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Virus-induced gene silencing for functional analysis of *eds1* gene in tomato infected with *Ralstonia solanacearum*

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Abstract

The efficiency of virus-induced gene silencing (VIGS) can be identified by monitoring the level of silencing of the target genes in plants. In this study, enhanced disease susceptibility 1 (*eds1*) gene was silenced in tomato (*Lycopersicon esculentum* L.) plants by VIGS, using *Tobacco rattle virus* (TRV) expression vector. Tomato plants at different growth stages (fully-opened cotyledon and eight-leaf) were co-infected with *Agrobacterium tumefaciens* C58C1 strain containing *eds1* complementary deoxyribonucleic acid (cDNA) at different (20°C and 28°C) temperatures and bacterial concentrations with 1.0×10^6 and 1.5×10^8 colony forming unit (CFU) mL^{-1}. The reverse transcriptase-polymerase chain reaction (RT-PCR) method was used to analyse the suppression level of the *eds1* gene and the VIGS efficiency assay. Data analysis showed a high *eds1* gene silencing at fully-opened cotyledon stage at 20°C using a *A. tumefaciens* at a concentration of 1.0×10^6 CFU rather than other conditions. In addition, the percentage of wilted plants was significantly varied in different conditions and the most wilted plants were observed at the 1.0×10^6 CFU and 20°C.

Based on our results, we suggest that *eds1* gene silencing can be used as a possible screening marker to select bacterial wilt-resistant species.

Key words: *Agrobacterium tumefaciens*, agro-infiltration, *eds1*, *Ralstonia solanacearum*, virus-induced gene silencing.

Introduction

Virus-induced gene silencing (VIGS) is known as an effective RNA-mediated gene silencing mechanism to suppress the transcriptional and post-transcriptional expression of targeted genes in plants (Holoch, Moazed, 2015). The VIGS occurs when a homologous RNA similar to host gene is introduced to a host cell by a virus plasmid and leads to degradation of the target mRNA (Unver, Budak, 2009; Vaistij, Jones, 2009). Recently, many recombinant vectors have been derived from viral genomes and have been adapted for a wide range of plant species. A novel construct of *Tobacco rattle virus* (TRV) vector is one of the most efficient VIGS vectors and has been widely used in various plants, such as *Arabidopsis thaliana* (Bond, Baulcombe, 2015), *Nicotiana benthamiana* (Senthil-Kumar, Mysore, 2011) and *Lycopersicon esculentum* (Kandoth et al., 2007). Optimization of *Tobacco rattle virus* (TRV)-induced gene silencing has showed that some parameters, including concentration and pre-incubation of *Agrobacterium*, the ecotypes, the growth stages and temperature of agro-inoculated plants, have potentially affected the VIGS efficiency (Wang et al., 2006; Bhaskar et al., 2009). The optimal temperature for plant growth is very variable in different plant species (Abrami, 1972; Hatfield, Prueger, 2015). Tomato plants show drastically reduced VIGS efficiency at temperatures above 21°C (Patil, Fauquet, 2015). Additionally, the growth temperature of agro-inoculated plants was identified as a factor which can cause a faster viral amplification, more formation of short-interfering RNAs and plant growth (Szittya et al., 2003; Ali et al., 2013).

Ralstonia solanacearum is known as the second important plant bacterial pathogen in the world after *Pseudomonas syringae* pathovars (Mansfield et al., 2012) and has been considered as a model in the plant pathology, biotechnology and genetic engineering studies (Hu et al., 2008; Wicker et al., 2012). In signalling pathway of plant disease, resistance is often mediated by corresponding gene pairs in the plant (resistance or *R* gene) and pathogen (a virulence or *Avr* gene). Gene *eds1* primarily mediates

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signalling derived from the TIR-NB-LRR class of *R* proteins and is indispensable for the function of these *R* genes (Narusaka et al., 2013). The activated eds1 gene is located in the up-stream of salicylic signalling pathway (Ding et al., 2015). So *eds1* gene over-expression could increase salicylic acid accumulation in infected plant tissues in response to pathogen attacks. In tomato, *eds1* gene has a key role in mediating resistance to a broad range of pathogens (viral, bacterial and fungal pathogens), yet shows imperative specificity for the function of *R* genes and is required in the receptor-like *R* gene class (Ercolano et al., 2012; Schön et al., 2013).

The aim of this study was to determine *Tobacco rattle virus* virus-induced gene silencing (TRV-VIGS) efficiency system through morphological and molecular indicators by measuring the suppression of the *eds1* gene expression and percentage of wilted tomato plants under different conditions, including growth development, temperature and bacterial concentration, to obtain the best conditions for reaching the highest VIGS efficiency.

Materials and methods

Plant materials and growth conditions. Tomato (*Lycopersicon esculentum* L., cultivar 'Khorram') seeds were provided by the Agricultural Research Institute, Yazd, Iran and were grown in green-house conditions during the summer 2016. The seeds were surface-sterilized in 1.5% sodium hypochlorite for 5 min, washed three times with sterile distilled water, and then were cultured in pots containing vermiculite perlite (1:1) mixture. The plants were regularly irrigated in greenhouse conditions at 20°C and 28°C under 16-hour light, 8-hour dark cycles with relative humidity of 75%. Tomato plants at the fully-opened cotyledon and eight-leaf stages were used for agro-infection assay.

Construction of Tobacco rattle virus (*TRV)-derived vectors.* The Gateway entry clones (pENTR11) containing pUC *ori* and attL1 and attL2 regions were used in this work. Enhanced disease susceptibility 1 (*eds1*) as target gene, beta-glucuronidase (*gus*) and phytoene desaturase (*pds*) genes as negative and positive controls respectively, were PCR-amplified using specific primers (Table 1) using tomato complementary deoxyribonucleic acid (cDNA) sources from infected tomato by *Ralstonia solanacearum*.

Inftection of tomato plants by Ralstonia solanacearum. In this study, 20 mL of a *R. solanacearum* suspension with the optical density (OD) at a wavelength of 600 nm (OD_{600}) of 1.0 was freshly prepared and poured over the surface of soil at 2–4 tomato leaf stage of growth. The scores of the disease symptoms in the plants were recorded six times from the sixth day after the infection to the end of the experiment.

Table 1. The characteristics of the primers used in this study

Gene	Forward sequence (5'-3')	Fragment length bp
eds1-F *eds1*-R	GAGGCTGTTGCACGATC GCTCTTGTACTCAGGCCAA	450
pTRV2-F pTRV2-R	CGTTGAGAATCCAGATGCTGT ACGAAGACACAACCTTGCTC	283
gus-F *gus*-R	CGCCATTTGAAGCCGATGTCAC AGAGATAACCTTCACCCGGTTGC	414
pds-F *pds*-R	CGGTCTAGAGGCACTCAACTTTATAA CGGGGATCCCTTCAGTTTTCTGTC	409

Total RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Total RNA extraction was carried out using a RNX-Plus solution (SinaClon, Iran) according to the manufacturer's protocol. First strand cDNA synthesis was made by using 500 ng of total RNA, random hexamer primers and reverse transcriptase enzyme (SinaClon, Iran). Then, cDNA was recombined into pENTR11 by BP (BP Clonase™ II enzyme) recombination reaction. Thereafter, LR (Gateway® LR Clonase™) recombination reaction was applied to transfer the inserts from pENTR11 clones into pTRV-RNA2-Gateway. pENTR11 carrying the cDNA of *eds1* gene (50 to 150 ng) was flanked between attL1 and attL2 regions and mixed with the destination vector (pTRV-RNA2-Gateway) 150 ng μl^{-1} (Fig. 1). Briefly, pENTR11 was mixed with 150 ng μl^{-1} of the destination vector DNA (pTRV-RNA2-Gateway) and 2 µl of LR Clonase II enzyme, then filled up to 10 µl of TE buffer (Tris-EDTA, pH 8.0). The mixture was then kept in a sealed glass vial at room temperature for 1 hour. Enzymes were inactivated with 1 µl of the proteinase K solution (2 µg μl^{-1}) for 10 min at 37°C. The presence of insert was checked by PCR method using specific primers related to each gene.

Virus-induced gene silencing (VIGS). These experiments were performed by using the *Agrobacterium tumefaciens* strain C58C1 containing pTRV1 and pTRV2. Bacterial strains were separately grown in an LB medium containing 10 mM MES and 20 mM acetosyringone with appropriate antibiotics. After one day, bacterial cells were harvested and re-suspended in infiltration buffer (10 mM $MgCl_2$ 10 mM MES, pH 5.6 and 150 mM acetosyringone) to obtain a final OD_{600} of 1.0 and 2.0 in both pTRV1 and pTRV2. Tomato plants infiltration was done in fully-opened cotyledon and eight-leaf stages at 20°C and 28°C with a 1 mL needleless syringe. The plants inoculated with pTRV2 alone were used as a control.

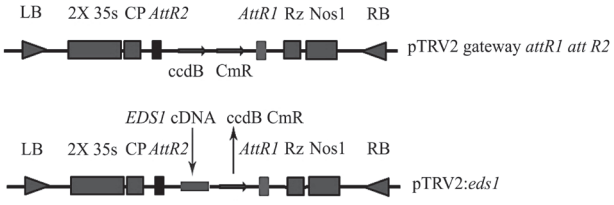


Figure 1. Schematic figure of Gateway LR reaction and recombinant of *eds1* gene into pTRV2

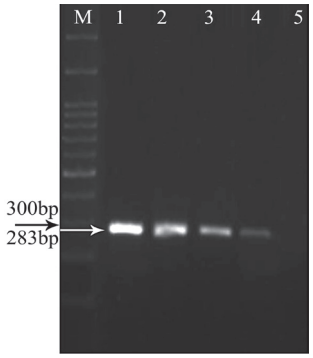
Percentage of the wilted plants. The percentage of the wilted plants was calculated by the following formula: PWP = Σ (percent of wilted plants) / total plants.

Statistical analysis. The data in this study were analysed using the analysis of variance (*ANOVA*) protocol to determine any significant difference among the treatments. The software *SPSS*, version 22.0 was used for the statistical analyses. The statistical significance level was considered to be $\alpha = 0.05$. This study was carried out in three replications each containing 15 plants.

Results

Detection of pTRV2 constructs. pTRV2 detection was done using specific primers based on a conserved sequence of the partial coat protein of *Tobacco rattle virus*. The expected size of pTRV (283 bp length) was detected in all agro-inoculated plants at different conditions.

The expression level of pTRV2 was significantly increased in the fully-opened cotyledon stage when compared to eight-leaf stage in infected tomato plants with *Agrobacterium* at 20°C (Fig. 2). So based on these results, we suggest that the replication of pTRV2 could be affected by different stages of plant growth and temperature. The most replication of pTRV2 was observed in the fully-opened cotyledon stage at 20°C.



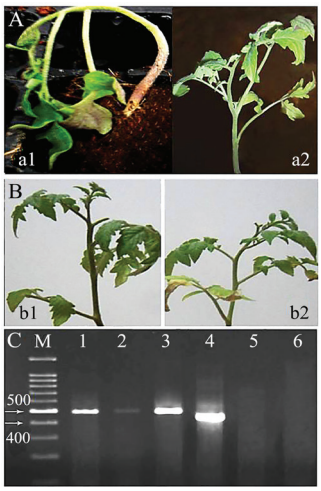
M – ladder (1k bp); line 1 – fully-open cotyledon stage at 20°C, line 2 – eight-leaf stage at 20°C, line 3 – fully-opened cotyledon stage at 28°C, line 4 – eight-leaf stage at 28°C, line 5 – non agro-inoculated plants (negative control)

Figure 2. The amplification of pTRV2 using RT-PCR analysis in tomato plants infected via ago-infection method

Tomato wilting and suppression of the eds1 gene. Tomato plants were infected with *Agrobacterium tumefaciens* at a concentration of 1.0×10^6 CFU. Two growth stages (i.e. fully-opened cotyledon and eight-leaf stages) and two temperatures (20°C and 28°C) were used to analyse agrobacterium-mediated virus infection. The results indicate that different growth stages and temperature conditions can change the efficiency of gene silencing in tested groups. The *eds1* gene expression was completely silenced in fully-opened cotyledon stage in approximately all agro-infected plants at 20°C. High percentage of wilting in infected tomato plants by *R. solanacearum* confirms *eds1* gene suppression (Fig. 3).

Percentage of wilted plants in the eds1 gene-silenced plants. The highest percentage of wilting was observed in the fully-open cotyledon stage at 20°C temperature compared to eight-leaf stage at the same temperature. At 28°C, the wilting symptoms were significantly reduced when compared to 20°C at both growths stages (Table 2). At this temperature slight wilting was observed. These results indicate that low temperature could influence the efficiency of gene silencing at different growth stages.

The progress of disease was surveyed 6–21 days post infection (DPI) in silenced tomato plants infected with *R. solanacearum*. Our data showed that the high wilting percentage occurred at fully-opened cotyledon stage at 20°C at 16–21 days post infection with *R. solanacearum*. At 6th day post infection, plant wilting percentage was reduced but this reduction was not significant between different growth conditions (Fig. 4). The pTRV2 empty plasmid was used as control for monitoring disease progression.



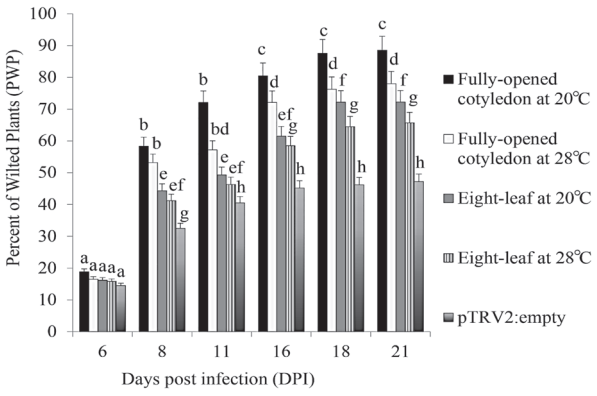
Note. The effect of growth stage and temperature on gene silencing in tomato plants infected by *Agrobacterium tumefaciens*: (A) agro-infected tomato plants: a1 – in fully-opened cotyledon stage at 20°C, a2 – eight-leaf stage at 20°C; (B) agro-infected tomato plants: b1 – in fully-opened cotyledon stage, b2 – eight-leaf stage at 28°C; (C) efficiency of VIGS based on suppression of the *eds1* gene: M – ladder (1k bp), line 1 – eight-leaf stage at 20°C (semi-efficiency), line 2 – fully-opened cotyledon stage at 28°C (high efficiency), line 3 – eight-leaf stage at 28°C (low efficiency), line 4 – *gus* gene (negative control), line 5 – fully-opened cotyledon stage at 20°C (highest efficiency), line 6 – *pds* gene (positive control).

Figure 3. Presence of wilting symptoms and level of *eds1* gene silencing

Table 2. Wilting percentage at different plant growth stages and temperature conditions in the infected tomato plants with normal concentration (10^6 CFU mL^{-1}) of *Agrobacterium tumefaciens* C58C1 strain

Plant growth stage	Wilting (%) in two temperatures	
	C°20	C°28
Fully-opened cotyledon	85.4 ± 1.1 a	79.9 ± 0.9 b
Eight-leaf	76.7 ± 1.6 c	66.4 ± 1.6 d

Note. The different letters show what means are significantly different according to the LSD at $p < 0.05$; mean + SD.



Note. The susceptibility of *eds1* gene-silenced plants infected with *R. solanacearum* from 6th to 21st day post infection at different growth stage and temperature; more-severe wilting symptom caused by *R. solanacearum* in fully-opened cotyledon stage at 20°C compared to other conditions; each value is the mean of three biological replicates; letters show data that are of equal variance but significantly different ($p < 0.05$; all data are mean ± standard error).

Figure 4. Effect of *eds1* gene silencing on the percentage of wilting symptoms in tomato plants infected with *Ralstonia solanacearum* at eighth-leaf stage

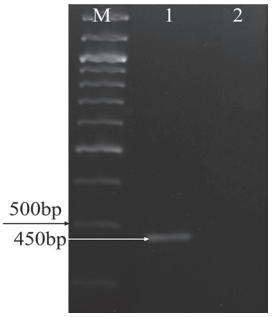
Bacterial concentration and efficiency of virus-induced gene silencing (VIGS). The effect of bacterial concentration on gene silencing efficiency was investigated in silenced plants against *eds1* gene by agro-infection assay. Based on results of wilting assay, we used just infected tomato plants in the fully-opened cotyledon stage. For this experiment, two concentrations (1.0×10^6 and 1.5×10^8 CFU mL^{-1}) of *A. tumefaciens* were used. All experiments were done at 20°C. The results of plant infection and wilting assay showed that plant wilting percentage is significantly decreased at high concentration of *A. tumefaciens* in *eds1* gene-silenced plants when compared to normal concentration (Table 3).

Table 3. Percentage of wilted tomato plants at fully-opened cotyledon stage at 20°C infected by different concentrations of *Agrobacterium tumefaciens* C58C1 strain

Plant growth stage	Temperature	Wilting (%) at two concentrations		*P* value
		1.0×10^6 CFU mL^{-1}	1.5×10^8 CFU mL^{-1}	
Fully-opened cotyledon	20°C	85.4 ± 1.1 a	79.9 ± 0.9 b	0.004

Note. The values following different letters in a column are significantly different from each other at $p \leq 0.05$; each value is the mean of three replications, including 15 plants per repetition; mean + SD.

RT-PCR method was used to measure the silencing level of *eds1* gene in agro-infected tomato plants with two different concentrations of *A. tumefaciens*. The result of RT-PCR analysis showed no-amplified fragment related to *eds1* gene in infected plants with *A. tumefaciens* at normal concentration (1.0×10^6 CFU mL^{-1}) while a clear band related to *eds1* gene was observed in infected tomato plants with high bacterial concentration (1.5×10^8 CFU mL^{-1}) (Fig. 5).



M – ladder (1k bp); silencing level in infected plants with: line 1 – bacterial concentration at 1.5×10^8 CFU mL^{-1}, line 2 – bacterial concentration at 1.0×10^6 CFU mL^{-1}

Figure 5. Bacterial concentration and silencing level of *eds1* gene

These results indicate a positive correlation between bacterial concentration and gene silencing. Thus, we confirmed that silencing of *eds1* gene is noticeably affected by the low bacterial concentration.

Discussion

Tomato wilting disease, caused by *R. solanacearum*, is one of the most destructive diseases of tomato which lead to crop loss in greenhouse cultures in Iran. Use of resistant plants for disease control is the main objective of many studies in the vegetables science research laboratory. Virus-induced gene silencing (VIGS) has been set up successfully to understand the functional analysis of different pathogenesis-related genes involved in plant disease resistance against pathogenic agents (Ramegowda et al., 2014; Lee et al., 2015). In this work, we attempted to investigate the key role of *eds1* gene in resistance of tomato to bacterial wilting disease caused with *R. solanacearum* by using VIGS approach. Firstly, we detected pTRV2 by specific primers and the PCR-amplifying showed the expected 283-bp-fragment of *eds1* gene. These observations indicated that pTRV2 could be noticeably replicated in infected tomato plants. Relative transcript fragments were differentially detected in infected tomato plants. Secondly, silencing efficiency of *eds1* gene was assayed in different conditions. Growth conditions such as developmental stage and photoperiodic conditions are the most important factors for determination of VIGS efficiency. The efficiency of virus-induced gene silencing with Soybean yellow common mosaic virus (SYCMV) in soybeans has been investigated by using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) method at different plant growth stages. These results showed a high VIGS efficiency at the primary leaves stage and low efficiency at closed cotyledon stage (Kim et al., 2016). We found that silencing of *eds1* gene was more effective in the inoculated seedlings at fully-opened cotyledon stage, whereas at the eight-leaf stage, VIGS efficiency was significantly reduced.

The effect of temperature on enhancement of VIGS in infected tomato plants has showed that the VIGS efficiency was affected by low temperature and low humidity (30%) and at high temperature (21°C), VIGS efficiency was significantly reduced (Fu et al., 2006). The host and environmental factors have an important role in viral RNA recombination that could lead to increase of VIGS efficiency (Jaag, Nagy, 2010). The role of temperature in VIGS efficiency has been studied on different plants using agro-infection method and has suggested that temperature can influence silencing efficiency by affecting vector DNA accumulation and the spread of endogenous gene silencing in tomato (Fu et al., 2006; Cai et al., 2007), cotton (Tuttle et al., 2008) and *Nicotiana benthamiana* (tobacco) and *Solanum lycopersicon* (tomato) as well as *Arabidopsis* (Padmanabhan, Savithramma, 2009). Also the role of environmental factors, including temperature, light intensity, humidity and plant growth stages, has been strictly demonstrated on VIGS efficiency (Liscombe, O'Connor, 2011; Kim et al., 2016). The optimal temperature to achieve a high VIGS efficiency has been typically determined in soybean cyst nematode (27°C), bacterial leaf pustule (28°C) and rust bioassays (25–30°C) (Zhang et al., 2010). Our observation showed that the optimal temperature is 20°C with a high VIGS efficiency and this temperature did not show any tomato growth limitation. In SYCMV-based VIGS system this parameter has been determined at 27°C (Kim et al., 2016). The high-efficiency VIGS at the low temperature is due to the high susceptibility of plants to viruses because at the high temperature, siRNAs is slowly accumulated and then RNA silencing will be displayed to lower level (Tuttle et al., 2008; Sung et al., 2014). However, we suggest use of low temperature and normal bacterial concentration to achieve a high VIGS efficiency.

A down expression and complete silencing of *eds1* gene was determined at 9 days post infection using semi-quantitative PCR analysis. Down regulation of

pds gene in California poppy (*Eschscholzia californica* Cham.) has been shown by VIGS technique. Two week after agro-infiltration, 92% of the manipulated plants showed intense reduction of *pds* gene expression level (Wege et al., 2007). In this study, we also showed strong *eds1* gene silencing at 16–21days post infection with *R. solanacearum*.

Replication of viral genome is critical to reach a high silencing efficiency. It has been shown that silencing in *N. benthamiana* plant by using *Agrobacterium tumefaciens* harbouring pTRV1, pTRV2 and pTRV2-*pds* is more efficient than tomato plant. Also, choice of suitable strains of *A. tumefaciens* is pivotal to increase VIGS efficiency. Usually, in tomato, the *A. tumefaciens* GV3101 strain is used while in two *N. benthamiana* GV3101 and GV2260 strains are used (Liu et al., 2002) but in this study, we used the *A. tumefaciens* C58C1 strain for agro-infection and a high silencing efficiency was observed. The difference of bacterial strains for agro-infection is related to resistance to antibiotics and use of suitable strains is dependent on the protocol of each laboratory and their experience.

Silencing efficiency of *SpPDS* and *SpMPK1* genes in *Solanum pimpinellifolium* has been evaluated with two bacterial concentration (OD_{600} = 1.0 and 2.0). Evaluation method included four indexes as: (1) silencing frequency of plants, (2) silencing efficiency of a plant, (3) silencing frequency of each leaf and (4) silencing efficiency of each gene. A high silencing efficiency of both targeted genes was determined at OD_{600} of 2.0 (Wang et al., 2006).

In this study, we calculated some of these indexes such as silencing efficiency in leaf and total plant and also gene expression level in the infected tomato plants with two bacterial concentrations.

Our results showed that a normal bacterial concentration (1.0×10^6 CFU mL^{-1}) could increase wilting symptoms that indicate a high silencing efficiency of *eds1* gene, whereas at the high concentration of *A. tumefaciens* (OD_{600} = 2.0) silencing efficiency was significantly reduced when compared to normal concentration (OD_{600} = 1.0). Our data showed that bacterial concentration of 1.0×10^6 CFU mL^{-1} will be more efficient for VIGS in tomato plants infected with *R. solanacearum.* These data are not in line with the results of Li et al. (2006), which show that bacterial concentration with OD_{600} = 2.0 led to high VIGS efficiency. This contradiction can result from two parameters: first, their high temperature (24°C) compared to our temperature conditions at 20°C, and second, the presence of *R. solanacearum* in our infected tomato plants.

Conclusion

In this work, we showed that the efficiency of virus-induced gene silencing (VIGS) approach can be affected by bacterial concentration and plant growth conditions. Infecting of tomato seedlings by *Tobacco rattle virus* (TRV) vector can induce gene silencing in the youngest leaf at the open cotyledon stage. Based on these results, we could conclude that *eds1* gene has a pivotal role to confer resistance to tomato plants. High wilting intensity in *eds1* gene-silenced tomato plants using VIGS approach confirmed our claim. Therefore, VIGS could be used as a powerful tool to achieve a constant transformation in different varieties of tomato. It seem that despite growth conditions and temperature, change of pH, nutrient, irrigation and salinity will help us to increase the efficiency of this technique.

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Viruso sukelto genų nutildymo panaudojimas *eds 1* geno funkcinei analizei *Ralstonia solanacearum* užkrėstuose pomidoruose

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Santrauka

Viruso sukelto genų nutildymo efektyvumą galima įvertinti nustatant pasirinkto geno nutildymo laipsnį augaluose. Tyrimo metu valgomojo pomidoro (*Lycopersicon esculentum* L.) augaluose jautrumo ligoms genas *eds1* buvo nutildytas taikant viruso sukeltą genų nutildymą, naudojant tabako garbanotosios dryžligės viruso (*Tobacco rattle virus*) raiškos vektorių. Pomidorai įvairiais augimo tarpsniais (pilnai atsidarius sėklaskiltei ir aštuntojo lapo) esant skirtingoms temperatūroms (20 bei 28 °C) buvo infekuoti $1,0 \times 10^6$ ir $1,5 \times 10^8$ mL^{-1} koncentracijos *Agrobacterium tumefaciens* C58C1 paderme, turinčia *eds1* cDNR geną. Atvirkštinės transkripcijos ir polimerazės grandininės reakcijos (AT-PGR) metodas buvo panaudotas analizuojant *eds1* geno slopinimą ir tiriant viruso sukelto genų nutildymo efektyvumą. tyrimo duomenų analizė parodė didelį *eds1* geno nutildymą visiškai atsidariusios sėklaskiltės tarpsniu esant 20°C temperatūrai ir panaudojus $1,0 \times 10^6$ KFV koncentracijos *A. tumefaciens* padermę, lyginant su kitomis sąlygomis. Be to, esant įvairioms sąlygoms nuvytusių augalų procentas smarkiai skyrėsi, o daugiausia jų buvo esant $1,0 \times 10^6$ mL^{-1} bakterijos koncentracijai ir 20° C temperatūrai.
Remiantis tyrimo rezultatais galima teigti, kad *eds1* geno nutildymą galima naudoti kaip galimą žymeklį, siekiant atrinkti bakteriniam vytuliui atsparias rūšis.

Reikšminiai žodžiai: *Agrobacterium tumefaciens*, agroinfiltracija, *eds1*, *Ralstonia solanacearum*, viruso sukeltas geno nutildymas.