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Differentially expressed genes in resistant and susceptible *Pistacia vera* L. Cultivars in response to *Pseudomonas fluorescens* and *Phytophthora parsiana*

Najmeh Hajabdollahi[a], Roohallah Saberi Riseh[a], Pejman Khodaygan[a], Mohammad Moradi[b] and Kobra Moslemkhani[c]

[a]Department of Plant Protection, Faculty of Agriculture, Vali –e- Asr University of Rafsanjan, Iran; [b]Pistachio Research Center, Horticultural Sciences Research Institute, Agricultural Research, Education and Extension Organization, Rafsanjan, Iran; [c]Seed and Plant Certification and Registration Institute, Agricultural Research, Education and Extension Organization, Tehran, Iran

ABSTRACT

Gummosis (Phytophthora root and crown rot) is an important and destructive disease that affects fruit trees like pistachio (*Pistacia vera* L.). Although many studies have been performed on the possibility of biological control of gummosis, up to now few studies have been done on transcriptionally induced resistance genes in pistachio against Phytophthora disease. In the present research, the cDNA-AFLP method was used to analyze the transcriptionally regulated genes during the interaction between susceptible (Sarakhs) and resistant (Badami-Rize Zarandi) pistachio cultivars with *Pseudomonas fluorescens* and/or *Phytophthora parsiana*. Using 12 AFLP primer combinations, approximately 740 different cDNA-AFLP fragments with sizes ranged from 50 to 700 bp were generated for susceptible and resistant pistachio cultivars. Forty six TDFs (transcript derived fragments) were then selected for susceptible cultivar and 24 TDFs for resistant cultivar. Accordingly, each one of these TDFs was recognized by similarity search using the BlastX program against the NCBI GenBank nucleotide database. Notably, 18.5% of TDFs were hypothetical or uncharacterized proteins and 1.4% of them indicated no significant similarity to the known genes. Among the TDFs with known function, disease resistance proteins were identified, which were newly regulated in response to pathogen or rhizobacterium. Furthermore, we found that the expression level of different growth-regulatory or defense-related genes was higher after the exposure to *Pseudomonas fluorescens* and/or *Phytophthora parsiana*. To better understand the defense mechanism of pistachio plants and recognition of *P. fluorescens*-activated genes, which induce resistance against *P. parsiana*, would significantly assist in the development of new resistant pistachio cultivars.

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CDNA-AFLP; gummosis; *P. fluorescens*; induced resistance; pistachio

Introduction

In the past decades, the production of crops has been mainly based on the application of high-yielding cultivars as well as the extensive use of chemical pesticides and fertilizers.

CONTACT Roohallah Saberi Riseh r.saberi@vru.ac.ir

Despite several crop protection tactics, the losses caused by pathogens, insects, and weeds are estimated to be between 20% and 40% for the main crops worldwide (Baltes et al., 2017; Saberi-Riseh et al., 2004). Several pistachio diseases have been reported from different regions of the world, among which soil-borne diseases such as Phytophthora crown and root rot (gummosis), Verticillium wilt, and root rot nematode have destructive effects on commercial trees. In Iran, pistachio gummosis is considered as a serious disease that annually jeopardizes the production of pistachio. Using suitable fungicides is known as a way for controlling the disease, but the chemical products used have some adverse impacts on the environment and human health (Hirooka & Ishii, 2013; Nouri-Ganbalani et al., 2018). Hence, several researchers have focused on the use of novel tools for protecting the crop, with less reliance on hazardous chemicals (Baradar et al., 2015; Pour et al., 2019; Zeynadini-Riseh et al., 2018).

Many microbes in the soil are beneficial for plants, such as fungi [Plant Growth-Promoting Fungi (PGPF)] and rhizobacteria [Plant Growth-Promoting Bacteria or Rhizobacteria (PGPB or PGPR)], which can also enhance the adaptation of plants in undesirable situations and improve plant growth (Jankiewicz et al., 2012; Kudoyarova et al., 2017; Kwon et al., 2010). A significant number of studies have focused on inducible resistance by microbes (Pieterse et al., 2014 and references therein), and some studies have also been conducted by many groups on their applications to crop protection (Alizadeh et al., 2013; Bakker et al., 2003; Berendsen et al., 2012; Cameron et al., 2013; Contreras-Cornejo et al., 2011; Hossain et al., 2017). Accordingly, this resistance results in an accumulation of volatile compounds, generation of reactive oxygen species, expression of proteins and genes related to pathogenesis, and other unknown processes (Backer et al., 2019; Kim et al., 2015; Van Loon, 1997).

One of the researched forms of induced resistance is ISR (Induced Systemic Resistance), which was shown to be triggered by root-colonizing mutualistic microbes; such as *Pseudomonas* spp., *Trichoderma* spp., and *Paenibacillus polymyxa* (Mhlongo et al., 2018). Correspondingly, *Pseudomonas fluorescens* is a well-studied plant-beneficial rhizobacterium that, after the colonization of the roots, promotes plant growth as well as induces systemic resistance (Zamioudis et al., 2013). It has been shown that antimicrobial compounds and the related molecules are synthesized by several *Pseudomonas* spp., which can inhibit soil-borne pathogen growth (Hariprasad et al., 2013; Saraf et al., 2014). There are many reports on the expression of plant defense-related genes, especially microbial-related (MR)-protein genes, which are induced by infection with plant beneficial bacteria during the induction of ISR (Jones et al., 2019; Nie et al., 2017; Pieterse et al., 2014). In pistachio (*Pistacia vera* L.; 2n = 30), enlarged gene families(e.g. chitinase and cytochrome P450) and the pathways of jasmonic acid (JA) biosynthesis have been recognized and are expressed in a pattern that suggests they may be involved in stress adaptation (Zeng et al., 2019).

cDNA-amplified fragment length polymorphism (AFLP) is a sensitive, efficient, reproducible, and prevailing method used for the large-scale analysis of genome expression in plants (Pak et al., 2017) and to a lower-scale of study in fungi and bacteria (Dellagi et al., 2000; Lazzi et al., 2014). The cDNA-AFLP technique has been successfully examined in many quantitative expression types of research conducted on plants (Colling et al., 2013; Craciun et al., 2006; Huang et al., 2018; Xin et al., 2015; Xue et al., 2015; Yu et al., 2011).

In the present study, we used cDNA-AFLP to identify genes that were differentially expressed during *P. fluorescens* inoculation in gummosis-infected susceptible cultivar (Sarakhs), in order to find the potential defense-related genes from the early signaling of pathways in response to a microbe. Moreover, we have compared a susceptible cultivar treated with *Pseudomonas* with control plants to see possible genes that can be triggered by this bacterium in the plant. On the other hand, to investigate the reaction of susceptible and resistant cultivars of *Pistacia vera* against *Phytophthora* infection, the infected treatments were compared to their control plants. Also, we compared the differential expressions of these genes in the two tested cultivars.

Materials and methods

Cultivation of bacteria and fungi

Pseudomonas fluorescens strain VUPF5 was obtained from the laboratory of the Vali-E-Asr University of Rafsanjan (Kerman, Iran). The efficiency of this bacterial strain against gummosis had been previously shown in a study by Fathi et al. (2018). A virulent isolate of *Phytophthora parsiana* was obtained from the culture collection at the Pistachio Research Center (Rafsanjan, Kerman, Iran).

The bacterial strain used in the present study was cultured on nutrient agar (NA) plates at 28 °C. After 24–28 h, cells of bacteria were harvested from the Petri dishes and then suspended into distilled water. The density of the bacterial suspension was appropriately adjusted to the desired concentrations based on the optical density at 540 nm.

The fungal isolate was cultured on cornmeal agar (CMA) at 28 °C. Afterward, the inoculum was prepared on rice as described by Holmes and Benson (1994). Briefly, rice grains (25 g) and deionized water (18 ml) were added to a flask, which was then autoclaved twice for 30 min on two consecutive days. Thereafter, the autoclaved rice was inoculated with six plugs of 3-day old mycelium of *P. parsiana* and then incubated for two weeks at 28 °C.

Plant materials

In this study, the susceptible (Sarakhs cultivar) and resistant (Badami-rize Zarandi cultivar) pistachios were used. Seeds of pistachio were obtained from Pistachio Research Center (Rafsanjan, Kerman, Iran). To plant these seeds, a method introduced by Moradi (2015) was used with some modifications. Five seeds per each pot were sown into 4 kg plastic pots and the pots arranged using a randomized block design within the research greenhouse of the Vali-E-Asr University of Rafsanjan (Kerman, Iran), set at 26 °C–28 °C with a natural photoperiod. To prevent insect infestation, pots were protected by 100-mesh muslin.

Plant treatments

Six week-old pistachio seedlings were inoculated with 5 g of *P. parsiana* colonized rice around seedling roots, which were then covered with soil. Subsequently, the pots were

covered with larger plastic wrap to preserve the relative humidity. After 18 h, the plastic wrap was removed. In bacterial treatments, a 40-ml suspension of the bacterial strain was added to each pot for soil drenching (equal to 10^8 CFU g^{-1}). For bacterial plus fungal treatments, the pistachio seedlings were treated with both *P. fluorescens* and *P. parsiana* at the same time. The control plants were treated with 5 g sterile rice and 40 ml of sterile distilled water. In all cases, sampling was performed for subsequent experiments 2 days after treatment, because most of the enzymes and defense compounds reach the maximum level after two to three days of challenge inoculation.

RNA isolation and cDNA synthesis

In our study, mRNA was isolated from control, fungal-infected, bacterial-infected, and bacterial-fungal infected treated plants using the mRNA capture kit (Roche applied science, Germany). In this step, mRNA, which is the template for first-stranded cDNA synthesis, was isolated using Takara first-stranded cDNA synthesis kit, Japan as described earlier in the protocol of Roche Company. To synthesize doubled stranded cDNA, 7.5 µl of 10x cDNA buffer, 1 µl of DNA polymerase I (10 u/µl), 0.2 µl of RNaseH (5 u/µl), 2 µl of T4DNA Ligase, 0.25 µl of dNTP (10 mM), and 46.5 µl of water were added to 20 µl of the first-stranded cDNA and then kept for 2 h at 15 °C. The purification of double-stranded cDNAs was done using phenol/chloroform extraction and ethanol precipitation. The obtained samples were then stored at −20 °C until use.

cDNA-AFLP analysis

The preparation of the template for cDNA-AFLP was done using the method previously introduced by Bacham et al. (1998). Firstly, approximately 20 µl of cDNA obtained from each sample was digested by restriction enzyme EcoRI for 3 h at 37 °C, which was then digested with MseI for 2 h at 65 °C. Secondly, the digested products were ligated to the corresponding MseI and EcoRI adapters using T4 DNA ligase (Fermentase) at 16 °C overnight (Table 1).

Table 1. Sequences of adaptors and primers used in pre-amplification and selective amplification.

Adaptors/Primers	Sequence
EcoRI adaptor 1	5′- CTC GTA GAC TGC GTA CC-3′
EcoRI adaptor 2	5′- AAT TGG TAC GCA GTC-3′
MseI adaptor 1	5′- GAC GAT GAG TCC TGA G-3′
MseI adaptor 2	5´- TAC TCA GGA CTC AT-3′
MseI primer	5´ GAT GAG TCC TGA GTA AC3´
EcoRI primer	5´ GAC TGC GTA CCA ATT CA3´
EcoRI + A	5′- GAC TGC GTA CCA ATT CA-3′
EcoRI + AAG	5′- GAC TGC GTA CCA ATT CAAG-3′
EcoRI + ACC	5′- GAC TGC GTA CCA ATT CACC-3′
EcoRI + ACT	5′- GAC TGC GTA CCA ATT CACT-3′
Tru1I + C	5′- GAT GAG TCC TGA GTA AC-3′
Tru1I + CCA	5′- GAT GAG TCC TGA GTA ACCA-3′
Tru1I + CAC	5′- GAT GAG TCC TGA GTA ACAC-3′
Tru1I + CAT	5′- GAT GAG TCC TGA GTA ACAT-3′
Tru1I+ CAG	5′- GAT GAG TCC TGA GTA ACAG-3′

Thereafter, 1 µl of the diluted ligation sample (1:10 v/v) was used as the pre-amplification template in the reaction mixture (20 µl) containing double-distilled water (8 µl), *Taq10* 2×Master Mix Red (Amplicon) (10 µl), and 10 mM pre-amplification primers (0.5). In the initial step, the cycling parameters were for 5 min at 94 °C followed by 20 cycles for 30 s at 94 °C (denature), for 1 min at 56 °C (anneal), for 1 min at 72 °C (extend), and the final extension for 10 min at 72 °C.

The pre-amplified products were diluted with double-distilled water (1:50 v/v) for selective amplification using 12 AFLP selective primer combinations. The cycling parameters were initial step at 94 °C for 5 min followed by 13 cycles of 94 °C for 30 s, 65 °C for 30 s (decreased by 0.7 °C in each cycle), and 72 °C for 1 min; 23 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min.

The separation of amplified products was done on 7% denaturing polyacrylamide gel electrophoresis (PAGE) for around 90 min at a constant voltage of 90 V using 20 × 25 cm gel apparatus (Nojan Pars, Iran), and then visualized using the Silver staining method (Creste et al., 2001).

Isolation and sequencing of the amplified cDNA products

AFLP fragments based on differential display were cut from polyacrylamide gels using a clean razor blade, and then incubated in 100 ml of Tris-EDTA buffer [Tris (10 mM) and EDTA (1 mM; pH 8.0)] within a boiling water bath for 10 min. The fragments were re-amplified under the same conditions of selective amplification protocol as described earlier with the corresponding primers. The resulting products were sent to TOPAZ GENE Company (Iran) to perform direct sequencing and then to ascertain their similarity to known proteins compared to the GenBank database using BLASTX.

Results

Screening of defense-related genes in pistachio by cDNA-AFLP

In infected and non-infected tissues of resistant and susceptible pistachio cultivars, differential transcript profiles of the cDNA-AFLP analysis were performed (Figure 1).

Using 12 AFLP primer combinations, in each cultivar, approximately 740 different cDNA-AFLP fragments with sizes ranging from 50 to 700 bp were generated. Differential intensity derived from differentially expressed fragments after the inoculation of the pistachio plants compared to their non-infected plants, was then selected for analysis. The up and down-regulated bands, which were newly induced or inhibited after the infection were re-amplified and purified to perform direct sequencing. A total of 70 transcript derived fragments (TDFs) (46 TDFs for cultivar Sarakhs and 24 TDFs for cultivar Badami) were selected for conducting further studies in which the differential TDFs accounted for 9.5% of the total TDFs. In addition, 18.5% of TDFs were hypothetical or uncharacterized proteins and 1.4% of them showed no significant similarity with known genes. Each one of these fragments was identified by similarity search using the basic local alignment search tool (BLAST) program against the NCBI GenBank nucleotide database.

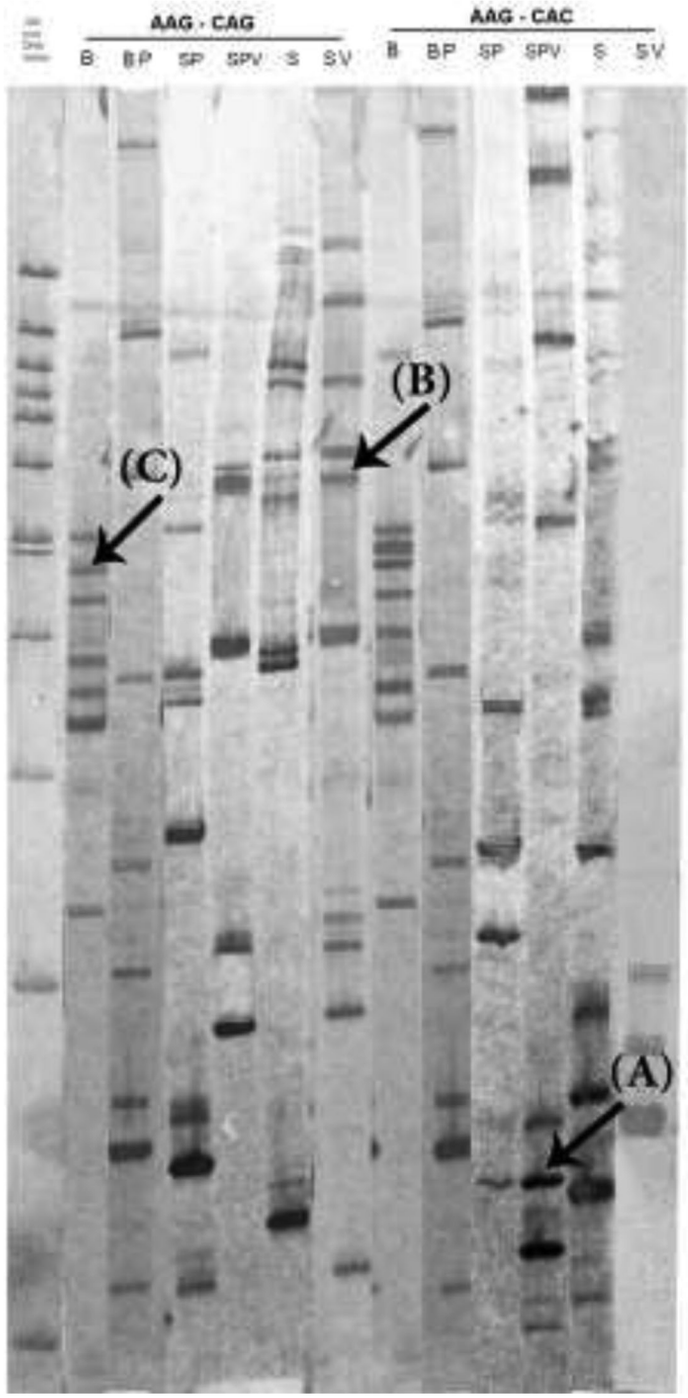

Figure 1. Differential display of transcript-derived fragments (TDFs). Products of 2 primer combination pairs (E-AAG/T-CAG, E-AAG/T-CAC) are shown. Arrows indicate the following results: up regulating after bacterial inoculation (A and B), turned off after exposure to a pathogen (C). B: Badami cultivar; BP: Badami cultivar + *Phytophthora parsiana*, SP: Sarakhs cultivar + *Phytophthora parsiana,* SPV: Sarakhs cultivar + *Phytophthora parsiana* + *P. fluorescens* strain VUPF5, S: Sarakhs cultivar.

Functional classification and homology analysis of transcript-derived fragments (TDFs)

In Sarakhs cultivar, out of the TDFs with homology to the known genes; 8, 3, 2, 18, and 8 fragments were known as parts of protein synthesis and modification-related genes, chloroplast-related genes, transport-related genes, gene expression and RNA metabolism-related genes, and defense and stress response-related, respectively and the function of 7 TDFs was unknown.

In Badami cultivar, out of the TDFs with homology to the known genes, 5, 1, 1, 5, and 5 fragments were known as parts of protein synthesis and modification-related genes, chloroplast-related genes, transport-related genes, gene expression and RNA metabolism-related genes, and defense and stress response-related ones, respectively. Also, the function of 7 fragments was unknown (Figure 2). After performing the similarity search, the treated pistachios had more up-regulated TDFs across the primary functional categories compared to the control plants.

In Badami-Rize Zarandi (resistant cultivar) after inoculation with the pathogen, photosynthesis, and respiratory chain-related genes were observed as follows: photosystem I chlorophyll an apoprotein A1, cytochrome c oxidase subunit ii, and NADH dehydrogenase subunit I. Additionally, two resistance genes were identified as *Pistacia vera* disease resistance-like protein DSC1 and *Pistacia vera* putative disease resistance protein At4g11170, which are expressed in response to *Phytophthora parsiana*.

In Sarakhs cultivar (susceptible), similar to Badami-Rize Zarandi, after its exposure to the pathogen, the genes associated with photosynthesis and respiratory chain were upregulated. Interestingly, the expression level of cytochrome c oxidase was higher in plants

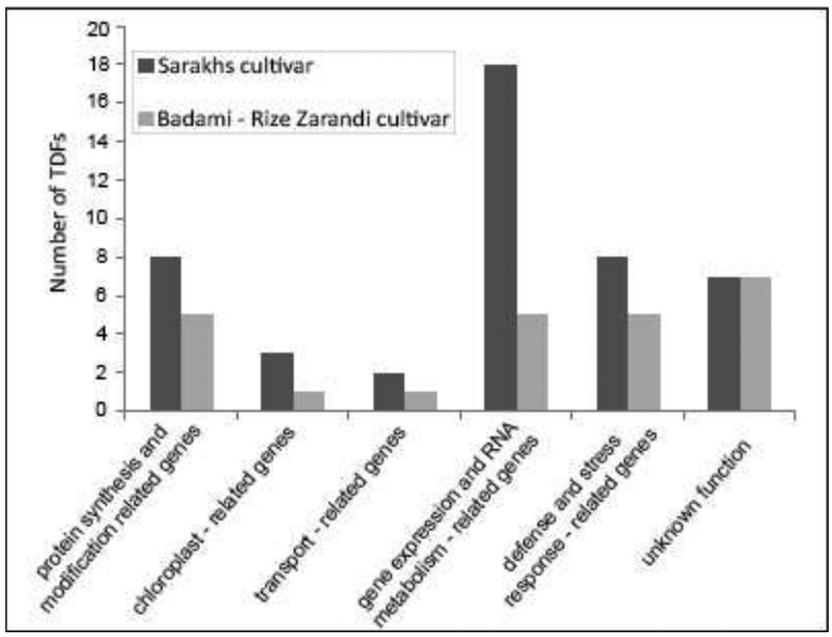

Figure 2. Functional classification of transcript-derived fragments (TDFs).

treated with both *Phytophthora parsiana* and *Pseudomonas fluorescens* compared to those plants treated with *Phytophthora parsiana* alone.

In Sarakhs cultivar after *Pseudomonas fluorescens* inoculation, some genes such as *Pistacia vera* transcriptional corepressor SEUSS and *Pistacia vera* MLP-like protein 28 were expressed. Moreover, *Pistacia vera* MLP-like protein 28 was also expressed in susceptible control plants, plants treated with *Phytophthora parsiana* alone, and plants with both *Phytophthora parsiana* and *Pseudomonas fluorescens*. However, the intensity of expression was higher in microbe treated plants. The complete list of TDFs identified in cDNA-AFLP analysis is shown in Table 2.

Discussion

The present study showed that the annotation of microbe- and/or pathogen-responsive TDFs indicate genes that probably act in early cascades of plant defense responses including signal transduction or pathogen distinction (Asai et al., 2002; McHale et al., 2006). Our data have also indicated that the differentially expressed TDFs identified were up-regulated during the infection of susceptible and resistant cultivars of pistachio, which possibly reflect different managements of cellular resources and/or the repression of defensive responses in these cultivars (Grenville-Briggs & Van West, 2005).

Resistance genes are known as the main components of plant-pathogen interactions, which can consequently activate immunity or resistance responses against pathogen invasion. It is noteworthy that most of the plant resistance genes against diseases encode NBS-LRR proteins (Li et al., 2015). Proteins of the interleukin-1 family were also found to play important roles in host defense, injury, and stress. In Sarakhs (susceptible) cultivar, the expression of interleukin-1 receptor antagonist (IL1RN) was identified. The protein encoded by this gene inhibits the activities of interleukin-1. Therefore, it may be considered as a reason why this cultivar is susceptible to Phytophthora attack. On the other hand, we have found two disease resistance genes in the resistant cultivar after *P. parsiana* exposure, which are as follows: *Pistacia vera* disease resistance-like protein DSC1 and *Pistacia vera* putative disease resistance protein At4g11170. DSC1 is a member of the TIR-NB-LRR family, which plays a major role in plant defense. This protein acts as a stimulator of hypersensitive response (HR) and as a guard for CAMTA3, which is a negative regulator of immunity during pathogen attack. Li et al. (2015) in their study considered a role for *DSC1* in *Gossypium hirsutum* resistance against Verticillium wilt. They reported a higher expression of *GhDSC1* in response to *Verticillium* wilt and jasmonic acid (JA) treatments in resistant cotton varieties in comparison to susceptible varieties, and its product was localized to the nucleus. This resistance response was shown to be related to the accumulation of reactive oxygen species (ROS) and higher expression of JA-signaling-related genes. Putative disease resistance protein At4g11170, which belongs to NB-ARC domain R proteins, plays some roles in defense response, response to ozone, and signal transduction. In another study, Wen et al. (2015) characterized a putative disease resistance gene in grapevine after a challenge of leaves with *Erysiphe necator* (Schw.) as a causal agent of powdery mildew. This gene encodes a protein with an NB-ARC domain from wild Chinese grapevine *Vitis pseudoreticulata* accession 'Baihe-35-1. Altogether, our study suggests that these resistance genes

Table 2. Homology of differentially expressed TDFs identified in the cDNA-AFLP analysis.

TDF	Primer combination	length (bp)	Similarity to gene	Identities	Regulation
B327	E + ACT/T + CCA	265	Gossypium raimondii isolate D5-4 chromosome D5_06	97%	Down
B404	E + ACT/T + CAC	375	ZR3 ATP-dependent Zn protease	70%	Newly expressed
BP53	E + AAG/T + CCA	381	Eukaryotic synthetic construct chromosome 17	85%	Newly expressed
B46	E + AGG/T + CCA	346	Pistacia vera uncharacterized protein LOC116120957	85%	Newly expressed
B136	E + AAG/T + CAC	239	Pistacia vera peptidyl-prolyl cis-trans isomerase FKBP53-like	89%	Up
B173	E + ACC/T + CAG	208	Pistacia vera serine/threonine-protein kinase-like protein ACR4	95%	Up
B200	E + ACC/T + CAC	259	Sciadopitys verticillata small subunit ribosomal RNA gene, partial sequence; chloroplast	100%	Up
B233	E + ACC/T + CCA	252	Pan troglodytes BAC clone CH251-516F10 from chromosome x, complete sequence	100%	Up
B372	E + ACT/T + CAC	592	Hypothetical protein	100%	Newly expressed
B373	E + ACT/T + CAC	378	choline O-acetyltransferase (CHAT)	84%	Up
B374	E + ACT/T + CAC	380	Pistacia vera chromatin remodeling protein SHL	98%	Up
BP57	E + AAG/T + CCA	212	Photosystem I chlorophyll an apoprotein A1	89%	Newly expressed
BP140	E + AAG/T + CAC	280	Pistacia vera disease resistance-like protein DSC1	91%	Newly expressed
BP141	E + AAG/T + CAC	285	Pistacia vera putative disease resistance protein At4g11170	89%	Newly expressed
BP227	E + ACC/T + CAT	497	Uncultured Azospirillum sp. clone OTU237 16S ribosomal RNA gene, partial sequence	84%	Newly expressed
BP237	E + ACC/T + CCA	215	Pistacia vera uncharacterized LOC116144813	85%	Newly expressed
BP241	E + ACC/T + CCA	447	Hypothetical protein	100%	Up
BP301	E + ACT/T + CAT	457	Cytochrome c oxidase subunit ii	54%	Up
BP330	E + ACT/T + CCA	497	Cytochrome c oxidase subunit ii	68%	Up
BP325	E + ACT/T + CCA	450	Pistacia vera glycosyltransferase BC10-like	74%	Newly expressed
BP402	E + ACT/T + CAC	154	Pistacia vera uncharacterized LOC116133414	94%	Down
BP408	E + ACT/T + CAC	307	Pistacia vera uncharacterized LOC116133414	95%	Down
BP409	E + ACT/T + CAC	342	NADH dehydrogenase subunit I	55%	Newly expressed
BP410	E + ACT/T + CAC	148	Hypothetical protein	72%	Newly expressed
S30	E + AAG/T + CAT	383	Pistacia vera MLP-like protein 28 (LOC116109470)	98%	Down
S32	E + AAG/T + CAT	366	Pecten Maximus genome assembly, chromosome: 2	97%	Newly expressed
S42	E + AAG/T + CAT	262	Sesbania rostrata cDNA-AFLP fragment	100%	Newly expressed
S275	E + ACC/T + CCA	339	Pistacia vera protein transport protein Sec61 subunit gamma-1	97%	Down
S276	E + ACC/T + CCA	103	Pistacia vera protein transport protein Sec61 subunit gamma-1	99%	Down
S350	E + ACT/T + CCA	256	Unknown		Down

(*Continued*)

Table 2. Continued.

TDF	Primer combination	length (bp)	Similarity to gene	Identities	Regulation
S351	E + ACT/T + CCA	200	Homo sapiens interleukin 1 receptor antagonist (IL1RN)	93%	Newly expressed
SV126	E + AAG + T +CAG	206	Pistacia vera acyl-protein thioesterase 2-like	100%	Up
SV157	E + AAG/T + CAC	168	Cnaphalocrocis medinalis clone 31 microsatellite sequence	94%	Newly expressed
SV220	E + ACC/T + CAC	330	Propionibacterium phage Wizzo, complete genome	92%	Newly expressed
SV279	E + ACC/T + CCA	455	Uncultured bacterium clone OTU_722 16S ribosomal RNA gene	77%	Newly expressed
SV280	E + ACC/T + CCA	327	Pistacia vera transcriptional corepressor SEUSS	95%	Up
SV282	E + ACC/T + CCA	330	Pistacia vera transcriptional corepressor SEUSS	98%	Up
SV283	E + ACC/T + CCA	315	Pistacia vera transcriptional corepressor SEUSS	99%	Up
SV284	E + ACC/T + CCA	618	Hypothetical protein	75%	Newly expressed
SV285	E + ACC/T + CCA	616	Hypothetical protein	64%	Newly expressed
SV287	E + ACC/T + CCA	601	Hypothetical protein	100%	Newly expressed
SV398	E + ACT/T + CAC	129	Vitis riparia x Vitis cinerea genome assembly, organelle: mitochondrion	95%	Down
SV121	E + AAG/T + CAG	419	Sphaeramia orbicularis genome assembly, chromosome: 3	81%	Down
SV318	E + ACT/T+ CAT	426	Pistacia vera MLP-like protein 28	94%	Down
SP67	E + AAG/T + CCA	84	Fusarium fujikuroi strain I1.3 chromosome VI	88%	Newly expressed
SP71	E + AAG/T + CCA	232	Photosystem I P700 apoprotein A1	47%	Up
SP96	E + AAG/T + CAG	278	Aegiceras corniculatum voucher YG209 chloroplast	99%	Newly expressed
SP99	E + AAG/T + CAG	276	Nyctanthes arbor-tristis voucher M:C. Parma 9713 chloroplast	100%	Newly expressed
SP147	E + AAG/T + CAC	279	Pistacia vera peptidyl-prolyl cis-trans isomerase FKBP53-like	95%	Down
SP210	E + ACC/T + CAC	252	Pistacia vera uncharacterized protein LOC116136329	75%	Newly expressed
SP211	E + ACC/T + CAC	280	Bacillus subtilis strain mmb14 16S ribosomal RNA gene	72%	Down
SP214	E + ACC/T + CAC	288	Leishmania major isolate MHOM/CN/2015/CPOLM-1 internal transcribed spacer 1 and 5.8S ribosomal RNA gene	96%	Newly expressed
SP333	E + ACT/T + CCA	436	elongation factor tu (tufA) gene	82%	Up
SP339	E + ACT/T + CCA	684	elongation factor tu (tufA) gene	97%	Up
SP413	E + ACT/T + CAC	399	elongation factor tu (tufA) gene	79%	Up
SP332	E + ACT/T + CCA	422	ZR3 ATP-dependent Zn protease gene	87%	Newly expressed
SP336	E + ACT/T + CCA	274	Cytochrome c oxidase subunit ii	59%	Up
SP377	E + ACT/T + CAC	273	Oryzias latipes strain Hd-rR chromosome 5 sequence	90%	Newly expressed
SPV22	E + AAG/T + CAT	282	tetratricopeptide repeat protein [Gemmatimonadetes bacterium]	36%	Down

(*Continued*)

Table 2. Continued.

TDF	Primer combination	length (bp)	Similarity to gene	Identities	Regulation
SPV24	E + AAG/T + CAT	193	Bemisia tabaci putative uncharacterized protein DDB_G0271606	82%	Newly expressed
SPV27	E + AAG/T + CAT	612	Uncultured bacterium isolate DGGE gel band Sup-3 16S ribosomal RNA gene	83%	Newly expressed
SPV107	E + AAG/T + CAG	285	Pistacia vera thylakoid membrane protein TERC	97%	Up
SPV345	E + ACT/T + CCA	435	choline O-acetyltransferase (CHAT)	90%	Up
SPV415	E + ACT/T + CAC	296	Elongation factor tu	75%	Up
SPV103	E + AAG/T + CAG	305	Pseudomonas yamanorum strain LBUM636 chromosome, complete genome	83%	Newly expressed
SPV183	E + ACC/T + CAG	201	Pistacia vera serine/threonine-protein kinase-like protein ACR4	96%	Up
SPV341	E + ACT/T + CCA	313	Pseudomonas oryzihabitans hypothetical protein	40%	Newly expressed
SPV342	E + ACT/T + CCA	476	Cytochrome c oxidase subunit ii	74%	Up
SPV343	E + ACT/T + CCA	263	Oryctolagus cuniculus coiled-coil domain containing 121 (CCDC121)	94%	Newly expressed
SPV269	E + ACC/T + CCA	198	VWA domain-containing protein	50%	Newly expressed

are involved in gummosis resistance, and hence may be valuable candidates for breeding gummosis-resistant pistachios.

In this study, we detected *Pistacia vera* MLP-like protein 28 in all treatments of Sarakhs cultivar, but the transcriptomic level of this gene was significantly elevated in pistachio after the *Phytophthora* and *Pseudomonas* infections. Major latex proteins (MLPs) belong to the MLP subfamily in the Bet v 1 protein family which respond to stresses caused by both biotic and abiotic factors and play key roles in the resistance of plants to different diseases. Yang et al. (2015) in their study reported MLP 28 as a positive regulator of the ethylene-responsive factor 6 in cotton in response to *Verticillium dahlia* infection. In addition, the expressions of genes of *PDF1.2* and *PR5* were upregulated in *GhMLP28*-overexpressing tobacco plants. In a study performed by Song et al. (2020), the role of MLP28 protein in the resistance of *Nicotiana benthamiana* against Potato Virus Y was investigated. Correspondingly, the profile gene expression showed that MLP28 protein is responsive to PVY infection and molecules of defense-related signaling such as JA, SA, and ET. Therefore, defense response mediated by MLP28 may play an important role in the protection against gummosis, and *Pseudomonas fluorescens,* as a rhizobacterium can trigger the plant to upregulate this gene.

Regulation of choline O-acetyltransferase (CHAT) was observed in the control resistant plants and susceptible plants that were exposed to *P. fluorescens* and *P. parsiana*. It was shown that acetylcholine has an important role in bacteria-plant interactions (Tretyn & Kendrick, 1991). Accordingly, acetylcholine may have a definite function in the symbiosis of *Rhizobium* with leguminous plants (Fluck & Jaffe, 1976), and its chemotactic effects on the bacterium *Pseudomonas fluorescens* have been shown (Fitch, 1963a, 1963b). In a study, Mugford et al. (2009) reported the role of some groups of acetyltransferases in the synthesis of antimicrobial compounds and the resistance of oats to diseases. The expression intensity of this gene was high in Badami-Rize Zarandi (resistant) control

plants, and this gene in Sarakhs (susceptible) cultivar, was expressed only after the treatment with *P. fluorescens* and *P. parsiana*. Therefore, it can be concluded that CHAT may play a role in resistance against *Phytophthora* disease.

In Sarakhs cultivar inoculated with *Pseudomonas fluorescens*, *Pistacia vera* transcriptional corepressor SEUSS and *Pistacia vera* acyl-protein thioesterase 2-like genes were induced. Transcriptional corepressor SEUSS takes part in different biological processes such as cellular response to external biotic stimulus, response to fungus, and response to bacteria. Moreover, acyl-protein thioesterases are involved in the depalmitolation of proteins and biosynthesis of fatty acids. Notably, fatty acids in the saturated form are necessary for the stability of the membrane under increased or normal temperatures (Gibson et al., 1994). Furthermore, they act as precursors for the biosynthesis of wax esters and the other components of surface lipid (Post-Beittenmiller, 1996). On the other hand, *Pistacia vera* glycosyltransferase BC10-like was expressed in resistant pistachios infected by *Phytophthora parsiana*. This enzyme is required for the regulation of cellulose biosynthesis in the cell wall, biosynthesis of hexoses in both cellulosic and non-cellulosic components of cell walls, and for the formation of arabinogalactan proteins contributing to the strengthening of the cell wall. In plant-pathogen interaction, the lipid bilayer plasma membrane acts as a scaffold to prevent the invasion of a pathogen, so its integrity and stability are of significant importance in confronting the pathogen. Following these findings, we postulated that a thicker and stronger cell wall may be one of the factors causing resistance in the Badami cultivar compared to Sarakhs cultivar. Also, inoculation with *Pseudomonas fluorescens* may strengthen the defense mechanism and prime pistachio plants to combat subsequent pathogen attack.

In pistachios that were exposed to *Phytophthora parsian* and *Pseudomonas fluorescens,* we observed the upregulations of photosynthesis and respiratory chain-related genes as follows: photosystem I chlorophyll an apoprotein A1, *P. vera* thylakoid membrane protein TERC, cytochrome c oxidase subunit II, and NADH dehydrogenase subunit I. Notably, mitochondria have a major role in the perception and transduction of some signals triggered by the cell upon recognition of the pathogen, including mitochondrial activities and especially respiration. This organ is also able to affect various steps of the immune responses of plants that are distinguished by profound redox changes (Colombatti et al., 2014). Many studies have reported a cytochrome c release as an event preceding the death of plant cells (Balk et al., 1999; Sun et al., 1999). Generally, in response to biotic stress, the expression of mitochondrial genes decreases, but in our study, interestingly, an increase was observed in the expression level of cytochrome c oxidase, especially in those plants treated with both *Phytophthora parsiana* and *Pseudomonas fluorescens*. One possibility is that, under the conditions of certain stresses, due to a dysfunctional mitochondrial cyanide-sensitive respiration, structural proteins and/or cytochrome c oxidase assembly accumulate. This accumulation actively replaces damaged or inactive COX subunits to diminish or avoid ROS generation (Mansilla et al., 2018). In algae and higher plants, photosynthesis and main cascades in the synthesis of hormones that are related to defense occur in chloroplasts. The chloroplast also is a key generator of nitric oxide and reactive oxygen species and a site for signaling calcium. These signaling molecules are necessary for the defense reaction of plants, as well. These cases demonstrate the activation of defense pathways in pistachio's inoculated plants.

In conclusion, we showed that *P. fluorescens* infection in susceptible pistachio plants induces the expression of several genes to regulate the defense mechanisms against *P. parsiana*. These mechanisms may be specifically limited to microbial infection, as some genes were only expressed in *P. fluorescens*-treated plants. Moreover, some genes were specifically expressed in the *P. parsiana*-infected plants. We assumed that these genes account for a major part of the induced resistance when plants are treated by microbes. These genes may also play important roles in regulating signaling pathways that are responsible for plant resistance to pathogens. Also, we found that the type and level of genes expressed in the resistant plants (Badami cultivar) had some differences from those in susceptible plants (Sarakhs cultivar). These findings will improve our understanding of the molecular mechanisms of plant defense. However, future studies should be performed by focusing on revealing the exact functions of the genes identified under the infection. Additionally, it would be interesting to explore the possibility of engineered pistachio plants to resist disease by the use of resistance genes.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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