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The transcriptional response to yellow and wilt disease, caused by race 6 of *Fusarium oxysporum* f. sp. *Ciceris* in two contrasting chickpea cultivars

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Abstract

Background Chickpea (*Cicer arietinum* L.) ranks as the third most crucial grain legume worldwide. Fusarium wilt (*Fusarium oxysporum* f. sp. *ciceri* (Foc)) is a devastating fungal disease that prevents the maximum potential for chickpea production.

Results To identify genes and pathways involved in resistance to race 6 of Foc, this study utilized transcriptome sequencing of two chickpea cultivars: resistant (Ana) and susceptible (Hashem) to Foc race 6. Illumina sequencing of the root samples yielded 133.5 million raw reads, with about 90% of the clean reads mapped to the chickpea reference genome. The analysis revealed that 548 genes (332 upregulated and 216 downregulated) in the resistant genotype (Ana) and 1115 genes (595 upregulated and 520 downregulated) in the susceptible genotype (Hashem) were differentially expressed under Fusarium wilt (FW) disease stress caused by Foc race 6. The expression patterns of some differentially expressed genes (DEGs) were validated using quantitative real-time PCR. A total of 131 genes were exclusively upregulated under FW stress in the resistant cultivar, including several genes involved in sensing (e.g., *CaNLR-RPM1*, *CaLYK5-RLK*, *CaPR5-RLK*, *CaLRR-RLK*, and *CaRLP-EIX2*), signaling (e.g., *CaPP7*, *CaEPS1*, *CaSTY13*, and *CaPR-1*), transcription regulation (e.g., *CaMYBs*, *CaGLK*, *CaERFs*, *CaZAT11-like*, and *CaNAC6*) and cell wall integrity (e.g., *CaPGI2-like*, *CaEXLs*, *CaCSLD* and *CaCYP73A100-like*).

Conclusions The achieved results could provide insights into the molecular mechanism underlying resistance to FW and could be valuable for breeding programs aimed at developing FW-resistant chickpea varieties.

Keywords Chickpea, Biotic stress, Fusarium wilt (race 6), RNA sequencing

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Background

Chickpea (*Cicer arientinum* L.), classified as the third most important grain legume, is commonly known as a low-cost protein source for both livestock feed and human consumption [1]. It contains many carbohydrates, lipids, minerals, vitamins, and other nonnutritive compounds that are beneficial to health [2]. Moreover, it has a significant impact on sustaining agricultural systems through nitrogen fixation, similar to other legumes [3]. Chickpea is produced about 16 million tons annually [4]. It is mainly cultivated in 50 countries and grown in a variety of ecological conditions; however, its yield and quality are affected by several challenges, such as fungal diseases [5, 6].

Among the fungal diseases, Fusarium wilt (FW) induced by *Fusarium oxysporum* f. sp. *ciceris* (Foc) is a widespread soil-borne disease that causes a significant reduction in chickpea production [7]. According to reports, Foc penetrates the roots through epidermal cells, and subsequently, hyphae spread to the root cortex region, where they colonize the xylem vessels. All of these factors prevent the upward movement of water and essential solutes, which results in wilting. The initial symptom of the infection is wilting, which ultimately results in death [8, 9]. Fusarium wilt epidemics can result in the destruction or complete loss of crops in fields that are highly infested under favorable conditions [10].

Eight distinct physiological races of the pathogen, identified as 0, 1 A, 1B/C, 2, 3, 4, 5, and 6, have been found to infect chickpea [10]. Races 1 A, 2, 3, 4, 5, and 6 cause wilting symptoms in chickpea, characterized by flaccidity, severe chlorosis, and vascular discoloration, eventually leading to plant death. In contrast, races 1B/C and 0 are less virulent, causing only yellowing symptoms [11].

Despite the availability of complete sequencing of desi and kabuli chickpea genomes [12, 13], the genes responsible for Fusarium resistance are poorly understood. Recently, researchers have employed the RNA-seq technique to identify stress-related genes involved in the response to FW. These authors reported several overrepresented genes related to the defense signaling pathway, disease resistance, and cell wall biogenesis in root tissues [14–19]. Various attempts have been made to dissect the genetic basis of resistance to different FW races in chickpea. Thus, some genes and quantitative trait loci (QTLs) have been identified in various chickpea genotypes to be associated with resistance to certain Foc races. However, limited data are available regarding the genetics of resistance to some Fusarium races, such as races 6 and 1B/C [16, 19].

Herein, we conducted a comparative transcriptome study of root tissues of Iranian contrasting genotypes named Ana (a resistant genotype) and Hashem (a susceptible genotype) to Foc race 6. This research focused on root tissues as the initial organ, which serves as the first line of defense against phytopathogens and is crucial for sensing and signaling during Fusarium infection. Our results revealed differential expression patterns between resistant and susceptible cultivars 48 h after the onset of infection. This study investigated transcriptome dynamics linked to FW disease in chickpea, highlighting crucial factors that influence disease resistance. Specific and differential expression patterns of genes were identified between resistant and susceptible cultivars. These findings provide a basis for further investigations to elucidate the molecular mechanisms underlying resistance in cultivars compared with susceptibility during Fusarium infection.

Results

Phenotyping

The first Fusarium disease symptoms appeared on the lower leaves of the plants approximately 12 days after inoculation. The symptoms started with slight yellowing at the leaf edges and progressed to twisting and complete yellowing. Finally, the leaves become necrotic and fall. The reaction of two cultivars, Ana and Hashem, to Fusarium disease (Race 6) under controlled conditions was marked by early onset of wilt symptoms (distinct and consistent disease phenotypes), based on the method proposed by Sharma et al. in 2005 [20]. The percentages of FW incidence in the Ana and Hashem cultivars were 20% (resistant) and 80% (susceptible), respectively (Supplementary Figure S1 and S2).

Sequencing metrics and mapping results

In total, 133.5 million raw reads were obtained from the root samples of the Ana and Hashem cultivars under control and FW stress conditions, and more than 89.15% of the raw reads presented Phred quality scores at the Q30 level. Additionally, almost 90% of the clean reads were mapped to the chickpea reference genome (Table 1). The assembly of mapped reads led to the recognition of 61,997 transcript isoforms and 31,177 genes. The results indicated that 548 (332 up- and 216 downregulated) and 1115 (595 up- and 520 downregulated) genes were differentially expressed under Foc race 6 stress in the resistant and susceptible cultivars, respectively (Fig. 1).

Gene ontology classification analysis for DEGs

Based on the GO analysis, significant GO terms were assigned to 193 (of 548) DEGs in Ana and 333 (of 1115) DEGs in Hashem. GO classification of the upregulated genes demonstrated that several biological procedures, including response to stimulus, response to stress, response to biotic stimulus, defense response,

Table 1 Summary statistics of the transcriptome reads and their mapping to the reference genome

Reads mapping	Reads number (%)		
Sample	Ana Control	Ana Treated 1	Ana Treated 2
Total reads	20,974,728	22,881,594	21,195,569
Total mapped reads	19,069,583 (90.92%)	20,879,587 (91.25%)	19,082,588 (90.03%)
Unique match	18,203,933 (86.79%)	19,924,028 (87.07%)	18,397,275 (86.8%)
Multiposition match	865,650 (4.13%)	955,559 (4.18%)	685,313 (3.23%)
Total unmapped reads	1,905,145 (9.08%)	2,002,007 (8.75%)	2,112,981 (9.97%)
Sample	Hashem Control	Hashem Treated 1	Hashem Treated 2
Total reads	23,529,811	23,872,344	21,044,139
Total mapped reads	21,229,993 (90.22%)	21,429,429 (89.77%)	18,890,896 (89.76%)
Unique match	20,299,725 (86.27%)	20,633,219 (86.43%)	18,132,422 (86.16%)
Multiposition match	930,268 (3.95%)	796,210 (3.34%)	758,474 (3.6%)
Total unmapped reads	2,299,818 (9.77%)	2,442,915 (10.23%)	2,153,243 (10.23%)

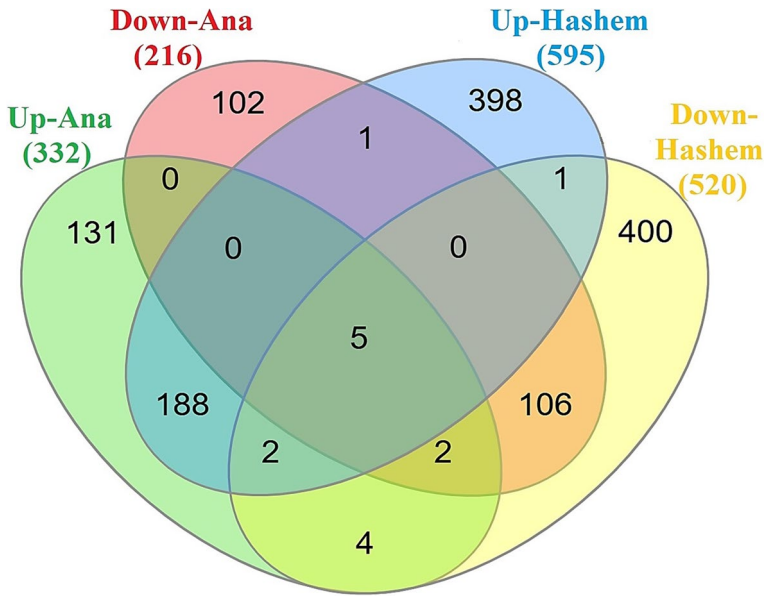

Fig. 1 Venn diagram of the DEGs under Fusarium wilt stress revealed the genes that were either exclusively or commonly differentially expressed in the resistant (Ana) and susceptible (Hashem) cultivars. Up: Upregulated; Down: Downregulated

immune response, response to fungus and lignin biosynthetic/metabolic process were significantly enriched in both cultivars, with their percentages being greater in the resistant cultivar than in the susceptible cultivar (Fig. 2A). Remarkably, some biological processes, such as the response to ethylene stimulus, and systemic acquired resistance, were significantly enriched only in the resistant cultivar (Fig. 2A). Likewise, some molecular function (MF) terms, such as catalytic activity, transferase activity, glycosyltransferase activity, cellulose synthase activity, and chitinase activity were enriched among the upregulated genes of both cultivars; however, their percentages in the Ana cultivar were greater than those in the Hashem cultivar (Fig. 2A). The terms lyase activity, lipase activity, triglyceride lipase activity, cell wall biogenesis, intramolecular lyase activity and chitinase activity were significantly enriched only in the Ana cultivar (Fig. 2A and B). On the one hand, GO classification of the downregulated genes revealed that several biological procedures (e.g., response to biotic stimulus, response to oxidative stress, and lignin metabolic/ biosynthetic process), molecular functions (e.g., transporter activity, transcription regulator activity, hydrolase activity and electron carrier activity), and cellular components

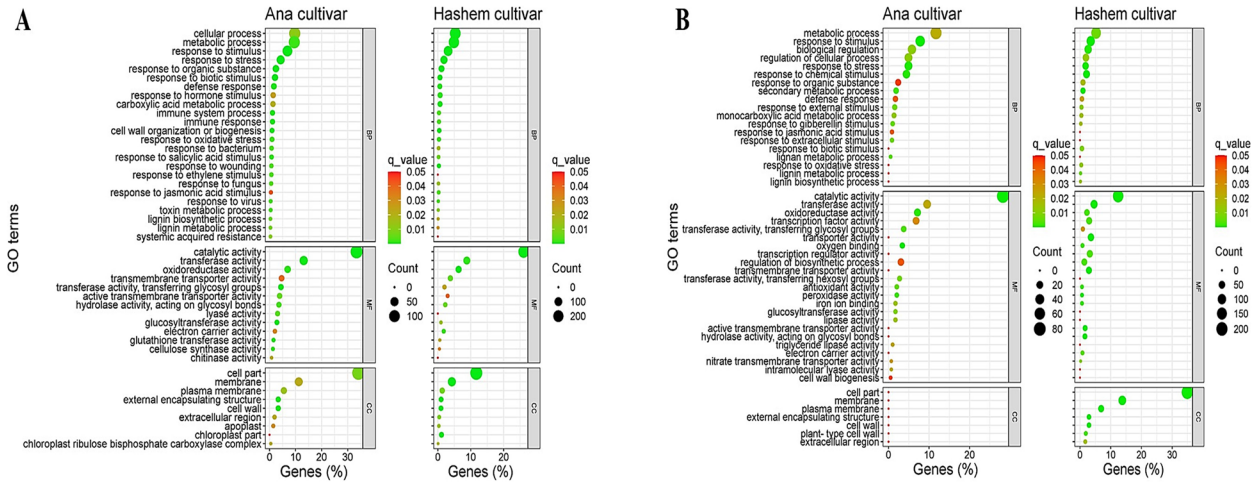

Fig. 2 Gene Ontology (GO) classification of differentially expressed genes (DEGs) in the Ana and Hashem cultivars based on three main categories: biological process (BP), molecular function (MF) and cellular component (CC). (**A**) bubble diagram: upregulated genes, (**B**) bubble diagram: downregulated genes: The X-axis represents the percentage of genes (%), and the Y-axis represents the GO terms. The color scales indicate the different thresholds for the q-values, and the sizes of the dots represent the number of genes corresponding to each GO term

(e.g., cell part, membrane, cell wall, external encapsulating structure, and extracellular region) were exclusively enriched in the hashem cultivar (Fig. 2B). This implies an inefficient defense response against fungal pathogens, leading to disease susceptibility.

Pathway analysis of DEGs

To achieve a more comprehensive understanding of the pathways involved in the response of each cultivar to FW stress, a BLAST search against the KEGG protein database was carried out [21, 22]. In total, 218 of the 548 Ana significant DEGs were categorized into 121 KEGG pathways, and 443 of the 1115 Hashem significant DEGs were categorized into 173 KEGG pathways, consisting of five main KEGG classes: genetic information processing, cellular processes, metabolism, environmental information processing, and organismal systems. These genes mainly belong to the following KEGG pathways: MAPK signaling pathway-plant, plant–pathogen interaction, plant hormone signal transduction, glycolysis/gluconeogenesis, glutathione metabolism, phenylpropanoid biosynthesis, flavonoid biosynthesis, cytochrome P450, nitrogen metabolism, ubiquinone, ABC transporters, other terpenoid–quinone biosynthesis, transcription factors, glycosyltransferases, protein kinases, transporters and exosomes (Fig. 3). The phenylpropanoid pathway, which had the most number of genes, is a metabolic pathway responsible for the synthesis of various plant secondary metabolites, containing lignin, flavonoids, lignans, phenylpropanoid esters, sporopollenin and hydroxycinnamic acid amides [23, 24]. The cytochrome P450 pathway, which had the second most number of genes in the Ana cultivar, is a transcription factor linked to plant stress responses [25, 26]. Additionally, the percentages of genes belonged to cytochrome P450, glutathione metabolism and flavonoid biosynthesis pathways in the Ana cultivar were higher than those in the Hashem cultivar (Fig. 3). "Proteomic and metabolomic analyses of chickpea–Foc interactions showed that numerous metabolic pathways, phenylpropanoid, isoflavonoid, and flavonoid biosynthesis pathways, were significantly upregulated in the resistant genotype. Proteomic and metabolomic studies of chickpea–Foc interactions revealed that many metabolic pathways, including the phenylpropanoid, isoflavonoid, and flavonoid biosynthesis pathways, were significantly upregulated in the resistant genotype [9, 27, 28].

Overview of the biotic stress pathway

To comprehend the various defense responses in the resistant and susceptible cultivars at the initial stage of Foc race 6 infection, MapMan analysis was used to investigate fluctuations in the significant DEGs in both cultivars under biotic stress. Also, the putative involvement of significant DEGs in the biotic stress response pathways was visualized using MapMan software (Supplementary Figures S4 and S5). On the basis of the MapMan analysis results, several genes located in the cell wall, such as pectinesterase 2-like (LOC101508209), probable pectinesterase/pectinesterase inhibitor 7 (LOC101489717) and expansin-like B1 (LOC101489892 and LOC101507544), were more highly upregulated in the resistant cultivar (Ana) than in the susceptible cultivar (Hashem). In addition, some genes related to the cell wall were upregulated exclusively in the resistant cultivar

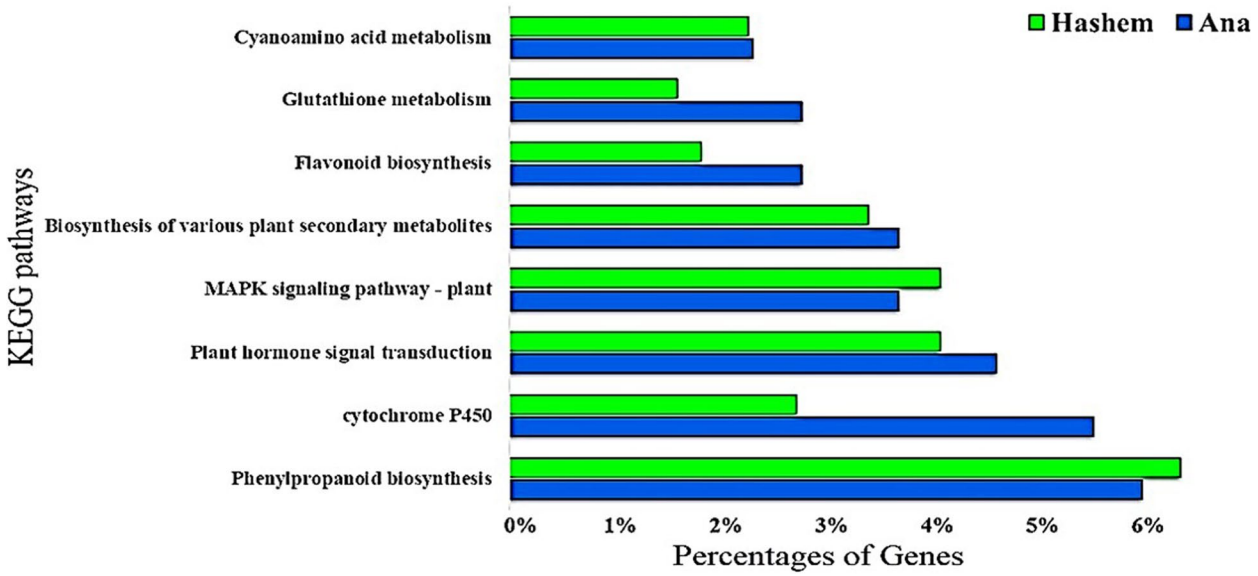

Fig. 3 The top eight pathways with the greatest number of genes in the KEGG protein database. The X-axis represents the ratio of genes with significant differential expression (DEGs) involved in the specified KEGG pathway to the total number of DEGs assigned to KEGG pathways in each genotype, and the Y-axis represents the KEGG pathways

(Supplementary Figure S4 and S5), including polygalacturonase inhibitor 2-like (LOC105852278) and probable xyloglucan endotransglucosylase/hydrolase protein 23 (*CaXTH23*: LOC101489989 and LOC101490102), which have logFCs of 3.87, 4.55 and 2.9, respectively (Fig. 5D). On the other hand, in the GO analysis, some cellular component terms assigned to the cell wall category were more sharply enriched in the resistant cultivar than in the susceptible cultivar (Fig. 2).

Two genes encoding glutathione S-transferase (GST; LOC101494465 and LOC113787225) were upregulated only in the resistant cultivar. One gene encoding the transcription factor MYB41 (LOC101497118) was highly upregulated only in the resistant cultivar under FW stress (logFC = 4.71). Furthermore, several genes encoding pathogenesis-related proteins (LOC101499251, LOC105851085, and LOC101510493) were exclusively upregulated in the resistant cultivar. Some individual genes encoding proteolysis components, such as basic 7 S globulin-like (*CaBg7S-like*: LOC101509822), senescence-specific cysteine protease Ca*SAG39-like* (LOC101497435) and uncharacterized (ncRNA; LOC113785693), were upregulated only in resistant plants under Fusarium wilt disease stress (Figs. 5 and 6; Supplementary Figure S4 and S5).

Interestingly, a gene encoding the cytochrome P450 CYP73A100-like (*CaCYP73A100-like*: LOC101503511) was upregulated (logFC = 3.89) only in the Ana cultivar under Fusarium wilt (race 6). On the other hand, analysis of biotic stress by MapMan software revealed that in the hormone signaling part (Auxins), several genes encoding cytochrome P450 (LOC101510162, LOC101494883, and LOC101510201) were downregulated only in resistant plants.

The results of MapMan showed that genes encoding the protein EXORDIUM-like (LOC101501686 and LOC101502011) were more upregulated in the Ana cultivar (resistant) than in the Hashem cultivar (susceptible). The protein LYK5 (LOC101494861) on chromosome 2 was upregulated only in the resistant cultivar, but another gene encoding the protein LYK5-like (LOC101501405) was downregulated in the susceptible cultivar. On the other hand, we found some uncharacterized genes that were upregulated only in the resistant cultivar (LOC113785810, LOC101496586 and LOC113788231), and some of them were ncRNAs.

The secondary metabolism pathway analysis of differentially expressed genes by MapMan determined that several genes encoding laccase-7-like (LOC101515697), 3-isopropylmalate dehydratase large subunit, chloroplastic-like (LOC101494361) and uncharacterized (LOC101511595) genes were more highly upregulated in the Ana cultivar than in the Hashem cultivar. Moreover, a gene encoding shikimate O-hydroxy cinnamoyl transferase-like (LOC101501659) was upregulated only in the Ana cultivar in response to Fusarium wilt disease. Although a gene encoding chalcone-flavonone isomerase 2-like (LOC101508130) was highly downregulated in the resistant cultivar under Fusarium wilt disease (logFC = −10), another gene encoding chalcone-flavonone

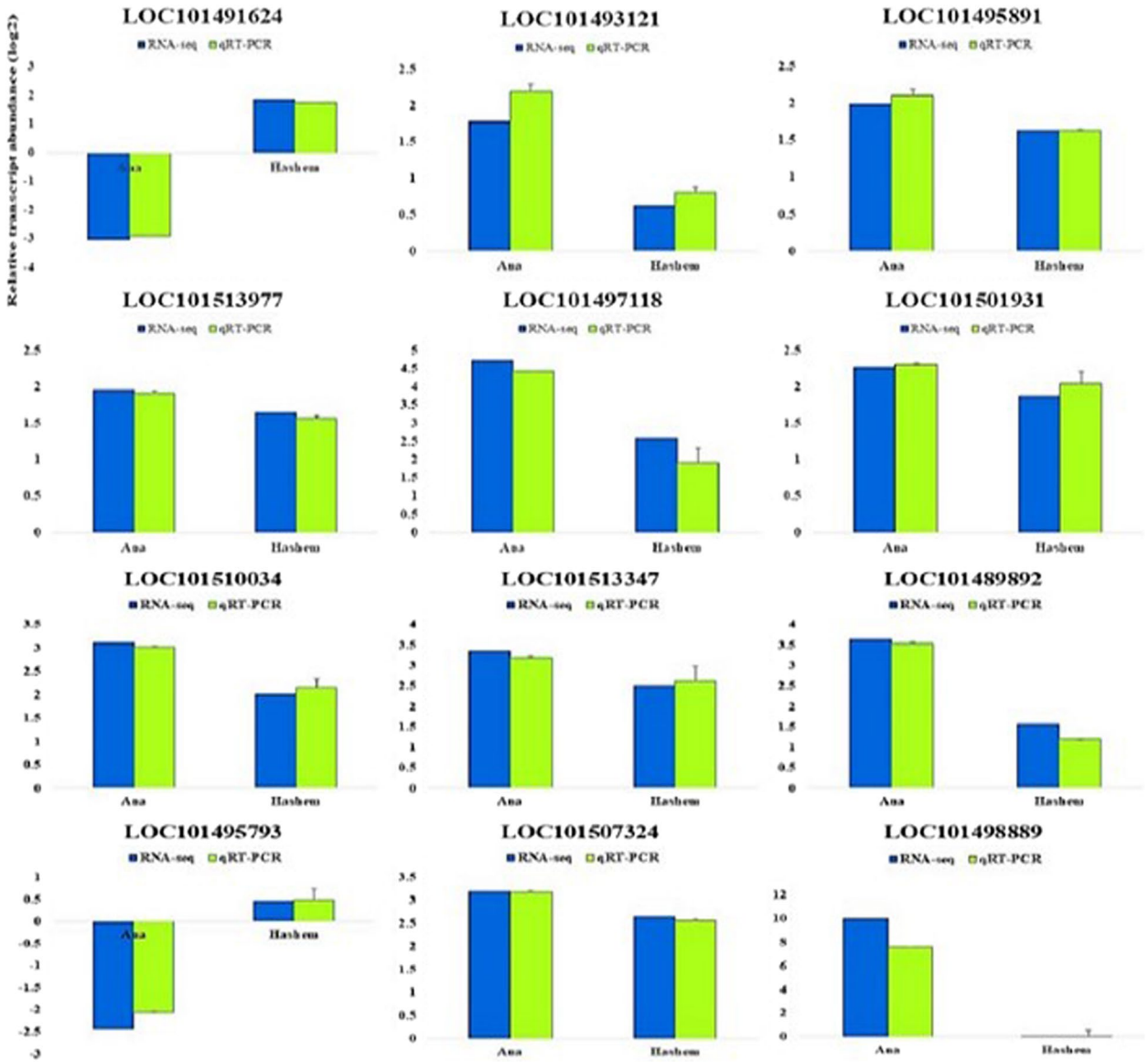

Fig. 4 Validation of twelve candidate genes via qRT–PCR in root tissue of Ana and Hashem under FW disease stress; Bar graphs illustrate the relative transcript abundance of the selected genes in the chickpea cultivars under different conditions (RNA-seq (blue) and qRT-PCR (light green)). Data points are demonstrated as log2 fold change values

isomerase 2 (LOC101500746) was slightly upregulated in the susceptible cultivar (logFC = 1.59).

Confirming DEGs using qRT–PCR

In order to validate the RNA-seq results, the expression patterns of twelve FW-responsive candidate genes were inspected by qRT–PCR (quantitative real-time PCR) in the resistant and susceptible cultivars (Fig. 4). The selected genes were as follows; LOC101491624 (linoleate 9 S-lipoxygenase-like), LOC101501931 (heat shock cognate 70 kDa protein 2), LOC101495891 (1-aminocyclopropane-1-carboxylate oxidase), LOC101513977 (probable leucine-rich repeat receptor-like serine/threonine-protein kinase), LOC101493121 (protein ENHANCED PSEUDOMONAS SUSCEPTIBILTY 1), LOC101497118 (transcription factor MYB41), LOC101510034 (basic 7 S globulin-like), LOC101513347 (spermidine hydroxycinnamoyl transferase-like), LOC101489892 (expansin-like B1), LOC101495793 (MATH and LRR domain-containing protein PFE0570w), LOC101507324 (putative protein TPRXL) and LOC101498889 (carbonic anhydrase 1) (Figs. 4 and 5).

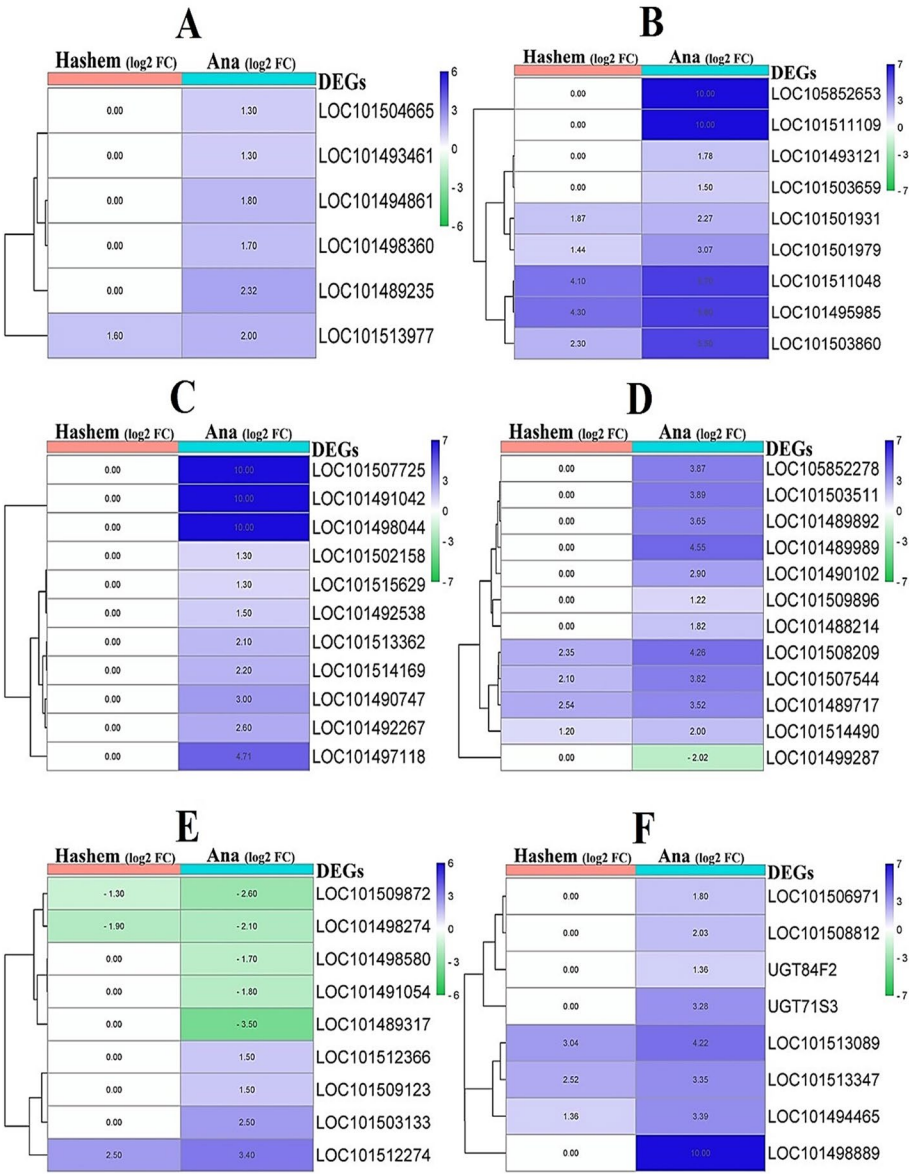

Fig. 5 Heatmap analysis of DEGs involved in disease resistance pathways with a Q value cutoff of ≤ 0.01 and a − 1 ≤ Log2-fold change ≥ 1 under Fusarium wilt (race 6) stress conditions at 48 hpi in the resistant versus susceptible chickpea cultivars. (**A**) pathogen sensing, (**B**) signaling pathways, (**C**) transcription regulation, (**D**) cell wall integrity, (**E**) Transport-related DEGs, (**F**) metabolism. Blue indicates upregulated expression, and green indicates downregulated expression upon stress. Cluster method and distance method was single and Euclidean, respectively

The results of the qRT–PCR confirmed the RNA–Seq results for both chickpea cultivars (in Ana; $R^2 = 0.999$ and in Hashem; $R^2 = 0.987$).

Discussion

Chickpea is an excellent source of protein for a large population worldwide, especially in Asia. It can serve as an alternative to fallow periods in cereal crop rotations, but FW disease causes significant economic losses in its production. Therefore, identifying the resistance mechanism to this disease in chickpeas is crucial [10]. Next-generation RNA-seq provided a comprehensive comparison between Ana (a resistant cultivar) and Hashem (a susceptible cultivar) in this research. Through transcriptome analysis, we identified differential gene expression patterns between Ana and Hashem. Several genes involved in disease resistance pathways that have been previously reported in chickpea and/or other plants were differentially expressed in Ana (Figs. 5 and 6 and Supplementary Table S2), as discussed below.

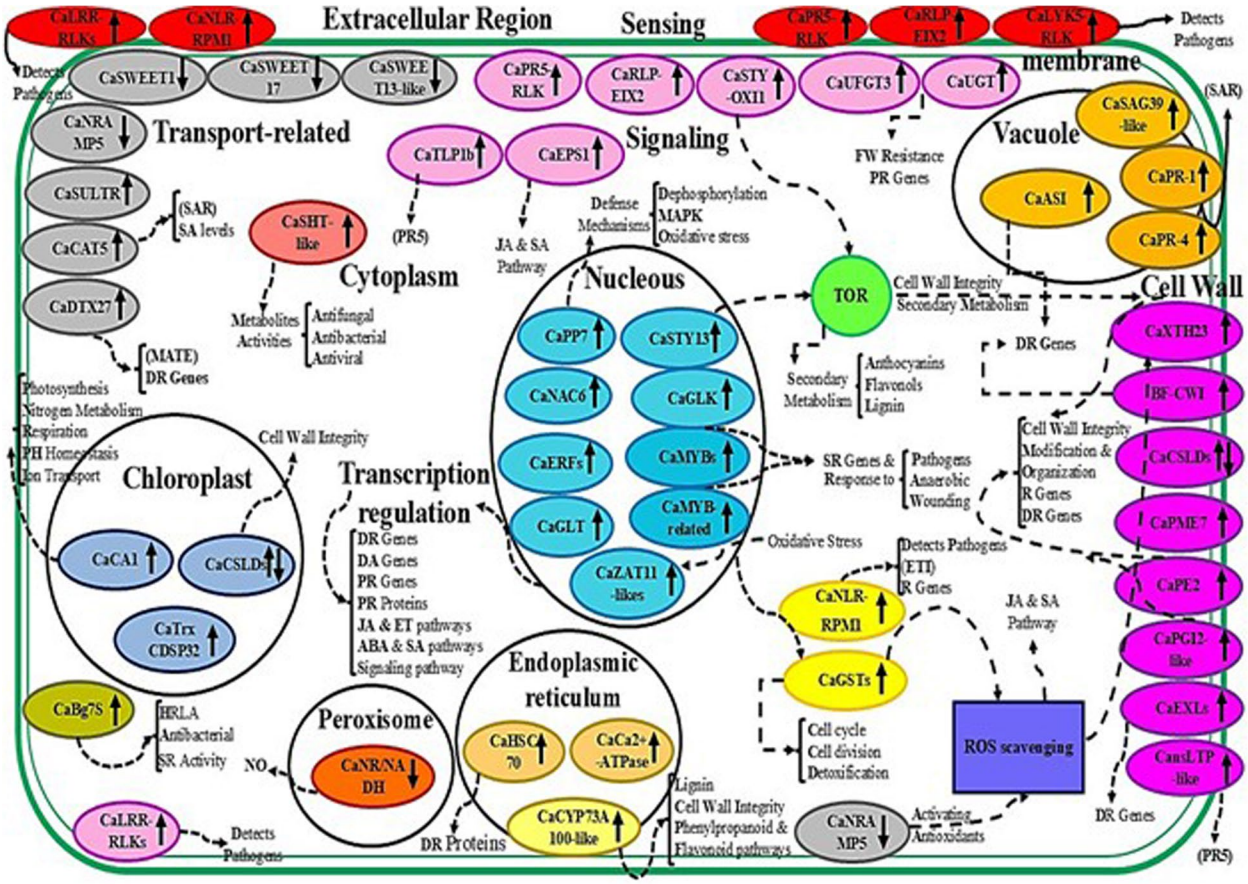

Fig. 6 A schematic representation of candidate genes and pathways might be involved in resistance to race 6 of Fusarium wilt in the resistant cultivar (Ana). Up arrow: upregulation; down arrow: downregulation under FW disease stress

DEGs involved in pathogen sensing

To sense pathogens in a timely manner, resistant plants express many RLKs and RLPs as recognition receptors (PRRs), which act as the first layer of inducible defenses during the early stages of tension [29]. The downstream defense signaling cascades are activated on time when the resistant cultivar detects the pathogen early [19]. Interestingly, several receptor genes, including *CaNLR-RPM1* (Nucleotide-binding site leucine-rich repeat-disease resistance protein RPM1; LOC101504665), *CaLYK5/PR5-RLK* (Lysin Motif Receptor-Like Kinase5/Pathogenesis related5-receptor like kinase; OC101494861 and LOC101493461), *CaLRR-RLK* (Leucine-rich repeat-receptor like kinases; LOC101489235 and LOC101513977) and *CaRLP-EIX2* (Receptor-like protein-Ethylene inducing xylanase2; LOC101498360), were significantly induced by FW in the root tissues of Ana (Fig. 5A and 6). It is noteworthy that transcripts of *CaNLR-RPM1* (LOC101504665) and *CaLYK5-RLK* (LOC101494861) were exclusively detected in Ana, and considerably increased under FW stress conditions (Figs. 5A and 6).

NLR-RPM1 is a plant intracellular immune receptor that specifically detects pathogen-released effectors, initiating effector-triggered immunity (ETI). This activation of ETI by NLR-RPM1 leads to a hypersensitive response (HR), which is a type of localized cell death that helps to restrict pathogen spread and boost disease resistance [30, 31]. LYK5-RLK is a major type of chitin receptor from the LRR-RLK class. Upon the detection of chitin, a constituent of fungal cell walls, LYK5 forms a complex with another receptor kinase to stimulate immune signaling pathways [32]. PR5-RLK is involved in distinguishing pathogen-associated molecular patterns (PAMPs) and triggering immune responses in plants [33]. RLP-EIX2 is known for recognizing and responding to the fungal protein ethylene-inducing xylanase (EIX). This interaction triggers defense mechanisms in plants, helping them fend off fungal pathogens [34]. RLP-EIX2 is structurally similar to other receptor-like proteins found in various plants,

which rely on pattern recognition receptors (PRRs) to detect PAMPs and initiate defense responses [35].

DEGs involved in signaling pathways

Several genes involved in signaling pathways were upregulated under FW stress either exclusively in the resistant cultivar or to a greater extent than in the susceptible cultivar (Figs. 5B and 6). For example, *serine/threonine-protein phosphatase 7* (*CaPP7*: LOC105852653) and *heat shock cognate 70 kDa protein 2* (*Ca HSC70s*; LOC101501931) might play roles in MAPK (mitogen-activated protein kinase) signaling. PP7 is implicated in the dephosphorylation of specific proteins, which can activate or deactivate signaling pathways related to plant defense mechanisms such as MAPK and oxidative stress signaling [36]. HSC70 belongs to the heat shock protein 70 (Hsp70) family and operates as a molecular chaperone. It can interact with various components of the MAPK pathway, maintaining its activity [37]. It has been reported that *HSC70s* are highly upregulated in chickpea, sunflower, and cabbage after infection with Foc [16, 38, 39].

Several genes associated with the hormone signaling pathway, including components of jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), ethylene (ET) and auxin (AUX), were differentially expressed after Foc infection (Figure S4). Among them, some components of SA were found in Ana, such as *ENHANCED PSEUDOMONAS SUSCEPTIBILITY 1* (*CaEPS1*: LOC101493121) (log2 FC = 1.78), whereas it was not expressed in Hashem. *EPS1* is an isochorismate-9-glutamate pyruvoyl-glutamate lyase that can degrade N-pyruvoyl-L-glutamate to generate SA [40]. Salicylic acid, an important signaling molecule, can induce resistance to diseases such as Fusarium. SA is reported to be a mediator between plants and microbes, which activates resistance against Fusarium [41].

Moreover, two genes encoding pathogenesis-related (PR) proteins (*CaPR-1*: LOC101503659, *CaPR-4*: LOC101511048) were significantly induced in Ana (Fig. 6). PR-1-like proteins are part of a larger family of PR proteins that are typically induced in response to pathogen attack and are involved in SAR (systemic acquired resistance). They are often associated with the SA signaling pathway, which is crucial for activating defense responses [42]. PR-4 proteins constitute another class of PR proteins that are often associated with the JA signaling pathway, which is also crucial for defense against pathogens [43]. Both the PR-1-like and PR-4 proteins have antimicrobial activity. PR-4 proteins often exhibit chitinase activity, which allows them to break down chitin. This activity helps prevent the growth and spread of fungi [42, 43].

Furthermore, we identified several genes associated with the PR5 (Pathogenesis-related 5) family in Ana that are known as thaumatin-like proteins (TLPs; *CaTLP1b* (LOC101495985) and *CansLTP-like* (LOC101503860)). TLPs have been increasingly demonstrated to contribute to resistance against various fungal diseases, including Fusarium, in numerous crop plants, especially legumes. They may also play a role in the crosstalk between different hormone responses [44].

Several genes related to TOR (target of rapamycin) signaling pathway, including *CaSTY13* (LOC101511109) and *CaSTY-OXI1* (LOC101501979), were highly upregulated in the resistant cultivar (Figs. 5B and 6). Serine/threonine-protein kinase TOR proteins are reported to be significantly expressed during infection by pathogens in chickpeas and regulate both catabolic and anabolic processes, such as cell cycle regulation, cell growth, mitochondrial signaling, secondary metabolism and apoptosis, cell wall structure, and development [17, 45]. The overexpression of serine/threonine-protein kinases in chickpea-Foc1 interaction has also been reported in various resistant genotypes at different time points [16, 17]. This excessive expression was predominantly found in the cell wall, which causes cell wall integrity after the onset of infection [46].

DEGs involved in transcription regulation

Transcription factors (TFs) are essential in plant defense, regulating the expression of genes that react to pathogen attacks [47]. Transcriptional reprogramming happens after triggering a signaling cascade by various transcription factors (TFs), which activate genes involved in hormonal control and other processes, including PR genes and structural genes [19]. GO analysis showed that the term 'transcription regulator activity' was dramatically enriched in the Ana cultivar (Fig. 2).

Among them, some MYBs (*CaMYB41*: LOC101497118; *CaMYB108*: LOC101490747; LOC101492267), MYB-related (LOC101507725) and G2. Like (*CaGLK*: LOC101491042) was significantly upregulated by FW only in the resistant cultivar (Figs. 5C and 6;). MYBs are a major group of TFs that have a variety of roles in eukaryotes, including responses to wounding and pathogens [48]. *MYB108* was previously reported in Arabidopsis, which controls the infection of two pathogens by stimulating defense-related genes (DR Genes); *PDF1–2* and *PR-1*, through ethylene and JA signaling pathways [49]. *AtMYB108* is an *R2R3MYB* protein that modulates responses to both biotic and abiotic stresses [49]. R2R3MYB has been found to increase resistance to *Bipolaris sorokiniana* root rot fungus, as well as enhancing the expression of PR1a, PR2, and TLP4 genes through the SA and ABA signaling pathways [50]. Furthermore,

overexpression of *AtMYB41* has been reported to activate monolignol and suberin-associated wax biosynthesis, as well as the formation of suberin-like lamellae in the cell walls of both epidermal and mesophyll cells, which eventually protects against biotic and abiotic stresses [51]. Furthermore, we found a gene coding GOLDEN2-LIKE (GLK) belonging to the MYB family in Ana. *GOLDEN2-LIKE* (*GLK*) plays a significant role in regulating plastid development and stress tolerance [52]. GLKs play a positive role in virus resistance against cucumber mosaic virus (CMV) in Arabidopsis by modulating the expression of defense-associated genes and the antioxidant system, which is partially associated with the accumulation of JA and SA [53].

Some ERFs (*CaERF9*: LOC101502158, *CaERF113*: LOC101515629, and *CaERF113-like*: LOC101513362) were also significantly upregulated by FW, specifically in Ana (Figs. 5C and 6). The ERF (ethylene-responsive transcription factor) is a subfamily of the AP2/ERF family and plays essential roles in the regulation of biotic stress responses. Utmost *ERF* proteins can bind to GCC box-containing promoters, which are found in the promoters of many pathogenesis-related (*PR*) genes, such as PRb-1b (PR1), β−1,3-glucanase (*PR2*), chitinase (*PR3*) and osmotin (*PR5*), and mediate the vital role of these genes in plant responses to biotic stress [54, 55]. It has been reported that ethylene signaling acts a vital role in cucumber resistance against Foc [56]. Consistent with the findings of a previous study [19], we also found that the *CaERF113* gene (LOC101515629) was upregulated in the resistant cultivar under FW stress.

Furthermore, some *C2H2s* (*CaZAT11-like*: LOC101498044 and LOC101492538) were exclusively shown significant upregulation by FW in Ana (Figs. 5C and 6). C2-H2-type proteins (TFs) are involved in regulating the signaling pathway during biotic stress [57]. Previous reports have demonstrated that the transcript abundance of *ZAT11* can be highly induced by H2O2; *ZAT11* was shown to be involved in paraquat-induced oxidative stress, which leads to programmed cell death [58].

CaNAC6 (LOC101514169) was significantly FW inducible only in Ana (Figs. 5C and 6). NAC transcription factors are encoded in plants by a gene family with proposed functions in both biotic and abiotic stress adaptation, also in developmental procedures [59]. It is reported that expression of *OsNAC6* (*Oryza sativa NAC6*) in rice is induced by blast disease [60]. Upregulation of the *NAC6* gene has previously been reported in barley, which promotes basal resistance against the virulent *Blumeria graminis* f. sp. *hordei* [59, 61].

DEGs involved in cell wall integrity

Some genes associated with cell wall modification and organization were either exclusively upregulated in the resistant cultivar (Ana) or more strongly upregulated in the resistant cultivar than in the susceptible cultivar, including polygalacturonase inhibitor 2-like (*CaPGI2-like*: LOC105852278) (Figs. 5D and 6). Fungal pathogens often produce polygalacturonases (PGs) to break down pectin polymers and weaken the plant cell wall. Polygalacturonase-inhibiting proteins (PGIPs) are cell wall glycoproteins that can recognize and inhibit PGs, thereby reducing their hydrolytic activity and helping control fungal progression [62, 63]. PGIPs interact with PGs via the Leucine-rich repeat (LRR) structure. This characteristic of PGIPs is evolutionarily linked to many plant resistance genes (R genes) [64, 65]. The PG–PGIP interaction also navigates the production of oligogalacturonides that elicit diverse defense responses (DR genes), including the expression of PR1 and salicylic acid-regulated genes [62, 63]. The upregulation of a PGI gene has been previously reported in the FW-resistant desi landrace of chickpea in response to Foc2 [19].

Pectinesterase 2-like (*CaPE2*: LOC101508209) and probable *pectinesterase/pectinesterase inhibitor 7* (*CaPME7*: LOC101489717) were upregulated in both cultivars while the induction was higher in the resistant cultivar. It has been reported that a *Pectinesterase-like* gene is involved in the synthesis and modification of cellulose, lignin, and other components found in different layers of the cell wall [66, 67]. A pectinesterase-like gene was significantly upregulated in *Musa acuminata* under *Pseudocercospora musae* disease [68]. It is related to epicuticular wax, pectin biosynthesis, cell wall organization, and cell wall biogenesis [68]. *Pectinesterase inhibitor* genes play a role in reorganizing the cell wall and in the plant's defense against pathogen attacks [69, 70].

Several *expansin-like* genes (*CaEXLB1*: LOC101489892 and LOC101507544; *CaEXLA2*: LOC101514490) were exclusively expressed in Ana or were more highly upregulated in Ana than in Hashem (Figs. 5D and 6). Expansins (EXPs) are involved in plant development and responses to diverse stresses [71]. They are extracellular proteins that loosen plant cell walls in novel ways [72]. A role in the disease response was reported for *EXLB1*, and the expression pattern of most *EXP* genes significantly changed during diverse infection times [73].

Three genes encoding cellulose synthase (*CaCSLD*, such as LOC101509896, LOC101488214, and LOC101499287) were exclusively expressed/induced in Ana (Figs. 5D and 6). *CSLD* (cellulose synthase) contributes to glucan deposition and maintaining cell wall integrity during pathogen invasion [74]. It has been reported

that the overexpression of type 3 *CSLD* prevents damage to the cell wall in resistant genotypes [17].

A gene encoding the cytochrome P450 *CYP73A100-like* (LOC101503511) was upregulated (logFC = 3.89) only in the Ana cultivar under Fusarium wilt (race 6). It has been reported that *CYP73A100* is upregulated during *Fusarium oxysporum* infection and contributes to lignin synthesis and accumulation [75]. The expression of some CYP genes is controlled in response to environmental stresses, and they play a significant role in the crosstalk between biotic and abiotic stress responses [26]. CYPs hold significant potential as candidates for engineering crop species that are resilient to both abiotic and biotic stresses [26].

Transport-related DEGs

The transport of essential elements to primary locations and during critical hours after infection affects the resistance network, where various groups of transporters play fundamental roles [17]. Several transport-related genes were upregulated in Ana, including *calcium-transporting ATPase* (*CaCa^{2+}-ATPase*: LOC101512366), cationic amino acid transporter 5 (*CaCAT5*: LOC101509123), and sulfate transporter 3.5 (*CaSULTR$_{3.5}$*: LOC101512274) (Figs. 5E and 6). Interestingly, the *Ca^{2+}-ATPase* gene was also upregulated in previous studies on Foc races 1, 2, and 4 in resistant chickpea cultivars [18, 19]. Some amino acid transporters (AATs), such as *AtCAT1* (*Arabidopsis thaliana cationic amino acid transporter 1*), positively affect the plant immune system. It has been reported that the overexpression of *AtCAT1* results in the continuous expression of PR1 and SA-related genes, along with an increase in SA rates. Given that *AtCAT1* expression rapidly responds to infection, AtCAT1 plays a role in plant systemic resistance [76]. Additionally, sulfur (SULTR) is considered a crucial macronutrient for plant growth, development, and response to several abiotic and biotic stresses [77]. In addition, GO analysis of molecular function showed that the term 'nitrate transmembrane transporter activity' was more enriched in Ana compared to the susceptible cultivar (Fig. 2). It has been reported that the tolerance of cucumbers to Fusarium wilt is enhanced by nitrate, which controls the production and distribution of fungal toxins [78].

Several transport-related genes were downregulated in Ana including nitrate reductase [NADH]-like (*CaNR/NADH*: LOC101498580), bidirectional sugar transporter *SWEETs* (*CaSWEET13-like*: LOC101491054, *CaSWEET17*: LOC101509872 and *CaSWEET1*: LOC101498274) and metal transporter Nramp5-like (*CaNRAMP5*: LOC101489317) (Figs. 5E and 6). Nitrate reductase (NIA2) is recognized for its role in regulating the biosynthesis and transport of nitric oxide (NO). It has been reported that the downregulation of *NR/NADH* in resistant plants is a probable strategy of the resistant host to counteract drought stress caused by phenolic deposition due to Foc1 invasion [17]. Furthermore, the regulation of sugar transporter and *SWEET* genes may play a role in plant defense against pathogen infection by adjusting the availability of sugar in the apoplasm [79]. Similar results were also reported during Foc race 2 infection in resistant and susceptible chickpea genotypes [19]. Additionally, the natural resistance-associated macrophage protein (NRAMP) gene family facilitates the transport of metal ions (NRAMP5 is a good example) in plants [80]. It has been reported that the downregulation of NRAMP5 significantly decreases the uptake and transport of manganese (Mn), which in turn activates enzymatic antioxidants. This enhances the capacity for ROS scavenging and boosts photosynthesis activity, thereby alleviating Mn toxicity in peach plants [80].

Moreover, the protein DETOXIFICATION 27-like (*CaDTX27*: LOC101503133), located in the plasma membrane, was upregulated only in Ana. It belongs to the multiantimicrobial extrusion (MATE) family. MATE genes have been shown to be associated with disease resistance in Arabidopsis [81, 82]. Likewise, it has been suggested that the protein DETOXIFICATION 48-like, encoding a MATE family protein, is related to defense activity against Foc (race 5) [15].

DEGs involved in metabolism

Upon infection, the plant and pathogen compete to utilize the host's sugar metabolism, which in turn triggers either resistant or susceptible responses. Sugar-metabolizing enzymes are differentially regulated during plant–pathogen interactions. In the present study, two genes encoding putative UDP-glucose glucosyltransferase (*CaUFGT3*: UGT71S3 and *CaUGT*: UGT84F2) were upregulated in the resistant genotype (Figs. 5F and 6). This finding is consistent with previous reports showing that UDP-glucosyltransferases (UGTs) are involved in FW resistance in wheat and barley through glycosylating the deoxynivalenol produced by the Fusarium spp. fungus [83]. Furthermore, a cell wall isozyme-like beta-fructofuranosidase (*CaBF-CWI*: LOC101513089) was upregulated in Ana (Figs. 5F and 6). It belongs to a class of sucrose-hydrolyzing enzymes known as invertases whose role in plant disease resistance has already been reported [84]. It is believed that *CWINV1* (cell wall invertase 1) is the enzyme that plays a crucial role in the reconstruction of damaged cell walls [85]. Based on the results of the present study, alpha-amylase/subtilisin inhibitor-like (*ASI*; LOC101508812) was also induced in Ana (Figs. 5F and 6). *ASI* proteins play a significant role in plant defense mechanisms against pathogens,

including fungi. They inhibit the activity of enzymes such as alpha-amylase and subtilisin, which are substantial for the growth and development of many pathogens [86]. In particular, these inhibitors can prevent the degradation of plant cell walls by fungal enzymes, thereby limiting the ability of pathogens to invade and cause disease [87].

Glutathione S-transferase (*CaGST*: LOC101494465, LOC101506971, and LOC113787225) was upregulated in the resistant cultivar (Figs. 5F and 6). *GSTs* are involved in the detoxification of a vast variety of xenobiotic compounds [88]. It has been reported that the increase in GSTs after Foc1 attack impacts the maintenance of redox balance [17, 89]. Also, *GSTs* combat toxin challenges that directly affect the cell cycle and cell division [14]. KEGG pathway analysis showed that the percentage of genes involved in the glutathione metabolism pathway in the resistant cultivar (Ana) was dramatically higher than that in the susceptible cultivar (Hashem) (Fig. 3).

Trihydroxycinnamoyl spermidines (*CaSHT-like*; LOC101513347) were also upregulated in both cultivars, while the increase was more in the resistant cultivar (Figs. 5F and 6). THCSpds are specialized plant metabolites known for their significant pharmacological properties, including antifungal, antibacterial, and antiviral activities [90]. Moreover, the carbonic anhydrase 1 (*CaCA1*: LOC101498889) gene was found to be involved in the nitrogen metabolism pathway [14]; it has also been reported via transcriptome analysis that *CA1* was identified in maize in response to Fusarium ear rot [91]. CAs are widespread enzymes that play crucial roles in essential processes such as photosynthesis, respiration, ion transport, and pH homeostasis [92].

Furthermore, CYP73A100, which is involved in the phenylpropanoid and flavonoid biosynthesis pathways, was detected (Figs. 5F and 6). It has been reported that some genes, such as *CaCYP73A100*, are upregulated during treatment with exogenous melatonin and *F. oxysporum*. These genes are involved in the synthesis of p-coumaric acid, flavonol 3-O-ethyltransferase, and 4-coumarate-CoA ligase, which contribute to the accumulation of lignin [75]. Melatonin is regarded as a polyfunctional master regulator in both higher plants and animals. Studies have shown that exogenous melatonin treatment can efficiently manage cucumber green mottle mosaic virus (CGMMV) infection and enhance resistance to *F. oxysporum* in plants [75, 93]. Upregulation of the *CYP73A100* gene in other plants under biotic stress has also been shown; for instance, it was expressed in soybeans during infection with soybean cyst nematodes [94].

Conclusion

Comparative analysis of the transcriptomic response of resistant and susceptible chickpea cultivars (Ana and Hashem, respectively) to race 6 of *F. oxysporum* f. sp. *ciceris* infection has provided some insights into the molecular mechanisms of resistance to FW in *Cicer arietinum*. Recognition of fungal pathogens by plants is the first critical step, which can lead to prompt activation of downstream defense signaling cascades and finally result in resistance. Remarkably, two receptor genes (i.e., *CaNLR-RPM1* and *CaLYK5-RLK*) were exclusively expressed in Ana and upregulated under FW stress conditions. Some other RLKs and RLPs (including *CaPR5-RLK*, *CaLRR-RLK*, and *CaRLP-EIX2*) were also significantly induced by FW in the root tissues of Ana. Moreover, several genes involved in signaling (such as *CaPP7*, *CaHSC70s*, *CaEPS1*, *CaSTY13* and *CaSTY-OXI1*) and transcription regulation (*CaMYBs*, *CaGLK*, *CaERFs*, *CaZAT11-like*, and *CaNAC6*) were found to be overrepresented by FW stress in the resistant cultivar. A rich set of genes related to defense responses (e.g., *CaPR-1* and *CaPR-4*) and cell wall integrity (e.g., *CaPGI2-like*, *CaPE2*, *CaPME7*, *CaEXLs*, *CaCSLD*, *CaCYP73A100-like*) were further identified in Ana, whereas they were not expressed in Hashem. Conclusively, the resistant genotype employs a subtle gene network, which may help in the early detection of pathogens, triggering prompt signaling pathways, leading to the activation of an efficient defense response against fungal pathogens, thereby enhancing its disease resistance (Fig. 6). The achieved results could facilitate the use of genetic engineering or molecular breeding approaches to develop chickpea varieties resistant to Fusarium wilt. However, the comparative study of the molecular response at different time points after *F.* oxysporum infection from different races, in various resistant and susceptible chickpea cultivars would be very fruitful to enhance the presented results.

Methods

Plant material

The present study employed two Kabuli chickpea cultivars, designated as Ana (pedigree: FLIP 98–130 C × FLIP 97–23 C) and Hashem (pedigree: FLIP94–110C × FLIP93–128C). The Ana and Hashem cultivars were among lines those crossbreeding was conducted at the International Center for Agricultural Research in the Dry Areas (ICARDA) and subsequently released as variety by the Dryland Agricultural Research Institute (DARI) of Iran in 2018 and 1995 [95], respectively. The seeds of the two cultivars were kindly provided by the GenBank of the DARI. Furthermore, the two cultivars displayed contrasting resistance and susceptibility to Foc Race 6 (Supplementary Figures S1 and S2).

The seeds of the two cultivars were sterilized for 10 min in 0.5% sodium hypochlorite (NaClO), rinsed with distilled water, and placed on dampened filter papers. On the third day, the uniformly germinated seeds were transferred to pots (6×6×8 cm) filled with pasteurized perlite in trays (41×56×12 cm). The plants were grown under controlled conditions at 25 ± 2 °C with a 16/8 (day/night) photoperiod under fluorescent light and a relative humidity of 75%.

Inoculation of *Fusarium oxysporum* f. sp. *ciceris* race 6 and evaluation of the pathogenicity response

The race 6 isolate of Foc [96] was used in this study. The fungus was cultured in potato dextrose broth (PDB, 200 g potato: 20 g dextrose: 1 L water) at a temperature of 28 to 30 °C for 3 to 4 days on a shaker. The medium was then filtered through four layers of clean cloth and centrifuged at 7500 rpm for 14 min. The resulting conidia were used to prepare a spore suspension with a concentration of 10^6 conidia per ml. Plants with replicating of each cultivar along with the same number of susceptible control varieties, Kaka, were inoculated with the pathogen according to the methods of Pouralibaba et al. (2015) [97]. Approximately 1–3 cm of the root tips of the plants at the 4–5-leaf stage (approximately 12–14 days after planting) were cut with sterilized scissors. The root tips were subsequently immersed in the spore suspension for 10 min before being planted in pots filled with sterilized Perlite. The control plants were subjected to the same procedure but were given sterile water instead. Following inoculation, the pots were irrigated with a complete NPK 20–20–20 + TE solution (20 g/10-liter water). The reaction to the pathogenic fungus was scored based on the percentage of mortality [98]. The first data recording was done immediately after observing the first symptoms of yellow/wilt on the susceptible control plant, and the plants were evaluated at one-week intervals until the death of all control plants (approximately one month after the first disease evaluation). The last data recorded were considered the final plant reaction to the disease.

For genotyping, root samples were collected at 48 h post-inoculation (hpi) from two biological replicates (each replicate included at least three individual plants) of inoculated and noninoculated plants. Previous experiments by several researchers revealed significant transcriptomic and proteomic changes at 48 hpi in chickpea-Foc1 interactions [17, 99]. Therefore, a 48-hour period was selected as the ideal time point for sample collection and transcriptome analysis. The root samples from each cultivar were promptly frozen in liquid nitrogen and subsequently stored at −80 °C.

RNA extraction and mRNA sequencing

For each biological replicate, equal amounts of root samples (collected at 48 hpi) from three individual plants were pooled and ground. Total RNA was extracted from both inoculated and non-inoculated Ana and Hashem cultivars using TRIzol (Bio Basic, Canada) based on the manufacturer's guidelines. The quality, quantity, and RNA integrity were evaluated by a NanoDrop ND-1000® spectrophotometer, agarose gel electrophoresis, and an Agilent 2100 Bioanalyzer system (Agilent Technologies Co. Ltd., Beijing, China). To avoid any genomic DNA contamination, RNA samples were treated with RNase-free DNase I (Thermo Scientific™) and subjected to PCR. Additionally, paired-end reads of 150 bp were generated with the Illumina HiSeq™ 2500 sequencing platform at Novogene Bioinformatics Institute (Beijing, China) for total root samples.

RNA-seq data analysis

The quality of the raw sequencing reads in FASTQ format was distinguished by FASTQC [100] software, and quality reads were confirmed based on phred score ≥ 30 (Q30). The high-quality paired-end reads were then mapped against the chickpea reference genome (https://www.ncbi.nlm.nih.gov/assembly/GCF_000331145.1) utilizing HISAT2 [101]. A reference annotation-based transcript (RABT) assembly and the genome GFF were created by Cufflinks [102, 103] using the aligned reads from each sample. The single assemblies were merged into a complete assembly using Cuffmerge with default parameters. Additionally, Cuffmerge was used to identify novel transcripts [101]. Differentially expressed genes (DEGs) were identified using Cuffdiff from the Cufflinks package, with thresholds set at $-1 \leq \log2\text{-fold change} \geq 1$ and a Q value cutoff ≤ 0.01. Also, Blastx was utilized for functional annotation of significant DEGs against the TAIR protein database using the ENSEMBL Genome Browser (https://ensembl.gramene.org/Arabidopsis_thaliana/Tools/Blast).

Functional annotation and pathway analysis of significant DEGs

For each cultivar, GO terms were assigned to significant DEGs using AgriGO website (http://systemsbiology.cau.edu.cn/agriGOv2/) with an FDR cutoff ≤ 0.05. The contributions of significant DEGs to KEGG pathways were identified via the online KEGG automatic annotation server (KAAS) (https://www.genome.jp/kegg/kaas/). Moreover, for pathway analysis of significant DEGs, MapMan (version 3.5.1) with a Q value cutoff of ≤ 0.01 and $-1 \leq \text{Log2-fold change} \geq 1$ was used [104]. Mapping significant DEGs to Arabidopsis pathway

genes led to the identification of genes involved in specific pathways [104].

Realtime PCR analysis

Real-time PCR was applied to confirm the RNA-seq results. Twelve genes were selected from the panel of Fusarium wilt-responsive genes identified through the RNA-seq results. Gene-specific primers were designed using Oligo 7 (version 7.60; Molecular Biology Insights, Inc.; USA). The primers used for the selected genes are provided in Supplementary Table S1. cDNA synthesis was done using a SinaClon cDNA synthesis kit (Cat. No: RT5201). Quantitative real-time PCR (qRT–PCR) was performed on three biological replicates of both noninoculated and inoculated root samples using a LightCycler® 96 Real-Time PCR System (Roche Life Science, Germany) and HS–qPCR Mix, 2x (SinaClon, Iran). GAPDH served as an internal control gene to normalize the gene expression values. The relative expression of the candidate genes was analyzed using the $2^{-\Delta\Delta Ct}$ method [105].

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11308-3.

Supplementary Material 1.

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Authors' contributions

"Z-S.S. designed the lab experiments and supervised the molecular part of the research. H.H-K. and H-R.P. supervised the plant culture, inoculation and phenotyping part of the research. A.F. performed the experiments and drafted the manuscript. A.F., S.D. and R.M-M. analyzed the data. Z-S.S. and R.M-M. revised the manuscript. All authors read and approved the final manuscript."

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Data availability

All the sequencing reads generated from Illumina HiSeq 2500 RNA-Seq are available from NCBI SRA under BioProject ID: PRJNA1050654 (SRR27173947, SRR27173948, SRR27173949, SRR27173950, SRR27173951, SRR27173952). All other data sets supporting this study are included in the article and its supplementary data.

Declarations

Ethics approval and consent to participate

We declare that the plants used in this study complied with local or national guidelines. All plant materials used in this article are cultivated crop varieties (i.e. two Iranian chickpea cultivars) belonging to the Iranian Dryland Agricultural Research Institute (DARI). According to the rules of this institute, the researchers are permitted to use the plant materials for research purposes.

Competing interests

The authors declare no competing interests.

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