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Research Article

# Stability of resistance against beet curly top disease in the presence of cucumber mosaic virus in *Arabidopsis thaliana*

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**Abstract:** Curly top is one of the most important viral diseases of sugar beet. Use of resistance sources is a promising strategy for control of this disease. In the present study, the efficiency of four gene silencing constructs (OUT-hp ،IN-hp ، sense and antisense) against two major causes of curly top disease in Iran, beet curly top virus-Svr (BCTV) and beet curly top Iran virus (BCTIV), were evaluated in transgenic plants. Selection of transgenic plant seeds was carried out on selective medium 1/2MS containing glufosinate-ammonium (Basta) and the results showed that the pBCTV-IN-hp construct resulted in the highest germinated seeds. Selected plants were transferred to greenhouse and evaluated for resistance to basta and detection of silencing constructs in the transgenic plants. Afterwards, resistance of the selected transgenic plants to beet curly top viruses and resistance stability against cucumber mosaic virus (CMV) was evaluated in a completely randomized design with six treatments in a factorial experiment. The results showed that the transformed lines with each of four constructs were significantly different in severity of symptoms, plant height and number of flowering stems compared to their respective controls. Although these transgenic plants were resistant to BCTV-Svr and BCTIV, in their challenge inoculation experiments it was shown that this resistance was suppressed by CMV infection.

**Keywords:** silencing constructs, transgenic plant, sugar beet, Geminiviruses

## Introduction

Beet curly top disease caused by *Geminiviridae* is one of the most important viral diseases of sugar beets (Brown *et al*., 2011). These viruses are transmitted by leafhoppers of the genus Circulifer, in which they circulate without multiplying (Soto and Gilbertson, 2003; Soto *et al*., 2005; Chen *et al*., 2010). Curly top viruses have a very broad host range of more than 300 plant species from 44 families, including crops, ornamentals, and weeds (Velasquez-valle *et al*., 2012). The major crops affected include common bean, pepper, spinach, sugar beet, and tomato (Briddon *et al*., 1998; King *et al*., 2011). So far beet curly top virus (BCTV-Svr) belonging to the genus *Curtovirus* and beet curly top Iran virus (BCTIV) belonging to the genus *Becurtovirus* have been reported to induce beet curly top disease in Iran (Hosseini Abhari *et al*., 2005; Heydarnejad *et al*., 2007; Bolok-Yazdi *et al*., 2008). Also mixed infections of BCTV-Svr and BCTIV have been reported in sugar beet from many regions of Iran (Ebadzad *et al*., 2008).

Control of curly top disease has been difficult due to the complex epidemiology of the disease. Actually, the wide host range of the viruses and the unpredictable annual migratory behavior of the vector contribute to the difficulty (Bennett, 1971; Wintermantel and Kaffka, 2006). Also, the fight against the vector, in addition to the cost and environmental risks, cannot effectively

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control the disease. As a result, like other plant viruses, it could be managed through integrated pest management using resistant plants and vector control. However, until now, there has not been a suitable resistance source for beet curly top disease, and the few reported resistance sources are "quantity resistance" with low heritability and low yields.

Viral genome sequences induce RNA silencing with various strategies, including the use of sense or antisense gene sequence of the virus, the production of dsRNA using frequency-reverse sequencing and miRNA patterning and generating a miRNA-induced silencing against target viruses. RNA silencing is an RNA-directed gene regulatory system involved in different fundamental processes, such as gene regulation, de novo histone and DNA methylation, establishment of heterochromatin, defense against viruses, and control of transposon mobility (Rodríguez-Negrete *et al*., 2009). Silencing pathways are complex and partially overlapping, but at least three basic classes can be distinguished: cytoplasmic RNA silencing (or post-transcriptional gene silencing; PTGS) mediated by small interfering RNAs (siRNAs), silencing mediated by microRNAs (miRNAs), and transcriptional gene silencing (TGS) mediated by siRNA-directed methylation of DNA and histone proteins (Bisaro, 2006). Silencing pathways involve the cleavage of a double-stranded, or an imperfect stem-loop, RNA molecule into a short 21- to 24-nucleotide (nt) RNAs by a Dicer-like enzyme. These RNAs, known as short interfering RNAs (siRNAs) and microRNAs, direct the silencing process in a sequence-specific manner (Rodríguez-Negrete *et al*., 2009).

Although RNA silencing has evolved to be a potential antiviral defense strategy, most of the plant viruses encode at least one suppressor protein to circumvent the defense mechanism. Thus, viruses intrude with the host silencing machinery, resulting in increased viral replication and/or repression of systematic silencing. These viral suppressors (VSRs) interfere with either single or multiple steps in silencing pathway to enhance virus replication, eventually restraining the production of siRNA. For instance, the V2 protein of tomato yellow leaf curl virus inhibits the generation of dsRNA by binding to SGS3 (cofactor of RDR6). This ultimately distracts the siRNA production and increases the susceptibility of tomato plant. Cucumber mosaic virus protein 2b binds with the AGO1 and blocks its cleaving activity (Diaz-Pendon *et al*., 2007; Burgyán and Havelda, 2011).

There are numerous reports of successful use of gene silencing strategies in the development of resistance to plant viruses, including Geminiviruses (Pooggin *et al*., 2003; Vanitharani *et al*., 2003). But the fact that plant viruses possess RSS implies that resistance to a specific virus via RNA silencing of the transgene would be lost when transgenic plants were coinfected with other viruses that also exhibit RSS. Actually, Nomura *et al*. (2014) reported that cucumber mosaic virus (CMV) infection breaks down the turnip mosaic virus (TuMV) resistance of transgenic *Arabidopsis thaliana* plants carrying the TuMV coat protein (CP) transgenes. For potato virus Y (PVY)-resistant transgenic tobacco plants carrying the nuclear inclusion a (NIa) transgene, Mitter *et al*. (2003) showed that PVY resumed infectivity when the transgenic tobacco plants were pre-infected by a severe strain of CMV. The ability of a CMV severe strain to break down the transgenic resistance to a virus was also demonstrated in transgenic plum pox virus (PPV)-resistant *N. benthamiana* plants containing the 50-region of the NIa gene, while tobacco vein mottling virus could not (Simo´n-Mateo *et al*., 2003). However, whether mild CMV strains would suppress the RNA silencing and whether the virulence level would affect the efficiency of RNA silencing has not been determined.

The aim of this study was to confirm the resistance of gene silencing constructs to beet curly top virus disease and to investigate their resistance to coinfection of beet curly top viruses and a mild strain of CMV in transgenic *Arabidopsis thaliana*. These constructs were designed by Montazeri *et al*. (un-published). Previously, they reported that silencing constructs showed complete resistance to the two causal agents of curly top disease in Iran (BCTV-Svr and BCTIV).



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## Materials and Methods

### Silencing constructs and viruses source

The gene silencing constructs designed by Montazeri *et al*. (un-published) were used in this study. They include pFGC-5941 as expression vector, the pBCTV-sense and pBCTV-antisense constructs each have a piece of cassette but are in different directions and two pBCTV-OUT-hp and pBCTV-IN-hp hairpin constructs, each with two versions of the cassette which are reverse complementary sequence and inverted around the intron sequence.

The viral sources used in this study included the BCTV-Svr strain (Ebadzad *et al*., 2008) donated by Dr Behjatnia from Shiraz University, Shiraz, Iran, the BCTIV strain (Heydarnejad *et al*., 2012) was donated by Dr. Heidarnejad of Shahid Bahonar University, Kerman, Iran and the CMV strain isolated from Shiraz (Azizi and Shams-bakhsh, 2014).

### Transmission of silencing constructs

The transfer of gene silencing constructs using *in planta* method (Bent, 2006) was carried out by immersing unopened buds of flowering stems of Arabidopsis plants into suspension of Agrobacterium containing constructs. For this aim, LBA4404 strain of *Agrobacterium tumefaiens* was used.

The treated plants were kept in a greenhouse at a temperature of 22 ± 2 °C and their seeds were collected after physiological examination.

### Sterilizing and cultivating seeds on a selective culture medium

In order to sterilize the seeds, 0.2 grams of seeds were poured into a 1.5 ml vial and mixed with isopropanol for 30 seconds to 1 minute. After removing isopropanol, sodium hypochlorite 3% was added with a Tween-20 drop and continuously mixed for 5 minutes. Seeds were washed three to four times with sterile distilled water and finally, one milliliter of sterile agarose solution 1% was added to it. The agarose containing the seeds was spread over the surface of the plates of the selective medium, and then the petri dishes were coated with an aluminum foil. For homogeneity and synchronization of seed germination, petri dishes were stored at 4 °C for 48 hours. In this experiment, seed of sterile Col-0 ecotype cultivated on the selected medium was used as a negative control, and the seeds were cultured on a selective medium without ammonium glufosinate herbicide (BASTA) as a positive control. Within 1 and 2 days after the transfer of petri dishes to 24 degrees Celsius and 16 hours of brightness, all seeds germinated. For two weeks, culture plots were observed on a daily basis and transgenic healthy seedlings were recorded at each turn.

The selective medium (base medium, ½ MS without hormone, no sugar, 0.6% agar) containing 10 mg/L herbicides of ammonium glufosinate (BASTA). The concentration of herbicide in Basta was based on the report of Montazeri *et al*. (un-published).

### Bioassay of Arabidopsis plants to ammonium glufosinate herbicide

In order to investigate the susceptibility of Arabidopsis plants to Basta (ammonium glufosinate) herbicide, bioassay test was carried out on herbicide susceptibility. Determination of resistance to herbicide was used to select transgenic plants with herbicide resistance gene. Six concentrations of 0.001, 0.005, 0.01, 0.1, 0.5 and 1 mg/ml were first selected to obtain the appropriate concentration. In this test, the herbicide was strained by cotton on the blade of the fourth leaf of plants in a 4-6 leaf stage. Daily observations were made to investigate possible changes. The lowest effective herbicide concentration was about 0.1 milligrams per milliliter with the least amount of necrosis, then 12 concentrations of 0.007, 0.008, 0.009, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08 and 0.09 mg/ml were checked. Finally, the lowest effective herbicide concentration (0.08 mg/ml) was used as an indicator for the separation of transgenic plants from non- transgenic plants.

### DNA extraction from Arabidopsis and Polymerase Chain Reaction (PCR)

Total DNA was extracted from plant tissues by CTAB method (Doyle, 1987) and subjected to



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PCR for detection of constructs in Arabidopsis plants. Inoculation of viruses of beet curly top disease was by injection method using insulin syringe and cucumber mosaic virus was inoculated mechanically. For mechanical inoculation a portion of freshly infected leaves were extracted in sterile mortar containing phosphate buffer (pH = 7.5). Carburandom powder was used on leaf blade at inoculation. The virus-containing extract was kept on ice until the end of inoculation. Specific primers, pFGC-F/pFGC-R and pFGC-F3503/pFGC-R4291, were designed by Montazeri *et al* (un-published), were used to detect gene silencing constructs and specific primers BCTV-Svr (Ebadzad *et al*., 2008) and BCTIV (Heydarnejad *et al.*, 2013) with a size of 750 and 792 bp, respectively, were used to detect inoculated viruses. In order to make semi-quantitative measurements of the accumulation of viral genomes in different plants, 18S RNA primers (Faria *et al.,* 2006) with a 500-bp band, as internal control, with viral specific primers were used in a semi-quantitative polymerase chain reaction (semi-Q-PCR).

The amplification of DNA fragments was done in a programmable DNA thermal cycler (Eppendorf Gradiant, Germany) for 25 cycles after an initial denaturation for 3 min at 94 °C and 30 seconds denaturation at 90 °C, annealing at 45 °C for BCTV-Svr and at 59 °C for BCTIV for 30 seconds and extension at 72 °C for 1 min. The final extension was for 5 min at 72 °C.

PCR and semi-quantitative PCR electrophoresis using a 1% agarose gel in a TBE buffer (containing Tris-Base 54 gr (pH = 8), Boric Acid 5/27 g and 20 ml EDTA (0/5M)) and in an electrical field with a voltage of 100 V was applied for 30 min in a horizontal electrophoresis apparatus (Pharmacia, EPS-500/400). The agarose gel was photographed using the Gel Doc (UVIDOC HD5, France) device. The molecular marker (Fermentas, Lithuania) GeneRuler™ 1kbp DNA was used to determine the size of the amplified parts.

**Evaluation of the severity of symptoms and growth parameters**

For the BCTV-Svr, BCTIV, BCTIV + BCTV-Svr viral treatments at the sixth week and for CMV/BCTIV, CMV/BCTV-Svr and CMV viral treatments, during the second week of growth of the Arabidopsis plants, parameters such as scoring, plant height, number of flowering stems, fresh and dry weights, and incubation period were evaluated in four lines of OUT-hp, IN-hp, sense, antisense constructs and pFGC carrier and Col-0 (wild-type). Arabidopsis Plants were pulled out of soil and left to dry for 3-4 days at room temperature. In this study, for all tests, a completely randomized design with 10 replications for hairpin constructs and 5 replications for sense and antisense constructs was used. The incubation period of viruses of beet curly top disease was investigated in gene silencing constructs for six viral treatments for 35 days.

**Data analysis**

The variance analysis of qualitative data was carried out based on Mumford (1974) and Strausbaugh *et al*. (2007). Data analysis was performed using SAS software 2002 (SAS Institute 2002) by Proc GLM (General Linear Model procedure) and LSD (Least Significant Difference) test and comparing the mean of data at a probability level of one percent. Total lab software was used to quantify the bands resulting from virus replication.

**Results**

The percent yield of transgenic seedlings selected for pBCTV-antisense and pBCTV-sense constructs was 0.5% and for pBCTV-OUT-hp, pBCTV-IN-hp and pFGC-5941 carrier, this was 0.46, 1 and 32% respectively; while all Col-0 seedlings (wild-type) were necrotic after two weeks. The results of the evaluation for the pBCTV-IN-hp construct was consistent with the results of Bent (2006). View of the selected seedlings in the pFGC-5941 carrier represents the resistance gene expression to ammonium glufosinate herbicide (Fig. 1 and Table 1).

Evaluation of Arabidopsis plants containing silencing constructs upon ammonium glucosamine herbicide treatment is shown in Table 2.



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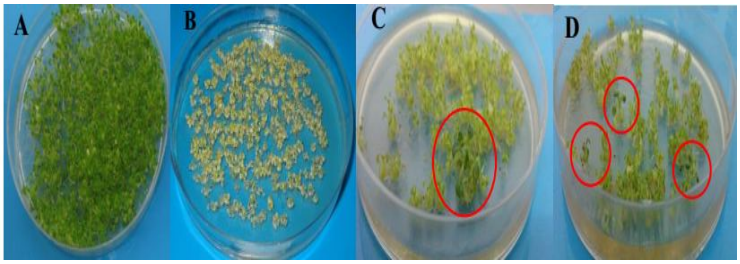


**Figure 1** Media plates containing wild type in a culture medium without basta (A) wild type in a culture medium containing basta (B) and seedlings with silencing constructs in a culture medium containing Basta (C and D).

**Table 1** Percentage of seedlings survived in a culture medium containing ammonium glufosinate herbicide (BASTA) for pBCTV-antisense, pBCTV-sense, pBCTV-OUT-hp, pBCTV-IN-hp constructs and pFGC-5941 carrier.

| Constructs | Survived seedlings (%) |
|---|---|
| pBCTV-sense | 0.5 |
| pBCTV-antisense | 0.5 |
| pBCTV-IN-hp | 1 |
| pBCTV-OUT-hp | 0.46 |
| pFGC-5941 | 0.35 |

**Table 2** Responses of Arabidopsis plants containing silencing constructs and wild type to ammonium glucosamine herbicide.

| Constructs | Total | Number of plants[1] |
|---|---|---|
| pBCTV-sense | 5 | 0 |
| pBCTV-antisense | 5 | 0 |
| pBCTV-IN-hp | 10 | 1 |
| pBCTV-OUT-hp | 10 | 0 |
| pFGC-5941 | 10 | 1 |
| Non-transgenic (Wild type) | 10 | 10 |

[1]The number of plants susceptible to herbicide ammonium glufosinate.

Results indicated that PCR analysis showed fragments of approximately 1940 bp in Arabidopsis thaliana carrying pBCTV-antisense and pBCTV-sense constructs (primer used: pFGC F/R) and 1156 bp in Arabidopsis thaliana carrying pBCTV-OUT-hp and pBCTV-IN-hp constructs (primer used: pFGC-3505/4291) (Fig. 2). The replication of these fragments in plants indicated the transformation of the plants with these constructs.

In this study, symptoms such as leaf curling, flowering stems deformity, a little anthocyanin accumulation (Lee *et al*., 1994; Park *et al.*, 2004), dwarfing, rapid collapse and death were observed in infection with BCTV-Svr, BCTIV + BCTV-Svr and CMV/BCTV-Svr (Fig. 3).

Symptoms of the disease were evaluated at 42 day post inoculation (dpi). The time of appearance of symptoms in plants containing four gene silencing inducing constructs and control of Col-0 and pFGC was different when infected with different viral treatments. Four construct treatments in infection with BCTIV and BCTV-Svr showed more delay (five and four weeks after inoculation, respectively) in symptoms appearance. While in simultaneous treatment with BCTIV + BCTV-Svr, this delay was less (three weeks after inoculation), symptoms were detected in treatment with either BCTIV and BCTV-Svr seven days after the CMV pre-inoculation (CMV/BCTIV and CMV/BCTV-Svr) (Fig. 4). On the other hand, in viral treatments of each curly top virus alone and simultaneously, all tested plants in pFGC and wild type showed symptoms, while symptoms were observed in only a number of transgenic plants with silencing constructs, this would indicate the efficiency of the silencing constructs. Also, the results showed that the number of plants with symptoms in hairpin transgenes was less than those in sense and anti-sense transgenes, and these constructs were weaker than hairpin constructs. In addition, the number of symptomatic plants in the BCTIV viral treatment was lower than BCTV-Svr, this is due to the difference in the biology of the viruses, and usually in case of BCTV-Svr the symptoms of disease appear earlier and are more severe than in BCTIV (Heydarnejad *et al.*, 2013; Montazeri *et al.*, 2016). When Arabidopsis plants were inoculated simultaneously with both curly top viruses, due to the synergistic effect of the two



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viruses (Taheri *et al*., 2014) the number of plants with symptoms was greater such that in combination viral treatment, all tested plants with sense and antisense constructs showed symptoms, this also reflected the poor performance of these constructs (Table 3). In super-infection with CMV, all of the transgenic plants of four silencing constructs showed clear symptoms after a week.

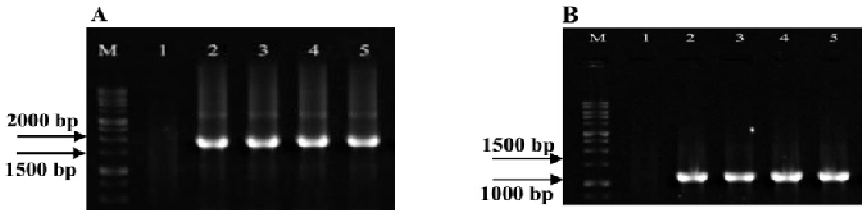


**Figure 2** PCR product electrophoresis pBCTV-sense and pBCTV Arabidopsis transgenic plants antisense with pFGC F/R primers in 1% gel electrophoresis. M: molecular weight marker (GeneRuler™ 1kbp, DNA ladder; Fermentas, Lithuania). (A) Lanes 1: non-transgenic plant. Lanes 2 and 3: Transgenic plants of Arabidopsis pBCTV-sense. Lanes 4 and 5: Transgenic plants of Arabidopsis pBCTV-antisense. (B) Lanes 1: non-transgenic plant. Lanes 2 and 3: Transgenic plants of Arabidopsis pBCTV-OUT-hp. Lanes 4 and 5: Transgenic plants of Arabidopsis pBCTV-IN-hp.


**Figure 3** Symptoms observed in Arabidopsis plants inoculated with various viral treatments. (A) Mock. (B) pFGC-5941 in single inoculation with BCTIV and BCTV-Svr. (C) sense and antisense constructs in single inoculation with BCTIV. (D) IN-hp and OUT-hp constructs in single inoculation with BCTIV. (E) IN-hp and OUT-hp constructs in single inoculation with BCTV-Svr. (F) sense and antisense constructs in single inoculation with BCTV-Svr. (G) sense and antisense constructs in simultaneous and mixed inoculation with BCTIV and BCTV-Svr (BCTIV + BCTV-Svr). (H) IN-hp and OUT-hp constructs in simultaneous and mixed inoculation with BCTIV and BCTV-Svr (BCTIV + BCTV-Svr). (I) sense and antisense constructs in pre-inoculated with a mild strain of Cucumber mosaic virus (CMV) and seven days after inoculation with BCTV-Svr and BCTIV (CMV/ BCTV-Svr and CMV/BCTIV). (J) IN-hp and OUT-hp constructs in pre-inoculated plants with a mild strain of Cucumber mosaic virus (CMV) and seven days after inoculation with BCTV-Svr and BCTIV (CMV/BCTV-Svr and CMV/BCTIV). (K) Arabidopsis plants inoculated with CMV. (L) pFGC-5941 in co-infection with BCTIV + BCTV-Svr, CMV/BCTV-Svr and CMV/BCTIV.



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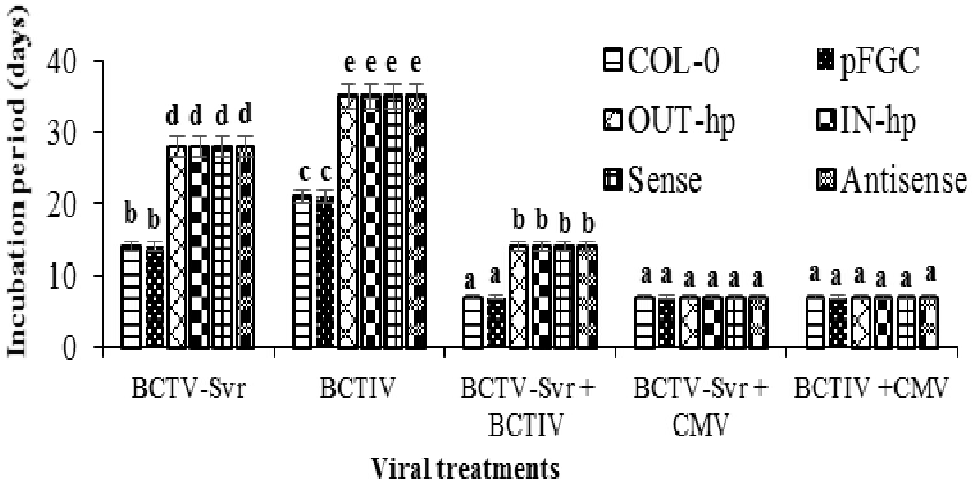


**Figure 4** The incubation period of viruses of beet curly top disease in COL-0 as wild type, pFGC as expression vector, sense and antisense constructs, OUT-hp and IN-hp hairpin constructs.

**Table 3** The number of transgenic Arabidopsis plants showing symptoms.

| Transgenic plant | Total | Viral treatments | | | | |
|---|---|---|---|---|---|---|
| | | BCTV-Svr | BCTIV | BCTV-Svr + BCTIV | CMV/ BCTV-Svr | CMV/ BCTIV |
| pBCTV-sense | 5 | 4 | 2 | 5 | 5 | 5 |
| pBCTV-antisense | 5 | 4 | 2 | 5 | 5 | 5 |
| pBCTV-IN-hp | 10 | 8 | 5 | 8 | 10 | 10 |
| pBCTV-OUT-hp | 10 | 6 | 5 | 8 | 10 | 10 |
| pFGC-5941 | 10 | 10 | 9 | 10 | 10 | 10 |
| Non-transgenic (Col-0) | 10 | 10 | 10 | 10 | 10 | 10 |

Two treatments of pBCTV-IN-hp and pBCTV-OUT-hp due to delayed initial symptoms as well as a lower number of symptomatic plants at 15 dpi had a better relative efficacy in silencing BCTV-Svr infection. In this viral treatment, the performance of pBCTV-OUT-hp was better because this construct had a smaller number of plants with symptoms compared to pBCTV-IN-hp. But in BCTIV, BCTIV + BCTV-Svr, CMV/BCTIV and CMV/BCTV-Svr treatments both of pBCTV-OUT-hp and pBCTV-IN-hp constructs showed the same performance. Two constructs of sense and anti-sense also had the same efficiency in all viral treatments. In the simultaneous inoculation of BCTIV and BCTV-Svr (BCTIV + BCTV-Svr) was observed any symptomless plants in sense and antisense constructs unlike hairpin constructs. It seems that hairpin constructs had a better performance. In CMV/BCTIV and CMV/BCTV-Svr treatments none of the plants containing the plant resistance were symptomless, which could be due to breaking the resistance by CMV (Table. 3).

The results of variance analysis showed that there was a significant difference in viral treatments, inter-treatments, lines, and interactions of lines with viral treatments at 1% level (Table. 4).

The results of the comparison of the mean score of height and number of flowering stems parameters showed that in evaluating the symptoms of four structural treatments in BCTIV and BCTV-Svr viral treatments alone and simultaneous treatment of these two



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viruses had a significant difference with control PFGC and Col-0 and the mean score of symptoms in these four treatments was significantly lower than the mean of control. In treatment of lines with CMV/BCTV-Svr, four treatments with control lines were in a statistical group and had a severity of the symptoms mean similar to symptoms of pFGC and Col-0 controls. In the treatment of CMV infection, the treatments were in a same statistical group with controls.

The average severity of symptoms in treatment of BCTIV in both control and treatments was less than BCTV-Svr viral treatment and the simultaneous treatment of the two viruses, due to the difference in the biology of the two viruses. Four structural treatments with common alphabets are located in the three BCTIV, BCTV-Svr and BCTV-Svr + BCTIV treatments and have significant differences with their controls. This result showed that the silencing constructs designed in infections with the two viruses were efficient. Sense and antisense constructs in the simultaneous treatment of the two viruses with BCTIV treatment were not statistically significant; which showed that these two constructs were less effective in severe and co-infection of the two viruses than the hairpin constructs (Fig. 5).

Comparison of mean growth parameters of plant height showed that in transgenic plants with BCTV-Svr, BCTIV and BCTV-Svr + BCTIV viruses, OUT-hp, IN-hp, sense and antisense had the highest height and compared to controls COl-0 and pFGC were significantly different. In treatment of BCTV-Svr, BCTIV and BCTV-Svr + BTIV, Col-0 and pFGC were treated in a separate statistical group and in CMV/BCTIV and CMV/BCTV-Svr viral treatments, four treatments and controls were all in a statistical group. In the treatment of CMV inoculation, all of the constructs tested fell in a statistical group with controls. Comparison of the average height of transgenic plants with four silencing constructs in the BCTIV + BCTV-Svr treatment, despite having a mean high elevation, with controls of BCTIV and BCTV-Svr was in a same statistical group. This result showed that the four silencing constructs were weaker in the severe and simultaneous infection of the two viruses in causing curly top disease. The highest mean plant height of the four silencing constructs was in BCTIV treatment. This is probably due to the fact that the BCTIV generally produces delayed symptoms in the host plant, which is an opportunity for the host plant to have a more general growth than in BCTV-Svr infected plants. According to the results of comparison of plant height in lines and different viral treatments, it can be concluded that viruses causing curly top disease affect plant height and meanwhile, the BCTV-Svr has been more affected than BCTIV. In addition, when the Arabidopsis plants were co-infected with the both viruses due to the synergistic effect of the two viruses of curly top disease (Taheri *et al*., 2014) this reduction in height was substantial. The results of the comparison of the mean plant height indicate that the induced resistance by the four silencing constructs can subdue plant height reduction effected by curly top viruses.

**Table 4** Analysis of variance of growth factors and symptom score.

| Variable | Degree of Freedom | Mean Square | | | | |
|---|---|---|---|---|---|---|
| | | Plant height | Number of flowering stems | Symptom score | Fresh weight | Dry weight |
| Line (L) | 5 | 740.85** | 52.05** | 18.61** | $0.43^{ns}$ | $0.05^{ns}$ |
| Viral treatments (VT) | 5 | 1097.17** | 152.76** | 31.56** | $0.19^{ns}$ | $0.04^{ns}$ |
| L × VT | 25 | 147.97** | 10.70** | 3.87** | $0.06^{ns}$ | $0.03^{ns}$ |
| Error | 264 | 0.005 | 0.46 | 0.61 | 0.01 | 0.01 |
| Coefficient of variation % | - | 1.00 | 24.68 | 35.08 | 10.12 | 11.25 |

** Significant difference at 1% level



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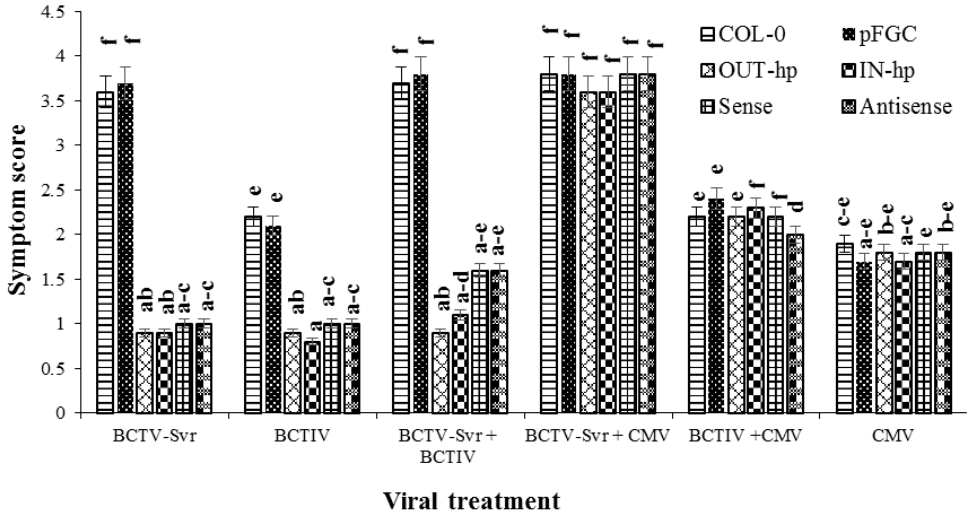


**Figure 5** Graph of the mean symptom score of various viral treatments in COL-0 as wild type, pFGC as expression vector, sense and antisense constructs, OUT-hp and IN-hp hairpin constructs.

In the CMV/BCTIV and CMV/BCTV-Svr treatments, four treatments of the constructs and controls were placed in the same statistical group, in fact, they had no meaningful difference and the average of plant height in this group had a significant decrease compared to CMV treatment. The height of four treatments in CMV/BCTIV and CMV/BCTV-Svr treatment compared to BCTIV, BCTV-Svr and BCTIV + BCTV-Svr treatments, were almost up to the level of control lines without silencing constructs and also compared with control treatment CMV had declined. Which indicates that the resistance of these four treatments was suppressed by mild strain of CMV (Fig. 6) number of flowering stems in transgenic treated plants showed that OUT-hp, IN-hp, sense and antisense lines in terms of number of flowering stems in treatment of BCTIV and BCTV-Svr viruses had a significant difference with the control of Col-0 and pFGC, they were placed in a statistical group (g). In the co-infection with both viruses (BCTV-Svr + BCTIV), the OUT-hp and IN-hp lines were grouped in a same statistical group (f), and the lines of sense and antisense were placed in a same statistical group (e) and were placed separately from the control lines (Col-0 and pFGC). In treatments of BCTV-Svr and BCTIV mixed with CMV, there were no significant differences between treatments with controls and were categorized in one statistical group. In CMV infected plants, control and silencing constructs were placed in a same statistical group. It is noteworthy that OUT-hp, IN-hp, sense and antisense lines with treatments of BCTIV, BCTV-Svr and CMV, were grouped in the same statistical group, indicating the efficacy of silencing constructs for resistance to curly top viruses. In BCTIV + BCTV-Svr treatment, the average number of flowering stems in the Col-0 and pFGC controls was significantly different from the four silencing constructs, indicating the activation of silencing in structural treatments compared to controls. However, the OUT-hp and IN-hp lines were grouped in one statistical group and separated from the sense and antisense lines. This indicates better performance of two hairpin construct in severe infection and co-infection of the two viruses of curly top, in the parameter of number of flowering stems, compared with sense and antisense constructs. In the parameters of the flowering stem, it was also observed that the co-infection of two viruses of curly top with the CMV inactivated the efficiency of the four constructs and the high activity of curly top viruses (Fig. 7).



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The results were also evaluated molecularly, and evaluation of the results of PCR and sqPCR tests showed that the relative expression of DNA in treatment with each of the curly top viruses and simultaneous treatments of both viruses in four constructs was less than controls. This finding could be indicative of the resistance of the gene silencing inducing constructs. While in the treatment of curly top viruses and CMV, the relative levels of expression of DNA viruses in four constructs and controls were almost identical. Therefore, CMV was able to suppress the resistance of silencing constructs. The results of PCR showed that fragments of approximately 750 bp and 792 bp were detectable in all viral treatments of BCTV-Svr and BCTIV, respectively (Figs. 8 and 9).

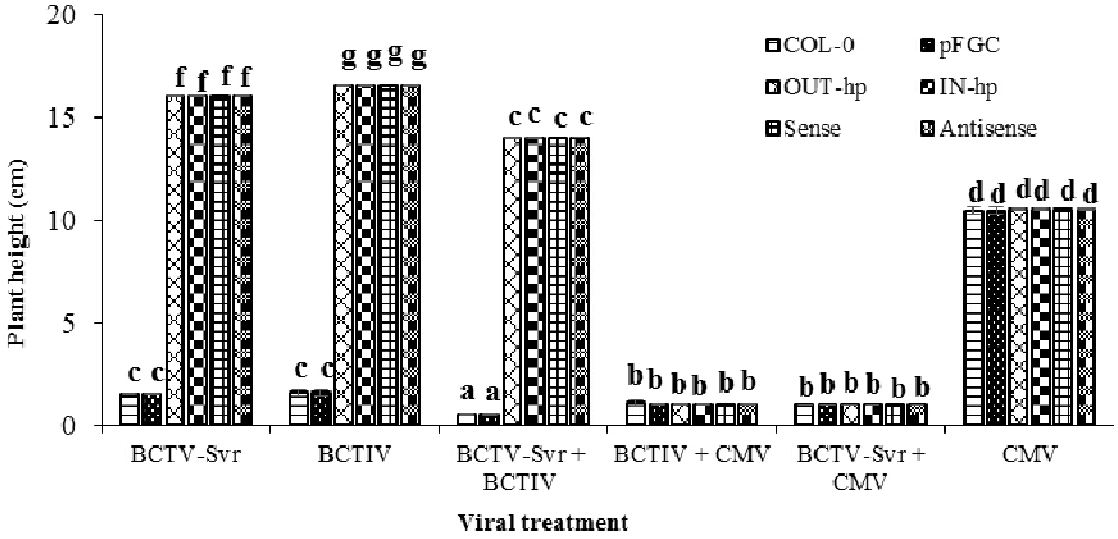


**Figure 6** Graph of the mean of plant height in various viral treatments in COL-0 as wild type, pFGC as expression vector, sense and antisense constructs, OUT-hp and IN-hp hairpin constructs.

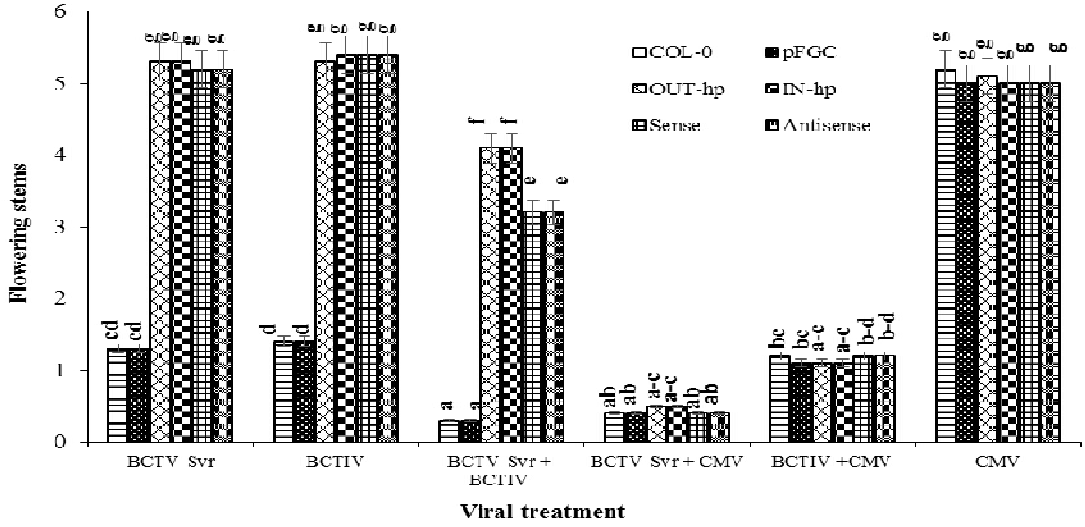


**Figure 7** The average number of flowering stems of various viral treatments in COL-0 as wild type, pFGC as expression vector, sense and antisense constructs, OUT-hp and IN-hp hairpin constructs.



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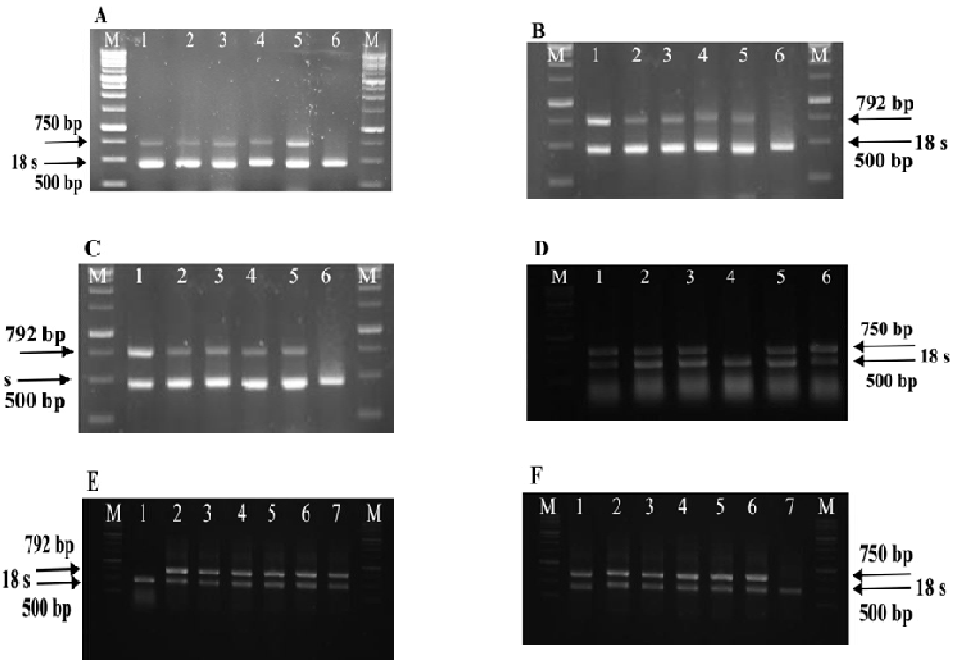


**Figure 8 (A)** Detection of the 750-bp DNA virus of BCTV-Svr by PCR in transgenic Arabidopsis plants with pFGC-5941 and pBCTV-antisense, pBCTV-sense, pBCTV-OUT-hp, pBCTV-IN-hp constructs using BCTV-Svr Primers V1V/V1C electrophoresis in a gel 1% M: Molecular weight marker (1kbp, DNA ladder; Fermentas, Lithuania). Lanes 1: Transgenic plants of Arabidopsis pBCTV-OUT-hp, Lanes 2: Transgenic plants of Arabidopsis pBCTV-IN-hp, Lanes 3: Transgenic plants of Arabidopsis pBCTV-antisense, Lanes 4: Transgenic plants of Arabidopsis pBCTV-sense, Lanes 5: Non-transgenic plant, Lanes 6: Non-inoculated plant.
**(B)** Detection of the 792 bp DNA fragment of BCTIV virus by PCR in transgenic plants with pFGC-5941 and pBCTV-antisense, pBCTV-sense, pBCTV-OUT-hp, pBCTV-IN-hp using BCTIV F/R primers in a 1% gel electrophoresis, M: Molecular weight marker (1kbp, DNA ladder; Fermentas, Lithuania). Lanes 1: Non-transgenic plant, Lanes 2: Transgenic plants of Arabidopsis pBCTV-sense, Lanes 3: Transgenic plants of Arabidopsis pBCTV-antisense, Lanes 4: Transgenic plants of Arabidopsis pBCTV-IN-hp, Lanes 5: Transgenic plants of Arabidopsis pBCTV-OUT-hp, Lanes 6: Non-inoculated plant.
**(C)** Detection of the 792 bp DNA fragment of BCTIV virus in mixed infections BCTIV + BCTV-Svr by PCR in Transgenic plants with pFGC-5941 and pBCTV-antisense, pBCTV-sense, pBCTV-OUT-hp, pBCTV-IN-hp using BCTIV F/R primers in a 1% gel electrophoresis, M: Molecular weight marker (1kbp, DNA ladder; Fermentas, Lithuania). Lanes 1: Non-transgenic plant, Lanes 2: Transgenic plants of Arabidopsis pBCTV-sense, Lanes 3: Transgenic plants of Arabidopsis pBCTV-antisense, Lanes 4: Transgenic plants of Arabidopsis pBCTV-IN-hp, Lanes 5: Transgenic plants of Arabidopsis pBCTV-OUT-hp, Lanes 6: Non-inoculated plant.
**(D)** Detection the 750 bp DNA fragment of BCTV-Svr virus in mixed infections BCTIV + BCTV-Svr by PCR in transgenic plants with pFGC-5941 and pBCTV-antisense, pBCTV-sense, pBCTV-OUT-hp, pBCTV-IN-hp using BCTV-SvrV1V/V1C primers in a 1% gel electrophoresis, M: Molecular weight marker (1kbp, DNA ladder; Fermentas, Lithuania). Lanes 1: Transgenic plants of Arabidopsis pBCTV-sense, Lanes 2: Transgenic plants of Arabidopsis pBCTV-antisense, Lanes 3: Non-transgenic plant, Lanes 4: Non-inoculated plant, Lanes 5: Transgenic plants of Arabidopsis pBCTV-OUT-hp, Lanes 6: Transgenic plants of Arabidopsis pBCTV-IN-hp.
**(E)** Detection the 792 bp DNA fragment of BCTIV virus in mixed infections CMV/BCTIV by PCR in transgenic plants with pFGC-5941 and pBCTV-antisense, pBCTV-sense, pBCTV-OUT-hp, pBCTV-IN-hp using BCTIV F/R primers in a 1% gel electrophoresis, M: Molecular weight marker (1kbp, DNA ladder; Fermentas, Lithuania). Lanes 1: Non-inoculated plant, Lanes 2: Non-transgenic plant, Lanes 3: Transgenic plants with pFGC-5941, Lanes 4: Transgenic plants of Arabidopsis pBCTV-antisense, Lanes 5: Transgenic plants of Arabidopsis pBCTV-sense, Lanes 6: Transgenic plants of Arabidopsis pBCTV-OUT-hp, Lanes 7: Transgenic plants of Arabidopsis pBCTV-IN-hp.
**(F)** Detection of the 750 bp DNA fragment of BCTV-Svr virus in mixed infections CMV/BCTV-Svr by PCR in Transgenic plants with pFGC-5941 and pBCTV-antisense, pBCTV-sense, pBCTV-OUT-hp, pBCTV-IN-hp using BCTV-SvrV1V/V1C primers in a 1% gel electrophoresis, M: Molecular weight marker (1kbp, DNA ladder; Fermentas, Lithuania). Lanes 1: Transgenic plants of Arabidopsis pBCTV-IN-hp, Lanes 2: Transgenic plants of Arabidopsis pBCTV-OUT-hp, Lanes 3: Transgenic plants of Arabidopsis pBCTV-sense, Lanes 4: Transgenic plants of Arabidopsis pBCTV-antisense, Lanes 5: Transgenic plants with pFGC-5941, Lanes 6: Non-transgenic plant, Lanes 7: Non-inoculated plant.



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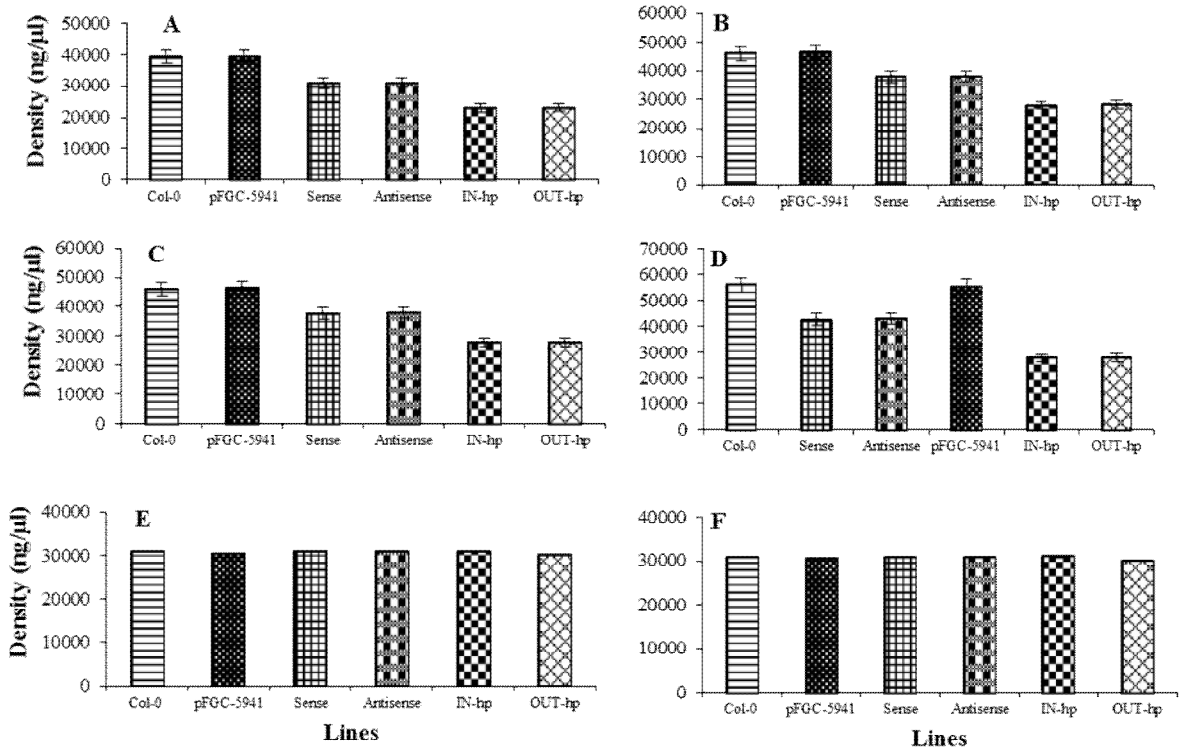


**Figure 9** The average DNA of **(A)** BCTV-Svr**, (B)** BCTIV**, (C)** BCTIV, **(D)** BCTV-Svr, **(E)** BCTIV, **(F)** BCTV-Svr (ng/µl) in viral treatments of BCTV-Svr, BCTIV, BCTIV + BCTV-Svr for C and D, CMV/BCTIV, CMV/BCTV-Svr, respectively in COL-0 as wild type, pFGC as expression vector, sense and antisense constructs, OUT-hp and IN-hp hairpin constructs.

## Discussion

BCTIV symptoms are similar to BCTV-Svr, but delay in the appearance of BCTIV symptoms has been observed in this study on Arabidopsis and other recent studies (Heydarnejad *et al.*, 2013; Montazeri *et al.*, 2016). On the other hand, the incubation period in simultaneous infections of the both BCTIV and BCTV-Svr compared to single infections was decreased. Actually, due to the synergistic effect of the two viruses of curly top disease (Taheri *et al.*, 2014), the incubation period was reduced to nearly half.

BCTIV symptoms appear to be delayed, so BCTIV is expected to be a mild virus with an effect similar to BCTV-Svr. This time lag difference in symptoms appearance of the two closely related viruses is probably due to the difference in the genes of the viruses. As previously reported, BCTV-Svr (BCTV-CFH) and BCTV-H (BCTV-Logan) strains are different in pathogenicity on susceptible host (Stenger *et al.*, 1990). The two viruses in the amino acid sequence of ORFs of viral sense are more than 95% similar but in the ORFs of the complementary sense domain, there is a significant difference between them and only 58 to 87% of the amino acid sequences are similar (Stenger *et al.*, 1994).

The main evidence to prove that RNA silencing in plants is an antiviral defense, would be to use plant viruses (not all of them) from silencing suppressors to counter, reduce, or escape this defense. The production of RNA silencing suppressors (viral suppressors of RNA silencing (VSRs)) is widely used by plant viruses as an anti-defensive strategy (Ding and Voinnet, 2007). These factors (VSRs) vary in the sequence, structure and activity within and



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among viral families, which indicates a convergent evolution (coevolution) between virus and plant for immune response based on RNA silencing. Although VSRs use different strategies in the anti-virus silencing pathway, their action is grouped into three groups. The first is to connect to the large strand of dsRNA, which results in preventing the Dicer process. Connection to the large strand of dsRNA has been observed in the P25 protein of the potato virus X (PVX), which by this interconnection is prevented from Dicer function and, as a result, it is not cut into smaller pieces (Jaubert *et al*., 2011). Second, the binding and decomposition of sRNA duplex and preventing the formation of RISC, which is the most common strategy for viral inhibitors. Protein P19 of cymbidium ring-spot virus is directly associated with the siRNA duplex. Protein P19 acts as a head-to-tail homodymer that specifically binds to a 21-nucleotide siRNA. It is noteworthy that point mutation causes a change in this construct and prevents the binding of siRNA to prevent its inhibitory activity. The binding of P19 to siRNA prevents from its binding to Argnaute proteins (AGO) and, as a result, prevents the formation of RISC. Also, this protein prevents from cell-to-cell transmission of siRNA. This inhibitory mechanism has also been reported in several other viruses (Havelda *et al*., 2003). P21 from beet yellows virus, HC-Pro from potyviruses, P15 from peanut clump virus, 2b from CMV, P38 from turnip crinkle virus, many of these proteins have multiple functions and also, prevent cell-to-cell transmission of siRNA (Lakatos *et al*., 2006). Third, the direct effect of factors interfering in silencing suppression. P6 from cauliflower mosaic virus which is an expression enhancer in this virus, has also been shown with physical connection to DRB4, protein bound to dsRNA (dsRNA-binding protein DRB4), which results in the prevention of dsRNA processing (Al-Kaff *et al*., 1998). Some viruses produce proteins that bind with Argonuate proteins and prevent its function. This will be done in two ways. First, it prevents that single strand with the 24-21-nucleotide binding to Argonuate proteins. Second, preventing the binding of Argonuate proteins containing the 21-nucleotide fragment to the target RNA. P38 binds to Argonautes and inhibits them from binding to RNA duplexes. P0 from beet western yellows *virus* binds to Argonuate proteins containing siRNA and prevents them from binding to target RNA (Csorba *et al*., 2010). Viruses with the RNA genome only disrupt the RNA in the silencing pathway but viruses with the DNA genome have both PTGS and RdDM pathways. Thus, in this way, the inhibitory pathways have evolved so that both of the paths of the immune response can be interrupted by silencing. For example, βC1 protein from β satellite of tomato yellow leaf curl China virus prevents DNA methylation. This protein also blocks the methyl cell enzyme required for methylation of cytosols by binding to S-adenosyl homocysteine hydrolase, resulting in a decrease in methylation of DNA (Yang *et al*., 2011).

Silencer suppressors in plant viruses, like other proteins, have multiple functions in plant viruses and can interfere with various stages of the silent cycle. The 2b gene of CMV can also by binding to AGO1 (Zhang *et al.*, 2006) and binding to siRNA (González *et al.*, 2010) interfere with their spread to other cells. Significantly, the 2b silencing suppressors of CMV, a DNA-free RNA viral virus in its life-cycle, has shown that it interferes with binding to the 24-nucleotides in RNA dependent DNA methylation pathway so preventing AGO4 activity (Duan *et al.*, 2012). Consequently, the contradictory results showed that 2b, in addition to preventing the induction of TGS and DNA methylation, facilitated the promoter gene sequences of the host (Kanazawa *et al.*, 2011).

Although the sense, anti-sense and hairpin constructs have the proper efficacy in inducing of resistance to beet curly top viruses, yet these results also showed that in the plant infected with CMV could lead to suppression of silencing and restore transgenic plants to a sensitivity phenotype. Therefore, more efficient and sustainable methods should be used for the production of transgenic plants, so that the gene silencing inducing constructs are designed in



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such a way that the transgenic plants are simultaneously resistant to the CMV and beet curly top viruses.

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# پایداری مقاومت به بیماری ویروسی پیچیدگی بوته چغندرقند در حضور ویروس موزاییک خیار در گیاه آرابیدوپسیس

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**چکیده:** پیچیدگی بوته یکی از بیماری‌های ویروسی مهم چغندرقند و استفاده از منابع مقاومت یکی از روش‌های مؤثر در کنترل این بیماری است. در پژوهش حاضر کارایی چهار سازه خاموشی (OUT-hp، IN-hp، Sense و Antisense) در برابر دو عامل اصلی بیماری پیچیدگی بوته چغندر در ایران ( beet curly top virus-Svr و beet curly top Iran virus) در گیاهان تراریخته آرابیدوپسیس ارزیابی شد. گزینش بذرهای تراریخت در محیط کشت 1/2MS حاوی علف‌کش گلوفوسینت (باستا) نشان داد که گیاهان تراریخت با سازه pBCTV-IN-hp بیش‌ترین بذر سبز شده را داشت. بوته‌های تراریخت انتخاب شده به گلخانه منتقل و برای مقاومت به علف‌کش باستا و ردیابی سازه‌های القاکننده خاموشی ارزیابی شدند. سپس مقاومت گیاهان تراریخت انتخاب شده نسبت به ویروس‌های عامل بیماری پیچیدگی بوته چغندرقند و پایداری مقاومت در آلودگی مخلوط با ویروس موزاییک خیار (cucumber mosaic virus) در آزمایش فاکتوریل در قالب طرح کاملاً تصادفی با شش تیمار ارزیابی شد. نتایج به‌دست آمده نشان داد که لاین‌های چهار تیمار سازه با شاهدهای خود از نظر شدت علایم، ارتفاع و تعداد ساقه گل‌دهنده تفاوت معنی‌داری داشتند. هرچند گیاهان تراریخت به ویروس‌های پیچیدگی بوته چغندر مقاوم بودند اما آلودگی به ویروس موزاییک خیار بر این مقاومت غلبه کرد.

**واژگان کلیدی:** سازه خاموشی، گیاهان تراریخت، چغندرقند، جیمینی ویروس‌ها

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