3 Biotech (2018) 8:111
https://doi.org/10.1007/s13205-018-1128-z

ORIGINAL ARTICLE

Analysis of differential transcript expression in chickpea during compatible and incompatible interactions with *Fusarium oxysporum* f. sp. *ciceris* Race 4

Parasappa R. Saabale[1,2] · Sunil C. Dubey[1,3] · Kumari Priyanka[1] · Tilak R. Sharma[4]

Received: 22 August 2017 / Accepted: 19 January 2018 / Published online: 5 February 2018

Abstract
The present study reports the transcriptome analysis of resistance (WR315) and susceptible (JG62) genotypes of chickpea in response to *Fusarium oxysporum* f. sp. *ciceris* (*Foc*) race 4 using the method of suppression subtractive hybridization. Altogether, 162 chickpea-expressed sequence tags (ESTs) were identified from two libraries and analyzed to catalog eight functional categories. These ESTs could be assembled into 18 contigs and 144 singletons with 10 contigs and 68 singletons from compatible and 8 contigs and 70 singletons from incompatible interaction. The largest category consisted of ESTs which encode for proteins related to hypothetical proteins (22.8%), followed by energy and metabolism (20.3%)-related genes, defense and cell rescue-related genes (17.9%) and signal transduction-related genes (16%). Among them, 17.1 and 18.7% were defense-related genes in compatible and incompatible interaction, respectively. These ESTs mainly includes various putative genes related to oxidative burst, pathogenesis and secondary metabolism. Induction of putative superoxide dismutase, metallothionein, 4-coumarate-CoA ligase, heat shock proteins and cysteine proteases indicated oxidative burst after infection. The ESTs belonged to various functional categories which were directly and indirectly associated with defense signaling pathways. Quantitative and semi-quantitative polymerase chain reaction exhibited differential expression of candidate genes and detected higher levels in incompatible interaction compared to compatible interaction. The present study revealed partial molecular mechanism associated with the resistance in chickpea against *Foc*, which is the key to design a strategy for incorporation of resistance via either biotechnological means or introgression of resistance genes.

Keywords Suppression subtractive hybridization · Chickpea · *Fusarium oxysporum* f. sp. *ciceris* · RT-PCR · Differential proteins

Introduction

Plants have an ability to combat stresses of diverse nature by modulating defense responses physiologically and morphologically through induced and preformed defensive strategies. Plants also show such defense response to combat pathogen infection. The mechanism involves complex interplay of signaling cascades such as nitrous oxide, salicylic acid, ethylene and jasmonic acid. In most cases, the host arrests the growth of the pathogen at the site of penetration; such defense mechanism is termed as pattern triggered immunity (PTI) which includes morphological barriers such as defense structures produced before infection and antimicrobial compounds such as phytoanticipins and phytoalexins produced after infection. However, in few cases, the pathogen bypasses host immunity in the absence of host-resistant proteins or R proteins by secretion of effecter molecules, which lead

✉ Parasappa R. Saabale
sparashu@gmail.com

Sunil C. Dubey
scdube2002@yahoo.co.in

[1] Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi 110 012, India

[2] Present Address: Regional Research Centre, Indian Institute of Pulses Research, Dharwad 580005, India

[3] Present Address: Division of Plant Quarantine, ICAR-National Bureau of Plant Genetic Resources, New Delhi 110 012, India

[4] National Research Centre on Plant Biotechnology, LBS Centre, IARI, New Delhi 110 012, India

to effector-triggered susceptibility (ETS) (Jones and Dangl 2006). On the other hand, effector proteins produced by the pathogen are recognized by host proteins and inhibit pathogen growth, which is known as effector-triggered immunity (ETI) (Jørgensen 1994). However, the defense mechanism of ETI or PTI is due to altered protein synthesis and its time bound degradation. Hence, a change in protein level is believed to be an indicator of host–pathogen interaction. Transcriptome analysis at time intervals of infection helps in the identification and isolation of the genes expressed in the host.

Chickpea (*Cicer arietinum* L.) is the third most important pulse crop of the world after peas and common bean and is grown under arid and semi-arid environmental conditions. India is a major producer, accounting for approximately 70% of world's chickpea production with an average productivity of 889 kg/ha (Directorate of Economics and Statistics DAC 2016). The susceptibility of the crop to many biotic and abiotic factors is the main reason for its low yield (Dubey and Suresh 2006). Among biotic factors, wilt caused by *Fusarium oxysporum* f. sp *ciceris* (Padwick) Matuo and K. Sato (*Foc*) is considered as one of the main causes for low productivity of chickpea. In India, this disease is prevalent in all the chickpea-growing states and causes an estimated annual loss of 10% (Singh and Dahiya 1973). However, the losses due to Fusarium wilt depends on the stage of the crop infected. Early wilting causes 77–94% losses, while late wilting causes 24–65% losses (Haware and Nene 1980). All growth stages of the crop are susceptible to the disease, but its incidence is more severe at flowering and pod formation stages when temperature is relatively high (> 24 °C), particularly under drought conditions (Govil and Rana 1994). Global distribution of the disease is correlated with the presence of designated races of *Foc* (Jimenez-Gasco and Jimenez-Diaz 2003), and eight races of the pathogen (race 0, 1A, 1B/C, 2, 3, 4, 5 and 6) have been reported worldwide. Out of these, 0 and 1 B/C cause yellowing and rest of the races cause the wilting syndrome (Haware and Nene 1982; Jimenez-Diaz et al. 1993). Recently, Dubey et al. (2012) identified eight races of *Foc* from India with a new set of chickpea differential cultivars.

Studies on host response to pathogen at the molecular level are poorly understood in chickpea. The suppression subtractive hybridization (SSH) allows rapid identification and isolation of differentially expressed genes in a single experiment. The SSH is a powerful technique widely used in comparing two populations of mRNA and to isolate clones of differentially expressed genes (Diatchenko et al. 1996). It has been utilized widely to isolate and characterize differentially expressed genes during defense response against various pathogens in different crops, viz, coffee against *Hemileia vastatrix* (Fernandez et al. 2004), chickpea against *Ascochyta rabiei* (Coram and Pang 2005; Jaiswal et al. 2011), wheat against *Puccinia striiformis* (Yu et al. 2010) and cotton against *Verticillium dahliae* (Xu et al. 2011). Hence, the present study has been designed to comprehend chickpea–*Fusarium* interaction at the transcriptome level by the method of SSH. This study was aimed to identify and validate differentially regulated genes predicted to be involved in defense response in chickpea against *Foc* race 4 during compatible and incompatible interaction at early stage of the infection. Since differences in race are expected to yield specific results; it is therefore necessary to study against different races.

Materials and methods

Plant cultivars, fungal strain and inoculation

Chickpea (*Cicer arietinum* L.) cultivars, JG 62 (susceptible) and WR 315 (resistant), were selected for the present studies. Liquid inoculation method developed by Gurjar et al. (2012) was followed with little modification. Chickpea seeds were wrapped in wet blotter paper and stored at room temperature (24–26 °C) till germination. The germinated seedlings were transferred to trays containing half-strength Hoagland's nutrient solution. These seedlings were kept under glasshouse conditions with 16 and 8 h photoperiod of day and night, respectively, at 25 °C and relative humidity of 40% at the National Phytotron Facility (NPF), Indian Agricultural Research Institute, New Delhi, India. Twelve days old seedlings were inoculated by root dipping with freshly prepared spore suspension of a highly virulent isolate, *Foc*53 (Race 4) of *F. oxysporum* f. sp. *ciceris* at 1×10^6 spores/ml for 10 min. The inoculated seedlings were shifted to Hoagland's solution by adding 0.01% dextrose. The seedlings grown on a tray without pathogen inoculation served as the control. Chickpea root was collected from pathogen-inoculated and -uninoculated samples at 48, 72, 96 and 120 h post-inoculation (hpi).

RNA extraction and cDNA library construction

A total of 200 µg RNA was isolated by the RNA purification mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Equal amounts of total RNA from the pathogen-inoculated and -uninoculated samples harvested at different intervals were pooled separately. Two microgram (µg) of poly(A)$^+$ mRNA was purified by mRNA isolation midi kit columns (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol.

An SSH library was constructed using a PCR select cDNA subtraction kit (Clontech., Palo Alto, CA, USA) following the manufacturer's protocol. The subtracted cDNAs were then immediately cloned directly into pGEM-T Easy

Vector (Promega, Madison, WI, USA) and transferred into DH5α *E. coli* electrocompetent cells. Detection of recombinant cells was carried out based on blue/white colony selection. Positive transformants were arrayed on 96-microtiter deep well plates and resultant cDNA clones were stored at − 80 °C.

cDNA inserts and differential screening

The presence of cDNA inserts was checked by PCR amplification in a 96-well Bio-Rad thermal cycler (Bio-Rad, CA, USA) using adapter primers (Table 1). PCR products (1.0 µl) of recombinant clones were arrayed onto Hybond-N+ nylon membranes (Millipore). The pathogen-inoculated and -uninoculated cDNA was labeled with ά-P32 radioisotope and hybridized with recombinant clones. The results from the hybridizations were recorded for each clone, and clones showing an intense hybridization signal with probes were selected for sequencing.

Sequencing and sequence analysis

The positive clones were sequenced in both forward and reverse direction with T7 and SP6 primer sequence (Sigma, USA) and assembled into contigs using CAP3 sequence assembly tool (Huang and Madan 1999). These sequences thus generated were used for homology search on National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tools (BLASTx and BLASTn) (Altschul et al. 1990). *E* value higher than 10^{-5} were designated as nonsignificant homology with databases. The functional classification of annotated expressed sequence tags (ESTs) was done according to the classification described by Bevan et al. (1998).

Validation of representative defense-related genes

Total RNA was extracted from pathogen-inoculated and -uninoculated samples and the genomic DNA was removed from total RNA using RNase free DNase I (Fermentas, USA). The primers were designed with Primer3 plus software according to homology cDNA sequences and synthesized by Sigma, USA (Table 1). Synthesis of cDNA was done from 500 ng of RNA using Thermo Scientific Verso™ cDNA Synthesis Kit (ABgene, UK) according to the manufacturer's protocol. Thirty nanogram of final cDNA concentrations was used for semi-quantitative PCR analysis and 18S ribosomal gene was used as the reference gene. The PCR conditions were 94 °C for 5 m and 20–25 cycles at 94 °C for 30 s, 58–62 °C for 30 s and 72 °C for 45 s and final extension at 72 °C for 10 m. The expression level based on the intensity of ethidium bromide staining was determined by visual observations (Fernandez et al. 2004).

Quantitative real-time PCR (qRT-PCR) experiments were performed on Bio-Rad iCycler (Bio-Rad, CA, USA) with SyBr green. Reaction mix (20 µl) containing 1 µl of cDNA (30 ng), 1 µl of each primer (10 µM) and 10 µl of SyBr Green PCR master mix (2×) and 7 µl of water. The following PCR conditions were performed: 95 °C for 10 min, followed by 40 cycles of 95 °C for 20 s, 50–55 °C for 30 s and 68 °C for 30 s. The PCR obtained products were subjected for melting curve analysis to evaluate primer specificity. Negative control without cDNA template was run for each analysis. *Cicer arietinum* 18S ribosomal gene was used as the housekeeping gene to normalize the expression of target genes and as a calibrator. The relative fold change was calculated from three replicates using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001).

Table 1 Primer sequences of stress related genes used in semi-quantitative and quantitative PCR to analyze the expression patterns in chickpea against *Fusarium oxysporum* f. sp. *ciceris*

ESTs	Primer (5′ → 3′)	Primer (5′ → 3′)
Proline-rich cell wall protein gene	F-TTGAAGTTGGGTGCATGTGT	R-TGCCACAGTCAATGAGAAGC
Squalene monooxygenase	F-CTGGGCCTTGAAGATTGTGT	R-TACATTGGGAAGGGAAGCAG
Trans-cinnamate4-monooxygenase	F-CACATGAACCTTCACGATGC	R-CGGGCAGGTACCTAAAGTCA
PR4 gene	F-GGTGAACAATGCGGTAGACA	R-TTCACCACTTTCACCACTGC
Serine/threonine protein phosphatase	F-TGTGCCGGAGACAAATTACA	R-ATCGCCAAGCATTAGCATTT
Superoxide dismutase	F-TCCCTCTCACTGGACCAAAC	R-TATCCCGGAGTTGAGAGTGG
Cysteine proteinase	F-TTTGGATCATGGGGTTCTTC	R-ACCACCAGAACCAGCATCTC
PR 10 gene	F-CACCCTCGACGAAAGTGAGT	R-GCAAGAAACCGCCTCTACTG
BTB/POZ domain-containing protein	F-TTTGGATCAGAGTGGGAAGG	R-CGTGACCTCACAACACCAAC
Chalcone synthase	F-TCGTTTGGCTAAGGATTTGG	R-TCTGGAGCAATTGTTTGTGC
Glutamine synthetase	F-GGTGGTTATCCTGGTCCTCA	R-ACTCCCACTGTCCAGGCATA
18S ribosomal gene	F-ACGTCCCTGCCCTTTGTACAC	R-CACTTCACCGGACCATTCAAT
Adapter primers	F-TCGAGCGGCCGCCCGGGCAGGT	R-AGCGTGGTCGCGGCCGAGGT

Results

Isolation of differentially expressed genes

Two cDNA libraries were constructed using resistant and susceptible cultivar against *Foc* to capture a wide spectrum of differentially expressed genes. These libraries contained 1143 (~ 90%) and 989 (~ 92%) cDNA inserts from compatible and incompatible interaction, respectively. The results also showed that the insertion size also ranged between 200 and 1000 bp and was mainly from 350 to 500 bp (Fig. 1). In Southern blot technique, clones whose intensity of hybridization was relatively different between two probes were selected for sequencing. A total of 202 ESTs were sequenced from 2132 recombinant clones, among which 102 were from compatible and 100 from incompatible interaction. After processing these sequences, 15.8% of the sequences were found redundant and 3.9% genes of fungal origin proteins were eliminated. As a result, a total of 162 unique sequences with 18 contigs and 144 singletons were obtained, of which 10 contigs and 68 singletons from compatible and 8 contigs and 70 singletons incompatible interaction were identified.

Characterization of expressed sequenced tags (ESTs)

Sequence analysis of the clones showed that more than 90% were of good quality and matched with the ESTs of different plant species of the NCBI database involved in a variety of cellular processes. Representative 162 ESTs were grouped according to their putative physiological functions, viz., defense-related genes, signal transduction-related genes, energy and metabolism-related genes, transcription and translation-related genes, cellular transport-related genes and hypothetical proteins. A total of 11.1% from compatible and incompatible library did not show homology with the known sequences of the gene bank database. The percent distribution of various classes of genes is depicted in Fig. 2. The largest category consisted of genes, which encode for proteins related to hypothetical proteins (22.8%), followed by energy and metabolism (20.3%)-related genes, defense and

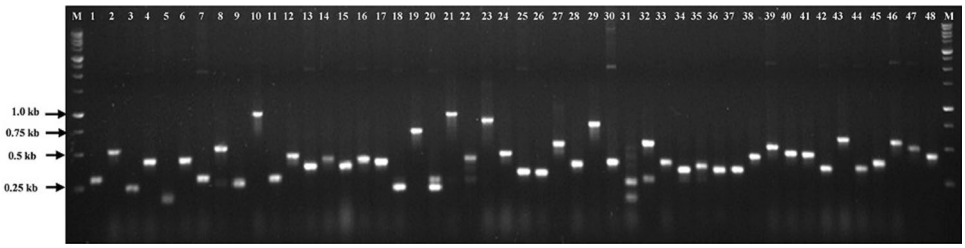

Fig. 1 Agarose gel (1.25%) profile showing the amplified cDNA inserts of chickpea cultivar WR-315. Lanes 1–48 inserts of subtractive library; M—1 kb DNA ladder at both sides

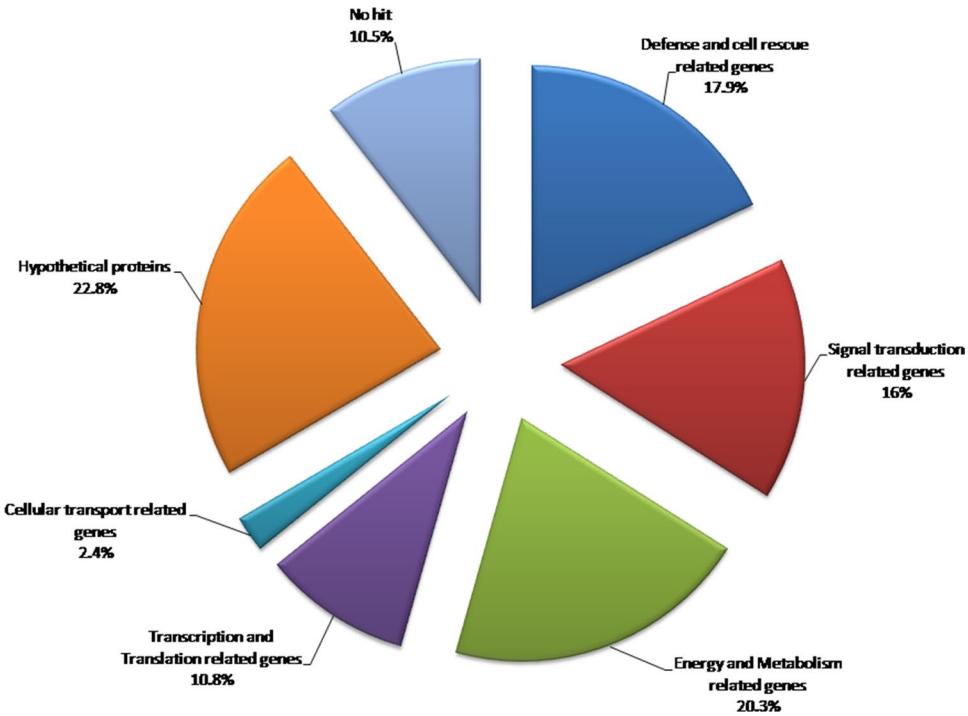

Fig. 2 Functional classification and relative distribution of identified genes in compatible and incompatible interactions

cell rescue-related genes (17.9%) and signal transduction-related genes (16%). The list of genes encoding for various functional categories for incompatible interaction are given in Table 2 and for compatible interaction in Table 3. Conserved domain search proved that the ESTs contains coiled coil, CCCH, leucine-rich repeats, U box, glycosyl hydrolase family 18, DnaJ/HSP40 family and Bet v 1 domain-containing proteins. These family proteins are reported to be involved in defense-related activity. Among both the libraries, some of the putative genes were found common.

Validation of differentially expressed genes

Twelve ESTs encoding defense-related proteins were further analyzed for the temporal expression by semi-quantitative and quantitative PCR analysis. In the present study, expression of putative genes encoding for superoxide dismutase (SOD), pathogenesis-related protein 4 (PR4), pathogenesis-related protein 10 (PR10), leucine rich repeat protein kinase, proline-rich cell wall protein, squalene monooxygenase, cysteine proteinase and cinnamate 4 hydroxylase expression was much higher in incompatible compared to compatible interaction (Fig. 3). These candidate genes were up-regulated at 48, 72 and 96 h after infection in the incompatible interaction. The expression of a putative superoxide dismutase expression was induced as early as 48 hpi in both the interactions. A putative PR10 protein expression was induced at 72 hpi and the highest expression was observed at 120 hpi in the incompatible interaction. The transcript level of a putative protein kinase expression in the present study was up-regulated at 48 and 72 hpi in the compatible and the incompatible interaction, respectively, and then dropped abruptly. The transcript level of a putative PR 4 protein expression was observed to be uniform in the incompatible interaction at all the hpi, and in the compatible interaction its expression was much higher at 48 and 96 hpi. In the resistant cultivar, proline-rich cell wall protein, cysteine proteinase and cinnamate 4 hydroxylase expression was maximum at 72 hpi, followed by 96 hpi and sharply declined at 120 hpi, whereas, squalene monooxygenase peak was observed at 96 hpi followed by 48 hpi. On the other hand, the expression of 18S ribosomal RNA was uniform throughout the experiment (Fig. 4).

Discussion

Chickpea root transcript was studied to understand molecular mechanism involved in resistance and susceptibility upon pathogen infection. For the first time, an attempt was made to develop the cDNA library in chickpea against *Foc* race four, a predominant race of North India. The diversity of partial sequences identified in the present studies would provide valuable insights into the biology of chickpea crop against Fusarium wilt. Based on previous work of Chatterjee et al. (2014) on transcriptome analysis in chickpea against *Foc* race 1, samples were collected at 48, 72, 96 and 120 h time points for capturing early response genes Gupta et al. (2009) also reported that *Foc* pathogen colonizes xylem vessels 96 h after infection, while significant transcriptomic alterations were observed at 48 h after infection. In the present study, homology search showed that the maximum hits were with *Medicago truncatula* which is the closest neighbor of legume. However, functional annotation is constantly being updated in this legume crop; hence, it reflected in the scores of chickpea. Though a complete draft genome sequence of chickpea has been reported recently, the functional annotations of genes need to be carried out to understand the host–pathogen interaction in the crop. A total of 162 unique sequences with 18 contigs and 144 singletons were taken into consideration for functional clustering. Out of these, 17.1 and 18.7% in compatible and incompatible interactions, respectively, encode for proteins related to defense processes, including regulation of oxidative burst, antimicrobial compounds and protein degradation. These genes include PR 10, PR 4, cysteine proteinase, superoxide dismutase (SOD), proline-rich cell wall protein, HSP 70, cytochrome 450, metallothionein, chalcone synthase, mitogen-activated protein kinases (MAPK), 4-coumarate-CoA ligase, and chitinases. These genes were also reported in the previous studies as differentially expressed in several host–pathogen interactions (Fernandez et al. 2004; Coram and Pang 2005; Jaiswal et al. 2011; Yu et al. 2010; Xu et al. 2011). Candidate genes identified in the present study such as superoxide dismutase, ubiquitin and 26S proteasome, cysteine proteases, metallothionein and BTB/POZ domain-containing protein at the early stage of the infection suggests the presence of reactive oxygen species during host–pathogen interaction. Some of the genes of this category have been shown to be involved in cross talk in various defense pathways (He et al. 1998). During stress conditions, peroxidation of polyunsaturated fatty acids occurs leading to cross talk of various genes and results in generation of reactive oxygen species (ROS). Putative BTB/POZ domain-containing gene was also shown to participate in defense mechanism in cotton against *Verticillium dahliae* (Xu et al. 2011) and BTB/POZ domain in NPR1 protein plays key role in regulation of systemic acquired resistance (Rochon et al. 2006). Genes encoding for ubiquitin and 26S proteasome play a key role in hormone signaling, oxidative burst and gene induction and programmed cell death (Jrujillo and Shirasu 2010). Putative cysteine proteases identified during early stage of infection in the present study were reported to play an active role in programmed cell death in *Arabidopsis* (Clarke et al. 2000). An oxidative burst takes place by production of ROS which was proven to cause cellular damage to both host plant and

Table 2 Representative resistance and stress related genes obtained by SSH of the incompatible interaction between chickpea and *F. oxysporum* f. sp. *ciceris* race 4

Clone ID	Accession no.	Putative annotation	*E* value	Size (bp)
Defense and cell rescue-related genes				
CH_FOC R-1	JZ585549	Wound-induced protein (*Glycine max*)	1e−34	198
CH_FOC R-4	JZ585551	Pathogenesis-related protein 10 (*Cicer arietinum*)	2e−101	463
CH_FOC R-5	JZ585552	Disease resistance response protein (DRRG49-c) (*Pisum sativum*)	4e−91	465
CH_FOC R-7	JZ585554	Cysteine proteinase (*Cicer arietinum*)	5e−103	477
CH_FOC R-8	JZ585555	Universal stress protein (*Medicago truncatula*)	8e−51	283
CH_FOC R-9	JZ585556	Chalcone synthase (*Cicer arietinum*)	9e−157	690
CH_FOC R-10	JZ585557	Senescence-associated protein (*Picea abies*)	2e−54	310
CH_FOC R-11	JZ585572	Superoxide dismutase (*Cicer arietinum*)	4e−108	456
CH_FOC R-12	JZ585565	U-box domain-containing protein 4 (*Glycinemax*)	2e−139	281
CH_FOC R-13	JZ585560	Avr9 elicitor response protein (*Medicago truncatula*)	2e−76	412
CH_FOC R-14	JZ585561	Cinnamate 4-hydroxylase (*Astragalus mongholicus*)	1e−92	518
CH_FOC R-16	JZ585563	Pathogenesis-related protein PR10 (*Medicago truncatula*)	2e−68	367
CH_FOC R-18	JZ585568	Cytochrome P450(*Cicer arietinum*)	3e−134	287[a]
CH_FOC R-21	JZ585566	Chaperone protein DnaJ/HSP70 (*Medicago truncatula*)	1e−125	515[a]
CH_FOC R-78	JZ585553	Stem-specific protein	1e−171	331
Signal transduction related genes				
CH_FOC R-15	JZ585562	Kunitz proteinase inhibitor-1	4e−176	339
CH_FOC R-17	JZ585567	FK506-binding protein (*Medicago truncatula*)	3e−47	230
CH_FOC R-24	JZ585570	MAP3K delta-1 protein kinase, putative (*Ricinus communis*)	5e−72	263
CH_FOC R-25	JZ585571	SAL1 phosphatase-like (*Glycine max*)	1e−98	568
CH_FOC R-26	JZ585573	Calcium-regulated/ATP-independent forisome protein (*Pisum sativum*)	2e−47	411
CH_FOC R-28	JZ585575	Rna-binding protein (*Medicago truncatula*)	1e−80	418
CH_FOC R-29	JZ585564	Zinc finger-containing protein 53 (*Glycine max*)	3e−53	400
CH_FOC R-30	JZ585559	Metallothionein (*Cicer arietinum*)	4e−158	331[a]
CH_FOC R-31	JZ585574	Serine/threonine protein kinase (*Glycine max*)	1e−147	755
CH_FOC R-35	JZ585534	Proteasome subunit beta type (*Medicago truncatula*)	5e−96	423
CH_FOC R-79	JZ585576	Nuclear RNA-binding-like protein(*Medicago truncatula*)	4e−145	533[a]
Energy and metabolism related genes				
CH_FOC R-32	JZ585535	Enolase (*Prunus armeniaca*)	1e−69	353
CH_FOC R-33	JZ585547	Cytosolic malate dehydrogenase (*Cicer arietinum*)	0.0	386[a]
CH_FOC R-34	JZ585545	Presequence protease (*Medicago truncatula*)	9e−180	801
CH_FOC R-35	JZ585534	Proteasome subunit beta type (*Medicago truncatula*)	5e−96	423
CH_FOC R-36	JZ585542	Citrate synthase (*Medicago truncatula*)	7e−90	442
CH_FOC R-37	JZ585541	Glyceraldehyde 3-phosphate dehydrogenase, cytosolic (*Cicer arietinum*)	1e−67	334
CH_FOC R-38	JZ585540	Probable beta-1,3-galactosyltransferase 10-like (*Glycine max*)	1e−53	291
CH_FOC R-39	JZ585537	Alcohol dehydrogenase class-3 (*Medicago truncatula*)	6e−69	441
CH_FOC R-40	JZ585544	Proteasome subunit beta type (*Medicago truncatula*)	6e−73	342
CH_FOC R-41	JZ585569	Casein kinase II subunit beta-like isoform X2 (*Cicer arietinum*)	9e−109	576
CH_FOC R-42	JZ585546	Beta-galactosidase (*Cicer arietinum*)	9e−141	602
CH_FOC R-43	JZ585548	Adenine phosphoribosyltransferase (*Trifolium repens*)	9e−27	175
CH_FOC R-44	JZ585547	Cytosolic malate dehydrogenase precursor (*Medicago sativa*)	1e−144	408[a]
CH_FOC R_50	JZ585578	PHR1like (*Arachis duranensis*)	5e−78	542
Transcription and translation related genes				
CH_FOC R-45	JZ585580	Transcription factor (*Vicia faba* var. *minor*)	5e−48	533
CH_FOC R-46	JZ585577	40S ribosomal protein (*Medicago truncatula*)	1e−78	354
CH_FOC R-47	JZ585543	60S ribosomal protein (*Medicago truncatula*)	3e−80	385
CH_FOC R-48	JZ585580	Transcription factor Pur-alpha (*Medicago truncatula*)	7e−64	317
CH_FOC R-49	JZ585581	60S ribosomal protein (*Cicer arietinum*)	3e−54	249

Table 2 (continued)

Clone ID	Accession no.	Putative annotation	*E* value	Size (bp)
CH_FOC R-51	JZ585584	Probable histone H2B.1-like isoform 1 (Vitis vinifera)	1e−63	323
CH_FOC R-52	JZ585585	DEAD box RNA helicase (*Pisum sativum*)	1e−173	750
CH_FOC R-53	JZ585579	Elongation factor 1-alpha (EF1-a) (*Cicer arietinum*)	2e−149	648
CH_FOC R-56	JZ585587	Poly(A)-binding protein (*Medicago truncatula*)	6e−112	313[a]
Cellular transport mechanism related genes				
CH_FOC R-58	JZ585588	Calreticulin (*Medicago truncatula*)	2e−74	393
CH_FOC R-59	JZ585589	Transmembrane channel protein (*Cicer arietinum*)	4e−77	342
CH_FOC R-60	JZ585591	Putative water channel protein (*Cicer arietinum*)	7e−166	320[a]
CH_FOC R-61	JZ585593	Putative tonoplast intrinsic protein (*Pisum sativum*)	1e−42	252

[a]Blastn result

pathogen during various abiotic and biotic stresses (Barna et al. 2003). ROS precede the hypersensitive reaction, which is associated with the defense process during host–pathogen interaction. In plants, ROS was reported to damage carbohydrates, lipids, proteins and nucleic acids (Blokhina et al. 2003). The putative SOD obtained in the present study is also reported to be involved in the production of H_2O_2 that functions as signal molecule for programmed cell death and protecting neighboring cells from ROS by oxidative cross-linking of cell wall (Borden and Higgins 2002). Further, metallothionein proteins are also involved in the protection of neighboring cells from oxidative damage by scavenging ROS (Kumari et al. 1998). Several heat shock protein gene induction was also correlated with oxidative stress (Scarpeci et al. 2008). This clearly indicates the crucial role of candidate genes in protecting the plants against oxidative burst. The GTP-binding protein and ADP ribosylation factor identified in the present study were involved in the signaling process (Hou et al. 2007). Putative LRR receptor protein kinase proteins are involved in the signaling process by activating downstream MAP kinases. These signaling events converge into an MAPK cascade through phosphorylation and dephosphorylation, which confers resistance to both fungal and bacterial pathogens (Asai et al. 2002). The pathogenesis-related protein 10 (PR10) identified in the study is involved in the defense against diverse groups of pathogens in chickpea (Saikia et al. 2005) and also reported to be induced in roots of rice by biotic and abiotic stress through jasmonic signaling pathway (Hashimoto et al. 2004), which was up-regulated upon infection of rice blast fungus. A strong induction of PR10 in response to bacteria, fungi, wounding, jasmonic acid and ABA treatment in *Lithospermum erythrorhizon* was observed (Hwang et al. 2003). During the study, several classes of genes were involved in the production of antimicrobial compounds. Pathogenesis-related 4 protein is reported to have chitinase activity (Legrand et al. 1987) and is involved in defense against biotic stress and abiotic stress in rice (Wang et al. 2011). Flavonoids obtained in this study are secondary metabolites produced during host–pathogen interaction and are involved in defense mainly through the production of phytoalexins. A chalcone synthase is a key enzyme involved in isoflovonoid production during biotic stress (Gurjar et al. 2012). Cinnamate 4 hydroxylase and 4-coumarate-CoA ligase play an important role in the a key reaction of the phenylpropanoid pathway, which leads to the production of several secondary metabolites. Similar findings were reported by Lu et al. (2006) and Soria-Guerra et al. (2010), where putative 4-coumarate-CoA ligase was also involved in the biosynthesis of jasmonic acid, which plays a vital role in plant defense. Aoki et al. (2000) reported that chytochrome 450 plays an important role in biosynthesis of (iso)flavonoids such as medicarpin, glyceollins, genistein and daidzein, which interns play a central role in plant defense mechanisms and also helps in the transportation of toxic materials formed during pathogen infection into the vacuole. Hence, a similar function might be proposed for chickpea responses to *Foc* infection. 3.9% of genes originated from the fungus, indicating the presence of fungal growth and proliferation in host plant. Similarly, Gurjar et al. (2012) obtained 18% fungal originated genes during chickpea and *Foc* race 1 interaction in resistance cultivar.

The quantitative and semi-quantitative analysis of defense-related genes such as PR 10, PR 4, LRR protein kinase, cinnamate 4 hydroxylase, proline-rich cell wall, cysteine proteinase, SOD and squalene monooxygenase gene expression was significantly higher in incompatible interaction. The majority of the genes showed peak expression at 48 and 72 hpi after inoculation. The findings of present studies are consistent with Coram and Pang (2005), who reported peak expression of these genes in chickpea against *A. rabiei* at 24 hpi, which returned to normal level of expression at 96 hpi. They also reported up-regulation of PR 10 protein in the incompatible interaction as compared to the compatible interaction. Lo et al. (1999) obtained peak expression of PR 10 at 36 h and at 48 h after inoculation with *Cochliobolus*

Table 3 Representative resistance and stress-related genes obtained by SSH of the compatible interaction between chickpea and *F. oxysporum* f. sp. *ciceris* race 4

Clone ID	Accession no.	Putative annotation	*E* value	Size (bp)
Defense and cell rescue-related genes				
CH_FOC S-2	JZ714722	Proline-rich cell wall protein (*Medicago sativa*)	4e−28	266
CH_FOC S-3	JZ714723	PR-4-like protein (*Galega orientalis*)	6e−31	182
CH_FOC S-4	JZ714724	U-Box domain-containing protein 13-like (*Glycine max*)	6e−170	917
CH_FOC S-5	JZ714725	Chitinase-related agglutinin (*Robinia pseudoacacia*)	1e−24	207
CH_FOC S-7	JZ714727	Chalcone synthase (*Cicer arietinum*)	8e−103	477
CH_FOC S-8	JZ714728	Glutamine synthetase PR1 mutant (*Lotus japonicas*)	6e−46	284
CH_FOC S-9	JZ714731	Immunophilin, putative (*Ricinus communis*)	1e−70	352
CH_FOC S-12	JZ714734	Avirulence Induced Gene1 (*Medicago truncatula*)	6e−159	929
CH_FOC S-14	JZ714736	Cinnamate 4-hydroxylase (*Astragalus chrysochlorus*)	2e−152	699
CH_FOC S-15	JZ714735	Prephenate aminotransferase (Petunia × hybrid)	2e−121	681
CH_FOC S-11	JZ714733	3-Phosphoshikimate 1-carboxyvinyltransferase (*Medicago truncatula*)	2e−70	418
CH_FOC S-31	JZ714729	BTB/POZ and MATH domain-containing protein (*Glycine max*)	5e−156	807
CH_FOC S-53	JZ714778	Coiled coil/BAH domain-containing protein (*Medicago truncatula*)	2e−100	491
CH_FOC S-55	JZ714753	4-Coumarate-CoA ligase (*Coffea arabica*)	5e−83	452
Signal transduction related genes				
CH_FOC S-10	JZ714732	FK506-binding protein 12 kD (*Arabidopsis lyrata* subsp. *Lyrata*)	9e−71	370
CH_FOC S-13	JZ714726	ADP ribosylation factor (*Hertia cheirifolia*)	4e−64	295
CH_FOC S-16	JZ714764	Protein phosphatase-2c (*Arabidopsis thaliana*)	2e−62	376
CH_FOC S-18	JZ714765	Glycine-rich RNA binding protein (*Medicago truncatula*)	1e−41	236
CH_FOC S-20	JZ714767	NAK-Serine/threonine protein phosphatase (*Glycine max*)	9e−97	418
CH_FOC S-22	JZ714770	Signal peptide peptidase family protein (*Arabidopsis lyrata*)	6e−97	495
CH_FOC S-25	JZ714772	Guanine nucleotide-binding protein (*Medicago truncatula*)	2e−79	380
CH_FOC S-26	JZ714763	Galactokinase like protein (*Medicago truncatula*)	7e−101	589
CH_FOC S-27	JZ714775	Phytosulfokine receptor 2-like (*Glycine max*)	5e−71	387
CH_FOC S-28	JZ714773	GTP binding/GTPase/protein binding protein (*Lepidium appelianum*)	5e−47	247
CH_FOC S-29	JZ714737	Ubiquitin-protein ligase listerin-like protein (*Medicago truncatula*)	4e−71	432
CH_FOC S-35	JZ714738	26S proteasome non-ATPase regulatory subunit (*Medicago truncatula*)	5e−46	164
CH_FOC S-66	–	Squalene monooxygenase 2 (*Medicago truncatula*)	4e−171	588[a]
Energy and metabolism related genes				
CH_FOC S-24	JZ714771	Probable lysine-specific demethylase JMJ14-like (*Glycine max*)	1e−55	425
CH_FOC S-21	JZ714769	Glycerol kinase (*Medicago truncatula*)	1e−82	486
CH_FOC S-30	JZ714774	Aminoacylase-1 (*Medicago truncatula*)	1e−120	624
CH_FOC S-34	JZ714760	Methionine synthase (*Cicer arietinum*)	1e−75	413
CH_FOC S-36	JZ714739	24-sterol C-methyltransferase (*Gossypium hirsutum*)	2e−71	365
CH_FOC S-37	JZ714741	Sucrose synthase (*Medicago sativa*)	5e−53	290
CH_FOC S-38	JZ714742	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (*Medicago truncatula*)	5e−143	638
CH_FOC S-39	JZ714743	Enolase-like (*Glycine max*)	1e−176	793
CH_FOC S-40	JZ714744	NAD-dependent malate dehydrogenase (*Prunus persica*)	3e−77	428
CH_FOC S-41	JZ714746	Protease like 7-like (*Glycine max*)	1e−104	570
CH_FOC S-42	JZ714758	Fructose-1,6-bisphosphate aldolase (*Pisum sativum*)	1e−75	368
CH_FOC S-43	JZ714757	ATP citrate lyase a-subunit (*Medicago truncatula*)	1e−137	614
CH_FOC S-44	JZ714740	Sterol 24-C methyltransferase 2-1 (*Medicago truncatula*)	1e−75	370
CH_FOC S-47	JZ714747	Sucrose synthase, putative (*Ricinus communis*)	9e−105	544
CH_FOC S-48	JZ714745	L-Asparaginase 2 (*Phaseolus vulgaris*)	3e−85	442
CH_FOC S-49	JZ714759	Acetyl-CoA acetyltransferase, cytosolic (*Medicago truncatula*)	4e−65	370
CH_FOC S-51	JZ714761	Ornithine aminotransferase (*Pisum sativum*)	4e−80	399
CH_FOC S-52	JZ714762	Argininosuccinate synthase (*Medicago truncatula*)	3e−124	579

Table 3 (continued)

Clone ID	Accession no.	Putative annotation	*E* value	Size (bp)
CH_FOC S-53	JZ714776	Transducin beta-like protein (*P. sativum*)		
Transcription and translation related genes				
CH_FOC S-56	JZ714777	Threonyl-tRNA synthetase, mitochondrial-like(*Glycine max*)	4e−37	192
CH_FOC S-58	JZ714779	RNA Binding Protein (*Medicago truncatula*)	2e−82	505
CH_FOC S-59	JZ714783	Eukaryotic initiation factor 4A (*Medicago truncatula*)	1e−85	413
CH_FOC S-60	JZ714781	Translation initiation factor IF-2 (*Medicago truncatula*)	4e−47	270
CH_FOC S-61	JZ714782	Elongation factor 1-alpha (EF1-a) (*Cicer arietinum*)	1e−157	873
CH_FOC S-62	JZ714784	Pre-mRNA-processing factor 40 homolog B-like (*Glycine max*)	2e−78	596
CH_FOC S-65	JZ714785	ATP-dependent RNA helicase DHX8-like (*Glycine max*)	1e−144	422[a]

[a]Blastn result

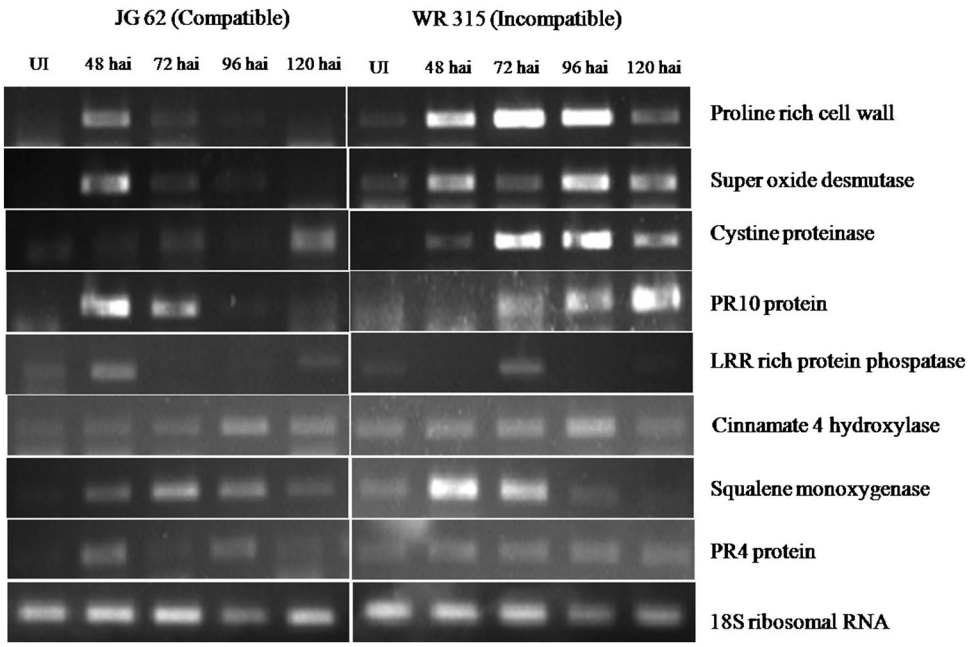

Fig. 3 Agarose gel (1.25%) showing RT-PCR analysis of putative genes of defense from susceptible (JG-62) and resistant (WR-315). *UI* uninoculated, *hai* hours after inoculation

sublineolum in sorghum. PR 10 and PR 3 genes were over-expressed in a partially field-resistant cotton cultivar compared to highly susceptible cultivar after inoculation with *F. oxysporum* f. sp. *vasinfectum* (Zambounis et al. 2012). Compared with their expression levels in uninfected and compatible-type fungal-infected roots, all of the genes were highly expressed in incompatible-type interaction (Wang et al. 2014).

Conclusion

The present investigation has provided insights into the pathogen-responsive genes in chickpea. This study reported the ESTs involved in defense mechanisms elicited in response to *Foc* race 4. The ESTs which are submitted to the GenBank database may be great genomic resources for academics working on chickpea. The ESTs will also serve as potential resources for future genetic improvement for resistance to Fusarium wilt in chickpea cultivars. Functional characterization of unknown ESTs can be further characterized by 5′ rapid amplification of cDNA ends (RACE) to know their role in the defense mechanism. Temporal and quantitative change in the expression of proteins involved in defense probably determines the net outcome of interaction. Thus, identification of root-specific promoters and driving the defense-related genes in the root tissue would be a promising strategy for Fusarium wilt management.

Acknowledgements The study was supported by the Indian Agricultural Research Institute, New Delhi, India.

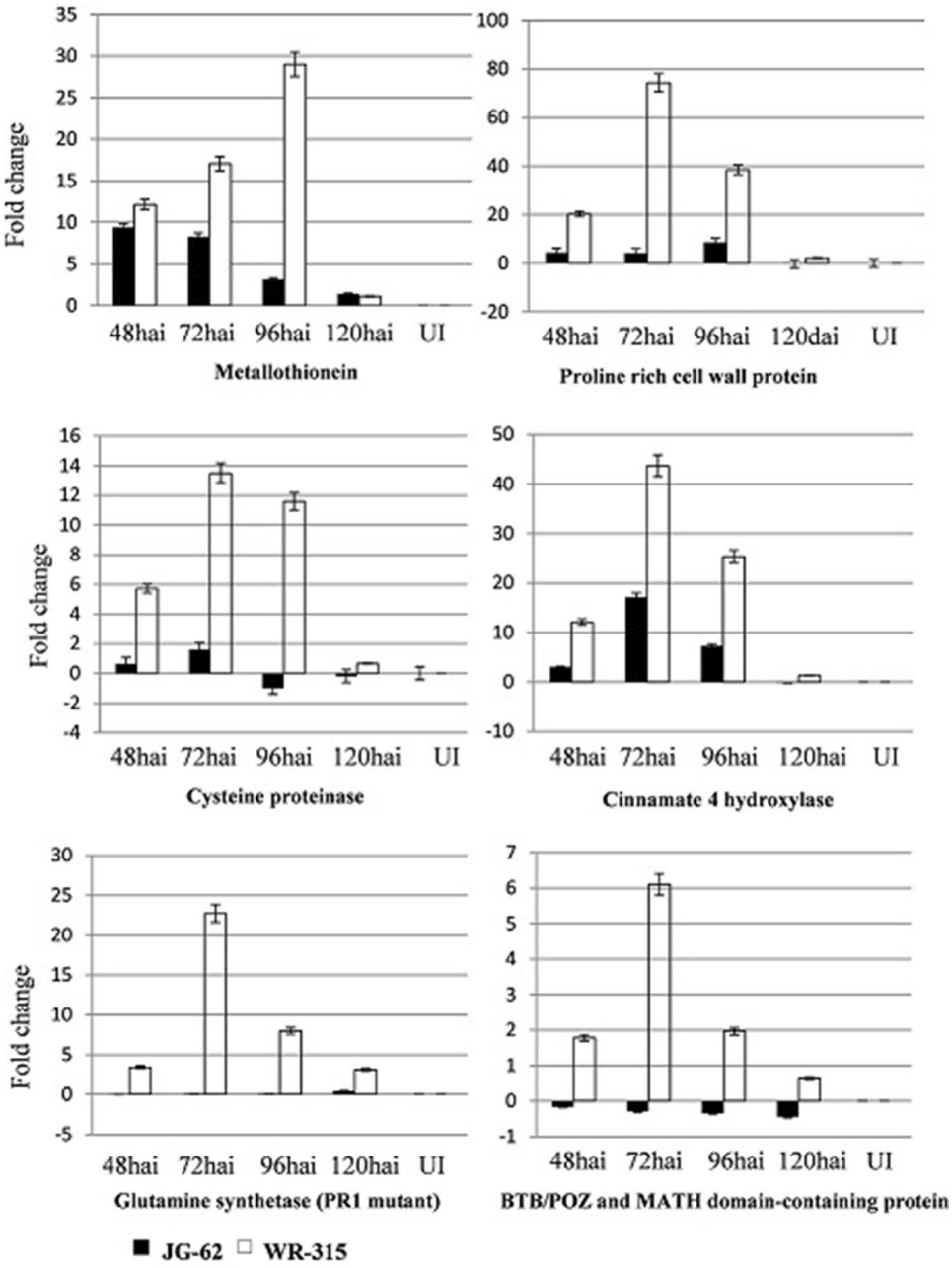

Fig. 4 Quantification of relative expression level of defense-related genes by qRT-PCR. *UI* uninoculated, *hai* hours after inoculation

Compliance with ethical standards

Conflict of interest No conflict of interest was declared by the authors.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Aoki T, Akashi T, Ayabe S (2000) Flavonoids of leguminous plants: structure, biological activity and biosynthesis. J Plant Res 113:475–488

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J (2002) MAP kinase signalling cascade in Arabidopsis innate immunity. Nature 415:977–983

Barna B, Fodor J, Pogany M, Kiraly Z (2003) Role of reactive oxygen species and antioxidants in plant disease resistance. Pest Manag Sci 59:459–464

Bevan M, Bancroft I, Bent E, Love K, Goodman H, Dean C, Bergkamp R, Dirkse W, Van-Staveren M, Stiekema W (1998) Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*. Nature 391:485–488

Blokhina O, Virolainen E, Fagerstedt KV (2003) Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann Bot 91:179–194

Borden S, Higgins VJ (2002) Hydrogen peroxide plays a critical role in the defense response of tomato to *Cladosporium fulvum*. Physiol Mol Plant Pathol 61:227–236

Chatterjee M, Gupta S, Bhar A, Chakraborti D, Basu D, Das S (2014) Analysis of root proteome unravels differential molecular responses during compatible and incompatible interaction between chickpea (*Cicer arietinum* L.) and *Fusarium oxysporum* f. sp. *ciceri* Race1 (Foc1). BMC Genom 15:949. https://doi.org/10.1186/1471-2164-15-949

Clarke A, Desikan R, Hurst RD, Hancock JT, Neill SJ (2000) No way back: nitric oxide and programmed cell death in *Arabidopsis thaliana* suspension cultures. Plant J 24:667–677

Coram TE, Pang ECK (2005) Isolation and analysis of candidate Ascochyta blight defence genes in chickpea. Physiol Mol Plant Phathol 66:201–210

Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, Siebert PD (1996) Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci 93:6025–6030

Directorate of Economics and Statistics (2016) Department of agriculture, cooperation and farmers welfare. Agriculture statistics at a glance, p 110

Dubey SC, Suresh M (2006) Randomly amplified polymorphic DNA markers for Trichoderma species and antagonism against *Fusarium oxysporum* f. sp *ciceris* causing chickpea wilt. J Phytopathol 154:663–669

Dubey SC, Priyanka K, Singh V, Singh B (2012) Race profiling and molecular diversity analysis of *Fusarium oxysporum* f. sp. *ciceris* causing wilt in chickpea. J Phytopathol 160:576–587

Fernandez D, Santos P, Agostini C, Bon MC, Petitot AS, Silva MC, Guerra- Guimaraes L, Ribeiro A, Argout X, Nicole M (2004) Coffee (*Coffea arabica* L.) genes early expressed during infection by the rust fungus (*Hemileia vastatrix*). Mol Plant Pathol 5:527–536

Govil JN, Rana BS (1994) Stability of host plant resistance to wilt (*Fusarium oxysporum* f. sp. *ciceri*) in chickpea. Int J Trop Pl Dis 2:55–60

Gupta S, Chakraborti D, Rangi RK, Basu D, Das S (2009) A molecular insight into the early events of chickpea (*Cicer arietinum*) and *Fusarium oxysporum* f. sp. *ciceri* (race 1) interaction through cDNA AFLP analysis. Phytopathology 99:1245–1257

Gurjar GS, Giri AP, Gupta VS (2012) Gene expression profiling during wilting in chickpea caused by *Fusarium oxysporum* f. sp. *ciceri*. Am J Plant Sci 3:190–201

Hashimoto M, Kisseleva L, Sawa S, Furukawa T, Komatsu S, Koshiba T (2004) A novel rice PR10 protein, RSJIOs PR10, specifically induced in roots by biotic and abiotic stresses, possibly via the jasmonic acid signaling pathway. Plant Cell Physiol 45:550–559

Haware MP, Nene YL (1980) Influence of wilt at different stages on the yield loss in chickpea. Trop Grain Legume Bull 19:38–40

Haware MP, Nene YL (1982) Races of *Fusarium oxysporum* f. sp. *ciceri*. Plant Dis 66:809–810

He ZH, He D, Kohorn BD (1998) Requirement for the induced expression of a cell wall associated receptor kinase for survival during the pathogen response. Plant J 14:55–63

Hou L, Li JB, Luo XY, Wang WF, Xiao YH, Luo M, Pei Y (2007) Cloning, expression and characterization of an ADP-ribosylation factor gene from cotton (*Gossypium hirsutum* L.). Acta Agron Sin 33:1226–1231

Huang X, Madan A (1999) CAP3: a DNA sequence assembly program. Genome Res 9:868–877

Hwang HJ, Kima H, Yu HJ, Oh MH, Lee I, Kim SG (2003) Gene encoding pathogenesis-related 10 protein of *Lithospermum erythrorhizon* is responsive to exogenous stimuli related to the plant defense system. Plant Sci 165:1297–1302

Jaiswal P, Cheruku RJ, Kumar K, Yadav S, Singh A, Kumari P, Dube SC, Upadhyaya KC, Verma PK (2011) Differential transcript accumulation in chickpea during early phases of compatible interaction with a necrotrophic fungus *Ascochyta rabiei*. Mol Biol Rep 39:4635–4646

Jimenez-Diaz RM, Alcala-Jimenez AR, Hervar A, Trapero- Casas JL (1993) Pathogenic variability and host resistance in the *Fusarium oxysporum* f. sp. *ciceri/Cicer arietinum* pathosystem. In: Arseniuk E, Goral T (eds) Third proceedings of European seminar: *Fusarium*-mycotoxins taxonomy, pathogenicity and host resistance, Rodzikov, Poland: Plant Breeding and Acclimatization Institute, pp 87–94

Jimenez-Gasco MM, Jimenez-Diaz RM (2003) Development of a specific polymerase chain reaction-based assay for the identification of *Fusarium oxysporum* f. sp *ciceris* and its pathogenic races 0, 1A, 5 and 6. Phytopathology 93:200–209

Jones JDG, Dangl JL (2006) The plant immune system. Nature 444:323–329

Jørgensen JH (1994) Genetics of powdery mildew resistance in barley. Crit Rev Plant Sci 13:97–119

Jrujillo K, Shirasu K (2010) Ubiquitination in plant immunity. Curr Opin Plant Biol 13:402–408

Kumari MV, Hiramatsu M, Ebadi M (1998) Free radical scavenging actions of metallothionein isoforms I and II. Free Radic Res 29:93–101

Legrand M, Kauffmann S, Geoffroy P, Fritig B (1987) Biological function of pathogenesis-related proteins: four tobacco pathogenesis-related proteins are chitinases. Proc Natl Acad Sci USA 84:6750–6754

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using realtime quantitative PCR and the $2^{\Delta\Delta}$C(T) method. Methods 25:402–408

Lo SC, Hipskind JD, Nicholson RL (1999) cDNA cloning of a sorghum pathogenesis-related protein (PR-10) and differential expression of defense-related genes following inoculation with *Cochliobolus heterostrophus* or *Colletotrichum sublineolum*. Mol Plant Microbe Interact 12:479–489

Lu S, Zhou Y, Li L, Chiang VL (2006) Distinct roles of cinnamate 4-hydroxylase genes in *Populus*. Plant Cell Physiol 47:905–914

Rochon A, Boyle P, Wignes T, Fobert PR, Despres C (2006) The coactivator function of Arabidopsis NPR1 requires the core of its BTB/POZ domain and the oxidation of C-terminal cysteines. Plant Cell 18:3670–3685

Saikia R, Singh BP, Kumar R, Arora DK (2005) Detection of pathogenesis-related proteins chitinase and 1,3-glucanase in induced chickpea. Curr Sci 89:659–663

Scarpeci TE, Zanor MI, Valle EM (2008) Investigating the role of plant heat shock proteins during oxidative stress. Plant Signal Behav 3:856–857

Singh KB, Dahiya BS (1973) Breeding for wilt resistance in chickpea. In: Symposium on problem and breeding for wilt resistance in bengal gram. IARI, New Delhi pp 13–14

Soria-Guerra RE, Rosales-Mendoza S, Chang S, Haudenshield JS, Zheng D, Rao SS, Hartman GL, Ghabrial SA, Korban SS (2010) Identifying differentially expressed genes in leaves of *Glycine tomentella* in the presence of the fungal pathogen *Phakopsora pachyrhizi*. Planta 232:1181–1189

Wang Y, Kim SG, Kim ST, Agrawal GK, Rakwal R, Kang KY (2011) Biotic stress-responsive rice proteome. Plant Biol 54:219–226

Wang Y, Kwon SJ, Wu J, Choi J, Lee YH, Agrawal GK, Tamogami S, Rakwal R, Park SR, Kim BG, Jung KH, Kang KY, Kim SG, Kim ST (2014) Transcriptome analysis of early responsive genes in rice during *Magnaporthe oryzae* infection. Plant Pathol J 30:343–354

Xu L, Zhu L, Tu L, Guo X, Long L, Sun L, Gao W, Zhang X (2011) Differential gene expression in cotton defence response to *Verticillium dahliae* by SSH. J Phytopathol 159:606–615

Yu X, Wang X, Wang C, Chen X, Qu Z, Yu X, Han Z, Zhao J, Guo J, Huang L, Kang Z (2010) Wheat defense genes in fungal (*Puccinia striiformis*) infection. Funct Integr Genom 10:227–239

Zambounis AG, Kalamaki MS, Tani EE, Paplomatas EJ, Tsaftaris AS (2012) Expression analysis of defense related genes in cotton (*Gossypium hirsutum*) after *Fusarium oxysporum* f.sp. *vasinfectum* infection and following chemical elicitation using a salicylic acid analog and methyl jasmonate. Plant Mol Biol 30:225–234