RESEARCH ARTICLE

Candidate genes expression profiling during wilting in chickpea caused by *Fusarium oxysporum* f. sp. *ciceris* race 5

Cristina Caballo[1], Patricia Castro[2]*, Juan Gil[2], Teresa Millan[2], Josefa Rubio[1], Jose V. Die[2]

1 Área de Genómica y Biotecnología, IFAPA, Alameda del Obispo, Córdoba, Spain, 2 Department of Genetics - ETSIAM, University of Córdoba, Campus de Rabanales, Córdoba, Spain

* patricia.castro@uco.es

OPEN ACCESS

Abstract

Chickpea production may be seriously threatened by Fusarium wilt, a disease caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *ciceris*. *F. oxysporum* race 5 is the most important race in the Mediterranean basin. Recently, the region responsible for resistance race 5 has been delimited within a region on chromosome 2 that spans 820 kb. To gain a better understanding of this genomic region, we used a transcriptomic approach based on quantitative real-time PCR to analyze the expression profiles of 22 selected candidate genes. We used a pair of near-isogenic lines (NILs) differing in their sensitivity to Fusarium race 5 (resistant vs susceptible) to monitor the transcriptional changes over a time-course experiment (24, 48, and 72 hours post inoculation, hpi). Qualitative differences occurred during the timing of regulation. A cluster of 12 genes were induced by the resistant NIL at 24 hpi, whereas a second cluster contained 9 genes induced by the susceptible NIL at 48 hpi. Their possible functions in the molecular defence of chickpea is discussed. Our study provides new insight into the molecular defence against Fusarium race 5 and demonstrates that development of NILs is a rich resource to facilitate the detection of candidate genes. The new genes regulated here may be useful against other Fusarium races.

Introduction

Chickpea (*Cicer arietinum* L.) is the second grain legume cultivated in the world with a total cultivated area of 14.5 million hectares [1]. It is mainly used for human consumption and is an essential constituent of the Mediterranean diet. Chickpea is a good and cheap source of protein and for this reason this crop is cultivated in the five continents.

One of the major biotic stresses limiting chickpea yield is its susceptibility to fungal diseases. Among these, Fusarium wilt caused by *Fusarium oxysporum* f. sp. *ciceris* (Foc) is a soil-borne disease widespread in most of chickpea growing areas. Foc is a saprophytic fungus that may survive in soil or debris up to six years causing big yield losses in years of severe outbreaks of the disease [2]. The fungus penetrates the plant via roots, the germ tube penetrates the

epidermal cells of plants and later the hyphae extend to root cortical region and colonizes the
xylem vessels, thus, preventing the upward translocation of water and essential solutes, result-
ing in wilt [3–5]. *F. oxysporum* f. sp. *ciceris* is pathogenic only on *Cicer* spp. [6] but can also
invade root tissues of other grain legumes such as faba bean (*Vicia faba*), lentil (*Lens culinaris*),
pea (*Pisum sativum*) and pigeonpea (*Cajanus cajans*) without causing external symptoms [5].

Eight physiological races of the pathogen (0, 1A, 1B/C, 2, 3, 4, 5 and 6) have been reported
to infect chickpea. Fusarium race 5 (Foc5) is one of the main problem in the Mediterranean
area. In chickpea, resistance reaction inheritance to different races has been reported to be
monogenic or oligogenic depending on the race or source of resistance [7,8]. Several studies
reported that resistance to Foc5 is controlled by a single gene located on linkage group 2
(LG2) of the chickpea genetic map [9,10]. This gene is clustered with other genes conferring
resistance to races 0, 1, 2, 3 and 4. The STMS (sequence tagged microsatellite site) marker
TA59 has been widely reported as the most associated with that cluster, and hence it has being
used in marker assisted selection [11,12]. A deeper knowledge of this genomic region could
provide new markers useful for breeding purposes as well as a better understanding of resis-
tance mechanisms. The recognition and defense by a host plant to its fungal pathogen and the
ability of the pathogen to overcome the plant defences, implies a very complex molecular net-
work. In the plant-pathogen interaction four phases can be highlighted: pathogen perception,
penetration, colonization and disease establishment [13]. In the last decade, numerous tran-
scriptome, metabolome and proteome studies have been carried out on chickpea-Foc interac-
tion to unveil the hidden clues behind the defense pathways, but all of them focused on Foc
race 1. In general, these studies reported that molecular changes in carbon and nitrogen
metabolism, in reactive oxygen species (ROS), in primary metabolites (amino acids and sug-
ars), in lignification and in phytoalexins are related with resistant reactions [13–22]. Neverthe-
less, as far as we know, there are not molecular studies reporting candidate genes for resistance
to Foc5.

In a previous study, the comparison between genetic and physical chickpea maps of segre-
gant plant material and near isogenic lines (NILs) made it possible to narrow down to 820 kb
the area of chromosome 2 (Ca2) where the resistance gene for Foc5 is located [23]. NILs pro-
vide plant material differing only in a small target region of the genome facilitating the detec-
tion of candidate genes underlying phenotypes. In chickpea, NILs have been used to produce
fine maps and targeting genomic regions associated with agronomic traits [24–26].

The aim of the present study was to determine differential expression of candidate genes
for resistance to Foc5. To achieve that goal, we selected a set of genes located within the region
of interest in LG2, and we analyzed their expression profile in a pair of NILs (resistant vs sus-
ceptible) at different time points after inoculation with Foc5.

Materials and methods

Plant material

In this study we used a pair of NILs—RIP8-94-5 resistant (R) / RIP8-94-11 susceptible (S)—
segregant to race 5 that were developed by searching residual heterozygosity in advanced RILs
(Recombinant Inbred Lines) derived from the cross ILC3279 x WR315 [11]. Line ILC3279 is a
kabuli type from the former Soviet Union maintained by ICARDA (International Center for
Agricultural Research in the Dry Area) that is susceptible to Foc5. WR315 is a desi type from
central India maintained by ICRISAT (International Crops Research Institute for the Semi-
Arid Tropics) resistant to all Foc races [27]. In addition, Cr5-9, a selection from *C. reticulatum*
(PI489777), susceptible to all Foc races, was included as a control.

Fusarium oxysporum f. sp. *ciceris* race 5 inoculation

Seeds of each NILs were sown in trays (41 x 56 x 12 cm) filled with perlite. Plants were grown in controlled conditions under a temperature regime of 25 and 22˚C and 12 h photoperiod under fluorescent light. Filter papers with fungus spores provided by Dr. W. Chen (Washington State University, Pullman, USA) were added at PDB medium (potato dextrose broth, 24 g l^{-1}) and grown in a minitron incubator chamber at 25˚C and 100 rpm under continuous fluorescent light. The liquid cultures were filtered through cheesecloth to remove the mycelium. The spore suspension was then pelleted by centrifugation at low speed (3,000 rpm) for 3 min. After that, the supernatant was discarded and the concentration of spores was adjusted to 1 x 10^6 spores ml^{-1}. Plants at the three to four node stage were inoculated following the method described by Bhatti [28]. After inoculation, the plants were watered daily and supplied with nutrient solution once a week. Root samples were collected and pooled from at least 4 inoculated and non-inoculated plants at 24, 48 and 72 hours post-inoculation (hpi). Samples were frozen in liquid nitrogen immediately after harvesting and stored at −80˚C. Two biological repetitions per time-point were performed. Ten inoculated plants remained non-harvested to verify that the inoculation was successful.

RNA isolation, cDNA synthesis and quality controls

Total RNA from all samples was isolated using the TRISURE reagent protocol (Bioline). RNA concentration was determined by measuring the optical density using a NanoDrop spectrophotometer. Only the RNA samples with A260/A280 ratio between 1.9 and 2.1 and A260/A230 greater than 2.0 were used in the analysis. To avoid any genomic DNA (gDNA) contamination, ~10μg of RNA extracts were treated with TURBO DNase I (Life Technologies) before to cDNA synthesis. Complementary DNAs was synthesized by priming with oligodT$_{12-18}$ (Life Technologies), using SuperScript III Reverse Transcriptase (Invitrogen) following the instructions of the provider. The cDNAs were diluted to a final volume of 20μl. Next, we tested the presence of genomic DNA (gDNA) contamination in the cDNA samples using a primer pair designed in two different exons of the NAD-dependent malic chickpea sequence XM_004510782 (gDNAF, 5'-GTTGATACCAGCAGCAGCAAC-3'; gDNAR, 5'-TTAGTGCCAAAGACAAAGGGGA-'3'). The primer pair was designed to amplify a product of 555 bp using gDNA as template or 180 bp using cDNA as template. In our tests for gDNA contamination, the 555 bp band was not amplified from any of the samples. To infer the integrity of the total RNA and assess the quality of the reverse transcriptase reaction we used a 3':5' amplification ratio assessment [29]. This assay aimed at measuring the integrity of the NAD-dependent malic sequence (XM_004510782). For this assay, we designed two primer pairs (malic_5'F, 5'-CGACCGTTGTCTGATTTTGTG-3'; malic_5'R, 5'-GGCCATTTTCAGAACCCCTAA-3'; and malic_3'F, 5'-GCTTCGAGCAGCAGTTGAAGA-3'; and malic_3'R, 5'-CTTTTGACATGTGTGCAAGTT-3') to amplify two cDNA fragments, one from the 5' end (81 bp) and one from the 3' region (80 bp) of the malic gene. The fragments are 1,180 and 460 bp, respectively, from the 3' end of the cDNA. The 3':5' amplification ratio of the malic cDNA fragments was calculated using the comparative Cq method [30]. The average ratio was 1.18 ± 0.59 (mean, sd). All ratios were < 1.5-fold. Only if ratios were > 4.4-fold would RNA quality be deemed inadequate [31]. Therefore, the cDNAs were judged to be suitable for qPCR analysis.

Primer design and quality controls

Primer sequences were designed to amplify 19 candidate genes within a genomic region of interest delimited between the markers TA59 and CaGM07922, covering 2 Mb, based on the

genetic fine-mapping of Foc5 susceptibility loci [23]. We also included three genes previously reported to be involved in molecular defence against Fusarium wilt obtained from a chickpea transcriptome upon Foc1 infection [32]. All PCR primers were tested for specificity using NCBI's BLAST software. Primers were designed using the following criteria: Tm of 60 ± 1°C and PCR amplicon lengths of 80–100 bp, yielding primer sequences with lengths of 19–23 nucleotides and GC contents of 40–80%. For predicting the secondary structure of the amplicons, we used MFOLD version 3.4 software with default settings of minimal free energy, 50 mM Na$^+$, 3 mM Mg^{2+}, and an annealing temperature of 60°C [33]. We chose primers that would yield amplicons with minimal secondary structures and melting temperatures that would not hamper annealing (S1 Fig). Designed primers were synthesized by Integrated DNA Technologies (Leuven, Belgium). Table 1 shows the primer sequence and the overall mean real-time PCR amplification efficiency of each primer pair (E) estimated from the data obtained from the exponential phase of each individual amplification plot and the equation $(1+E) = 10^{slope}$ using LinReg software and the criteria of including three-five fluorescent data points with R2≥0.998 to define a linear regression line [34].

Real-time qPCR assays

PCR reactions were carried out in a CFX Connect Real-Time System thermal cycler (Bio-Rad, Hercules, CA, USA) iTaq Universal SYBR Green Supermix (Bio-Rad) to monitor dsDNA synthesis. Reactions contained 1.5 μl of the diluted cDNA as a template and 0.2 μM of each primer in a total volume reaction of 10 μl. Master mix was prepared and dispensed into individual wells using electronic Eppendorf Xplorer$^{®}$ multipipettes (Eppendorf AG, Germany). The following standard thermal profile was used for all PCRs: polymerase activation (95°C for 3 min), amplification and quantification cycles repeated 40 times (95°C for 3 s, 60°C for 30 s). The specificity of the primer pairs was checked by melting-curve analysis performed by the PCR machine after 40 amplification cycles (60–95°C) and is shown in S2 Fig. Fluorescence was analyzed using Bio-Rad CFX Manager analysis software v2.1. All amplification plots were analyzed using a baseline threshold of 75 relative fluorescence units (RFU) to obtain Cq (quantification cycle) values for each gene-cDNA combination.

Reference genes selection and qPCR data analysis

For optimal normalization of data, we evaluated the stable gene expression of four references in our dataset. Two candidates encoding a phosphatase protein (*PP2A*), and pentatricopeptide repeat-containing protein (*PPR*) were chosen based on previous reports [35]. We also tested the expression of a transcription factor initiation IIA (*TFIIA*) which had happened to be one of the most stable genes under a variety of conditions [36]. Finally, we also designed primers to amplify a chickpea sequence (*Ca4g26410*) ortholog to an *Arabidopsi*s gene that had showed high stability values across developmental series in cross-species experiments [37,38]. To evaluate the stability of the reference genes, we used the geNorm algorithm and the coefficient of variation (CV) of normalized relative quantities based on the equations defined by the qBase framework [39,40]. Calculations were performed using the advanced quantification model with efficiency correction, multiple reference genes normalization and use of error propagation rules described by Hellemans [40]. The open-source interface for the statistics software R, RStudio (http://www.rstudio.com/), was used to perform exploratory data analysis and build clusters. To gain insights into the biological roles of genes used, we performed a search of Gene Ontology (GO) categories using the UniProt database [41].

Table 1. Primers for RT-qPCR. Chickpea identifiers, annotation NCBI, primer sequences, amplicon size and chromosome position. Primer PCR efficiency and PCR product Tm data represent mean values ± sd. PCR efficiencies (E) calculated according to the equation $(1 + E) = 10^{slope}$.

Chickpea ID NCBI	Annotation	Primer Sequence (5'-3')	PCR product size (bp)	Chr position	PCR E	PCR product Tm (°C)
LOC101503802	kinesin-like protein FRA1	F-TGCTTCCATTCCACCGAAGCCTG R-TTGCTTTTGCATGGCCCGTGGT	82	Ca2: 23082299–23082381	2.02 ± 0.09	75.77 ± 0.25
LOC101505941	serine/threonine-protein kinase CTR1	F-CGCCTGAGTGGATGGCTCCAGA R-GGTCACGAGTTCCCACAGGATCA	98	Ca2: 23219419–23219517	1.99 ± 0.07	78.06 ± 0.16
LOC101506693	GATA transcription factor 25-like	F-AGGATTCTGGGCAGGACGACAG R-CGCCGCATCATTGGGGTCGATT	85	Ca2: 23277155–23277240	1.94 ± 0.05	78 ± 0
LOC101507659	E3 ubiquitin-protein ligase UPL1-like	F-AGTGCTGCATCGCCAGTTATCCA R-GCCGAGCCTTGTCCTCCTTGCT	73	Ca2: 23441535–23441608	1.95 ± 0.08	77.88 ± 0.29
LOC101508507	probable rhamnogalacturonate lyase C	F-CCAACGGGGTAGCGTTTGTGGT R-GCATCTCCTGGTGGTGCCAATCC	108	Ca2: 23463108–23463216	1.96 ± 0.07	77 ± 0.11
LOC101509037	XIAP-associated factor 1-like	F-GGTGTCCGACGACGAAGG R-ACAGCAATACCGGTGATGGC	80	Ca2: 23476846–23476926	1.98 ± 0.09	77.5 ± 0
LOC101509359	MADS-box transcription factor 23-like	F-ACGGTTTGTTGAAGAAGGCGAAGG R-TGATCTCATGCTGGTGCTAGCGAA	116	Ca2: 23551043–23551159	1.95 ± 0.06	77.43 ± 0.17
LOC101510206	serine hydroxymethyltransferase, mitochondrial-like	F-GGCTCGAGGGTTGAGAAGGTGT R-TGCCCCCAGGAACCATAGCAGA	94	Ca2: 23674613–23674707	1.99 ± 0.07	77.16 ± 0.23
LOC101510544	26S protease regulatory subunit 8 homolog A-like	F-GCCAGGGAGCATGCACCATCAA R-GCGCTGCACCTCACTATCACCA	105	Ca2: 23699688–23699793	1.97 ± 0.07	78.52 ± 0.10
LOC101511605	CBL-interacting serine/threonine-protein kinase 8	F-TGCTTCGGACAACTTGCGGGAC R-CCCACACGACCAAACATCCGCT	98	Ca2: 23829711–23829809	1.97 ± 0.05	76.74 ± 0.23
LOC101495287	auxin-responsive protein IAA8-like	F-TGAGAGGCCTCCTGGTGTCTGTG R-CCTGTGCCTTGGTAGCTGGTGC	92	Ca2: 23950935–23951027	1.97 ± 0.07	76.71 ± 0.25
LOC101495941	MATE efflux family protein 5-like	F-GGTGGGGTGGCAATAGCAATGGT R-AGCTTGGTGGGACCCATGATTGT	109	Ca2: 24038256–24038365	1.94 ± 0.06	74.09 ± 0.21
LOC101496824	sucrose transport protein SUC4	F-ATCTGGCTTTGCGGCCCAGTTT R-GTCGACCGAATCGGCTTTGGCA	88	Ca2: 24044382–24044470	1.93 ± 0.06	79.64 ± 0.14
LOC101497351	bidirectional sugar transporter N3-like	F-ATGCATGGTTCCCTTCGAGTCCA R-GCCACAATGCATAGAGGTGCTGC	89	Ca2: 24085883–24085972	1.96 ± 0.08	76.84 ± 0.25
LOC101497678	glycerol-3-phosphate dehydrogenase [NAD(+)] 2, chloroplastic	F-AACCGGTCTGTCAGGAACTGGAG R-TCGCCTGATCCAAGACGCACAC	93	Ca2: 24092611–24092704	1.96 ± 0.07	76.7 ± 0
LOC101502928	WRKY transcription factor 55	F-AGCACCATTATCATATCCACCAC R-ACAATTGGGGAGAAATGGTGGT	80	Ca2: 24128013–24128093	1.96 ± 0.06	68.87 ± 0.10
LOC101499218	E3 ubiquitin-protein ligase SHPRH	F-CAACACGTGGTCCTTGTTGAGCC R-TGTCCAATCCGATGTACGCGACT	80	Ca2: 24209104–24209184	1.91 ± 0.07	77.7 ± 0.14
LOC101501552	probable inactive receptor kinase At5g58300	F-GGTGGGTGAAGTCTGTGGTTTCTGA R-TCTGAAGCATCTGCACCATCTCCTC	108	Ca2: 24365119–24365227	1.95 ± 0.10	76.29 ± 0.16
LOC101510320	pathogenesis-related protein 5	F-GCTGCACATTTGATGCAACGGGA R-TGGAGCTGCACCATTCCCATCAC	89	Ca2: 24740222–24740311	1.93 ± 0.05	76.88 ± 0.24
LOC101499873	TMV resistance protein N-like	F-TGACCTTACGTGGCACCGCTAT R-GCTGCGGATCATTTGACAACCCA	126	Scaffold	1.97 ± 0.06	76.74 ± 0.22
LOC101490851	20 kDa chaperonin, chloroplastic-like	F-CCAGGGTCTGTGGATGAGGAAGG R-GCCCTTGAAGTCATTCCCCGCA	99	Ca7: 3352808 - 3352906	1.96 ± 0.06	74.91 ± 0
LOC105852647	MLP-like protein 28	F-TGGTGGAGATATTGATGAGCACT R-CGGAAGCACTACCATCAGCC	80	Ca7: 45480380 - 45480459	1.97 ± 0.08	72.19 ± 0.25

https://doi.org/10.1371/journal.pone.0224212.t001

Results and discussion

In the present study, we compared the expression patterns of 22 potential defence-related genes in response to Foc5 inoculation by a real-time qPCR-based strategy. Over the past two decades, our laboratory has been working towards increasing the genetic variability of the cultivated chickpea species using a combination of inter- and intra-specific crosses that are evaluated for complex traits of agronomic importance, such as resistance to Fusarium wilt. Foc is the major soil-borne fungus affecting chickpeas globally and may cause important yield losses under favourable conditions [8]. Particularly, Foc0 and Foc5 races are present in Mediterranean basin, being Foc5 the most virulent. We have previously reported DNA markers targeting Foc0 and Foc5 resistance through linkage analyses, but those were not highly saturated areas [26,42–47]. Recently, we saturated a region located on Ca2, which is likely implicated in resistance to Foc5. That region is delimited by SNP markers and includes twenty-six genes [23]. In the present study we selected nineteen annotated genes within this genomic region. We also included three genes beyond the target region that have been related with chickpea resistance against Fusarium races 1, 2 and 4 in previous reports [32]. We selected a pair of NILs differing in their sensitivity to Foc5 to monitor the gene expression patterns during a time-course of 24-48-72 hours following the pathogen inoculation.

Disease development

We considered the experiment suitable for further molecular analysis when the susceptible genotype NIL (RIP8-94-11) and the positive control (Cr5-9) started developing a distinct overall yellow coloration, as compared to the normal appearance in non-inoculated plantlets about two weeks after inoculation with Foc5. Nearly five/six weeks after inoculation, the susceptible plants showed complete wilting, while the resistant inoculated NIL RIP8-94-5 and the non-inoculated plants showed normal healthy growth. Over the course of the experiment, the root length of non-inoculated samples was similar in both, resistant and susceptible lines. On the contrary, related to the inoculated plants, the resistant genotype increased the lateral root development, while the susceptible NIL showed a dark brown, and eventually, dead root system. Cross-sectioned stems of these samples revealed the xylem colonization by the pathogen, while resistant plants showed normal development (Fig 1).

qPCR assays

Before quantifying the transcript accumulation of the candidate genes, we paid close attention to the preparative stages, which were performed in compliance with the MIQE guidelines [48]. Thus, the main factors of the qPCR workflow that could affect the reliability of the data, such as RNA quality, DNAse treatment, two-step RT-qPCR, use of the same RT master mix that generated one cDNA batch, primer sequences avoiding secondary structures in the amplicon, and PCR efficiency correction, were carefully controlled during the experiment. Additional PCRs were also performed using chickpea genomic DNA as template and gene-specific primers confirming the absence of introns in the amplicon region. Reference genes selection was also a critical step in our analysis and we setup a pilot study to assess the best references to use as internal controls for the broad physiological and cellular changes that are expected to occur during the disease development. The pilot study indicated that *Ca4g26410* and *TFIIA* were the most stable reference genes with stability values lower than the threshold of $M < 0.5$ and $CV < 0.25$ as defined for acceptable reference genes [40] (S3 Fig). PCR efficiency (E) of the references was respectively: $E = 1.987 \pm 0.095$, $E = 1.943 \pm 0.071$ (mean ± sd). To validate the stable expression of the references in our material, we calculated the mean of their normalized

Fig 1. Cross-sectioned stems after six weeks of inoculation. (A) Resistant line to Fusarium race 5 (RIP8-94-5). (B) Susceptible line to Fusarium race 5 (RIP8-94-11).

relative quantities. Maximum expression difference between samples for *Ca4g26410* and *TFIIA* was 1.64-fold (S4 Fig).

Analysis of the interaction time course

We aimed to identify changes in expression levels related to the differential responses of the two NILs (S5 Fig). To best organize our findings, we first show the candidate genes changing in expression levels related to the differential responses against the Fusarium infection. This may be labelled as 'treatment-dependent regulation'. Next, we show the candidate genes that showed different levels between inoculated plants (resistant vs susceptible NIL; 'genotype-dependent regulation'). Their potential functions are also further discussed in the second part of this section.

Treatment-dependent regulation. Hierarchical clustering based on the expression levels of the ratios of the inoculated/non-inoculated plants is presented in Fig 2. Visualizing expression levels through hierarchical clustering gave a sense of the coordinated regulation of genes (Fig 2A). The analysis of their expression levels identified 2 major clusters (one gen remained outside these two clusters). The first cluster grouped 12 genes primarily induced by the resistant genotype at 24 hpi (Fig 2B). The pattern of these samples was similar to the profile exhibited by the susceptible plants at 72 hpi (Fig 2A). According to their GO term-based annotations, their encoded proteins are mostly connected to membranes and their integral components (GO:0016020 and GO:0016021), and associated with signal transduction activities (GO:0007165). The second cluster contained 9 genes mainly induced by the susceptible genotype at 48 hpi (Fig 2C). According to their GO term-based annotations, their proteins are
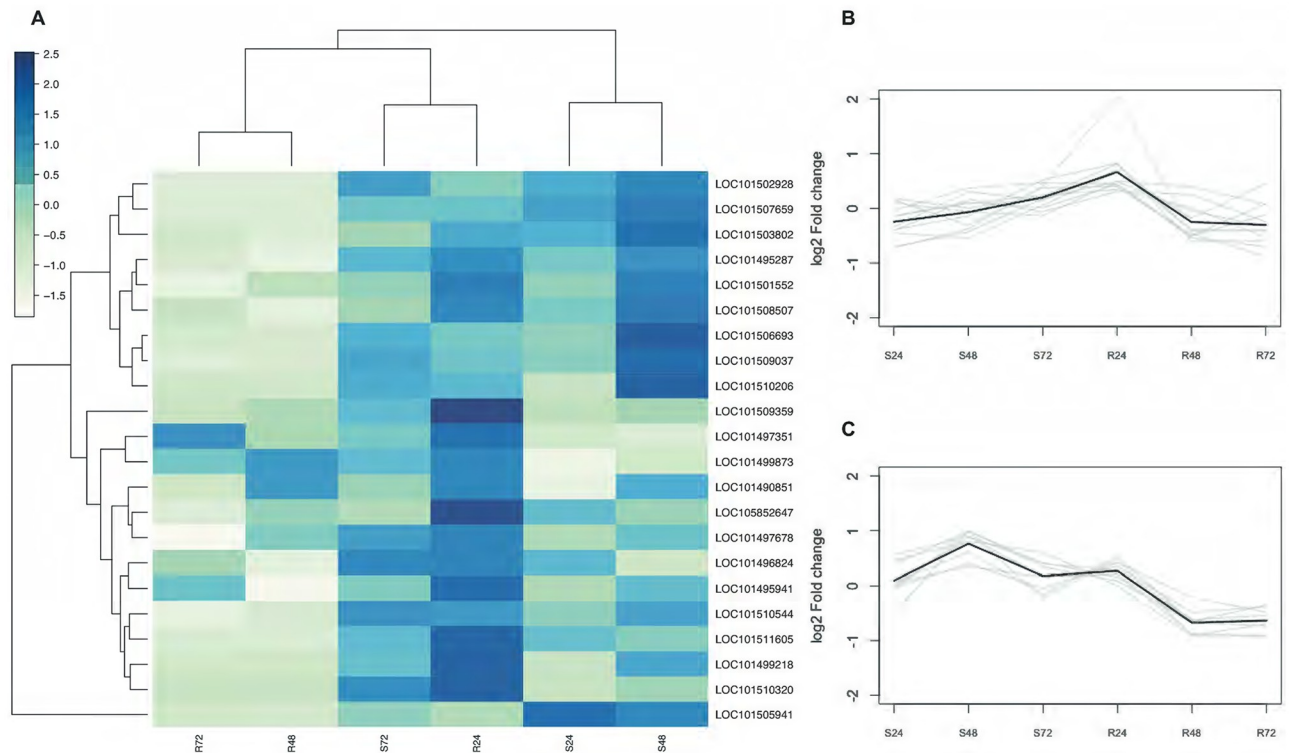
Fig 2. Hierarchical clustering analysis. (A) The heatmap was constructed using the log2-transformed expression levels. Columns represent sample comparisons, while rows represent candidate genes. Colour scale, representing log2 expression differences between inoculated/non-inoculated plants, is shown in the bar. R and S represent resistant and susceptible NILs; 24, 48 and 72 represent hours after inoculation. (B) Expression profiles of differentially expressed genes peaked at 24 hpi in roots of the resistant genotype. (C) Expression profiles of differentially expressed genes peaked at 48 hpi in roots of the susceptible genotype. The mean expression average of each cluster is shown in black.

mostly connected to nucleus (GO:0005634) and are involved in the regulation of transcription (GO:0006355).

Genotype-dependent regulation. The hierarchical expression profiling analysis revealed not only a differential set of genes expressed under the Foc5 infection, but also a distinct temporal pattern. Thus, we compared the expression profiles of inoculated plants, and found that genes with significantly higher levels in the resistant NIL RIP8-94-5 were mostly activated at 24 hpi, whereas other distinct genes showing higher levels in the susceptible NIL RIP8-94-11 were mostly regulated at 48 hpi. This may suggest that timing of gene regulation is relevant to pathogen recognition, so the host plant can trigger effective defense responses. The two groups of genes are discussed next.

In the first group some genes were found to show higher expression levels in the resistant NIL. The highest expression differences between inoculated plants—resistant vs susceptible—over the experiment were found in the genes LOC101509359 (encoding a MADS-box transcription factor), and LOC101499873 (TMV resistance protein). Both expression differences peaked at 24 hpi (S1 Table). At this time-point, in total 5 genes had higher expression levels in the resistant NIL (Fig 3). Three out of the five genes are located in the genomic region of interest in chromosome 2 [23]. As these loci (LOC101509359, LOC101495941 and LOC101510206) have not been previously linked to defence responses against Fusarium, we labelled them as novel genes. The other two genes have been shown to be induced under Foc1 infection and are located in chromosome 7 and one scaffold non-mapped to the genome [32].
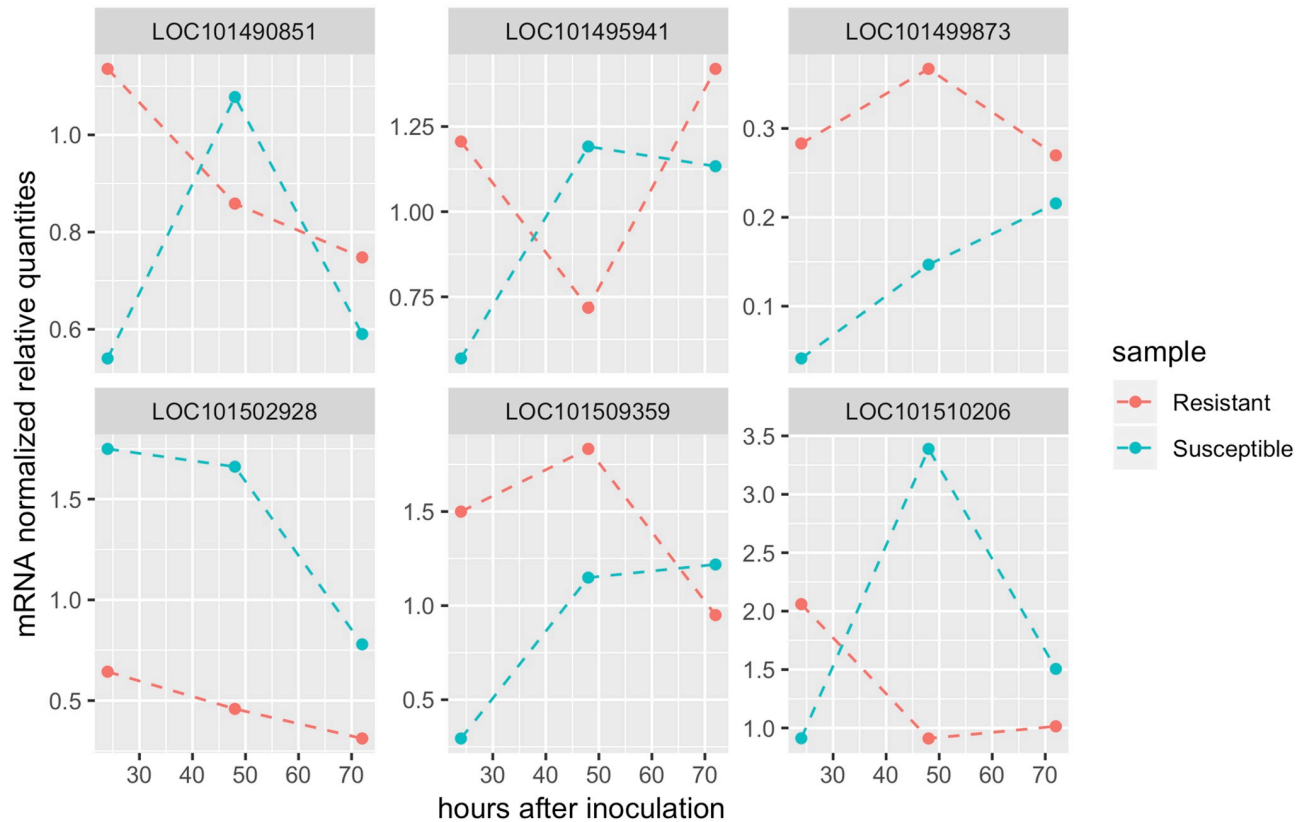
Fig 3. Expression profile of candidate genes in roots of chickpea over the time-course experiment. Red line shows the averaged expression level in resistant NILs, whereas blue line describes the averaged level in the susceptible NILs.

The novel genes, LOC101509359, encodes a MADS-box transcription factor. MADS-box genes are widely described as molecular signatures involved in developmental control and signal transduction in eukaryotes and play many critical regulatory roles [49,50]. In the past decade, a large set of plant MADS transcription factors has been demonstrated to function essentially in diverse biological processes, such as root architecture establishment, gametophyte differentiation, fruit ripening, flowering time regulation, and reproductive organ development [51]. However, stress resistance-related function of MADS-box genes is still not clear [52]. Some MADS-box genes have been found involved in stress-responsive processes beyond growth and development related functions in *Arabidopsis*, Chinese cabbage, wheat, rice, maize and tomato [53–58]. Besides, recent studies highlight that overexpression of a MADS-box transcription factor significantly promoted lateral root development [59,60]. This result is in agreement with the increase of lateral roots that we observed in the resistant inoculated plants.

Another differential expressed novel gene on Ca2 is LOC101495941, which encodes a protein belonging to the multidrug and toxic compound extrusion (MATE) family. MATE genes are involved in various biological activities such as leaf senescence, efflux of antibiotics, aluminium tolerance, vacuole sequestration of plant-derived alkaloids and flavonoids, iron homeostasis and regulation of local auxin biosynthesis [61]. These genes are important as secondary transporters that mediate chemical efflux and play significant roles in salt tolerance in plants [61–64]. In *Arabidopsis*, MATE genes have been shown to be associated with disease resistance [65–67]. Despite some genes in the MATE family have been related to seemingly

important physiological functions, most of them are still uncharacterized and the roles of MATE proteins in resistance to pathogens in legumes remains still unknown [68].

The third novel gene on Ca2 is LOC101510206, which encodes for a serine hydroxymethyl-transferase. This enzyme is responsible for interconversion of serine and glycine and is essential for cellular one-carbon metabolism providing one-carbon units for a series of important biosynthetic processes such as synthesis of methionine, thymidylates, and purines [69]. *Arabidopsis* mutants defective in serine hydroxymethyltransferase activities have been described more susceptible than control plants to infection with biotrophic and necrotrophic pathogens and have revealed interesting roles in influencing plant defense abilities [70–74]. In legumes, this gene class has been pointed out as responsible for resistance against the nematode *Heterodera glycines* [75]. The expression levels of LOC101510206 is an interesting finding as another locus from the same family mapped onto chromosome 8 has been found to be regulated in chickpea during Foc1 attack [17].

As mentioned above, two genes described previously in the literature as related to defence, LOC101499873 and LOC101490851, showed higher expression levels in the resistant than the susceptible plants at 24 hpi (Fig 3). LOC101499873 is located in a scaffold in the chickpea physical map and described like TMV resistance protein, while LOC101490851, located in chromosome 7, encodes a chaperonin, a molecule class reported to be essential for plants during biotic and abiotic stress conditions [76]. Despite the fact that these two genes are not located within the region of interest controlling the resistance against Foc5 in Ca2, this is an interesting outcome as their transcripts accumulation in early stages of the infection suggests a general network of defence independently of Foc race.

The second group was characterized by a number of genes showing higher expression levels in the susceptible NIL. Nine genes in cluster 2 (LOC101503802, LOC101505941, LOC101506693, LOC101507659, LOC101509037, LOC101510206, LOC101510544, LOC101501552, and LOC101502928; Fig 2C) showed significant higher expression levels in the susceptible NILs at 48 hpi than the resistant at the same time-point. This is the result of transcript induction by susceptible plants, whereas these genes, with the exception of LOC101502928, were not regulated by the resistant. However, LOC101502928, which encodes a WRKY transcription factor, was steadily down-regulated in the resistant over the course of the experiment (S1 Table). WRKY transcription factors may control the defence signalling cascade through a complicated network of genes [77,78]. The downregulation of a defence-related gene is intriguing but that pattern has been already observed in other studies, such as downregulation of the resistance gene GroES2 in chickpea inoculated with Foc1 [32]. Recently, the silencing of two apricot MATHd genes has been proved to confer resistance against the *Plum pox* virus [79]. There is a need to further explore the exact role of this gene and its interaction with others during defense to understand deeper the regulatory mechanism.

Concluding remarks

The comparative expression profile between resistant and susceptible inoculated plants showed that both lines are able to sense, and therefore, respond against the fungal attack. However, a number of quantitative and qualitative differences between the genotypes arose during the time-course. The susceptible NIL RIP8-94-11 induces a distinct set of genes that peaked at different time-point and we may speculate whether the response is not strong enough, or expressed at a non-appropriate timing. Conversely, the resistant NIL RIP8-94-5 activates early a defence signalling cascade to protect its primary metabolism from the harmful consequences of pathogenic mayhem, avoiding wilting, and highlighting that proper defence responses during the first hours of fungal attack are crucial to overcome the infection. In this study we have

shown that the resistant plants combine an active strategy of defence against Foc5 by up-regu-lating the expression of three genes (LOC101509359, LOC101495941 and LOC101510206) with a general defensive line independent of Foc race.

NILs, like those used in this study, have the advantage that only a small target region of the genome is segregating, consequently, the genetic background noise is uniform. Different expression patterns could be considered more reliable than other plant material. On the basis of this consideration future re-sequences of this pair of NILs could give us the opportunity to find key differences in their genomes.

Supporting information

S1 Fig. Modeling of secondary structures of the amplicons for the assays designed in this study. Thermodynamic stability (ΔG, kcal/mol) is presented in the figure. Primers are indicated by black arrows. Although some secondary structures might be present where primers anneal for some assays, they have a positive ΔG value and Tm < 60 ˚C, and hence will not influence the amplification efficiency.
(PDF)

S2 Fig. Dissociation curves for the 22 PCR products.
(PDF)

S3 Fig. Stability ranking of four reference genes from chickpea series. Numbers on top of the bars indicate the CV values of the reference involved in the normalization. References showing the highest stable expression ($M < 0.5$ and $CV < 0.25$) are represented in black colour.
(TIFF)

S4 Fig. Evaluation of reference genes for chickpea samples.
(TIFF)

S5 Fig. Alluvial diagram showing regulation of candidate genes over the time-course experiment in A) resistant, and B) susceptible plants.
(PDF)

S1 Table. Expression ratios of genes in resistant and susceptible plants over the time-course experiment. Values indicate log2 average expression ratios of infected plants (resistant / susceptible). Bold text indicates statistically significant regulation ($P < 0.05$).
(DOCX)

Acknowledgments

Author Contributions

Conceptualization: Cristina Caballo, Patricia Castro, Juan Gil, Teresa Millan, Josefa Rubio, Jose V. Die.

Formal analysis: Cristina Caballo, Patricia Castro, Jose V. Die.

Investigation: Cristina Caballo.

Methodology: Cristina Caballo, Patricia Castro, Josefa Rubio, Jose V. Die.

Supervision: Juan Gil, Teresa Millan, Josefa Rubio, Jose V. Die.

Writing – original draft: Cristina Caballo.

Writing – review & editing: Patricia Castro, Juan Gil, Teresa Millan, Josefa Rubio, Jose V. Die.

References

1. FAOSTAT (2017) Retrieved January 15, 2019 from http://faostat.fao.org/

2. Chen W, Sharma HC, Hari C, Muehlbauer FJ, Frederick J, American Phytopathological Society. Compendium of chickpea and lentil diseases and pests. St. Paul, Minn: APS Press; 2011.

3. Recorbet G, Steinberg C, Olivain C, Edel V, Trouvelot S, Dumas-Gaudot E, et al. Wanted: pathogenesis-related marker molecules for *Fusarium oxysporum*. New Phytol. 2003; 159: 73–92. https://doi.org/10.1046/j.1469-8137.2003.00795.x

4. Cho S, Muehlbauer FJ. Genetic effect of differentially regulated fungal response genes on resistance to necrotrophic fungal pathogens in chickpea (*Cicer arietinum* L.). Physiological and Molecular Plant Pathology. 2004; 64: 57–66. https://doi.org/10.1016/j.pmpp.2004.07.003

5. Jiménez-Díaz RM, Castillo P, Jiménez-Gasco M del M, Landa BB, Navas-Cortés JA. Fusarium wilt of chickpeas: Biology, ecology and management. Crop Prot. 2015; 73: 16–27. https://doi.org/10.1016/j.cropro.2015.02.023

6. Kaiser W, Alcala-Jimenez A, Hervas-Vargas A, Trapero-Casas J, Jiménez-Díaz R. Screening of wild Cicer species for resistance to races 0 to 5 of *Fusarium oxysporum* f. sp *ciceris*. Plant Dis. 1994; 78:962–967

7. Sharma KD, Muehlbauer FJ. Fusarium wilt of chickpea: physiological specialization, genetics of resistance and resistance gene tagging. Euphytica. 2007; 157: 1–14. https://doi.org/10.1007/s10681-007-9401-y

8. Jendoubi W, Bouhadida M, Boukteb A, Béji M, Kharrat M. Fusarium Wilt Affecting Chickpea Crop. Agriculture. 2017; 7: 23. https://doi.org/10.3390/agriculture7030023

9. Tekeoglu M, Tullu A, Kaiser WJ, Muehlbauer FJ. Inheritance and Linkage of Two Genes that Confer Resistance to Fusarium Wilt in Chickpea. Crop Sci. 2000; 40: 1247. https://doi.org/10.2135/cropsci2000.4051247x

10. Winter P, Benko-Iseppon AM, Hüttel B, Ratnaparkhe M, Tullu A, Sonnante G, et al. A linkage map of the chickpea (*Cicer arietinum* L.) genome based on recombinant inbred lines from a *C. arietinum×C. reticulatum* cross: localization of resistance genes for fusarium wilt races 4 and 5. Theor Appl Genet. 2000; 101: 1155–1163. https://doi.org/10.1007/s001220051592

11. Castro P, Pistón F, Madrid E, Millán T, Gil J, Rubio J. Development of chickpea near-isogenic lines for Fusarium wilt. Theor Appl Genet. 2010; 121: 1519–1526. https://doi.org/10.1007/s00122-010-1407-5 PMID: 20652529

12. Mannur DM, Babbar A, Thudi M, Sabbavarapu MM, Roorkiwal M, Yeri SB, et al. Super Annigeri 1 and improved JG 74: two Fusarium wilt-resistant introgression lines developed using marker-assisted backcrossing approach in chickpea (*Cicer arietinum* L.). Mol Breeding. 2019; 39: 2. https://doi.org/10.1007/s11032-018-0908-9 PMID: 30631246

13. Ashraf N, Basu S, Narula K, Ghosh S, Tayal R, Gangisetty N, et al. Integrative network analyses of wilt transcriptome in chickpea reveal genotype dependent regulatory hubs in immunity and susceptibility. Sci Rep. 2018; 8: 6528. https://doi.org/10.1038/s41598-018-19919-5 PMID: 29695764

14. Gupta S, Chakraborti D, Sengupta A, Basu D, Das S. Primary metabolism of chickpea is the initial target of wound inducing early sensed *Fusarium oxysporum* f. sp. *ciceri* race I. PLoS ONE. 2010; 5: e9030. https://doi.org/10.1371/journal.pone.0009030 PMID: 20140256

15. Gupta S, Bhar A, Chatterjee M, Das S. Fusarium oxysporum f.sp. ciceri race 1 induced redox state alterations are coupled to downstream defense signaling in root tissues of chickpea (*Cicer arietinum* L.). PLoS ONE. 2013; 8: e73163. https://doi.org/10.1371/journal.pone.0073163 PMID: 24058463

16. Gupta S, Bhar A, Das S. Understanding the molecular defence responses of host during chickpea–Fusarium interplay: where do we stand? Functional Plant Biol. 2013; 40: 1285. https://doi.org/10.1071/FP13063

17. Gupta S, Bhar A, Chatterjee M, Ghosh A, Das S. Transcriptomic dissection reveals wide spread differential expression in chickpea during early time points of *Fusarium oxysporum* f. sp. *ciceri* Race 1 attack. PLoS ONE. 2017; 12: e0178164. https://doi.org/10.1371/journal.pone.0178164 PMID: 28542579

18. Chatterjee M, Gupta S, Bhar A, Chakraborti D, Basu D, Das S. 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 Genomics. 2014; 15: 949. https://doi.org/10.1186/1471-2164-15-949 PMID: 25363865

19. Kumar Y, Dholakia BB, Panigrahi P, Kadoo NY, Giri AP, Gupta VS. Metabolic profiling of chickpea-Fusarium interaction identifies differential modulation of disease resistance pathways. Phytochemistry. 2015; 116: 120–129. https://doi.org/10.1016/j.phytochem.2015.04.001 PMID: 25935544

20. Kumar Y, Zhang L, Panigrahi P, Dholakia BB, Dewangan V, Chavan SG, et al. *Fusarium oxysporum* mediates systems metabolic reprogramming of chickpea roots as revealed by a combination of proteomics and metabolomics. Plant Biotechnol J. 2016; 14: 1589–1603. https://doi.org/10.1111/pbi.12522 PMID: 26801007

21. Sharma M, Sengupta A, Ghosh R, Agarwal G, Tarafdar A, Nagavardhini A, et al. Genome wide transcriptome profiling of *Fusarium oxysporum* f sp. *ciceris* conidial germination reveals new insights into infection-related genes. Sci Rep. 2016; 6: 37353. https://doi.org/10.1038/srep37353 PMID: 27853284

22. Upasani ML, Limaye BM, Gurjar GS, Kasibhatla SM, Joshi RR, Kadoo NY, et al. Chickpea-*Fusarium oxysporum* interaction transcriptome reveals differential modulation of plant defense strategies. Sci Rep. 2017; 7: 7746. https://doi.org/10.1038/s41598-017-07114-x PMID: 28798320

23. Caballo C, Madrid E, Gil J, Chen W, Rubio J, Millan T. Saturation of genomic region implicated in resistance to *Fusarium oxysporum* f. sp. *ciceris* race 5 in chickpea. Mol Breeding. 2019; 39: 16. https://doi.org/10.1007/s11032-019-0932-4

24. Ali L, Madrid E, Varshney RK, Azam S, Millan T, Rubio J, et al. Mapping and identification of a *Cicer arietinum* NSP2 gene involved in nodulation pathway. Theor Appl Genet. 2014; 127: 481–488. https://doi.org/10.1007/s00122-013-2233-3 PMID: 24247237

25. Ali L, Deokar A, Caballo C, Tar'an B, Gil J, Chen W, et al. Fine mapping for double podding gene in chickpea. Theor Appl Genet. 2016; 129: 77–86. https://doi.org/10.1007/s00122-015-2610-1 PMID: 26433827

26. Jendoubi W, Bouhadida M, Millan T, Kharrat M, Gil J, Rubio J, et al. Identification of the target region including the Foc0 1 /foc0 1 gene and development of near isogenic lines for resistance to Fusarium Wilt race 0 in chickpea. Euphytica. 2016; 210: 119–133. https://doi.org/10.1007/s10681-016-1712-4

27. Sharma KD, Chen W, Muehlbauer FJ. Genetics of Chickpea Resistance to Five Races of Fusarium Wilt and a Concise Set of Race Differentials for *Fusarium oxysporum* f. sp. *ciceris*. Plant Dis. 2005; 89: 385–390. https://doi.org/10.1094/PD-89-0385 PMID: 30795454

28. Bhatti MA. Effects of inoculum density and temperature on root rot and wilt of chickpea. Plant Dis. 1992; 76: 50. https://doi.org/10.1094/PD-76-0050

29. Die JV, Román B. RNA quality assessment: a view from plant qPCR studies. J Exp Bot. 2012; 63: 6069–6077. https://doi.org/10.1093/jxb/ers276 PMID: 23045609

30. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008; 3: 1101–1108. PMID: 18546601

31. Die JV, Obrero Á, González-Verdejo CI, Román B. Characterization of the 3':5' ratio for reliable determination of RNA quality. Anal Biochem. 2011; 419: 336–338. https://doi.org/10.1016/j.ab.2011.08.012 PMID: 21889484

32. Gurjar GS, Giri AP, Gupta VS. Gene Expression Profiling during Wilting in Chickpea Caused by *Fusarium oxysporum* F. sp. *Ciceri*. AJPS. 2012; 03: 190–201. https://doi.org/10.4236/ajps.2012.32023

33. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 2003; 31: 3406–3415. https://doi.org/10.1093/nar/gkg595 PMID: 12824337

34. Ramakers C, Ruijter JM, Deprez RHL, Moorman AFM. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett. 2003; 339: 62–66. https://doi.org/10.1016/s0304-3940(02)01423-4 PMID: 12618301

35. Castro P, Román B, Rubio J, Die JV. Selection of reference genes for expression studies in *Cicer arietinum* L.: analysis of cyp81E3 gene expression against *Ascochyta rabiei*. Mol Breeding. 2011; 29: 261–274. https://doi.org/10.1007/s11032-010-9544-8

36. Die JV, Gil J, Millan T. Genome-wide identification of the auxin response factor gene family in *Cicer arietinum*. BMC Genomics. 2018; 19: 301. https://doi.org/10.1186/s12864-018-4695-9 PMID: 29703137

37. Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible W-R. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol. 2005; 139: 5–17. https://doi.org/10.1104/pp.105.063743 PMID: 16166256

38. Die JV, Rowland LJ. Superior cross-species reference genes: a blueberry case study. PLoS ONE. 2013; 8: e73354. https://doi.org/10.1371/journal.pone.0073354 PMID: 24058469

39. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.

Genome Biol. 2002; 3: research0034. https://doi.org/10.1186/gb-2002-3-7-research0034 PMID: 12184808

40. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 2007; 8: R19. https://doi.org/10.1186/gb-2007-8-2-r19 PMID: 17291332

41. Consortium UniProt. UniProt: a worldwide hub of protein knowledge. Nucleic Acids Res. 2019; 47: D506–D515. https://doi.org/10.1093/nar/gky1049 PMID: 30395287

42. Rubio J, Hajj-Moussa E, Kharrat M, Moreno MT, Millan T, Gil J. Two genes and linked RAPD markers involved in resistance to *Fusarium oxysporum* f. sp. *ciceris* race 0 in chickpea. Plant Breeding. 2003; 122: 188–191. https://doi.org/10.1046/j.1439-0523.2003.00814.x

43. Cobos MJ, Fernández MJ, Rubio J, Kharrat M, Moreno MT, Gil J, et al. A linkage map of chickpea (*Cicer arietinum* L.) based on populations from Kabuli x Desi crosses: location of genes for resistance to fusarium wilt race 0. Theor Appl Genet. 2005; 110: 1347–1353. https://doi.org/10.1007/s00122-005-1980-1 PMID: 15806343

44. Iruela M, Castro P, Rubio J, Cubero JI, Jacinto C, Millán T, et al. Validation of a QTL for resistance to ascochyta blight linked to resistance to fusarium wilt race 5 in chickpea (*Cicer arietinum* L.). Eur J Plant Pathol. 2007; 119: 29–37. https://doi.org/10.1007/s10658-007-9121-0

45. Cobos MJ, Winter P, Kharrat M, Cubero JI, Gil J, Millan T, et al. Genetic analysis of agronomic traits in a wide cross of chickpea. Field Crops Res. 2009; 111: 130–136. https://doi.org/10.1016/j.fcr.2008.11.006

46. Halila I, Rubio J, Millán T, Gil J, Kharrat M, Marrakchi M. Resistance in chickpea (*Cicer arietinum*) to Fusarium wilt race '0'. Plant Breeding. 2009; https://doi.org/10.1111/j.1439-0523.2009.01703.x

47. Palomino C, Fernández-Romero MD, Rubio J, Torres A, Moreno MT, Millán T. Integration of new CAPS and dCAPS-RGA markers into a composite chickpea genetic map and their association with disease resistance. Theor Appl Genet. 2009; 118: 671–682. https://doi.org/10.1007/s00122-008-0928-7 PMID: 19034411

48. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009; 55: 611–622. https://doi.org/10.1373/clinchem.2008.112797 PMID: 19246619

49. Wang L, Yin X, Cheng C, Wang H, Guo R, Xu X, et al. Evolutionary and expression analysis of a MADS-box gene superfamily involved in ovule development of seeded and seedless grapevines. Mol Genet Genomics. 2015; 290: 825–846. https://doi.org/10.1007/s00438-014-0961-y PMID: 25429734

50. Theißen G, Gramzow L. Structure and evolution of plant MADS domain transcription factors. Plant Transcription Factors. Elsevier; 2016. pp. 127–138.

51. Smaczniak C, Immink RGH, Angenent GC, Kaufmann K. Developmental and evolutionary diversity of plant MADS-domain factors: insights from recent studies. Development. 2012; 139: 3081–3098. https://doi.org/10.1242/dev.074674 PMID: 22872082

52. Saha G, Park J-I, Jung H-J, Ahmed NU, Kayum MA, Chung M-Y, et al. Genome-wide identification and characterization of MADS-box family genes related to organ development and stress resistance in *Brassica rapa*. BMC Genomics. 2015; 16: 178. https://doi.org/10.1186/s12864-015-1349-z PMID: 25881193

53. Lee B, Henderson DA, Zhu J-K. The Arabidopsis cold-responsive transcriptome and its regulation by ICE1. Plant Cell. 2005; 17: 3155–3175. https://doi.org/10.1105/tpc.105.035568 PMID: 16214899

54. Wei B, Cai T, Zhang R, Li A, Huo N, Li S, et al. Novel microRNAs uncovered by deep sequencing of small RNA transcriptomes in bread wheat (*Triticum aestivum* L.) and *Brachypodium distachyon* (L.) Beauv. Funct Integr Genomics. 2009; 9: 499–511. https://doi.org/10.1007/s10142-009-0128-9 PMID: 19499258

55. Lee I, Seo Y-S, Coltrane D, Hwang S, Oh T, Marcotte EM, et al. Genetic dissection of the biotic stress response using a genome-scale gene network for rice. Proc Natl Acad Sci USA. 2011; 108: 18548–18553. https://doi.org/10.1073/pnas.1110384108 PMID: 22042862

56. Zhao Y, Li X, Chen W, Peng X, Cheng X, Zhu S, et al. Whole-genome survey and characterization of MADS-box gene family in maize and sorghum. Plant Cell Tiss Organ Cult. 2011; 105: 159–173. https://doi.org/10.1007/s11240-010-9848-8

57. Khong GN, Pati PK, Richaud F, Parizot B, Bidzinski P, Mai CD, et al. Osmads26 negatively regulates resistance to pathogens and drought tolerance in rice. Plant Physiol. 2015; 169: 2935–2949. https://doi.org/10.1104/pp.15.01192 PMID: 26424158

58. Yin W, Hu Z, Hu J, Zhu Z, Yu X, Cui B, et al. Tomato (*Solanum lycopersicum*) MADS-box transcription factor SlMBP8 regulates drought, salt tolerance and stress-related genes. Plant Growth Regul. 2017; 83: 55–68. https://doi.org/10.1007/s10725-017-0283-2

59. Liu W, Han X, Zhan G, Zhao Z, Feng Y, Wu C. A Novel Sucrose-Regulatory MADS-Box Transcription Factor GmNMHC5 Promotes Root Development and Nodulation in Soybean (*Glycine max* [L.] Merr.). Int J Mol Sci. 2015; 16: 20657–20673. https://doi.org/10.3390/ijms160920657 PMID: 26404246

60. Sun C-H, Yu J-Q, Wen L-Z, Guo Y-H, Sun X, Hao Y-J, et al. Chrysanthemum MADS-box transcription factor CmANR1 modulates lateral root development via homo-/heterodimerization to influence auxin accumulation in *Arabidopsis*. Plant Sci. 2018; 266: 27–36. https://doi.org/10.1016/j.plantsci.2017.09.017 PMID: 29241564

61. Tanaka Y, Iwaki S, Tsukazaki T. Crystal structure of a plant multidrug and toxic compound extrusion family protein. Structure. 2017; 25: 1455–1460.e2. https://doi.org/10.1016/j.str.2017.07.009 PMID: 28877507

62. Saier MH, Paulsen IT. Phylogeny of multidrug transporters. Semin Cell Dev Biol. 2001; 12: 205–213. https://doi.org/10.1006/scdb.2000.0246 PMID: 11428913

63. Moriyama Y, Hiasa M, Matsumoto T, Omote H. Multidrug and toxic compound extrusion (MATE)-type proteins as anchor transporters for the excretion of metabolic waste products and xenobiotics. Xenobiotica. 2008; 38: 1107–1118. https://doi.org/10.1080/00498250701883753 PMID: 18668441

64. Min X, Jin X, Liu W, Wei X, Zhang Z, Ndayambaza B, et al. Transcriptome-wide characterization and functional analysis of MATE transporters in response to aluminum toxicity in *Medicago sativa* L. PeerJ. 2019; 7: e6302. https://doi.org/10.7717/peerj.6302 PMID: 30723620

65. Diener AC, Gaxiola RA, Fink GR. Arabidopsis *ALF5*, a multidrug efflux transporter gene family member, confers resistance to toxins. Plant Cell. 2001; 13: 1625–1638. https://doi.org/10.1105/TPC.010035 PMID: 11449055

66. Nawrath C. EDS5, an Essential Component of Salicylic Acid-Dependent Signaling for Disease Resistance in Arabidopsis, Is a Member of the MATE Transporter Family. THE PLANT CELL ONLINE. 2002; 14: 275–286. https://doi.org/10.1105/tpc.010376 PMID: 11826312

67. Tiwari M, Sharma D, Singh M, Tripathi RD, Trivedi PK. Expression of OsMATE1 and OsMATE2 alters development, stress responses and pathogen susceptibility in *Arabidopsis*. Sci Rep. 2014; 4: 3964. https://doi.org/10.1038/srep03964 PMID: 24492654

68. Wang J, Hou Q, Li P, Yang L, Sun X, Benedito VA, et al. Diverse functions of multidrug and toxin extrusion (MATE) transporters in citric acid efflux and metal homeostasis in *Medicago truncatula*. Plant J. 2017; 90: 79–95. https://doi.org/10.1111/tpj.13471 PMID: 28052433

69. Wu X-Y, Zhou G-C, Chen Y-X, Wu P, Liu L-W, Ma F-F, et al. Soybean cyst nematode resistance emerged via artificial selection of duplicated serine hydroxymethyltransferase genes. Front Plant Sci. 2016; 7: 998. https://doi.org/10.3389/fpls.2016.00998 PMID: 27458476

70. Moreno JI, Martín R, Castresana C. Arabidopsis SHMT1, a serine hydroxymethyltransferase that functions in the photorespiratory pathway influences resistance to biotic and abiotic stress. Plant J. 2005; 41: 451–463. https://doi.org/10.1111/j.1365-313X.2004.02311.x PMID: 15659103

71. Voll LM, Jamai A, Renné P, Voll H, McClung CR, Weber APM. The photorespiratory Arabidopsis *shm1* mutant is deficient in *SHM1*. Plant Physiol. 2006; 140: 59–66. https://doi.org/10.1104/pp.105.071399 PMID: 16339799

72. Zhang Y, Sun K, Sandoval FJ, Santiago K, Roje S. One-carbon metabolism in plants: characterization of a plastid serine hydroxymethyltransferase. Biochem J. 2010; 430: 97–105. https://doi.org/10.1042/BJ20100566 PMID: 20518745

73. Engel N, Ewald R, Gupta KJ, Zrenner R, Hagemann M, Bauwe H. The presequence of Arabidopsis serine hydroxymethyltransferase SHM2 selectively prevents import into mesophyll mitochondria. Plant Physiol. 2011; 157: 1711–1720. https://doi.org/10.1104/pp.111.184564 PMID: 21976482

74. Wei Z, Sun K, Sandoval FJ, Cross JM, Gordon C, Kang C, et al. Folate polyglutamylation eliminates dependence of activity on enzyme concentration in mitochondrial serine hydroxymethyltransferases from *Arabidopsis thaliana*. Arch Biochem Biophys. 2013; 536: 87–96. https://doi.org/10.1016/j.abb.2013.06.004 PMID: 23800877

75. Liu S, Kandoth PK, Warren SD, Yeckel G, Heinz R, Alden J, et al. A soybean cyst nematode resistance gene points to a new mechanism of plant resistance to pathogens. Nature. 2012; 492: 256–260. https://doi.org/10.1038/nature11651 PMID: 23235880

76. Rutherford SL. Between genotype and phenotype: protein chaperones and evolvability. Nat Rev Genet. 2003; 4: 263–274. https://doi.org/10.1038/nrg1041 PMID: 12671657

77. Phukan UJ, Jeena GS, Shukla RK. WRKY transcription factors: molecular regulation and stress responses in plants. Front Plant Sci. 2016; 7: 760. https://doi.org/10.3389/fpls.2016.00760 PMID: 27375634

78. Song H, Sun W, Yang G, Sun J. WRKY transcription factors in legumes. BMC Plant Biol. 2018; 18: 243. https://doi.org/10.1186/s12870-018-1467-2 PMID: 30332991

79. Zuriaga E, Romero C, Blanca JM, Badenes ML. Resistance to *Plum Pox Virus* (PPV) in apricot (*Prunus armeniaca* L.) is associated with down-regulation of two *MATHd* genes. BMC Plant Biol. 2018; 18: 25. https://doi.org/10.1186/s12870-018-1237-1 PMID: 29374454