g.*, chitinase, glucanase), construction of defensive barriers (*e.g.*, lignin, suberin), and hypersensitive reaction (Hammond-Kosack and Jones 1996, Mellersh *et al.* 2002, Sindelar and Sindelarova 2005). The resulting ROS participate in the damage of the attacking pathogen (Peng and Kuc 1992), cross-link proteins into the plant cell wall (Bradley *et al.*1992), lignification of cell wall (Olson and Varner 1993), and induction of expression of a variety of defense related genes. A final result of the induced oxidative burst may be its participation in the hypersensitive response (Hückelhoven and Kogel 2003, Levine *et al.* 1994). The role of enzymes in plant-fungal pathogen interactions was summarized by Lebeda *et al.* (2001). Some of these responses as observed in my studies with chickpea-Fusarium system are discussed below.

Callose deposition

One of the earliest responses to *Fusarium* sp. infection is the deposition of additional wall callose material (papillae) within contact cells. The vascular plugging was found to seal off xylem elements of resistant pea cultivars, which serves as physical barriers (lignification) to retard or prevent vascular invasion and spread of pathogen (Kraft 1994). Earlier studies in our laboratory have associated the decrease in β-1,3-glucanase activity and increase in chitinase activity in root tissue of the resistant cultivar with a higher rate of callose deposition (Giri *et al*., 1998).

Oxidative burst

The oxidative burst, an early event of plant defense and/or signaling, appears rapidly in a number of plant-pathogen interactions (Lamb & Dixon, 1997). Plants have well-

developed defense systems against ROS, involving both limiting the formation of ROS as well as instituting its removal (*Alscher et al*., 2002). Plants usually keep the levels of ROS under tight control by the production of scavenging enzymes and non-enzymatic antioxidants (Wojtaszek, 1997; Kuzniak and Urbanek, 2000; Moller, 2001; Vranova *et al*., 2002). H_2O_2 also inhibits the growth and viability of diverse microbial pathogens (Wu *et al*., 1995). The oxidative potential of H_2O_2 also contributes to plant cell wall strengthening and may create additional barriers during plant-pathogen interactions through peroxidase-mediated cross-linking of proline-rich structural proteins and phytoalexins biosynthesis during oxidative burst.

Antioxidants

The ROS damages the host machinery also and to protect itself the host produces some antioxidants like glutathione. Glutathione is an abundant metabolite in plants that has many diverse and important functions (Noctor & Foyer 1998), including signal transduction (Noctor *et al*. 2002; Gomez *et al*. 2004). Activation of glutathione synthesis and its accumulation is a general feature of enhanced oxidation of the cytosol. Recent evidence suggests that the enzymes of GSH synthesis and metabolism are induced together in response to stress (Mittova *et al*. 2004). The pathway of glutathione synthesis is conserved in all organisms and involves two enzymes, γ-glutamylcysteine synthetase and glutathione synthetase. Mutations in the γ-glutamylcysteine synthetase gene resulted in decreased levels of glutathione (Cobbett *et al*. 2000).

Resistant (R) genes

Early recognition of potential pathogen is the key event in plant defense. The recognition is based on specific pathogen factors (avr) which are recognized by a cognate host factor (R) constituting most effective types of defense in plants, mediated by *R* genes (Keen, 1990; Dangl & Jones, 2001). This *R* gene-specified disease resistance (also termed gene-for-gene resistance) is often, but not always, accompanied by a rapid and localized cell death (hypersensitive response: HR) at the site of attempted infection. When corresponding *R* and *avr* genes are present in the plant and the pathogen, it results in disease resistance, and if either is inactive or absent, leads to disease. The *R* gene products are, therefore, capable of sensing the *avr*-dependent factor and subsequently triggering a chain of signaling events that activate defense mechanisms (Keen, 1990; Dangl & Jones, 2001). The largest class of *R* genes encodes a cytoplasmic protein with leucine rich repeats (LRRs) and a putative NB site termed the ‘NBS-LRR’ class. This class can be further subdivided into members that carry either N-terminal homology to the Toll protein and interleukin-1 receptor (TIR-NB-LRR), or to those that carry a

putative coiled-coil (CC) at the N-terminus (CC-NB-LRR). Resistance genes from both these subclasses are known to confer resistance against fungi. NBS-LRR genes are known that confer resistance against flax rust, maize rust, barley powdery mildew, rice blast, the tomato *Fusarium* wilt pathogen, and the downy mildew oomycete (Dangl & Jones, 2001).

14-3-3 via CDPK pathway

Transient changes in ion flux across the plasma membrane appear to be a common early event that triggers defense signaling. Camoni et al. (1998a), have identified a protein-protein interaction between 14-3-3 proteins and a CDPK. Three 14-3-3 isoforms were tested and all of them activated CDPK-1, with slight differences in their potency, suggesting that the ability to stimulate CDPK-1 is a general feature of 14-3-3 proteins. Receptor-mediated regulation of plasma membrane-located ion channels stimulates ion fluxes (Ca^{2+}/H^{+} influx, K^{+}/Cl^{-} efflux) immediately after challenge with avirulent pathogens or elicitors. One of the downstream targets for Ca^{2+} has been shown to be a calcium-dependent protein kinase (CDPK), a class of serine/threonine protein kinases, unique to plants and some protists. The large CDPK gene family suggests that isoenzymes confer different specificities and functions in multiple signaling pathways (Hong *et al*., 1997; Hrabak *et al*., 1996). CDPKs have been implicated in response to several environmental stresses, induction of CDPK mRNA, and its role in activating plant defenses in plant-pathogen interactions has been reported (Romeis *et al*., 2000). In tobacco cells expressing the tomato *Cf-9* gene, two K^{+} channels were shown to be differentially regulated (Blatt *et al*., 1999). In another study of *Cf-9*-transgenic tobacco cells, the Avr9 peptide induces a rapid activation of two isoforms of CDPK through phosphorylation (Romeis *et al*., 2000 & 2001). The plant defense is triggered by a signaling network of parallel pathways that may be interlinked at single components. The increase in the cytosolic Ca^{2+} concentration, which occurs within seconds after elicitation, appears to be a master regulator required for many subsequent signaling steps. ROS production, CDPK and MAP kinase activation, defense gene triggering, and phytoalexin production, singly or in combination, were shown to be compromised in the presence of Ca^{2+} -chelating or Ca^{2+} channel-inhibiting compounds (Scheel, 1998). The 14-3-3 appears to mediate HR by activating CDPK which in turn utilizes Ca^{2+} ions.

14-3-3 via MAPK pathway

The alternative pathway connecting 14-3-3 to HR is mediated through MAP kinase. MAP kinase cascades are also involved in early events of plant defense signaling. The tobacco MAPKs, SIPK and WIPK are rapidly activated upon challenge with avirulent

pathogens or elicitors (Zhang & Klessig, 1998; Romeis *et al*., 1999). Evidence of contribution of MAPK cascades to defense gene activation has also been shown using a gain-of-function approach (Yang *et al*., 2001). Expression of a constitutively active form of *NtMEK2* (a tobacco MAP kinase) activates both SIPK and WIPK, induces HR-like cell death, and induces genes for 3-hydroxy-3-methylglutaryl CoA reductase (*HMGR*) and L-phenylalanine ammonia-lyase (*PAL*), which are key enzymes in the phytoalexins and SA biosynthetic pathways, respectively (Yang *et al*., 2001).

WRKY transcription factors

MAPK also connects to WRKY transcription factor as demonstrated by Asai *et al*. (2002) in *Arabidopisis* where the MAPK cascades comprising MEKK1, MKK4/MKK5 and MPK3/MPK6 activate the WRKY22/29 in mesophyll cells challenged with the flagellin elicitor. The WRKYs are plant-specific transcription factors that bind to the W box DNA element (TGAC core sequence) found in the promoters of diverse classes of defense related genes (Eulgem *et al*., 2000). In parsley cells, WRKY1 is targeted to the nucleus upon treatment with Pep13 elicitor, and activates fungal elicitor-induced gene expression by binding to W box elements (Eulgem *et al*., 1999).

Aquaporins

Aquaporins are proteinaceous pores that facilitate the passive diffusion of water across membranes of living cells. Plant aquaporins are divided into four groups or clades based on amino acid sequence similarities: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin-like intrinsic proteins (NIPs) and small basic intrinsic proteins (SIPs). Some groups may be subdivided again (for review, see Luu and Maurel, 2005), as is the case with PIP proteins (divided into PIP1 and PIP2). Aroca *et al*., (2006) showed the upregulation of the PIP gene expression in roots and attributed it to a direct effect of the low water availability and the inherent fall of the soil water potential. In fact, Hill *et al*. (2004) have proposed that aquaporins could function as osmosensors in plant membranes.

5.3. Concluding remarks

The concept of “interactome” (Birch & Kamoun, 2000) arose with the development of EST libraries from plants as well as from the pathogen, where the analyses of both groups of transcriptomes were combined on the same array. Simultaneous analysis of genes from the two partners of the interaction should increase our understanding of the successive “attack and defense” steps leading to plant disease or resistance, as recently illustrated by Moy *et al*. (2004) through the interactome of the *P. sojae*/soybean

compatible pathosystem. This for the first time illustrated analysis of an interactome between a pathogen and a legume.

The field of plant-pathogen interactions is much more advanced in *A. thaliana* than in model legumes. Several transcriptomics analyses were performed to assess the regulation and coordination of gene networks for various aspects of compatible or incompatible interactions (for reviews see, Kazan *et al*., 2001; Wan *et al*., 2002). From these studies, concepts were drawn to predict plant responses according to the nature of the attacking pathogen. Salicylic acid (SA) signaling pathway and defense genes consecutively induced by this molecule, such as PR1, should preferentially be recruited by plants inoculated with biotrophic parasites. By comparison, resistance to necrotrophic parasites should essentially be triggered by gene expression associated with the jasmonic acid (JA) and ethylene pathways, notably by lipoxygenases (LOX), (Hammond-Kosack & Parker, 2003). Recent data gained from pathogen-legume interactions suggested that this division of plant defense responses is not that well-defined. Although soybean and *P. syringae* (Zou *et al*., 2005) seemed to have a good synteny of the responses described for the *A. thaliana*/*P. syringae* pathosystem (Tao *et al*., 2003), another legume-pathogen interaction was in contradiction with the proposed model. Indeed, the resistance of chickpea to *Ascochyta rabiei*, a necrotrophic pathogen, could be triggered by both SA- and MeJ-pathways (Cho & Muehlbauer, 2004). Here in the present study involving chickpea-Fusarium system few genes like 14-3-3 and WRKY were identified as being new to the said system. Other examples illustrate the fact that the type of plant responses might not be strictly correlated to the kind of parasitism. The defenses induced in *A. thaliana* inoculated with the hemibiotrophic fungal pathogen *Colletotrichum higginsianum* could rather be compared to a response to a necrotrophic pathogen (Narusaka *et al*., 2004). The conclusions were not so obvious from legume pathosystems. In an incompatible interaction between the hemibiotrophic *C. trifolii* and *M. truncatula*, five LOX genes were early induced (Torregrosa *et al*., 2004), indicating rather a necrotrophic-like response, however, SA-related genes such as PR1 were also found to be over expressed. Other comparisons of *M. truncatula* responses to *Erysiphe pisi*, a biotrophic parasite, and to *C. trifolii* during incompatible interactions showed that the modulation of plant defense-related genes looked very similar across the analyzed pathosystems (Foster-Hartnett *et al*., 2004).

All these comparisons indicated that even if a huge knowledge could be gained using *A. thaliana* as a model for plant-pathogen interactions, the identified resistance

mechanisms could not be fully extrapolated to legumes. Clearly, more transcriptomic analyses are needed to specifically characterize legume-pathogen interactions. Symbiotic N fixing organism's co-existence in legume root makes the interaction scenario more complex since pathogen and symbionts induce similar initial molecular responses. How and when the common theme of responses deviates to result in useful/harmful association is not very clear.

The transcriptomics studies of legume-pathogen interactions are still limited to a few reports, mainly focused on only two species. Whereas the increasing data gained on the model legumes can accelerate our understanding of the onset of disease or resistance, global approaches need to be transferred to major crops such as peas or beans. The availability of transcriptomic tools such as the Mt16k oligonucleotide arrays for *M. truncatula*, the 36k cDNA arrays for soybean and the Ps6k arrays for pea will induce a major change of scale in the analysis of many biological processes in legumes (Kuester & Bendahmane, 2004) and plant-pathogen interactions will directly benefit from these high throughput EST analyses.

As RNAs are only transmitters of the information, it might be also necessary to associate proteome or metabolome studies with the transcriptome results. Finally, one of the bottlenecks associated with the generation of transcriptome data is the analysis of genes with unknown function. High-throughput approaches in this field need to be developed for legumes. Several recent reports relating insertional mutagenesis with transposon in *M. truncatula* (d'Erfurth *et al*., 2003), TILLING strategy in *L. japonicus* (Perry *et al*., 2003) or supplementary silencing technologies for legumes such as virus-induced gene silencing in pea (Constantin *et al*., 2004) or tissue-specific gene silencing in soybean (Tuteja *et al*., 2004) gave hope to adapt some of these techniques to many other legumes.

5.4 Future directions

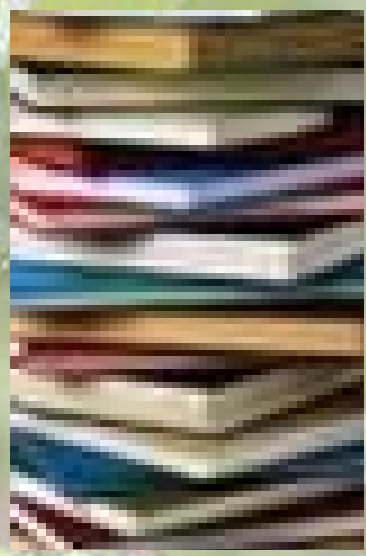
1) To increase the potential number of defense-related genes, will require generation and study of more cDNA libraries from chickpea plants inoculated with various pathogens or from elicitor-treated tissues or cells. Availability of such defense-related libraries would be a useful source of information to identify new genes involved in plant responses to pathogen attack. *In silico* analysis of such collections through "electronic Northerns", will help in identification of genes involved in plant responses to pathogen attack, in a similar way as reported for symbiosis (Fedorova *et al*., 2002; Journet *et al*., 2002).

2) Application of more transcriptomic studies (SSH- subtractive suppression hybridization; SAGE- serial analysis gene expression; cDNA-AFLP) to chickpea will help in getting an insight into mechanisms of stress-related (biotic or abiotic) or developmental processes. The impact of transcriptomics in chickpea breeding would be more with the inclusion of microarray techniques.

3) The transcriptomic data in combination with maps generated from markers and detecting SNPs in differentially expressed genes leading to single nucleotide amplification polymorphism markers (SNAP; Drenkard *et al*., 2000; Hayashi *et al*., 2004), would help to directly employ the gene of interest as markers as seen in mouse (Schadt *et al*., 2003).

4) The determination of genetic variability in these genes in the chickpea germplasm will then be the next step towards targeted molecular breeding and more efficient germplasm management.

5) Functional validation of the differentially expressed genes in chickpea using various methodologies like over expression or silencing (VIGS- viral induced gene silencing or more recently the RNAi- RNA interference) will help in consolidating their candidature for application to transgenics to develop new cultivars resistant to various pathogens or pest.



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Curriculum vitae

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Publications:

1. **Nimbalkar SB**, Harsulkar AM, Giri AP, Sainani MN, Franceschi V and Gupta VS (2006) Differentially expressed gene transcripts in roots of resistant and susceptible chickpea plant (*Cicer arietinum* L.) upon *Fusarium oxysporum* infection. Physiological and Molecular Plant Pathology 68: 176-188.

2. **Nimbalkar SB**, Harsulkar AM, Giri AP and Gupta VS (2007) Two 14-3-3 transcripts express differentially in roots of resistant and susceptible chickpea varieties upon *Fusarium* infection. (Communicated to Plant Science).

3. **Nimbalkar SB**, Harsulkar AM, Giri AP, and Gupta VS (2007) Transposons induced by biotic stress as revealed by cDNA-AFLP and the survey of database. (Communicated to Functional & Integretive Genomics).

4. **Nimbalkar SB**, Harsulkar AM, and Gupta VS (2007) cDNA-AFLP a method of choice to study interacting trascriptomes. Review Paper. (In preparation).

Presentations in International & National Conferences: 4

Awards/Honors:

- **Junior & Senior research fellowships** from Council of Scientific & Industrial Research (**CSIR, Govt. of India**) in Life sciences, awarded to conduct Ph.D. work.

- Qualified highly competitive National examination: Graduate Aptitude Test for Engineering (**GATE**).
- UAS Dharwad **merit scholarship** during B.Sc (Agri).

Co-curricular Activities:

- NCC 'B' Certificate holder.
- Attended many NCC training camps and led the platoon few times.
- Represented college team in football and hockey.

References:

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