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Pseudomonas putida induces resistance to Fusarium oxysporum f. sp. radicis-lycopersici in tomato plants by activating expression of defense-related genes

Birsen ÇAKIR AYDEMİR*[*1] Müge ŞAHİN[2] Ayşe GÜL[3]
Hatice ÖZAKTAN[4] Lalehan YOLAGELDİ[5]

[1, 3]Department of Horticulture, Faculty of Agriculture, Ege University, 35100, Bornova- İzmir/ TÜRKİYE
[2]Aegean Agricultural Research Institute, Menemen- İzmir/ TÜRKİYE
[4, 5]Department of Plant Pathology, Faculty of Agriculture, Ege University, 35100, Bornova-İzmir /TÜRKİYE

[1]https://orcid.org/0000-0003-4268-8547 [2]https://orcid.org/0000-0002-5570-9143 [3]https://orcid.org/0000-0003-1845-5284
[4]https://orcid.org/0000-0001-9971-6508 [5]https://orcid.org/0000-0003-2237-994X

**Corresponding author (Sorumlu yazar): birsencakir@hotmail.com*

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ABSTRACT: *Plant growth-promoting rhizobacteria (PGPR) may prevent attack from pathogenic microorganisms by eliciting induced systemic resistance (ISR). In the present work,* ***Pseudomonas putida*** *isolate TR21/1 showed significant biological control of tomato seedlings inoculated with* ***Fusarium oxysporum f.sp. radicis-lycopersici*** *(FORL). Here, the SA-responsive genes PR-1, PR-4, PR-6 and CH9 were downregulated upon induction of ISR by P. putida strain TR21/1 and induced when bacterized tomato roots were inoculated with FORL. This indicates that SAR involves the accumulation of SA-responsive genes but ISR does not. Similarly, expression of ET-regulated genes such as ACO1, ACO3, ACO4 were not induced in ISR-expressing tomato roots and* ***P. putida*** *treatment induced only ACO2 expression suggesting that ACO2 expression is involved in ISR-expressing tomato seedlings. In contrast, the infection of ISR expressing plants by FORL strongly induced ACO3, ACO2, and ACO1 indicating the transcriptional regulation of ACO genes in response to FORL attack which may be related to possible ethylene synthesis in response to pathogen. Here P. putida treatment increased ETR1 gene expression in roots and this induction was upregulated in presence of FORL indicating that ETR1 plays a role in the protection of plants against FORL by reducing ethylene sensitivity. Activation of SA-and ET- regulated genes in bacterized plants in the presence of FORL implies that not only SA but other signals as well, may play an important role in inducing resistance.*

Keywords: ***Pseudomonas putida****, systemic acquired resistance (SAR), induced systemic resistance (ISR),* ***Fusarium oxysporum f.sp. radicis-lycopersici*** *(FORL), gene expression.*

Pseudomonas putida, domates bitkilerinde savunma ile ilgili genlerin ekspresyonunu aktive ederek Fusarium oxysporum f.sp. radicis-lycopersici'ye karşı direnci indüklemesi

ÖZ: *Bitki büyümesini teşvik eden rizobakteriler (PGPR), indüklenmiş sistemik direnci (ISR) sağlayarak patojenik mikroorganizmaların saldırısını önleyebilir. Mevcut çalışmada,* ***P. putida*** *izolatı TR21/1,* ***Fusarium oxysporum f.sp. radicis-lycopersici*** *(FORL) ile enfekte olmuş domates fidelerinin önemli ölçüde biyolojik kontrolünü sağlamıştır. Burada SA-yanıt veren genler PR-1, PR-4, PR-6 ve CH9 ISR'nin* ***P. putida*** *streyn TR21/1 tarafından indüklenmesi üzerine ifadeleri baskılanmış ve bakterili domates kökleri* ***Fusarium oxysporum f.sp. radicis-lycopersici*** *ile enfekte edildiğinde bu genlerin ifadeleri indüklenmiştir. Bu sonuçlar, SAR'ın SA'ya yanıt veren genlerin birikimini içerdiğini ancak ISR'nin içermediğini göstermiştir. Benzer şekilde, ACO1, ACO3, ACO4 gibi ET tarafından düzenlenen genlerin ifadeleri, ISR gösteren domates köklerinde indüklenmemiş ve* ***P. putida*** *uygulaması, sadece ACO2 ekspresyonunu indüklemiştir. Buna karşılık, ISR gösteren bitkilerin FORL ile enfeksiyonu, ACO3, ACO2 ve ACO1 genlerinin ifadelerini güçlü bir şekilde indüklemesi FORL saldırısına yanıt olarak olası*

bir etilen sentezi ile ilgili ACO genlerinin transkripsiyonel düzenlemesini gösterir. Burada P. putida uygulaması köklerde ETR1 geninin ifadesini indüklemiş ve FORL ile inokülasyon bu genin ifadesinin indüksiyonunu daha da artırmıştır. Bu sonuçlar, ETR1'in etilen duyarlılığını azaltarak bitkilerin FORL'e karşı korunmasında rol oynadığını göstermiştir. FORL varlığında bakteri uygulanmış bitkilerde SA ve ET tarafından düzenlenen genlerin aktivasyonu, sadece SA'nın değil, diğer sinyallerin de direncin indüklenmesinde önemli bir rol oynayabileceği anlamına gelmektedir.

Anahtar kelimeler: ***Pseudomonas putida***, Sistemik kazanılmış direnç (SAR), indüklenmiş sistemik direnç (ISR**)**, ***Fusarium oxysporum* f.sp. *radicis-lycopersici*** (FORL), gen ekspresyonu.

INTRODUCTION

Fusarium crown and root rot of tomato (FCRR) caused by *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) is a disease observed in both greenhouse and field conditions and has been reported in many countries (Nutter *et al.,* 1978; Jones *et al.,* 1991). FCRR cannot be treated efficiently with fungicides. The use of biocontrol rhizobacteria is a promising treatment to prevent the disease.

During the past two decades there have been several reports on successful FCRR management on tomato by using different fungal and bacterial antagonists such as *Trichoderma harzianum* (Sivan *et al.,*1987), *Glomus intraradices* (Datnoff *et al.,* 1995), *Streptomyces griseoviridis* strain K61 (Minuto *et al.,* 2006) and non pathogenic isolates of *Fusarium* (Horinouchi *et al.,* 2008).

Plant growth-promoting rhizobacteria (PGPR) are free-living or rhizosphere bacteria that can promote plant growth and reduce plant disease (Ryu *et al.,* 2006). PGPR can promote plant growth and development either directly and indirectly (Ortiz-Castro *et al.,* 2009). PGPR direct stimulation of plant growth includes production of phytohormones, other plant stimulants and uptake of essential nutrients (Ardakani *et al.,* 2010) whereas, indirect stimulation comprises antibiotic production, synthesis of extracellular enzymes to hydrolyze the fungal cell wall and competition for niches within the rhizosphere (Zahir *et al.,* 2003). They are also able to degrade organic pollutants and reduce metal toxicity of contaminated soils (bioremediation), and facilitate phytoremediation (Janssen *et al.,* 2015). Inoculation with PGPR can protect the plant from abiotic stress by activating several mechanisms that induce systemic tolerance (Yang *et al.,* 2009; Timmusk *et al.,* 2014).

Plant defence mechanisms occur as two forms, systemic-acquired resistance (SAR) and induced systemic resistance (ISR) controlled by phytohormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Pieterse *et al.,* 2014). SA regulates SAR, while JA and ET regulate ISR (Pieterse *et al.,* 2014). SAR is induced by mechanical wounding, herbivory and pathogenic interactions and characterized by an increase in the synthesis of pathogenesis-related (PR) proteins and production of antimicrobials such as phytoalexins (Freeman and Beatie, 2008). SAR is accompanied by an increased level of SA which, activates the expression of a large set of PATHOGENESIS-RELATED (PR) genes, involved in defense responses (Pieterse *et al.,* 2014). In contrast, ISR is induced by non-pathogenic PGPRs and characterized by ethylene- and JA-dependence but SA-independence without PR gene activation (Ton *et al.,* 2002; Iavicoli *et al.,* 2003). However, these two independent signalling pathways converge through the same transcriptional regulator, nonexpressor of pathogenesis-related (PR) gene 1 (NPR-1) (Katagiri and Tsuda, 2010).

PGPRs are known to induce resistance called ISR against fungi, virus, bacteria, nemotodes and insects (Murphy *et al.,* 2003). Various non-pathogenic PGPR strains are able to trigger ISR in plants against broad spectrum phytopathogens (Van Wees *et al.,* 2008). Induction of systemic resistance by several strains of *Bacillus spp*. and *Pseudomonas fluorescens* has been already reported (Ryu *et al.,* 2004). Several reports have been published regarding *Pseudomonas* PGPR

strain efficiency in controlling FCRR (Bolwerk *et al.,* 2003). Recently, Baysal *et al.,* (2008) reported that *B. subtilis* strain EU07 was able to reduce FCRR.

In *Arabidopsis thaliana,* ISR triggered by root-colonising strains of *Pseudomonas fluorescens* was shown to be ethylene-and JA-dependent but SA-independent (Knoester *et al.,* 1999; Ton *et al.,* 2002). However ISR triggered by other strains of PGPR may also involve SA signalling (Zhang *et al.,* 2002). More recently, *Pseudomonas* strains, able to control FCRR under rockwool conditions, were isolated from tomato roots (Kamilova *et al.,* 2005; Validov *et al.,* 2007). However, the ability to develop ISR depends on the host/rhizobacterium combination (van Loon *et al.,* 1998). *Pseudomonas putida* WCS358n and *P. fluorescens* WCS374r perform differently on different plant species; *Arabidopsis* is responsive to *P. putida* WCS358r, whereas radish and carnation are not (Leeman *et al.,* 1995; Van Wees *et al.,* 1997), suggesting that specific recognition between the plant and rhizobacterium is required for the induction of ISR.

The expression of stress and defence genes in plants may be modulated by PGPR, which helps plants to grow actively under abiotic or biotic stress conditions (Akhgar *et al.,* 2014). Similarly, interactions between plants and pathogens are able to elicit the expression of certain PR genes (Wojtasik *et al.,* 2014). Upregulation of PR proteins, including chitinase and β-1,3-glucanase, in response to Fusarium has been reported in flax plants (Galindo-Gonzalez *et al.,* 2016).

There are only a few studies dealing with the molecular mechanism of the plant response to FORL or PGPR. Little is known about induction of the expression on various defence related genes due to *P. putida* treatment in tomato seedling infected with FORL. The aim of this study was to evaluate *P. putida* for protection of tomato against FORL and to investigate the induction of defence-related genes involved in SA and ET- dependent signalling pathway by *P. putida* against challenge inoculation with FORL. To our knowledge this is the first description of transcriptional changes that persist for several weeks. These results provide new insight into the molecular and biochemical response of FORL infected tomato plants to colonization by PGPR.

MATERIAL and METHODS

Plant Material

Tomato cultivar *Solanum lycopersicum* cv Kardelen F_1, which is a popular cultivar in Türkiye, and is susceptible to FORL, was used throughout the experiments. All seeds were sterilized by immersion in 1% (v/v) sodium hypochlorite for 30 min and rinsed three times in sterile distilled water prior to sowing.

Fungal culture and inoculation

A FORL strain, usaFORL was used (kindly provided by the Batı Akdeniz Agricultural Research Institute; originally from Dr. J.W. Scott, University of Florida). FORL strain usaFORL causing crown and root rot disease was maintained on potato-dextrose agar (PDA) slant. The pathogen inoculum was prepared by culturing the fungus in PD broth by shake culture for 7 days. The microconidial suspension was filtered through cheese cloth and the concentration was adjusted to 10^7 conidia ml^{-1}.

Tomato seedlings were inoculated by using the standard root-dip method (Menzies *et al.,* 1990). Tomato seedlings at the second true-leaf stage were carefully uprooted and freed from soil by immersion in water and gentle shaking. Then they were dipped into the microspore suspension for 30 min and transplanted to pots. Uninoculated plants were dipped in sterile distilled water. Plants were maintained in the growth chamber.

PGPR strain and inoculation

The bacterial strain, called TR21/1, isolated from the rhizosphere of tomato plants grown in south-eastern greenhouses in Türkiye was selected for this study according to the tests for *in vitro* plant growth promotion and biocontrol to FORL (Gul *et al.,* 2012). This strain was identified as *Pseudomonas putida* according to biochemical and physiological test results as described previously

(Schaad *et al.*, 2001). The GRAM reaction was performed by using a 3% KOH test (Suslow *et al.*, 1982). The presence of oxidase, fluorescent pigment production, gelatinase, arginine dihydrolase, levan sucrose, reduction of nitrate, pectinolytic activity on potato slices and hypersensitive reaction (HR) on tobacco leaves were ascertained (Lelliott and Stead, 1987; Schaad *et al.*, 2001). Previous study has revealed that the *P. putida* strain TR21/1 can produce auxins and siderophores, has phosphate-solubilizing capacity and does not enhance the growth of tomato plants compared to the non-bacterized control treatment (Gul *et al.*, 2012).

Bacterial inoculation was carried out prior to sowing as seed coating and two times after transplanting as substrate drenching (Cummings *et al.*, 2009). The culture was grown on King's medium B for 24 h at 24°C. Bacterial inoculant was suspended with 5 mL Carboxyl Methyl Cellulose (CMC, 1.5%). The concentration of bacterial cells in the suspension was adjusted by diluting with sterile deionized water, thus a final concentration of 109 CFU/mL was obtained (Callan *et al.*, 1990).

Surface sterilized seeds of tomato cultivar Kardelen F1 were soaked in 10 ml of bacterial suspension at a concentration of 109 CFU/mL in erlenmeyer flask by shaking for 30 min at 150 rpm. For rhizobacterium non-inoculated treatments (FORL and control), seeds were shaken with only CMC (1.5%). After shaking, seeds were left on blotting paper for 24 h under sterile cabinet before sowing. Seeds were sown manually in a medium (Klasmann TS1; a mixture of peat, perlite and vermiculite) in plastic viols in a germination room maintained at 25℃ with 80-90% relative humidity. At the second true-leaf stage, seedlings were inoculated with fungal pathogen. Then, seedlings were transplanted in 2 L plastic pots filled with peat. Bacterial inoculation was drenched just after transplanting by application of 30 ml of bacterial suspension per pot including 2 plants and repeated 7 days after transplanting. Water was applied to non-inoculated plants in the same way. Plants were kept in the growth chamber for four weeks at 24°C during the day and 20°C during the night with 16 h light and 8 h dark conditions, respectively.

Root colonization and population dynamics of rhizobacterium

Plants inoculated with rifampicin (200 µg/ml) resistant bacterium were used to determine root colonization and population dynamics of tested rhizobacterium on plant roots. Rifampicin-resistant mutants (Rif+) of rhizobacterium were isolated as previously described by Kloepper (1980) and Stockwell *et al.* (1996). TR21/1 (Rif+) population was monitored in both FORL-inoculated and non-inoculated plant roots. Root samples (0.5 g) were taken 2 and 4 weeks after transplanting and placed into sterile flasks, 49.5 ml of 0.5 M phosphate buffer was added to each flask. Flasks were placed on a rotary shaker at 150 rpm for 20 min. Samples were diluted, spread on two replicate petri dishes containing Kings medium B supplemented with rifampicin (200 µg/ml) and rifampicin resistant colonies were enumerated as mutants of PGPR (Stockwell *et al.*, 1996).

Evaluation of plant growth and disease scoring

Four weeks after the challenge of the tomato plants by FORL, the disease level was scored by indexing the disease severity. The lower stem and top root were evaluated for disease scoring. Each plant was rated for discoloration on a scale of severity, 0–5 as follows: 0 = healthy plants; 1 = light rot on root (less than 10% of total area); 2 = dark lesions on 25% of root; 3 = infection on half of the total root area, severe rot on taproot; 4 = infection on 75% of the total root area, lesions on crown, wilting of older leaves, and 5= severe infection on total root area, wilting and death of young leaves.

The disease index (DI) was calculated using the formula:

$$\text{Disease index} = \frac{\Sigma\ (\text{rating number} \times \text{number of plants in the rating})}{\text{Total number of plants} \times \text{highest rating}}$$

The growth promotion was evaluated by using 3 parameters: the leaf number, fresh weight, and dry weight of shoots and roots. The dry weight was measured after oven-drying the plant samples at 65°C to constant weight.

Data analysis

The experiment was set up according to completely randomized design consisting of three replication for each treatment. For each replicate (pot), the mean value of the six plants was used. Data related to disease severity and plant growth were analyzed by analysis of variance (ANOVA) and means were compared using Duncan's multiple range test at $P<0.05$. The statistical analysis was supported by SPSS 17.0 (SPSS, Chicago, IL).

For expression analysis, all data were normalized to actin gene expression. Relative changes in gene expression levels were analyzed. Three biological replicates were used to calculate the mean and standard deviation (SD) in the expression level of each gene.

RNA isolation and RT-PCR

Four weeks after inoculation with FORL or PGPR, roots from tomato plants were harvested and immediately frozen and ground in liquid nitrogen then stored at 80°C until use. Total RNA was extracted according to the method described by Bray (1988), with an additional step of selective precipitation with 2 M LiCl. Repeated reverse transcription–polymerase chain reaction (RT–PCR) assessment of gene expression was performed. RNA was then purified using the RNeasy purification kit (Qiagen, Germany) according to the RNA clean-up protocol. Prior to RT–PCR, the total RNA samples were treated with DNase I (Fermentas, USA) for 10 min and quantified by spectrophotometry and agarose gel electrophoresis. First-strand cDNA was synthesized using The RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) with 0.5 µg of total RNA plus oligo (dT) primers in a volume of 20 µL. The single-stranded DNA mixture was used as template in PCRs. PCR reactions were performed in a 25-µL volume containing 1 µL (5-fold dilution) of first-strand cDNA as template, 0.2 µM each primer, 0.2 µL 5 U μL^{-1} Dream -Taq polymerase, and 2.5 µL of 10X PCR buffer. PCR conditions included an initial denaturing step at 94°C for 3 min, and then 18 cycles of 95°C for 20 s, 62°C for 45 s, and 72°C for 2 min, followed by a final extension at 72°C for 10 min. The linear range of detection for each transcript was detected after 18 cycles. The resulting PCR products were separated on 1% agarose gels, and the band intensity was quantified using BiO1D software (Vilber Lourmat). Three independent experiments were repeated with similar results. The primer pair for Actin gene was used as an internal control. The gene specific primer pairs used are presented in Table 1.

Table 1. The specific primers used in RT-PCR reactions.
Çizelge 1. RT-PCR reaksiyonlaronda kullanılan primerlerin listesi.

Accession number	Gene name	Forward primers (5'-3')	Reverse primers (5'-3')
X58273	*ACO1*	5'-tcaaacagttgctattgggc-3'	5'-ccactsacyttgtcatcttgga-3'
Y00478	*ACO2*	5' ggaaaacactttaccaagaaattaag-3'	5'-ccactsacyttgtcatcttgga-3'
Z54199	*ACO3*	5'-cacacacaccaaaaaaagaaaactcac-3'	5'-ccactsacyttgtcatcttgga-3'
AB013101	*ACO4*	5'-catctcttcaatctcttgtataattcac-3'	5'-ccactsacyttgtcatcttgga-3'
Z15140	*CH9*	5'-aattgtcagagccagtgtcc-3'	5'-tccaaaagacctctgattgc-3'
AF043084	*ETR1*	5'-atgggatctcttctccggat-3'	5'- ggaagttgaatgggtacagt-3'
AJ011520	*PR1*	5'-ccaagactatcttgcggttc-3'	5'-gaacctaagccacgatacca-3'
M69247.1	*PR4*	5'-atggggttgttcaacatctcattgttact-3'	5'-ttaataaggacgttctccaacccagtt-3'
M69248.1	*PR6*	5'-atggggttgttcaacatctcattgttact-3'	5'- ttagtaaggacgttgtccgatccagttgc-3'
AB199316	*LeActin*	5'-atggggttgttcaacatctcattgttact-3'	5'-ttagtaaggacgttgtccgatccagttgc-3'

RESULTS and DISCUSSION

The beneficial effects of PGPR are attributed to several mechanisms, including enhanced efficiency of plant nutrition uptake (Anandham *et al.*, 2008), hormone production (Glick *et al.*, 2007), and anti-pathogen defense (Van Wees *et al.*, 2008). The present study investigated the third mechanism, specifically the ability of *P. putida* strain T21/1 for controlling FORL. In addition, we studied long-term effects of T21/1 on growth and defense-related gene expression in FORL-inoculated tomato plants.

Efficacy of *P. putida* strain TR21/1 in disease suppression of FCRR

P. putida strain TR21/1 showed significant biological control of tomato seedlings inoculated with FORL (Fig. 1A, 1B). Disease severity was significantly reduced compared with the non-treated control by *P. putida* treatment. The disease incidence percentage on plants treated with the *P. putida* strain was 40% which was significantly lower than (64%) the untreated control plants (only FORL-inoculated) (Fig. 1B). In addition, protection of tomato plants against FORL was significantly higher (37.5 %) compared to non-bacterized control plants. Similarly, *Pseudomonas putida* strain PCL1760 is capable of controlling Tomato foot and root rot (TFRR) (Validov *et al.*, 2007) and FORL (Validov *et al.*, 2009) through competition with fungi for "nutrients and niches," as illustrated by a significant decrease in the amount of fungal DNA. Another advantage of *Pseudomonas putida* strain PCL1760 is its lack of antibiotics production (Haas and Defago, 2005). We can speculate that the mode of action for TR21/1 is likely unrelated to antibiotics. Instead, TR21/1 is probably eliciting ISR, a plant defense mechanism characterized by the absence of direct toxic effects against pathogens.

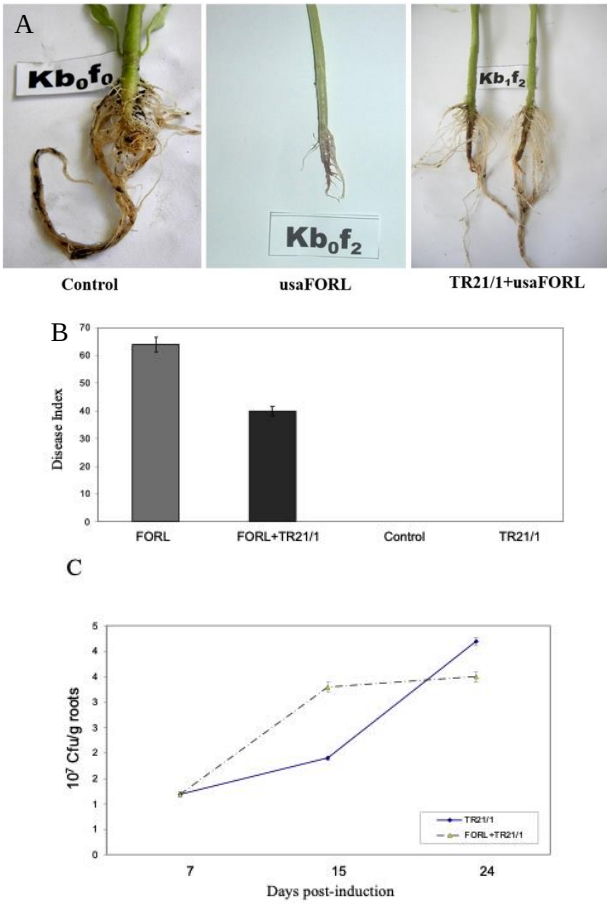

Figure 1. Disease symptoms and quantification of induced systemic resistance against *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL). Symptoms development of (FORL) (A) in roots at 4 weeks post-inoculation of tomato seedlings, inoculated with usaFORL strain. These pictures are representative of at least two independent biological experiments. Disease severity was measured 4 weeks after challenge inoculation by recording the percentage of total surface showing symptoms for each plant (B). Bacterial proliferation data on tomato roots are presented (C). Data points are means (cfu/g) with standard errors from three different experiments.

Şekil 1. Hastalık semptomları ve *Fusarium oxysporum f.sp. radicis-lycopersici* (FORL)'ye karşı indüklenen sistemik direncin ölçümü. usaFORL streyn ile inoküle edilmiş domates fidelerinin 4 hafta sonra köklerde (A) FORL 'nin belirtilerinin gelişimi. Bu resimler en az iki bağımsız biyolojik deneyi temsil etmektedir. Hastalık şiddeti, her bitki için semptom gösteren toplam yüzey yüzdesi kaydedilerek, inokülasyondan 4 hafta sonra ölçülmüştür (B). Domates kökleri üzerindeki bakteri üreme verileri (C). Veriler farklı üç deneyden elde edilen standart hatalara sahip ortalamalardır (cfu/g).

Colonisation of *P. putida* strain TR21/1 in soil and on tomato roots

The population densities of *P. putida* strain TR21/1 showed a slight decrease from inoculated population of $1x10^7$ cfu g-1 to 1,2 x 10^6 cfu g-1 after incubation for 7 days then increased to 4,2 $x10^6$ cfu g-1 after incubation for 24 days (Fig. 1C). Similarly, the population densities of *P. putida* strain TR21/1 in presence of FORL showed a slight decrease from inoculated population of $1x10^7$ cfu g-1 to 1,2 x 10^6 cfu g-1 after incubation for 7 days then increased to 3,5 $x10^6$ cfu g-1 after incubation for 24 days which is comparable to that of the control plants (Fig 1C). Thus, *P. putida* strain TR21/1 used in the study showed high colonising ability in presence or in absence of FORL on the tomato root system.

Effect of *P. putida* strain TR21/1 on growth promotion of tomato seedlings

The *P. putida* strain TR21/1 used in this study decreased weight of shoots and roots in both FORL inoculated and non-inoculated plants compared with the non-bacterized controls (Fig. 2A, 2B).

FORL inoculation gave rise to decrease shoot and root weight in both bacterized and non-bacterized plants. The difference between FORL inoculated and control plants was significant in respect to shoot fresh and dry weight in the presence of TR21/1, on the other hand it was not significant in the absence of TR21/1 (Fig. 2A, 2B). Root fresh and dry weight decreased significantly by FORL inoculation in both bacterized and non-bacterized plants (Fig. 2C, 2D). There were significant differences between treatments in respect to plant growth characteristics except leaf number (Fig. 2E).

In this study, the growth of TR21/1-treated tomatoes and non-bacterized control plants did not differ, indicating that ISR-mediated defense against FORL was not associated with plant growth promotion. This outcome is interesting given that TR21/1 produces auxins and siderophores, on top of exhibiting phosphate-solubilizing capacity (Gul *et al.*, 2012). Furthermore, it is well established that

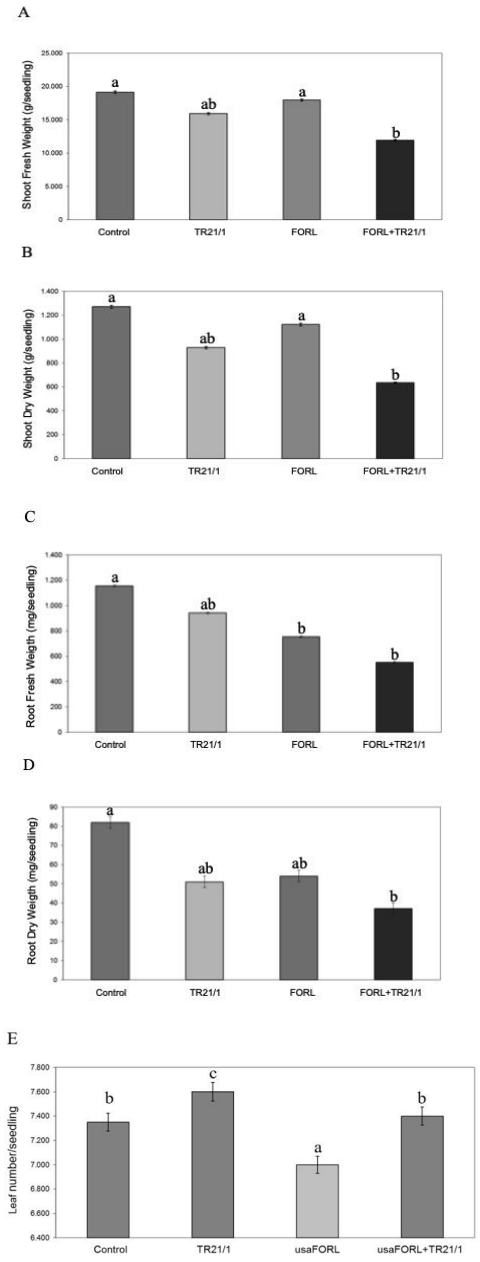

Figure 2. Influence of TR21/1 or *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) on tomato development. Average weight of fresh (A) and dry shoots (B), average weight of fresh (C) and dry roots, (D) and leaf number (E). Statistical comparison within all seedlings was performed by Duncan's test ($a < 0.05$). Different letters above the bars on the graphs indicate significantly different results.

Şekil 2. TR21/1 veya *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) streynlerinin domates gelişimi üzerine etkisi. Yaş (A) ve kuru sürgünlerin ortalama ağırlığı (B), yaş (C) ve kuru köklerin ortalama ağırlığı, (D) ve yaprak sayısı (E). Tüm fideler içinde istatistiksel karşılaştırma Duncan testi ile yapılmıştır ($a < 0,05$). Grafiklerdeki çubukların üzerindeki farklı harfler, önemli ölçüde farklı sonuçları gösterir.

PGPR can promote plant growth through atmospheric nitrogen fixation, nutrient uptake enhancement, soil iron chelation, phytohormone synthesis, and stimulation of enzymes that modulate plant growth/development (Arora *et al.,* 2001).

Expression analysis of PR genes

To investigate the long-term response of tomato seedlings to FORL with/without bacterial treatment at the transcriptional level, we performed expression analysis of the SA-regulated genes by gene-specific RT-PCR analysis. Gene expression was investigated locally in roots collected 4 weeks after transplanting to pots. *In vitro* synthesized single-stranded cDNAs from RNA isolated from tomato roots inoculated with *P. putida* strain TR21/1 in presence or in absence of FORL isolate were assessed using sets of specifically designed primers, which enabled the amplification of *PR1*, *PR4*, *PR6* and *CH9* genes in the roots (Fig. 3).

In the roots, *PR-1* transcripts were downregulated in bacterized roots of tomato plants whereas the gene was induced in FORL inoculated plants. Moreover, this induction was strongly increased in bacterized plants inoculated with FORL compared to untreated control plants (Fig. 3A). Concerning the level of *PR4* gene expression, transcripts were slightly downregulated in FORL-inoculated plants while its expression was almost inhibited in bacterized-plants (Fig. 3B). By contrast, in plants bacterized with *P. putida* strain TR21/1 in presence of FORL, *PR4* transcripts were upregulated compared to untreated control plants (Fig. 3B). Similarly, the *PR6* transcripts were downregulated in bacterized plants but upregulated in FORL-inoculated plants compared to untreated control plants (Fig. 3C). The induction of *PR6* expression in FORL inoculated plants was comparable to that in bacterized roots inoculated with FORL.

The *CH9* transcripts were regulated in response to bacterial and/or FORL treatments (Fig. 3D). Its transcripts were slightly induced by *P. putida* or FORL treatment compared to untreated control plants while its transcripts were strongly induced in bacterized plants treated with FORL (Fig. 3D).

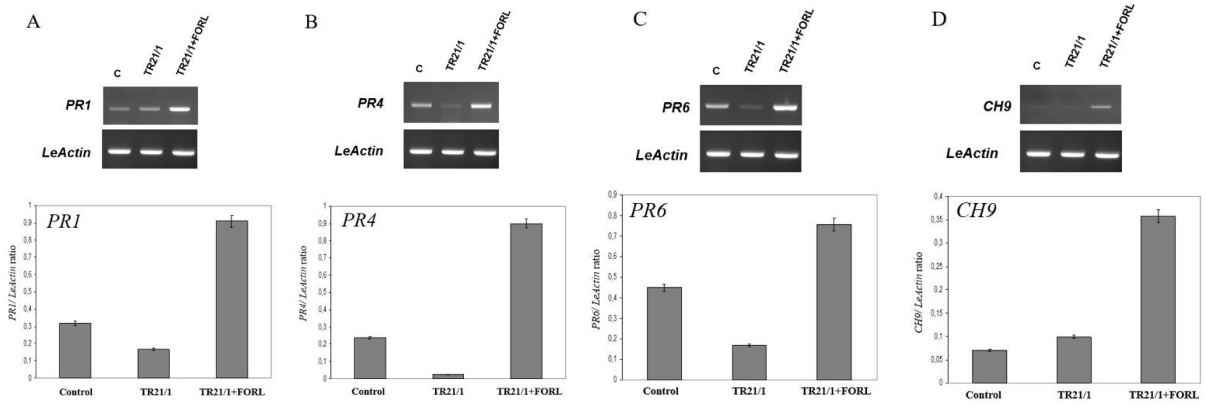

Figure 3. Expression of defense-related genes in SAR- and ISR-expressing roots of tomato plants. Tomato cultivar Kardelen F_1 grown for 4 weeks after treatment with either FORL strains of *F. oxysporum* f.sp. *radicis-lycopersici* or TR21/1 and FORL. From A to C, expression analysis of SA-regulated genes including *CH9, PR1, PR4* and *PR6.* The relative expression levels of the genes were normalized with respect to *LeActin* levels using Bio1D software (Vilber Lourmat). Independent data were analyzed in triplicate, and the standard deviations are shown with error bars.

Şekil 3. Domates bitkilerinde SAR ve ISR ifade eden köklerinde savunma ile ilgili genlerin ifadesi. Domates çeşidi Kardelen F1, *F. oxysporum* f.sp. *radicis-lycopersici* FORL veya TR21/1 ve FORL streynlerinin herhangi biri ile enfekte edildikten sonra 4 hafta boyunca yetiştirilmiştir. A'dan C'ye, *CH9, PR1, PR4* ve *PR6* dahil SA tarafından düzenlenen genlerin ekspresyon analizi. Genlerin nispi ekspresyon seviyeleri, Bio1D yazılımı (Vilber Lourmat) kullanılarak LeActin seviyelerine göre normallize edilmiştir. Bağımsız veriler üç kopya halinde analiz edilmiş ve standart sapmalar hata çubuklarıyla gösterilmiştir.

Simultaneous with ISR elicitation, TR21/1 and other PGPR may also elicit SAR through a separate signaling pathway involving SA or jasmonic acid and ethylene (ET) (Lawton *et al.*, 1996; Pieterse *et al.*, 1998). This inherent plant response is effective against a broad spectrum of pathogens and is correlated with SA- and ET-induced *PR* genes (Delaney *et al.*, 1994; Lawton *et al.*, 1996). Thus, in this study, we tested whether specific gene expression could act as an indicator of PGPR-triggered SAR. Our results comparing *PR* gene transcripts in TR21/1-treated tomatoes without FORL revealed that *PRs* were downregulated or not produced. This result indicated that *P. putida*-induced ISR in tomato does not involve *PR* accumulation.

We observed the induction of *PR1*, *PR4*, and *PR6* when bacterized plants were challenged with FORL. This result expands on previous reports of SA- and pathogen-elicited *PR1* expression against the fungal pathogen in *Arabidopsis* (van Wees *et al.*, 2000) and in tomato (Kavroulakis *et al.*, 2006). Here, we found that *PR4* expression was downregulated in TR21/1-treated tomato roots, suggesting that the gene is not activated under ISR. This result is in line with our previous study demonstrating a slight downregulation of *PR4* transcripts in FORL-inoculated plants (Çakır *et al.*, 2014).

Expression of ET-regulated genes

Pathogen infections leading to chlorotic or necrotic symptoms cause an increase in ethylene production with ACC oxidase activity being increased (de Laat and van Loon 1983). Ethylene responses can also be regulated by changes in ethylene perception in tomato. The ethylene-insensitive *Nr* mutant homologous to *ETR1* showed increased tolerance to virulent strains of *Fusarium oxysporum*, *Pseudomonas syringae* pv. tomato and *Xanthomonas campestris* pv. *vesicatoria* (Lund *et al.*, 1998).

Expression of the *ACO1* gene did not change in response to *P. putida* strain TR21/1 treatment while its expression was activated in response to FORL inoculation (Fig. 4A). Its expression was strongly induced when bacterized plants were inoculated with FORL (Fig. 4A). Regarding *ACO2* expression, its transcripts were strongly induced in both *P. putida* strain TR21/1 or FORL treated plants. Interestingly, *ACO2* transcripts were upregulated when bacterized plants were inoculated with FORL (Fig. 4B). By contrast, *ACO3* expression did not change in response to *P. putida* strain TR21/1 treatments while its expression was induced in the presence of FORL (Fig. 4C). *ACO3* expression was induced in comparison to FORL inoculated plants. While *ACO1* and *ACO3* expression were unaffected, TR21/1 treatment strongly induced *ACO2* expression, suggesting that *ACO2* is involved in ISR-expressing tomato seedlings. We also observed that FORL infection strongly induced *ACO3*, *ACO2*, and *ACO1* in bacterized plants, implying transcriptional regulation of *ACO* that may be related to pathogen-induced ET synthesis.

In contrast, expression of *ACO4* gene was downregulated in FORL treated plants, while in *P. putida* strain TR21/1 treated plants, its expression was slightly induced (Fig. 4D). Interestingly, the level of *ACO4* transcripts was higher when bacterized plants were inoculated with FORL (Fig. 4D).

Our results corroborate previous research in tomato showing elevated ET synthesis and *ACO1* expression under challenge from *Xanthomonas campestris* pv. *vesicatoria* (Ciardi *et al.*, 2000), along with AvrPto and AvrPtoB-induced *ACO* expression under *Pseudomonas syringae* infection (Cohn and Martin, 2005).

There was accumulation of transcripts encoding *ETR1* in bacterized plant roots or FORL inoculated plants roots compared to untreated control plants, however, transcript accumulation was greater in bacterized plant in the presence of FORL (Fig 4E). The induction of *ETR1* transcripts was increased when *P. putida* strain TR21/1 treated plants were inoculated with FORL (Fig. 4E).

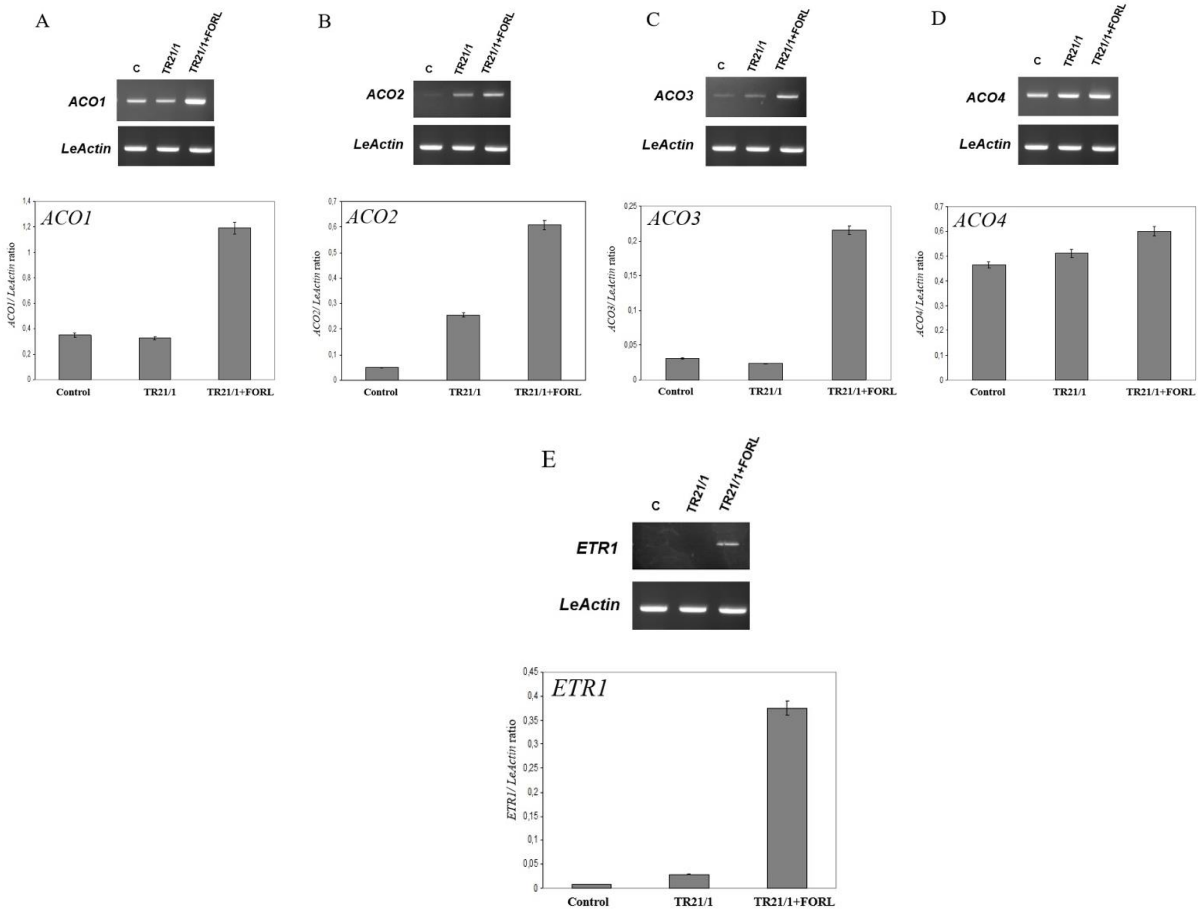

Figure 4. Expression of defense-related genes in SAR- and ISR-expressing roots of tomato plants. Tomato cultivar Kardelen F_1 grown for 4 weeks after treatment with either FORL strains of *F. oxysporum* f.sp. *radicis-lycopersici* or TR21/1 and FORL. From A to C, expression analysis of ET-regulated genes: *ETR1*, *ACO1*, *ACO2*, *ACO3*, and *ACO4*. The relative expression levels of the genes were normalized with respect to *LeActin* levels by using Bio1D software (Vilber Lourmat). Independent data were analyzed in triplicate, and the standard deviations are shown with error bars.

Şekil 4. Domates bitkilerinin SAR ve ISR ifade eden köklerinde savunma ile ilgili genlerin ifadesi. Domates çeşidi Kardelen F1, *F. oxysporum* f.sp. *radicis-lycopersici* FORL veya TR21/1 ve FORL streynlerinden herhangi biri ile enfekte edildikten sonra 4 hafta boyunca yetiştirilmiştir. A'dan C'ye, ET tarafından düzenlenen genlerin ekspresyon analizi: *ETR1, ACO1, ACO2, ACO3* ve *ACO4* genlerin nispi ekspresyon seviyeleri, Bio1D yazılımı (Vilber Lourmat) kullanılarak LeActin seviyelerine göre normalize edilmiştir. Üç farklı bağımsız veriler analiz edilmiş ve standart sapmalar hata çubuklarıyla gösterilmiştir.

In this study, we observed that TR21/1 treatment increased *ETR1* expression in roots, a response that was further elevated upon FORL challenge. This outcome suggests that ET receptors (e.g., ETR1, NR) are active in plant defense, specifically through a role in reducing ET sensitivity. Reducing ET sensitivity may increase tolerance to pathogens because the plant hormone can induce pathogenesis-related proteins or phytoalexins and strengthen cell walls through stimulating the phenylpropanoid pathway (Arshad and Frnakenberger, 1992).

In conclusion, our results represent the first report showing initial downregulation in defense-related gene expression when treated with *P. putida* strain TR21/1, followed by subsequent over-regulation when challenged with FORL. We recommend that future studies compare defense reactions between plants immediately after FORL inoculation and plants previously inoculated with FORL.

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