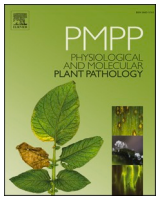
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Expression comparisons of defense-related genes in resistant and susceptible chickpea cultivars in response to *Ascochyta rabiei*

Gülsüm Palacıoğlu [a], Göksel Özer [b], Harun Bayraktar [c,*]

[a] Department of Plant Protection, Faculty of Agriculture, Şırnak University, Şırnak, 73000, Turkey
[b] Department of Plant Protection, Faculty of Agriculture, Bolu Abant Izzet Baysal University, Bolu, 14030, Turkey
[c] Department of Plant Protection, Faculty of Agriculture, Ankara University, Ankara, 06110, Turkey

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ABSTRACT

Ascochyta blight caused by *Ascochyta rabiei* is a destructive disease affecting chickpea crops worldwide. Numerous studies have sought to identify resistance genes involved in the defense response against Ascochyta blight, but the function of potential resistant genes across different chickpea genetic backgrounds remains poorly understood. This study evaluated the potential role of 16 genes involved in defense responses in partially resistant (ILC482) and susceptible (Sarı98) chickpea cultivars. The leaf samples were collected from 2-week-old seedlings at 6, 12, 24, 48, and 72 h post-inoculation (hpi) with *A. rabiei* and analyzed for differential expression of defense genes using qRT-PCR. Twelve out of 16 genes were differentially regulated between chickpea cultivars. The qRT-PCR analysis indicated that the expression of defense genes was significantly higher in the partially resistant cultivar than that in the susceptible cultivar. The time point of the highest upregulation ratio for defense genes was variable between the cultivars. The expression of glutathione S-transferase, serine/threonine-protein kinase, WRKY gene (*CaWRKY16*), and wall associated-receptor kinase genes reached maximum levels in the resistant cultivar at 6 hpi, while antimicrobial peptide precursor (*SNAKIN2*), polymorphic antigen membrane protein, ethylene receptor-like sequences (*CaETR1*), and two nucleotide-binding site-leucine-rich repeat genes showed the highest activity at 24 hpi. In particular, *CaWRKY16*, *CaWRKY50*, glutathione S-transferase, *CaETR1*, and wall-associated-receptor kinase genes provided valuable information for assessing chickpea cultivars with different resistance levels. The results indicated that the expression changes of these genes involved in defense pathways play a crucial role in enhanced resistance of chickpea plants against Ascochyta blight and, therefore could be potential candidate genes for future breeding studies.

1. Introduction

Chickpea (*Cicer arietinum* L.) is a vital legume crop with an annual production exceeding 14 million tonnes worldwide [1]. However, like other crops, chickpea suffers from numerous disease agents throughout its growth stages. Ascochyta blight, caused by *Ascochyta rabiei* (Pass.) Labr. is a biotic factor that can cause serious damage up to 100% in chickpea yield [2]. The pathogen affects all aerial parts of plants and can cause severe blight epidemics in the presence of susceptible hosts and environmental conditions favorable for disease development [3]. Although various methods have been used to suppress Ascochyta blight, utilizing host resistance has become the most economical and effective method [2,4]. To date, breeding programs have successfully developed some resistant and/or tolerant cultivars for Ascochyta blight [3,5]. The sexual stage of *A. rabiei* introduces a high degree of genetic and pathogenic variability [6–8]. A key approach in breeding programs is to explore and exploit defense genes related to host resistance, and multiple genetic sources of Ascochyta resistance have been identified against different pathotypes [9–12]. However, more research is needed to understand better the genetic mechanisms associated with host resistance.

Plants have developed a wide variety of defense mechanisms to defend themselves against pathogens during their evolutionary history. In response to pathogen infections, the activation of defense genes involved in signaling pathways associated with morphological barriers, secondary metabolites, and defense-related proteins changes to curtail the pathogen development and spread [13]. Pathogenesis-related (*PR*) proteins are acknowledged for their vital roles in host resistance to

* Corresponding author.
E-mail address: bayrakta@agri.ankara.edu.tr (H. Bayraktar).

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various biotic and abiotic factors [17]. Enhanced resistance to Ascochyta blight in chickpea has been observed with the overexpression of *PR*-proteins, chitinase, and β-1,3-glucanase [14,18]. The nucleotide-binding site-leucine-rich repeat (*NBS-LRR*) genes have been discovered to play significant roles in response to Ascochyta infection, functioning as signaling molecules [4,19–21]. Similarly, the role of defense genes associated with wall-associated-receptor kinases (*WAK*), zinc finger protein, copper amine oxidase, serine/threonine-protein kinases, Snakin-2 (*SN2*), ethylene receptor, polymorphic antigen membrane protein (*PAMP*) has been demonstrated in the resistance response of various chickpea genotypes [4,15,16,22]. Glutathione S-transferases (*GST*), which regulate cellular H_2O_2 have also been found to play a vital role in the response of chickpea plants to Ascochyta blight [15]. *WRKY* genes appear to have a critical role in the activation of defense pathways of chickpea during *A. rabiei* infection and salicylic acid treatment [23].

Many defense-related genes have been characterized at the transcriptional level for chickpea cultivars [15,19,23]. However, little information is still available about molecular mechanisms regulating gene expression during the infection process. To enhance the resistance of chickpea against fungal pathogens, it is crucial to identify the underlying resistance mechanisms. One effective strategy is to compare putative resistance gene transcripts between resistant and susceptible genotypes. Differential expression of defense genes can provide more insights into signaling pathways involved in host-pathogen interactions. In addition, the response of host plants to pathogen infection could suggest significant differences depending on the timing and magnitude of defense-related genes rather than the expression of various genes. In the present study, we evaluated the expression changes of defense-related genes and transcription factors at different times in resistant and susceptible cultivars of chickpea in response to *A. rabiei* and suggested that especially *WRKY*, *GST*, *CaETR1,* and *WAK* genes play a role in the resistance of chickpea plant in the early stage of pathogen infection. Significant findings from this study may contribute to a better understanding of the mechanisms underlying chickpea resistance to Ascochyta infection.

2. Material and methods

2.1. Plant material

Chickpea cultivars used in this study were obtained from the Central Research Institute for Field Crops in Ankara, Turkey. Cultivars ILC482 is partially resistant, while Sarı98 is susceptible to Ascochyta blight [12]. The surface of the chickpea seeds was sterilized with 1% sodium hypochlorite (NaOCl) for 2 min and then washed several times with distilled water. Five seeds were sown in pots (15 cm diameter) containing sterilized soil, river-bed sand, and manure (1:1:1 v/v/v). Three pots were used for each cultivar and for each assessed time point. Plants were left to grow in a growth chamber for two weeks under a 23 ± 2 °C and 12 h day/night photoperiod.

2.2. Plant inoculation

A virulent isolate, Asc3, belonging to *A. rabiei* pathotype 1, was obtained from the culture collection of Ankara University, Faculty of Agriculture, Turkey. Isolate Asc3 is highly virulent on Sarı98, and is moderately virulent on ILC482 [24]. The pathogen was grown on chickpea seed meal–dextrose agar (CSMDA) at 23 °C for 15 days. Spore suspension was prepared by collecting the spores from the agar surface and filtering through cheesecloth to remove mycelia. The concentration of the inoculum was adjusted to 1.5×10^5 spores per mL of sterile distilled water. Fourteen-day-old chickpea seedlings were inoculated with 20 mL of conidia suspension for each pot until run-off using a pressure hand sprayer, and the inoculated plants were covered with transparent plastic bags for 48 h to maintain moisture. The inoculated plants were grown at 23 ± 2 °C with a 12 h day/night photoperiod then the above-ground parts were detached to be used for RNA isolation at 6, 12, 24, 48, and 72 h time points after inoculation. The samples were immediately frozen in liquid nitrogen and stored at −80 °C until used for RNA isolation. The leaves and stem tissues from five plants for each replicate were pooled, and three biological repeats were conducted. Control plants were inoculated with sterile distilled water only. The efficacy of *A. rabiei* infection was confirmed by observing the expected disease symptoms in the plants after 21 days of inoculation [14]. Disease severity was assessed according to 1–9 scale (1: no visible lesions on any plants; 3: visible necrotic lesions on less than 10% of the plants; 5: visible necrotic lesions on up to 25% of the plants, stem girdling on less than 10% of the plants but little damage; 7: lesions on most plants, stem girdling on less than 50% of the plants resulting in the death of a few plants; 9: lesions profuse on all plants, stem girdling present in more than 50% of plants and the death of most plants), as described by Bayraktar et al. [24].

2.3. RNA extraction and cDNA synthesis

The samples collected at each time point were ground to a fine powder in liquid nitrogen using a pestle and mortar. Total RNA was extracted from 100 mg of grounded tissues using NucleoZOL RNA reagent (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. The total RNA samples were treated with *Dnase* I (Thermo Fisher Scientific Inc., Waltham, MA, USA) to eliminate residual DNA. The quality and quantity of RNA were evaluated using a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific Inc.). First-strand cDNA was synthesized using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions and then diluted 10-fold with sterile Millipore water for further studies.

2.4. Quantitative real-time PCR

The expression level of chickpea defense genes was analyzed in both ILC482 and Sarı98 cultivars (Table 1). *EF1-α*, *GADPH,* and *actin* genes were tested as the housekeeping gene, and *EF1-α* was selected as an internal control for relative quantification based on its consistency and efficiency in dilution series from different plant tissues (average C_T: 18.79 to 28.71, PCR efficiency: 1.05, slopes: 3.2, r^2: 0.99) [65]. Similarly, *EF1-α* gene was previously used as the housekeeping gene in the expression analysis of chickpea plants [4,16,64]. Real-time PCR was performed using a BioRad CFX96 Real-Time PCR System (Bio-Rad Laboratories, Inc.). PCR mixtures consisted of 2 μL diluted cDNA, 0.6 μM primer, 2 × Sso Advanced SYBR Green Supermix (Bio-Rad Laboratories, Inc.) in 20 μL volume. The PCR cycle conditions consisted of 45 cycles of 10 s at 95 °C and 10 s at 60 °C. Melting curves analysis (65–95 °C with a heating rate of 0.5 °C/s) was performed at the end of all real-time PCR assays to confirm the presence of non-specific products and primer dimers.

2.5. Statistical analysis

Chickpea plants were scored based on the 1–9 scale and the percentage of the disease severity was calculated. The data were compared by the least significant difference test (LSD, $P = 0.05$) using the Minitab 17 statistical software (Minitab Inc., State College, PA, USA). The relative quantification of gene expression was determined by applying the $2^{-\Delta\Delta CT}$ method [25] using Ct-values of the *EF1-α* gene for normalization. The obtained data were subjected to ANOVA analysis or Student's t-test analysis using Minitab 17 statistical software (Minitab Inc., State College, PA, USA). Three biological replicates for each sample and two technical replicates of each biological replicate were analyzed for real-time PCR analysis. A mean fold change expression level of 1.5 was used as a cut-off point and statistically significant.

Table 1
Gene-specific primers used in quantitative real time-PCR for amplifying defense-associated genes of chickpea cultivars inoculated *Ascochyta rabiei*.

Gene description	Gene ID	Primer name	Forward primers	Reverse primers	Literature
Polymorphic antigen membrane protein (*PAMP*)	DY475248	PAMP	CCGCTGATACAGTGGAGGTT	GTTTCCCCAATTTCCTCACC	Leo et al. [15]
Glutathione S-transferase (*GST*)	DY475250	GST	TCCCTCCAACCTACTAACAAGG	TTTGGATTGGATAAGATTTGGTTT	Leo et al. [15]
SNAKIN2 antimicrobial peptide precursor (*SN2*)	CV793608	SN2	CATGGCAACAAGACCAAGTGTC	GTTGGGAACAAAGTAGGGACTG	Leo et al. [15]
CaETR1 (Ethylene receptor-like sequences)	DY396400	CaETR1	TAGGGTTTGGACCAAGCAAG	CTTCTGAGACTGCTGCAACG	Madrid et al. [48]
serine/threonine-protein kinase	LOC101488744	Ca_05521	GTTGCCTTAGAGACTGCTAC	ACCTGGTGTTCCTTGTGG	Li et al. [4]
Wall-associated-receptor kinases (*WAK*)	LOC101489079	Ca_05520	GGAGAGTAGAACAGTTTG	ATGTAGATAAGCCAATGC	Li et al. [4]
LRR receptor-like kinase	LOC105851082	Ca_05515	CTGAAGCAATAGACGGTAG	CAGCAGTCCATTCCTCTC	Li et al. [4]
Nucleotide binding site and leucine rich repeat (*NBS-LRR*) gene-9145	LOC101509145	NBS-LRR-9145	TTGAAATTGATAGACCTCCCAAAC	CAGCAGCAACATCTAATTCTTGA	Sagi et al. [19]
Nucleotide binding site and leucine rich repeat (*NBS-LRR*) gene-5691	LOC101495691	NBS-LRR-5691	GGATTGCTGGATTGGTGGCT	CCAACTTGACTTTCCCAGGC	Sagi et al. [19]
Nucleotide binding site and leucine rich repeat (*NBS-LRR*) gene-8365	LOC101498365	NBS-LRR-8365	GGAAGAGAGAGTGACAGGAAAG	AGCCACCAATTCCCACTATG	Sagi et al. [19]
CaWRKY16	LOC101500706	RTWR-17F	AAGGATTCATGGACGAGAACAATAG	CTTGCCGTATTTTTTCCAGGTAA	Kumar et al. [23]
CaWRKY50	LOC101502574	RTWR-51F	ATCGGACGAAGATCCCAATATG	ACGTGGCTCAGTGACATTGTTT	Kumar et al. [23]
NADH-ubiquinone oxidoreductase (*Ar65*)	GW996388	Ar65RT1	CCGCCACATCAACATTCCTA	GGCCACCAGAGGAGCTTGT	Sing et al. [64]
E3 SUMO-protein ligase PIAS1 (*Ar9*)	GW996332	Ar9RT	GACCTGACGTTGAGCGATGA	TCTGCCCTTGCCTTTTTAGC	Sing et al. [64]
nucleic acid-binding-OB-fold, *ST47_g4184*	-	ST47_g4184 RT	CTGCTGCATGCCATTCTTTC	CGAGCGTGCGGTTAGTTCTC	Verma et al. [16]
C2H2 zinc finger, *ST47_g10012*	-	ST47_g10012 RT	CGTTCCGCAGCCTCATCTT	CCAACCCAAGGGTGAAGGA	Verma et al. [16]
Internal control gene	AJ004960	EF1α	TCCACCACTTGGTCGTTTTG	CTTAATGACACCGACAGCAACAG	Garg et al. [65]

3. Results

Disease symptoms on chickpea cultivars appeared 6 days after inoculation, but the partially resistant cultivar 'ILC482' exhibited a slower development of symptoms compared to the susceptible cultivar 'Sarı98'. The analysis of data by Student's t-test indicated significant differences between the two cultivars ($p < 0.01$). The ILC482 cultivar was determined to be partially resistant to Ascochyta blight, while the Sarı98 cultivar exhibited susceptibility with severity scores ranging from 45% to 90% at 21 days after inoculation, respectively (Fig. 1, Fig. 2).

To evaluate differences in the response of chickpea cultivars to Ascochyta blight, we selected 16 genes involved in different defense pathways such as cell signaling, cell growth, oxidative burst, SA accumulation, hypersensitive response, pathogenesis-related gene expression, ethylene pathway, detoxification of various compounds, biochemical changes, and plant immune responses based on a literature search on the defense responses of chickpea to Ascochyta blight. The expression levels of these genes were evaluated at five-time points after inoculation using qRT-PCR. The melting curve analysis produced a single peak with different melting point for each primer, confirming the expression of only one product (Table S1). Of the 16 defense genes selected, 12 genes exhibited detectable expression levels in the samples. Transcripts of *Ar65*, *Ar9*, *ST47_g4184*, and *ST47_g10012* were not detectable and therefore could not be analyzed.

Both cultivars showed significant variations in *NBS-LRR* gene transcript levels at different time points (Fig. 3). *NBS-LRR* gene-9145 demonstrated a significant yet slight upregulation (1.92 and 2.25 fold) in ILC482 at 6 and 72 hpi. The transcript amount at 12, 24, and 48 hpi remained below the baseline level. Similarly, Sarı98 did not exhibit any significant expression pattern for the *NBS-LRR* gene-9145 transcript at any inoculation point. *NBS-LRR* gene-5691 was significantly expressed in both cultivars at all the time points except for 48 hpi. The transcript of *NBS-LRR* gene-5691 was upregulated about 3.22 and 3.31 fold in ILC482 from 6 to 24 hpi, followed by a decrease (1.18 fold), respectively. The susceptible cultivar, Sarı98, showed a weak elevation of 1.76 fold at 6 hpi only. The expression enhancement of *NBS-LRR* gene-8365 in the partially resistant cultivar was significantly distinct from the susceptible cultivar at 12 and 24 hpi, with the highest expression in ILC482 reaching 3.73-fold at 24 hpi (Fig. 3). The *LRR* gene expression did not differ significantly between the two cultivars till 24 hpi, and the activity



Fig. 1. Disease symptoms observed on susceptible (Sarı98) (a) and partially resistant (ILC482) (b) chickpea cultivars 21 days after inoculation with *Ascochyta rabiei*.

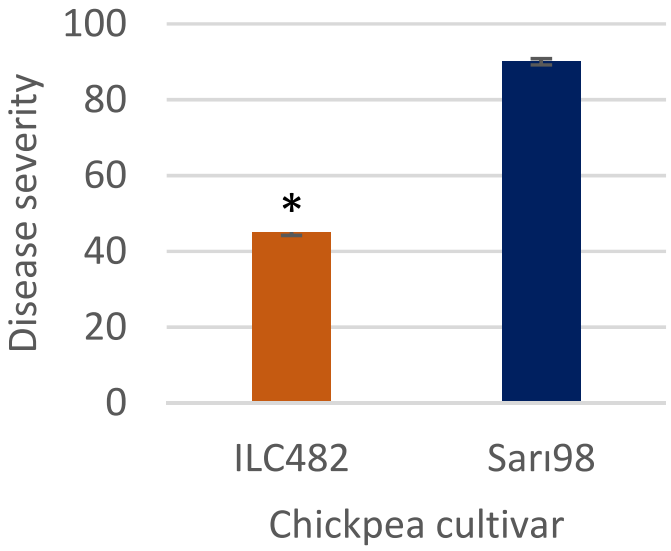


Fig. 2. Disease severity values of two chickpea cultivars; Sarı98 (susceptible), ILC482 (partially resistant) to *Ascochyta rabiei* based on the 1–9 scale. Data are presented as means ± standard deviation (SD) from three independent replicates. Asterisks indicate the statistical significance of differences using the Student's t-test ($p < 0.01$).

remained below the baseline level. Sarı98 exhibited a slight increase in transcript level at 48 hpi, peaking at 3.45-fold at 72 hpi. ILC482 showed a weak response at 24 hpi and a strong 5.23-fold induction of *LRR* gene at 72 hpi. *CaWRKY16* expression was significantly higher in ILC482 than in Sarı98 at all the time points, except for 72 hpi (Fig. 3). The expression of *CaWRKY16* gene in Sarı98 and ILC482 cultivars peaked at 8.84 and 15.89-fold, respectively, at 6 hpi, followed by a decrease at 12 hpi (2.35 and 6.23 fold). At 24 hpi, *CaWRKY16* levels increased again, reaching 3.82 and 6.89-fold. The *CaWRKY50* gene was significantly expressed in ILC482 at 6 hpi and thereafter, while no significant expression was determined in Sarı98 till 72 hpi. The transcript in ILC482 peaked at 12.16-fold at 72 hpi, while the expression rate in Sarı98 was 7.9-fold (Fig. 3).

The *CaETR1* gene exhibited a significant increase in both cultivars within the first 6 hpi, followed by a decrease at 12 hpi (Fig. 4). Expression levels at 24 hpi in Sarı98 and ILC482 were 2.99 and 5.7-fold, respectively. The expression elevation of *WAK* gene was notably higher in the partially resistant cultivar ILC482 compared to the susceptible cultivar Sarı98 with a maximum expression level observed at 6 hpi (Fig. 4). A slight attenuation of *WAK* gene transcripts (12.83-fold) was observed for ILC482 at 12 hpi, while the level in Sarı98 returned to basal level at this point. The expression of *WAK* was upregulated at 24 hpi in both cultivars, followed by downregulation at 48 and 72 hpi. Additionally, the serine/threonine-protein kinase gene in ILC482 exhibited a significant increase in expression at 6 hpi with a level approximately 10-fold higher than that observed in Sarı98 (Fig. 4). The expression level in ILC482 was upregulated at the other time points. The level of serine/threonine-protein kinase mRNA in Sarı98 was slightly induced at 6 and 12 hpi, and the maximum level was observed at 48 hpi.

SNAKIN2 expression was upregulated approximately 3.89 and 3.01-fold in susceptible and partially resistant cultivars at 6 hpi, respectively; succeeded by a weak downregulation at 12 hpi (Fig. 4). The highest level of *SNAKIN2* expression in ILC482 was observed at 24 hpi, with a 4.52-fold enhancement. The *SNAKIN2* transcript in both cultivars sharply reduced below the baseline at 48 and 72 hpi. *PAMP* expression in both cultivars was significant at 6, 12, and 24 hpi, with ILC482 exhibiting a higher transcript level than Sarı98. The maximum *PAMP* level occurred at 24 hpi with 5.7 and 9.9-fold increases for Sarı98 and ILC482, respectively. *GST* activity was significantly distinct between both cultivars at all inoculation points, with the maximum activity detected at 6 hpi (Fig. 4). The expression level at 12 hpi attenuated rapidly in both cultivars, followed by a 5.7 and 9.92-fold increase at 24 hpi. *GST* expression in ILC482 exceeded that of Sarı98 at all-time points except for 72 hpi.

4. Discussion

Host plant resistance is considered the most effective and practical approach in disease management strategies against *Ascochyta rabiei* [4,14]. Previous studies suggested that genetic resistance to Ascochyta blight is controlled by several genes in quantitative trait loci (QTL) of which are identified in the linkage loci groups [9–11,26–28]. However, the genetic background of Ascochyta resistance is not well established. Expression profiles using qRT-PCR offer valuable insights into the possible roles of defense genes related to plant response against pathogen infection. Earlier research on chickpea defense genes to Ascochyta has identified the changes of various defense genes, however, comprehensive analyses regarding the timing and magnitude of these genes across various host genetic backgrounds have not been conducted [16,23,64]. In the present study, the differential expression of several valuable genes associated with defense mechanisms in chickpea was evaluated in two chickpea cultivars with different levels of resistance. Significant differences were observed in the expression levels and timings of some transcripts linked to defense responses against Ascochyta infection.

NBS-LRR genes constitute a major class of *R* genes that detect pathogen presence and activate defense pathways [21,29]. The regulatory role of *NBS-LRR* proteins in plant defense has been confirmed in the genomes of different plant species [30–32]. Various *NBS-LRR* genes from chickpea have been identified to play significant roles in defense mechanisms [19,21,33]. This study evaluated the function of some *NBS-LRR* genes in response to chickpea cultivars against Ascochyta blight. The expression levels of *NBS-LRR* gene-9145 and *NBS-LRR* gene-5691 genes were significantly higher in the partially resistant cultivar, while the levels in Sarı98 mostly remained below the baseline level. The *NBS-LRR* gene-8365 and *LRR* transcripts in both cultivars exhibited significant differences. The overexpression of these transcripts suggests a crucial role of *NBS-LRR* genes in enhancing resistance to *A. rabiei*. This finding is consistent with the results of Sagi et al. [19], who indicated that the overexpression of *NBS-LRR* genes involved in the activation of plant defense was correlated with Ascochyta blight QTLs. Some *NBS-LRR* genes were more upregulated in partially resistant cultivar compared to susceptible cultivar to Ascochyta blight. The expression timing and magnitude of these genes in resistant plants have a more significant effect on host-pathogen resistance, reducing more pathogen growth and spread compared to susceptible plant. This is in agreement with the findings of Zhou et al. [20] who explored the differences in the expression of resistance gene analog (RGA) associated *NBS-LRR* genes in chickpea varieties with varying susceptibility to *A. rabiei* infection. They noted that a higher expression of certain RGAs was observed in the resistant genotype during the early stages following inoculation. Similarly, *NBS-LRR* and *WRKY* genes have been known to be associated with Fusarium wilt resistance of resistant and susceptible chickpea genotypes [66]. Correspondingly, various studies have demonstrated the possible involvement of *NBS-LRR* genes in resistance response to plant pathogens [34–39]. *NBS-LRR* genes from Arabidopsis confer resistance to *Fusarium oxysporum*, *Hyaloperonospora parasitica*, and *Pseudomonas syringae* [34,35]. Overexpression of the grapevine *TIR-NB-LRR* gene, *VaRGA1*, resulted in enhanced resistance to *Phytophthora capsici* in *Nicotiana benthamiana* [36]. *Pi64*, a *CC-NBS-LRR* protein, was found to play a significant role in resistance to leaf and neck blast in rice [37]. Differential expression of *NBS-LRR* resistance gene between cultivated peanut (*Arachis hypogaea*) and wild peanut (*A. duranensis*) played a role in resistance to *Aspergillus flavus* infection [38]. In soybean, a significant correlation between *NBS-LRR* genes and the disease resistance QTLs demonstrated the potential function of these

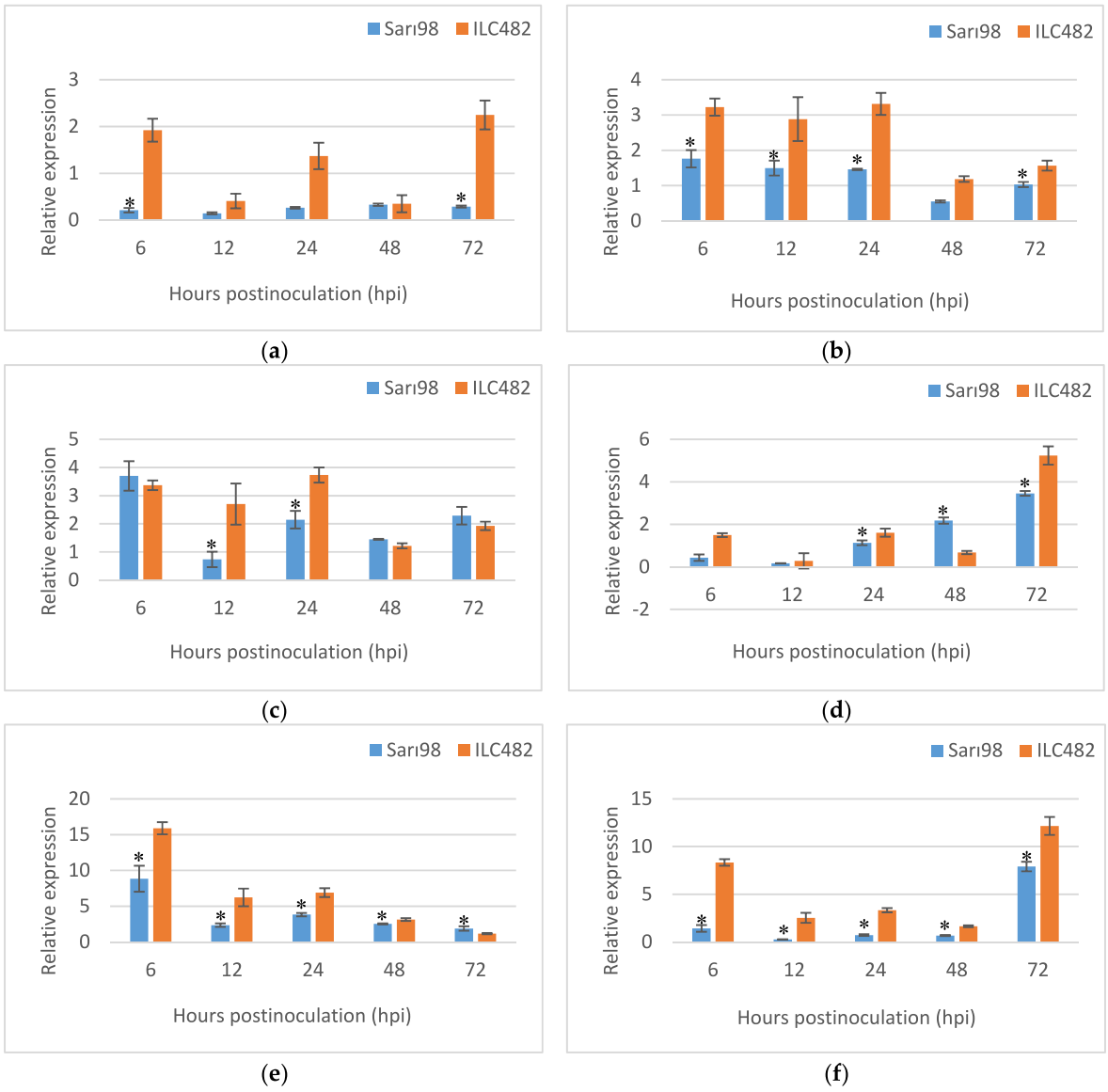


Fig. 3. Expression analysis of *NBS-LRR* gene-9145 (a), *NBS-LRR* gene-5691 (b), *NBS-LRR* gene-8365 (c), *LRR* (d), *CaWRKY16* (e) and *CaWRKY50* (f) genes in partially resistant (ILC482) and susceptible (Sarı98) cultivars of chickpea inoculated with *Ascochyta rabiei*. The expression data are expressed as normalized fold change to *EF1-α* expression. A fold change greater than 1.5 is considered biologically significant. Data are presented as means ± standard deviation (SD) from three independent replicates. Asterisks indicate the statistical significance of differences using the Student's t-test ($p < 0.01$).

resistance genes in plant defense pathways [39].

WRKY genes are known to play a key role in the regulation of various metabolic pathways in the response of plants to multiple stresses [40]. In recent years, several *WRKY* genes have been identified in the genome of chickpea using genome-wide analysis, and their expression patterns have been evaluated under biotic and abiotic stresses [23,41,42]. The present study aimed to determine the function of certain *WRKY* genes involved in the defense mechanism of Ascochyta blight. The findings revealed that *WRKY* genes, especially *CaWRKY16* and *CaWRKY50* had a significant role as potential candidate genes for improving chickpea resistance against Ascochyta blight. The expression patterns of *WRKY* transcripts exhibited variation differed between partially resistant and susceptible cultivars at an early stage of pathogen infection. The expression of these *WRKY* genes is also associated with stress mechanisms signaling by salicylic acid [23]. Similarly, earlier studies confirmed the role of *WRKY* genes in coping with biotic and abiotic stresses of various plant species. *WRKY genes* have been suggested to function in multiple defense-responsive pathways in chickpea to *F. oxysporum* f. sp. *ciceris* infection [41,43,66]. The overexpression of Arabidopsis *WRKY33* enhanced resistance against necrotrophic fungal pathogens, *Botrytis cinerea,* and *Alternaria brassicicola* [44]. In chili pepper, *CaWRKY1* enhanced resistance against *Pseudomonas syringae* and Tobacco mosaic virus [45]. The expression of *TaWRKY70* in wheat provided resistance against the stripe rust pathogen *Puccinia striiformis* f. sp. *tritici* [46].

CaETR1, an ethylene receptor-like sequence linked to QTL_{AR1}, has been identified as a candidate gene for differentiating between resistance and susceptible chickpea genotypes in response to Ascochyta blight [47,48]. In this study, the expression of *CaETR1* was higher in the partially resistant cultivar, suggesting that differential expression of this gene could offer considerable benefits for resistance screening of chickpea genotypes against the pathogen. This is in agreement with the results presented by Leo et al. [15], who observed notable differences in expression levels between resistant/moderately resistant and susceptible genotypes. Wall-associated-receptor kinases (*WAK*) and serine/threonine-protein kinases are known to regulate cellular pathways associated with resistance to stress conditions as well as plant growth and development. Our results indicated both genes have active

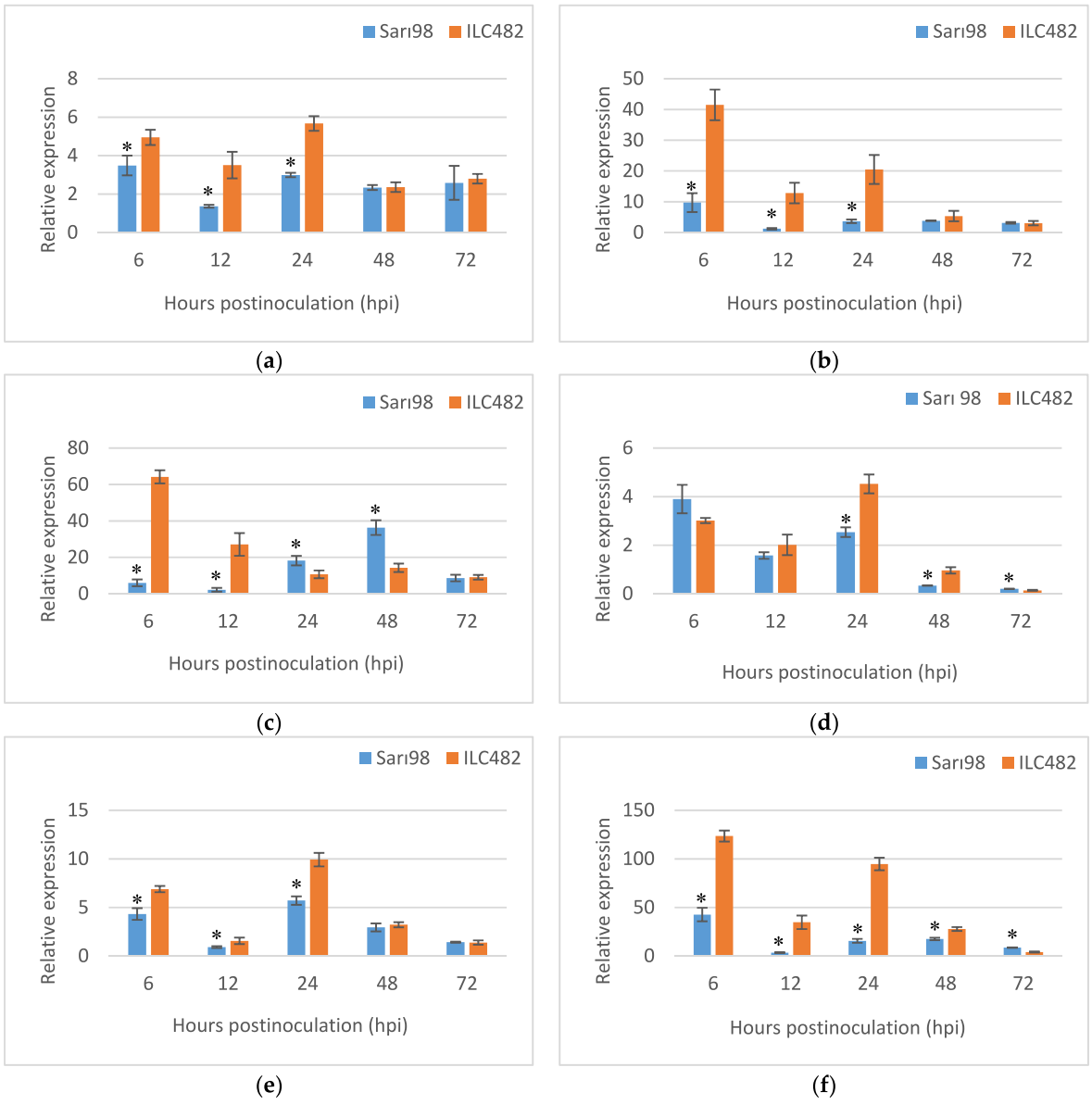


Fig. 4. Expression analysis of *CaETR1* (a), Wall-associated-receptor kinases (*WAK*) (b), serine/threonine-protein kinase (c), *SNAKIN2* (SN2) (d), Polymorphic antigen membrane protein (*PAMP*) (e) and Glutathione S-transferase (*GST*) (f) genes in partially resistant (ILC482) and susceptible (Sarı98) cultivars of chickpea inoculated with *Ascochyta rabiei*. The expression data are expressed as normalized fold change to *EF1-α* expression. A fold changes greater than 1.5 is considered biologically significant. Data are presented as means ± standard deviation (SD) from three independent replicates. Asterisks indicate statistical significance of differences using the Student's t-test ($p < 0.01$).

roles in resistance against Ascochyta blight and exhibited high expression levels in the partially resistant cultivar in the early stages of infection. Similarly, Li et al. [4] observed elevated expression of *WAK* and serine/threonine-protein kinases in resistant cultivars. Snakins, a class of cysteine-rich peptides, are involved in plant defense response associated with disruption of the cell membrane as well as the plant growth process [49,50]. *SNAKIN2*, sourced from tomato, has been well-characterized and showed broad-spectrum antifungal and antibacterial activity against plant pathogens [51,52]. In this study, the overexpression of *SNAKIN2* gene increased at 24 hpi in response to pathogen infection. This enhanced expression in the partially resistant cultivar is in accordance with the greater expression of *SNAKIN2* previously determined in resistant or moderately resistant chickpea genotypes [14,15]. Similarly, the overexpression of *SNAKIN2* gene in transgenic tomato enhanced tolerance to *Clavibacter michiganensis* sub. sp. *michiganensis* [53]. Conversely, silencing this gene in tobacco plants caused an increase in susceptibility to *C. michiganensis* [54]. *PAMP* is a surface protein with a leucine-zipper motif and is responsible for the transcription of defense-related genes [55]. This study found that *PAMP* gene was differentially expressed in the partially resistant and susceptible cultivars, with upregulation observed from 6 to 24 hpi. This result is consistent with the findings of Leo et al. [15], who detected higher gene expression in all genotypes except for the highly susceptible Kaniva genotype. Similarly, Coram and Pang [14] proved that *PAMP* expression was associated with disease resistance of chickpea genotypes against Ascochyta blight.

Glutathione S-transferase enzyme genes have active roles in plant defense responses against biotic and abiotic stresses as well as plant growth and development [56,57]. Previous studies on the possible functions of *GSTs* in diverse plant-pathogen interactions have indicated the upregulation of various *GST* genes in defense responses after pathogen infection [58,59]. In tomato, *GST* activity was upregulated more rapidly in resistant plants against powdery mildew disease, while *GST*-silenced plants showed increased susceptibility [60]. *GSTU13* from

Arabidopsis thaliana was considered a vital gene involved in the activation of the defense response, as the lack of functional *GSTU13* gene caused to enhance susceptibility to various pathogens [61]. Our finding confirmed the induction of *GST* gene in response to Ascochyta blight, with higher *GST* activity detected in the partially resistant cultivar till 72 hpi. Similarly, Leo et al. [15] noted that *GST* expression was higher in resistant chickpea genotypes than in susceptible ones. However, *GST* expression was downregulated during the interaction of banana -*F. oxysporum* f. sp. *cubense* and bean-*Rhizoctonia solani* [62,63].

5. Conclusions

The current study evaluated the changes in the expression of valuable genes involved in the defense responses of chickpea cultivars with various resistance levels at five-time points during the Ascochyta infection process. We identified stronger and earlier expression of defense genes in the partially resistant cultivar, suggesting that resistance to Ascochyta blight is associated with overexpression of these defense genes, in particularly *CaWRKY16*, *CaWRKY50*, *glutathione S-transferase*, *CaETR1*, and *WAK* genes. The expression patterns of these genes, which play an essential role in resistance response, varied significantly at different time points after infection and, therefore, may be used as potential candidates for breeding programs and new plant protection strategies. The results obtained from this study also provided us with a better understanding of the role of valuable genes involved in controlling Ascochyta blight, but more research is still necessary to gain more insight into the molecular mechanisms underlying Ascochyta-chickpea interactions.

Author contributions

Gülsüm Palacıoğlu: Investigation, Visualization; Göksel Özer: Resources, Methodology, Writing-review and editing; Harun Bayraktar: Funding acquisition, Software, Supervision, Writing-review and editing. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pmpp.2023.102107.

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