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RESEARCH ARTICLE

Transcriptional Response of Defensive and Regulatory Genes Involved in Tomato Plant Hormone Signaling Pathways against Fusarium Wilt

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ABSTRACT: Fusarium wilt caused by the fungus *Fusarium oxysporum* f. sp. *lycopersici* (FOL) is one of the major devastating diseases of tomato plants throughout the world. There is no information on the molecular response of tomato line, Early Urbana-Y to FOL. The present study was performed to study the line response to FOL using phytopathological (disease severity and shoot fresh weight) as well as molecular methodologies. The transcription of several genes responsive to jasmonic acid (JA; *Pin2*), salicylic acid (SA; *Chi3*, *Chi9* and *PR1*), the regulatory genes responsive to the signaling hormone JA (*MYC2* and *WRKY33*) and ethylene (ET; *ERF1*) were studied by qRT-PCR technique at different time points after FOL inoculation (6-96 hour after inoculation). Disease symptoms development and reduced shoot fresh weight of the inoculated plants despite up-regulation of SA-dependent defense genes at different time points after pathogen infection indicated that SA signaling pathway is involved in the susceptibility of the Early Urbana-Y line to FOL. In contrast, JA and ET pathway genes were not strongly induced in response to the pathogen suggesting the involvement of JA/ET-mediated defense responses in reducing disease susceptibility. However, to gain a better understanding of enhanced resistance to fusarium wilt, more detailed molecular mechanisms underlying susceptibility of Early Urbana-Y line to FOL need to be further investigated in the future.

KEYWORDS: Ethylene; Gene expression; Jasmonic acid; Salicylic acid; Systemic resistance

INTRODUCTION

Tomato wilt disease is one of the most prevalent and devastating diseases of tomatoes in the most growing areas of Iran [1]. The causal agent of the disease is *Fusarium oxysporum* f. sp. *lycopersici* (FOL) [2]. The symptoms of tomato fusarium wilt first appear as the chlorosis in some of the young leaves that gradually turn to yellow and occupy the whole leaf blade. As the disease progresses, the leaf tissue in the central part of the yellow areas is necrotized and dry off. Disease symptoms are also visible in vascular tissues of diseased plant stems. Root rot, crown rot, stem rot as well as the necrosis of vascular tissues lead to wilt and whole plant death. Therefore, the infected plants get desiccated under field conditions and destroyed [3].

The interaction between pathogen and host is a complex and dynamic interrelation. Disease develops when its causal pathogen can escape from various layers of host defense. Plant immunity systems can recognize aliens and activate severe defensive responses to minimize the injuries caused by hazardous factors [4]. The activation of

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such a system depends on particular signals recognized by a plant. Pathogen-Associated Molecular Patterns (PAMPs) associated with invading pathogens have been identified as potential signals involved in the activation of the plant immunity system. PAMP signals are recognized by Pattern Recognition Receptors (PRRs) of plants and a complex signal of PAMP-PRR leads to plant immunity system activation and defense genes expression [5]. Plant hormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) play an important role in systemic and inter-cellular signaling systems to induce the expression of different defensive genes [6].

SA is considered as an important endogenous immunity signal involved in the stimulation of plant defensive reactions to diseases. The infection of plants by pathogens leads to Systemic Acquired Resistance (SAR) induction via the stimulation of SA bioaccumulation [7]. SAR is a fortified defensive status against a wide range of pathogens that are triggered off throughout a plant following a local infection. The occurrence of SAR needs SA signaling [8] in such a way that SA plays a significant role in the activation of NPR1 transcription factor which is involved in the induction of Pathogenesis-Related Proteins (PRPs) genes and defensive genes expression [9]. On the other hand, JA-derived systemic resistance, known as Induced Systemic Resistance (ISR), is a counterpart of SA-derived SAR. ISR is mostly induced by Biological Control Agents (BCAs) and necrotrophic fungi. A jasmonate-derived metabolite, 7-isojasmonyl-L-isoleucine as a plant defensive system inducing signaling molecule has a notable role in the activation of MYC2 transcription factor and the induction of down-stream defensive genes [10]. ET signaling system is also regarded as an important part of plant intrinsic immunity system [11] in such a way which its biosynthesis is stimulated following a pathogenic attack and/ PAMP activation, and it gets involved in defensive response development via activation of the regulatory transcription factor, ERF1. ERF transcription factors include only a single DNA-binding domain, and their specific binding to GCC-box in the promoters of PRP gene and JA-, and ET-induced genes which have already been reported [12]. Because of their hydrolytic activities on pathogen cell wall and in plant defense signaling pathways, most PRPs exhibit antimicrobial properties [13]. These proteins have been classified into 17 classes based on their function and characteristics from which beta-1, 3-glucanases and chitinases are two relevant groups of hydrolytic enzymes encountered in many plant species after their infection by various kinds of pathogens [14]. The co-occurrence of PRPs and SAR reflects the share and the important cooperation of these proteins in the increment of the defensive potential of induced tissues [15]. On the other hand, proteinase inhibitors can also limit pathogen access to the sources of amino acids via the reduction of the pathogen capability to digest the host proteins. Therefore, proteinase inhibitors can be widely induced in the response to insects and pathogenic invasions [16]. Different sorts of proteinase inhibiting proteins are usually found in plants. Proteinase inhibiting proteins have been classified based on the target group of proteinases they affect. Four groups of proteinases have been identified including serine proteinases, cysteine proteinases, aspartic proteinases, and metalloproteinases [17].

The induction of SA-dependent resistance and JA-dependent resistance are antagonistically interrelated [18]. On the other hand, JA and ET as the major mediators of plant resistance to *F. oxysporum* [19] have positive interactions [20]. With some cases, JA and ET have induced SA activity and consequently increased expression of PRP genes [21]. Therefore, the signaling pathways dependent on these three plant hormones are interrelated in the precise regulation of defensive reactions that finally lead to the activation of plant defensive responses for the resistance to pathogens. The interaction between FOL and tomato is specifically controlled by race and cultivar dependent factors [22]. In this research, the transcriptional responses of the genes involved in signaling pathways dependent on JA/ ET and SA plant hormones were evaluated to get better understanding about the interactions between the Iranian line, Early Urbana-Y and the pathogenic fungus, FOL. Based on the available reports, this is the first report on the expression of these genes in tomato line, Early Urbana-Y against FOL.

MATERIALS AND METHODS

Culture of tomato plants

Plant material used in this study was Early Urbana-Y, which was kindly granted by. Prof. Farokhi Nejhad (Shahid Chamran University, Ahwaz). The seeds were superficially sterilized in 2% sodium hypochlorite solution for 5 min, rinsed twice with sterile deionized water and then were sown in 72-cell plastic seedling starter trays filled with a 1:1 W/W mixture of sterilized vermiculite: agronomical soil. Two seed were planted per

cell and then were kept at 25°C under photoperiodic conditions of 16 h: 8 h light: darkness for 2 weeks [23]. Then, the plantlets were transferred to 1 L plastic pots containing sterilized vermiculite and soil (1:1 W/W) and grown in greenhouse with the conditions of 24-28°C, 40% RH and 16/8 h (L/D) photoperiod. The 4-leaf stage plantlets were treated.

Preparation of fungal inoculum and treatments

A culture of the fungus, *Fusarium oxysporum* f. sp. *lycopersici* was taken from the Plant Pathology Laboratory, Department of Plant Protection, Faculty of Agriculture, Agricultural Sciences and Natural Resources University of Khuzestan, Mollasani, Khuzestan, Iran. To prepare spore suspension, the fungus was hyphal tipped into fresh potato dextrose plates, and the culture plates sealed with the parafilm, incubated at 26 °C in dark for five days. Then, three mL of sterile deionized water was pipetted into each plate under sterile conditions of a laminar flow chamber. The spores were washed off via repeated pipetting over the whole colony. The prepared suspensions were pipetted out of the culture plates and collected into a single sterile falcon tube. The suspension was shaken and the density of spores was determined using a hemocytometry lamella (Neubauer Improved, HBG, Germany) and a light microscope (Olympus, Japan). The density of the spores (almost all single-celled microconidia) was adjusted to 10^6 microconidia per mL [24]. The 4-leaf stage plantlets were treated by adding 20 mL spore suspension (10^6 spores/ mL) to the soil in the close vicinity of the plantlets just after transferring them to the pots considered for this purpose. For controls samples, only 20 mL sterile water was applied in the same way. Three apical leaves were sampled from treated and control plants in 6, 12, 24, 48, 72, and 96 hours post-inoculation (hpi). The samples were wrapped into aluminum foil pieces and immediately immersed into liquid nitrogen and stored at -80 °C until RNA extraction.

Pathological assays

In order to study the response of Early Urbana-Y to the treatment by FOL, a completely random design-based experiment was carried out. The experiment included a treatment and control. The preparation of fungal inoculum, the procedure of plant inoculation and incubation were as mentioned above. Three plants (one per pot) were applied as replicates for each treatment. The water need of plants was provided at the level of field capacity. The plants were applied for disease severity and shoot fresh weight analyses 40 days after inoculation. The disease severity was calculated based on the following formula [25]:

Disease severity=100 × ((Number of symptomatic leaves) ⁄(Total number of leaves))

The shoots were cut in the soil line, immediately wrapped in aluminum foil and transferred to the laboratory to determine the fresh weight of plants. The fresh weight of each shoot was measured using a digital balance. All data were statistically analyzed using SAS (version 9.1) software and the comparison of means was performed using Duncan's Multiple-Range Test method ($p < 0.05$). Shoot fresh weight decrease (%) was calculated as follow:

$$\Delta SFW\ (\%) = [{}^{SFW_{FOL} - SWF_C}/_{SWF_C}] \times 100$$

Where ΔSFW (%) is the decrease of tomato shoot fresh weigh in percent, SWF_{FOL} is the shoot fresh weigh of tomato plants treated with FOL, and SWF_C is the shoot fresh weight of control plants.

Table 1. The primers applied in qRT-PCR reactions.

Gene	Primer sequence (5'-> 3')	Amplicon length (bp)
PR1	tcttgcggttcataacgatg cagctcttgagttggcatag	104
Chi9	atctggttctggatgactcc tttgtgatgacaccgaatcc	125
Chi3	actatggcagaggacctatc tcgcaactaaatcagggttg	108
Act	ccatgttcccaggtattgc agcctccaatccagacac	120
PinII	gcactggttacaagggttg tttgccaatccagaagatgg	145
ERF1	ggtccttggtctctactcaat tcttgtgcttgactcttctagt	139
MYC2	gacgtgattcaatggctcct caggggaagcaatgaagaag	140
WRKY33	gtggaaagggcatcacaag tcggttaattgagtggttgc	138

Molecular and gene expression analysis

RNA extraction was performed following the guidelines of Super RNA Extraction (Cat. No. YT9080) kit manufactured by Yekta Tajhiz Azma Ltd. (www.yektatajhiz.com), Tehran, Iran, and cDNA synthesis was done following the instructions for Thermo kit (Cat. No. K1621). The primers for the studied genes (Table 1) were designed using Primer Quest software freely available online at www.idtdna.com. The primers were synthesized by SinaClone Company (www.sinaclone.ir), Tehran, Iran. The qRT-PCR

reactions were carried out using 2X Real-Time PCR Master Mix (SYBR Green) and Real-Time PCR System (ABI Company). The cycles' condition applied was as follow: primary denaturation at 95 °C for 10 min; 40 cycles of three successive steps i.e. denaturation at 95 °C for 15 sec, the annealing of primers at 60 °C, and extension at 72 °C for 20 sec. The threshold cycle (Ct) for each of the studied genes was normalized with the actin gene of *S. lycopersicum* used as the internal reference house-keeping gene. Fold changes in transcript expression were calculated by the $2^{-\Delta\Delta Ct}$ comparative method (compared to that of the control un-treated plants). The rate of $-\Delta\Delta Ct$ calculated as $\Delta\Delta Ct$ = [(Ct of gene of interest – Ct of reference gene) treated sample – (Ct of gene of interest – Ct of reference gene) untreated control]. The value of $2^{-\Delta\Delta Ct} < 1$ implied a reduction in the expression of interest gene as compared to control. The value > 1 implied that expression of interest gene was up-regulated compared to the control [26]. Relative expression software tool (REST®), the software described by Pfaffl [27], was used to analyze the obtained data. Three individual plants were used as biological replicates while two technical replicates (cDNA samples) were applied per biological replicate.

RESULTS

Pathological assays indicated the occurrence of fusarium wilt disease, where the calculated disease severity was 53.3 %. Also, the disease lead to a reduced fresh weight. The mean fresh weight of shoot of a control and diseased plants were respectively 19 and 14.16 g (-25.47 % reduced fresh weight compared to an average control plant shoot) (Fig. 1).

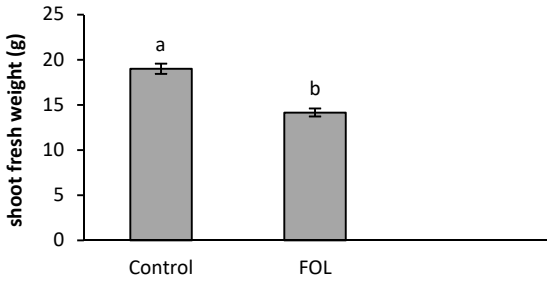

Figure 1. Shoot fresh weight of tomato plants treated with *Fusarium oxysporum* f. sp. *lycopersici* (FOL). Data were recorded 40 days after pathogen treatment. Different letters indicate significant differences between the treatment and control plants (Duncan's test using GLM procedure, P< 0.05).

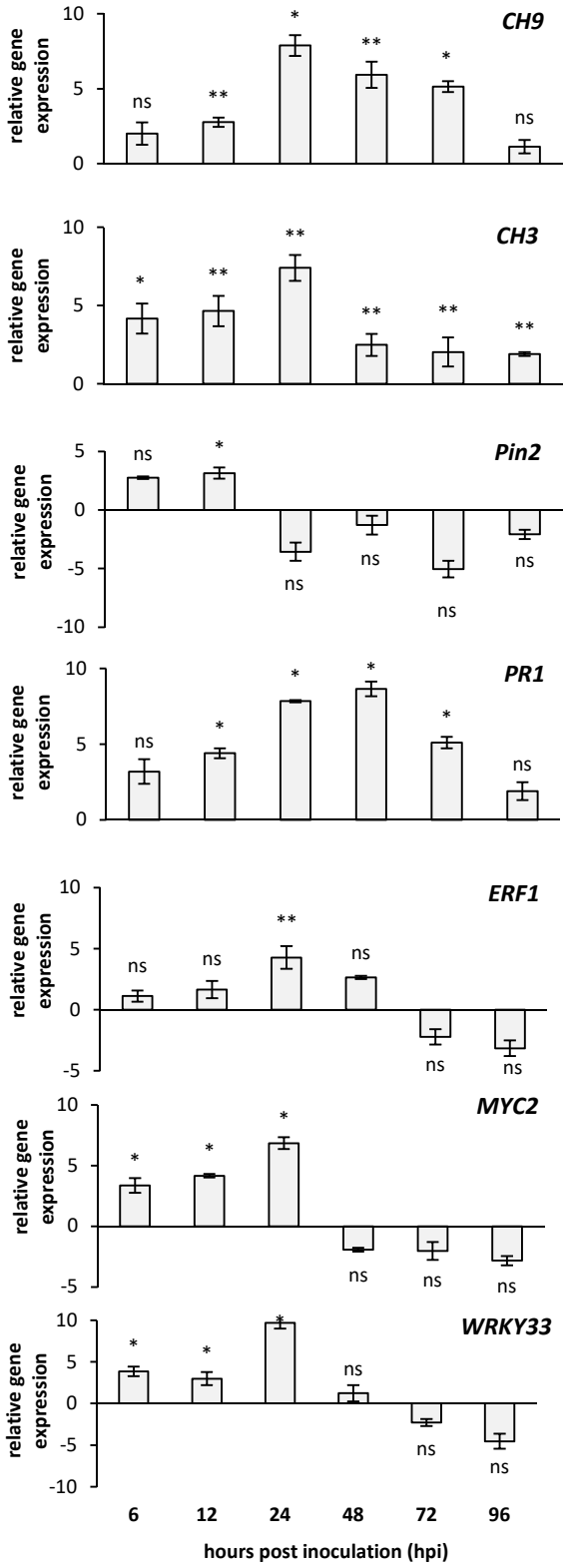

Figure 2. Effect of *Fusarium oxysporum* f. sp. *lycopersici* on expression of *Chi3*, *Chi9*, *Pin2*, *PR1*, *ERF1*, *MYC2*, and *WRKY33* genes of tomato (Early Urbana-Y) at 6, 12, 24, 48, 72, and 96 hpi in comparison with control plants.
*, and ** indicate the significance at P < 0.05 and P < 0.01, respectively. 'ns' exhibits the absence of statistically significant difference.

The plants treated with the pathogen exhibited a significant increase in the transcription of the *Chi3* gene in 6 to 96 hpi (Fig. 2). The increase attained it's maximum 24 hpi (an increase of 7.4 times compared with that in control plants). The transcription rate of *Chi9* and *PR1* genes 6 hpi did not change notably as compared to control plants, however, their transcriptions meaningfully increased 12 to 72 hpi. The peak of *Chi9* and *PR1* gene expression were respectively observed 24 hpi (7.8 times of that in control plants) and 48 hpi (8.6 times of that in control plants). The transcription rate of the proteinase inhibitor II gene (*Pin2*) exhibited a significant increase (compared to that in control plants) only 12 hpi, however, its transcription was not significantly different from that in control plants in the next hours. The genes *MYC2* and *WRKY33* indicated significant increases in their transcription rate till 24 hpi, while they showed no significant difference from that in control plants in the next hours. The transcription rate of the *ERF1* gene also exhibited a significant increase just in 24 hpi, however, no meaningful difference was found between its transcription rates in treated and untreated plants in the remaining hours after treatment (Fig. 2).

DISCUSSION

Induction of an appropriate level of immunity response after pathogen infection requires interaction between SA, JA and ET signaling networks, depending on path systems lifestyle categories, necrotroph or (hemi) biotroph [28]. According to previous reports, JA and ET can effectively elicit resistance to necrotrophic fungi. However, SA-dependent defensive signals are thought to be induced following inoculation with (hemi) biotroph fungi [29]. Moreover, the analysis of resistance of mutants defective in SA-related genes exhibited enhanced susceptibility to a set of (hemi) biotrophic fungi in Arabidopsis [30-31]. Our results showed that expression of SA-responsive genes including *Chi3*, *Chi9* and *PR1* was significantly up-regulated at different time points after FOL infection. In accordance with our results, Aimé et al [32] showed that transcript accumulation of *Chi3* and *Chi9* genes was up-regulated in Fusarium-infected tomato plants. The fungal cell wall chitin is a homopolymer of N-acetyl-D-glucosamine units. Despite the lack of chitin, plants possess chitinolytic enzymes [33]. Upon fungal infection, plant cells secrete chitinases that degrade chitin and release chitin fragments (chito-oligosaccharides or chitin oligomers) from wall of fungal cells. These fragments act as elicitors and induce plant immune responses [34]. It is reported that the overexpression of chitinase in tobacco plants conferred enhanced resistance to fungal pathogens [35]. Furthermore, similar to our results, the induction of *PR1* and *PR5* genes expression has been reported in Arabidopsis plants after infecting by *F. oxysporum* [36]. The transcription level of the *PR1* gene is mostly regulated by the SA pathway. In Arabidopsis, the role of SA signaling pathway in the activation of *PR1*, *PR2* and *PR5* gene expression was illustrated by analyzing the mutants deficient in SA-responsive pathway such as npr1 [37-38]. It is reported that, NPR1 (Non-expressor of pathogenesis-related genes1) as a transcriptional co-activator in controlling SA-mediated genes expression, promotes binding affinity of TGA transcription factor to the promoter elements and thereby leads to more activation of PR1 gene [39]. In the current study, as was expected, FOL induced plant SA-dependent defensive responses, however, these responses were not able to prevent the pathogen invasion. This failure showed itself as a significant decrease in the fresh weight of FOL-treated Early Urbana-Y plants and the disease severity of 53.3%.

Berrocal-Lobo and Molina [40] revealed that transcriptional factor ERF1, as an integrator of ET and JA signaling networks, mediates resistance to *F. oxysporum*. Hence, constitutive expression of *ERF1* following the inoculation of the fungus, resulted in the development of resistance to *F. oxysporum* f. sp. *conglutinans* and *F. oxysporum* f. sp. *lycopersici*. To determine the involvement of ET-dependent signaling pathway in controlling Early Urbana-Y tomato resistance to FOL, we evaluated *ERF1* transcript profile through a 90 h time span (6-96 hpi). Except for 24 hpi, no significant accumulation of *ERF1* transcripts was observed at other time points. Hence, it seems that induction of *ERF1* is not strong enough to confer enhanced resistance of tomato to FOL. Moreover, comparative analysis of transcriptional response of SA and JA-responsive genes in the evaluated tomato line showed the JA-dependent defense genes (*MYC2*, *WRKY33* and *Pin2*) were activated during the first hours after invasion (12-24 hpi) and suppressed at later time points. In contrast, SA-marker genes were constitutively up-regulated at different time points suggesting antagonistic effect of SA on JA-regulating defense genes expression. This result was consistent with Spoel et al. [41]. Furthermore, it has been demonstrated that suppression of JA-dependent defense genes

expression by pathogen-induced SA is mediated by NPR1 [42].

The temporal pattern of the expression of tomato signaling pathways in Early Urbana-Y and FOL interaction were similar to the susceptible tomato line, Momor [43]. In addition, our findings were in agreement with Morid and Hajmansoor [44], that showed Early Urbana-Y was determined as a FOL-susceptible cultivar based on the applied PCR-RFLP genetic markers. Surprisingly, it has been reported that overexpression of SA-responsive defense genes resulted in enhancing disease susceptibility to necrotrophic pathogens [45-46]. Herein, SA-dependent response also appears to act as a positive regulator of susceptibility to FOL. In contrast, it has been demonstrated that JA/ET-dependent defense responses negatively regulated susceptibility to necrotrophic pathogens [29]. Accordingly, our study confirmed reported findings of Swarupa et al. [47] that resistance to FOL is mainly mediated by ET and JA signaling pathways. Hence, temporal pattern of the transcriptional response of studied genes suggested that FOL is a necrotrophic rather than a hemibiotrophic phytopathogenic fungal parasite. Our result is in agreement with Edgar et al. [48]; Li et al. [19]; Makandar et al. [49] and Mandal et al. [50].

CONCLUSION

Our observation indicated the susceptibility of Early Urbana-Y to fusarium wilt and enlighten its molecular responses to FOL. The disease severity and reduced shoot fresh weight of the treated plants despite of the significantly up regulated SA marker genes indicated that SA-dependent defense genes play a vital role in regulating of susceptibility to FOL. In contrast, the temporal pattern and transcript level of other evaluated genes suggested the JA/ET-mediated defense responses were not enough to prevent the pathogen invasion. Furthermore, antagonistic effect of SA on JA- dependent defense gene expression was also observed. Finally, these findings revealed that Early Urbana-Y line resistance to FOL appears to require the ET/JA-mediated defense response. However more detailed molecular mechanisms about the interaction between Early Urbana-Y and FOL is needed to be further investigated.

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پاسخ رونویسی ژنهای دفاعی و تنظیمی موثر در مسیر سیگنالی هورمونی گیاه گوجه فرنگی بر علیه پژمردگی فوزاریومی

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چکیده

پژمردگی فوزاریومی ناشی از *Fusarium oxysporum* f. sp. *lycopersici* (FOL) یکی از بیماری‌های عمده‌ی مخرب گوجه فرنگی در سراسر جهان است. هیچ گونه اطلاعات مولکولی در ارتباط با اثر متقابل گوجه فرنگی ایرانی رقم ارلی اربانا Y با FOL وجود ندارد. بر این اساس، مطالعه‌ی حاضر برای بررسی پاسخ به FOL با استفاده از بیماری‌شناسی گیاهی (شدت بیماری و وزن تر اندام هوایی) و روش‌های مولکولی انجام شد. رونوشت‌های مربوط به ژن پاسخ دهنده به اسید جاسمونیک (*Pin2*)، ژن‌های پاسخ دهنده به اسید سالیسیلیک (*Chi3*، *Chi9* و *PR1*)، ژن‌های تنظیمی مسئول مسیر سیگنال‌دهی اسید جاسمونیک (*WRKY33* و *MYC2*) و اتیلن (*ERF1*) با استفاده از تکنیک qRT-PCR در بازه‌های زمانی منظم از ۶ تا ۹۶ ساعت بعد از مایه زنی FOL اندازه‌گیری شدند. توسعه نشانه‌های بیماری و کاهش وزن تر اندام هوایی در گیاهان تیمار شده، علیرغم افزایش بیان ژن‌های دفاعی وابسته به اسید سالیسیلیک در زمان‌های مختلف پس از تلقیح بیمارگر نشان داد که مسیر سیگنالی اسید سالیسیلیک در حساسیت ارلی اربانا Y نسبت به FOL موثر است. بالعکس، ژن‌های مسیر JA و ET در پاسخ به آلودگی بیمارگر بیان قدرتمندی نداشتند که می‌تواند نشان‌دهنده نقش ژن‌های دفاعی هدایت شده توسط جاسمونات و اتیلن در کاهش حساسیت به این بیمارگر باشد. با این‌حال، جهت فهم دقیق حفاظت بیشتر بر علیه FOL، لازم است که مکانیسم دقیق مولکولی حساسیت ارلی اوربانا Y نسبت به FOL در آینده بیشتر مورد بررسی قرار گیرد.

کلمات کلیدی: اتیلن؛ بیان ژن؛ اسید جاسمونیک؛ اسید سالیسیلیک؛ مقاومت سیستمیک