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Research Article

Response of tomato cultivars to agroinfection with Beet curly top Iran virus

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Abstract: *Beet curly top Iran virus* (BCTIV) is a member of the genus *Becurtovirus* (family *Geminiviridae*) which constrain host crop production in various geographical regions in Iran. This virus infects several crops such as sugar beet *Beta vulgaris* and tomato *Solanum lycopersicum*. BCTIV infected tomato shows leaf curling, leaf distortion and stunting. In this study, we investigated the response of various tomato cultivars to BCTIV infection using an infectious clone of BCTIV under glasshouse condition at the University of Zanjan in 2013-2014. Based on a completely randomized design experiment twelve tomato cultivars were tested for their reaction to BCTIV infection. The replication of virus and symptom production was recorded and analyzed. Based on the obtained coefficient of infection and disease severity index, none of the tested cultivars was found resistance to the virus infection. However, one of the tested cultivars, Super Chief, showed no leaf curling symptom and the virus replicated at a significantly lower level in this cultivar as compared to a susceptible cultivar, Alindi 811, using quantitative PCR. Tomato cultivars including Grosse Lisse and Early Eurbana were grouped as susceptible while the other cultivars including Super Star were moderately susceptible to the virus infection. Therefore, growing this cultivar shows promise for an increase of yields from tomato plants prone to BCTIV infection after complementary field experiments. The screening of more cultivars or wild-type tomatoes for the identification of gene/s providing resistance to this viral disease is suggested.

Keywords: Agroinfection, BCTIV, Resistance, Tomato

Introduction

Geminiviruses are characterized by their single-stranded DNA genome and their twinned icosahedra particles. They represent major constraints on production of various crops. Based on the sequence and genome organization, the family *Geminivirideae* was grouped into seven genera including the new genus *Becurtovirus* (Varsani *et al.*, 2014).

The genome of BCTIV encodes five open reading frames (ORFs), three of them (called V1, V2 and V3) on the virion-sense strand which are close to the corresponding ORFs in curtoviruses; while the other two ORFs (called C1 and C2) on the complementary-sense strand are close to those of mastreviruses.

BCTIV is a major pathogen in sugar beet *Beta vulgaris* and has been reported from other dicotyledonous crops such as tomato *Solanum lycopersicum*, cowpeas *Vigna unguiculata* and common bean *Phaseolus vulgaris* (Gharouni

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Kardani *et al.*, 2013). BCTIV-infected crops show various symptoms including leaf curling, yellowing, thickened leaves, upward leaf rolling leaf malformation, vein swelling and enation, rolling of the leaf margins and general stunting (Gharouni Kardani *et al.*, 2013).

The control of plant viruses including BCTIV is mainly based on physical barriers and/or chemical spraying to limit vector population but with limited success. In addition, chemical control often results in harmful environmental effects and may also result in insecticide-resistant in the vector. Therefore, using resistant tomato cultivars is the most environmentally supportable and economically practical approach to reduce BCTIV damages.

Resistance to both BCTV and BCTIV infection has been reported in sugar beet plants (Montazeri *et al.*, 2016). Various plant factors such as resistance genes and also pathways including the ubiquitin/26S proteasome pathway, small RNA pathways, cell division cycle components, and the epigenetic mechanism are known as defence responses participating in plant–geminivirus interaction, reviewed by Sahu *et al.* (2013). For example, a single dominant allele designated *Bct* was characterized in common bean plants that shows resistance to *Beet curly top virus* (BCTV) infection (Larsen and Miklas, 2004).

A practical screening system is required for evaluation of host plants for resistance to viral infection. For geminiviruses, agrobacterium-mediated inoculation offers an effective inoculation and also is comparable to inoculation by viral vectors (Bian *et al.*, 2007). In addition, this method also reduces the variation in inoculum pressure and onset of infection that occur in the field evaluations.

In tomato plants, BCTIV infection induces severe symptoms. The yield loss due to BCTIV infection can be affected using resistant or tolerant tomato cultivars. However, there is no report on the response of various tomato cultivars to BCTIV infection. The aim of this study was screening common tomato cultivars for their response to BCTIV infection and quantification of viral DNA in the infected tomato plants.

Materials and Methods

Plant material and virus isolate

Tomato cultivars were grown in pots containing loamy sand, vermiculite, and coco peat (1:1:1). Plants were maintained under 14/10-h light/dark periods, 24 ± 3 °C and 85 % relative humidity. Common tomato cultivars were used in this study including Rio Grande, Super Chief, Super Strain B, Mobil, Early Urbana Y, Super Star, Super A, Sadeen 95, Platero, Sylviana, Alindi 811 and Grosse Lisse.

The infectious clone of a pepper isolate of BCTIV-Kaf [IR:Kaf:.2016:Pepper] was constructed after isolating a full length BCTIV genome (GenBank accession number, KP410285) from pepper plants in Fars province, Iran (submitted in Molecular Biology Research Communications). Briefly, a head-to-tail partial dimer of BCTIV-Kaf was constructed and sub-cloned into a binary vector, pBin20 (Hennegan and Danna, 1998), to obtain the pBin20-1.4BCTIV-Kaf construct. The resulting construct was then introduced into *Agrobacterium tumefaciens* strain C58 by electroporation with a Gene Pulser apparatus (Bio-Rad, Germany). These cells were used for inoculation of tomato plants.

Experimental design, virus inoculation and symptom severity rating

To test the response of tomato cultivars to BCTIV-Kaf infection, twelve tomato cultivars were grown in pots in greenhouse at 24 ± 3 °C (14: 10 h, light:dark). Based on a completely randomized design experiment, for each cultivar 24 plants were included in three replicates. For agroinoculation, *A. tumefaciens* cells containing a partial dimer of BCTIV in a binary vector, pBin20-1.4 BCTIV (Eini *et al.*, 2016), were grown in Luria Broth medium containing Kanamycin (50 µg/ml) and Rifampicin (25 µg/ml) on shaker incubator at 28 ºC for 24 hours. The optical density of bacterial cells at 600 nm was measured and adjusted to 0.2. Five microliters of these cells were used to agroinoculated each plant at the

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four-leaf stage as described by Kheyr-Pour *et al*.(1991).

DNA extraction and detection of virus in tomato plants

Total DNAs were extracted from the newly emerged leaf tissues at 28 days after agroinoculation using a modified CTAB method (Rouhibakhsh *et al.*, 2008). Extracted DNAs were tested for BCTIV-Kaf infection using polymerase chain reaction (PCR) with a specific primer pair, BC CP-F/BC CP-R (Table 1). This was to amplify a DNA fragment (753bp) of coat protein gene. PCR assays were carried out in 10 µl reaction mixtures containing 100 ng of total DNA template, 6 mM MgCl2, 0.8 mM of each dNTP, 0.4 µM of each primer and 1 U of Platinum *Taq* DNA Polymerase (ThermoFisher Scientific) in the reaction buffer provided by the same source. The mixture was heated for 2 min at 95 °C and subjected to a 32 cycle-PCR program of 30 sec at 94 °C, 30 sec at 52 °C, and 40 sec at 72 °C followed by one step at 72 °C for five minutes.

Disease evaluation and data analysis

Symptom development was monitored from the second week and evaluated at 28 days after inoculation. Disease symptoms in the infected plants were scored according to the following scale as suggested by Friedmann (1998). Zero for symptomless; one for yellowing and mild leaf thickening; two for yellowing, leaf thickening and mild leaf curling; three for yellowing, leaf thickening and severe leaf curling; four for yellowing, leaf thickening; severe leaf curling, epinasty and stunting plants.

The disease incidence (DI), disease severity (DS) index and coefficient of infection (CI) was calculated using the following formulae as previously described (Arunachalam *et al.*, 2002).

%DS = Sum of numerical rating/ (total number of observed × maximum disease grade) × 100

%DI = Number of infected plants/ total number of plants observed × 100

CI = %DI × %DS/100

Based on the calculated CI, infected plants were grouped into five levels of resistance as suggested by Kanakala *et al*. (2013). In addition, an analysis of variance (ANOVA) for the calculated and normalized (Arc sinx$^{\wedge 0.5}$) PDS index was used to statistically differentiate (Duncan's Multiple Range Test, $P \leq 0.05$) the reaction of tomato cultivars to BCTIV-Kaf infection using SAS (9.1) software by applying Generalized Linear Models.

Real-time PCR for quantitation of BCTIV replication

To compare the replication level of BCTIV in tomato cultivars using Real-time PCR, two cultivars were included: Super Chief (a moderate resistant cultivar) and Alindi 811 (a susceptible cultivar). These two cultivars were inoculated at the four-leaf stage with agrobacterium cells containing pBin20-1.4 BCTIV. After 14 days, Leaf tissues were collected from both cultivars and then total DNAs were extracted and tested for virus infection by PCR. To test the level of BCTIV-Kaf in the infected plants, for each infected plant sample a triplicate reactions containing 50 ng of total DNA, 26.6 pmol of BCT1 F and BCT1 R primers and Absolute QPCR SYBR Green buffer (ABgene) was prepared. The qPCR reactions were carried out in a Rotor-Gene 2000 real-time PCR instrument (Corbett Research) using four biological repeats (Eini *et al.*, 2009).

Relative BCTIV copy number for each sample was calculated against a housekeeping gene, SlEFα1, using primers SlEFα1 F and SlEFα1 R primers (Table 1). The relative amount of virus for each sample was calculated using the ΔΔCt method as described by Mason *et al.* (2008). The identity of the PCR products was confirmed by sequencing.

Table 1 Oligonucleotide primers used in this study.

Primers	Size (nt)	Sequences (5' to 3')
BCT1 -F	19	CAGTATTGGCAACAGCAAC
BCT1 -R	19	TTACGAAATATATATTTTG
BC CP- F	25	CCAAGCTTAAGGTTAGTTTTAAGCG
BC CP-R	26	AAAAGCTTCAGCAATTTCTTCACTTC
SlEFα1-F	20	TACTGGTGGTTTTGAAGCTG
SlEFα1-R	24	AACTTCCTTCACGATTTCATCATA

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Results

Phenotypical reaction of tomato cultivars to BCTIV-Kaf infection

Tomato cultivars agroinoculated with BCTIV-Kaf showed various symptoms included yellowing, leaf thickening, leaf curling, epinasty and stunting (Fig. 1). More severe symptoms were observed in Alinidi 811, Early Urbana Y and Grosse Lisse. In these cultivars, the first symptoms including leaf curling were observed from 14 days after inoculation while in another cultivar, Super Chief, no clear symptom was appeared at this stage and even at 28 days after inoculation. In the other tested cultivars a range of symptoms were recorded.

Analysis the response of tomato cultivars to BCTIV-Kaf agroinfection

Disease incidence was calculated based on the PCR results from the extracted DNA of inoculated plants. Figure 2 shows a representative PCR result for the inoculated plants, Alinidi 811, in which a large number of plants were found to be infected with BCTIV-Kaf. These results were used to calculate the disease incidence. The highest disease incidence of 91% was observed in Grosse Lisse and the lowest number, 62%, was obtained for Super A cultivar. This means that BCTIV-Kaf was replicated and spread efficiently to the new leaf tissues in all tested cultivars.

Figure 1 Tomato plants infected with BCTIV-Kaf show various symptoms 28 days after infection. (a) A tomato plant, Alindi 811cv.shows stunting and severe leaf curling symptom as compared to the healthy plant on the right side. (b) A tomato plant, Super Chief, shows mild or lacks clear symptoms. (c) A tomato plant, Alindi 811, shows yellowing, leaf thickening and leaf curling.

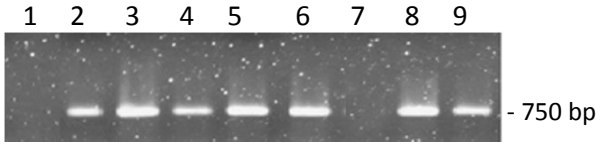

Figure 2 Electrophoretic pattern of amplified coat protein from BCTIV-Kaf by PCR from agroinoculated tomato plants, Alindi 811. Lanes 1 and 2 are a negative and positive DNA control, respectively; lanes 3 to 9 represent seven inoculated tomato plants. The size of amplified DNA is shown on the right side of the gel.

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A range of disease severity index between 30 to 75% was obtained for tested tomato cultivars in which the lowest number, 30%, was obtained for Super Chief and the highest number, 75%, for Alindi 811 (Table 2). In addition, analysis of variance for the calculated and normalized PDS index showed a significant ($P < 5\%$) variation between Super Chief and other tested cultivars including Alindi811 and Early Urbana Y in response to BCTIV-Kaf infection (Fig. 3).

Table 3 shows that based on the obtained CI numbers for each individual cultivar, none of the tested tomato plants was grouped as resistant or highly susceptible to BCTIV-Kaf, while Super Chief was relatively resistant. Tomato cultivars including Alindi 811, Grosse Lisse and Early Urbana Y cultivars were susceptible and other tested cultivars were moderately susceptible to the BCTIV infection (Table 3).

Replication level of BCTIV in tomato cultivars

Comparison of the DNA level of BCTIV in the moderated resistant, Super Chief, and susceptible cultivar, Alindi 811, by Real-time quantitative PCR showed that at early stages of virus infection, the level of virus replication was clearly lower in Super Chief, than that in Alindi 811 (Fig. 4). This may explain the induction of mild or lack of symptoms in Super Chief and more severe symptoms in susceptible cultivars such as Alindi 811.

Table 2 Coefficient of the infection rate based on the PDI and PDS for tested tomato cultivars.

Cultivar	CI[1]	PDI[2]	PDS[3]	Cultivar	CI	PDI	PDS
Alindi 811	66	88	75	Early Urbana Y	63.24	88	71.87
Grosse Lisse	61.32	91	66.66	Super strain B	32.39	79	41.66
Mobil	32	64	50	Super A	34.10	62	55
Super Chief	19.50	65	30	Platero	36.56	64	57.14
Super Star	33.72	71	47.50	Rio Grande	33	66	50
Sylviana	40.6	70	58.33	Sadeen 95	34.32	66	52.50

[1] Coefficient of Infection; [2] Percent Disease Incidence; [3]Percent Disease Severity.

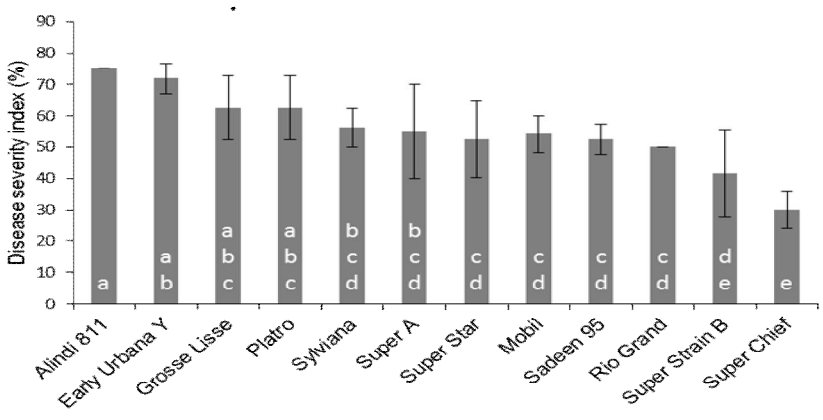

Figure 3 Response of tomato cultivars to BCTIV-Kaf infection based on the percentage of disease severity (PDS) index. Twenty four plants were tested for each individual cultivar in three replicates. The same letters on columns indicate no statically difference ($P < 0.5\%$) for the obtained PDS index using Duncan's Multiple Range Test. Error bars show the standard error.

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Table 3 Response of tomato cultivars to BCTIV infection based on coefficient of infection (CI).

Host reaction	CI	Tomato cultivars
Resistant	0-10	None
Moderately resistant	10.1-30	Super Chief
Moderately susceptible	30.1-60	Mobil, Super A, Platero, Sadeen 95, Rio Grande, Super Star, Sylviana, Super strain B
Susceptible	60.1-80	Alindi 811, Grosse Lisse, Early Urbana Y
Highly susceptible	80.1-100	None

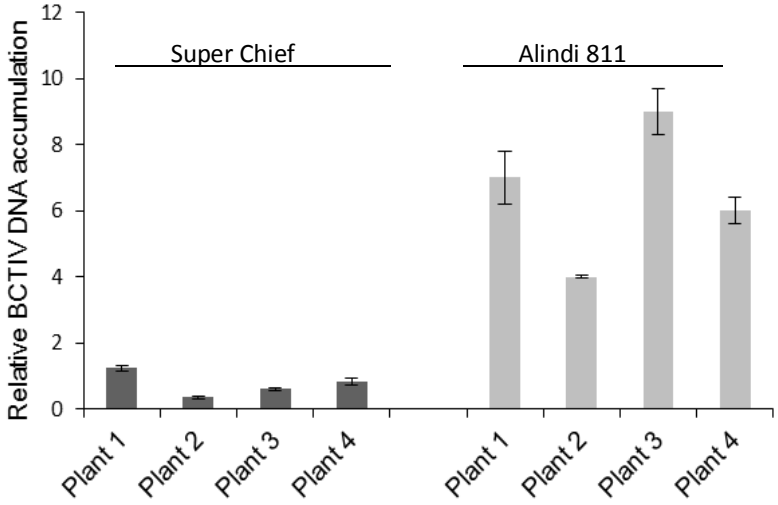

Figure 4 Real-time PCR shows replication level of BCTIV-Kaf in two cultivars of tomato. For each cultivar four infected plants were tested at 14 days after inoculation. The level of BCTIV-Kaf replication was normalised against elongation factor α1 (EFα1). Error bars represent experimental variation for each sample.

Discussion

BCTIV is a widespread and major constrain in crop production in Iran. It has been identified from important dicotyledonous crops such as sugar beet, tomato and common bean (Gharouni Kardani *et al.*, 2013; Heydarnejad *et al.*, 2007; Soleimani *et al.*, 2013). In this study, we found that tomato cultivars inoculated with BCTIV-Kaf infectious clone produced a range of symptoms including leaf curling, leaf distortion and stunting (Fig. 1). More severe symptoms were observed in Alindi 811, Grosse Lisse, Early Urbana Y; while no clear or mild symptoms were observed in Super Chief. This symptom variation reflects the difference of tomato cultivars in response to the virus infection. Based on the PCR results, BCTIV-Kaf was replicated and also moved to the new plant tissues in a large number (> 62%) of inoculated plants. Although the highest (88%) disease incidence was observed in susceptible cultivars including Alindi 811 and Early Urbana Y, a relatively high disease incidence (65%) was also obtained for Super Chief plants which produced mild symptoms. Therefore, in this cultivar, virus replication and spread were not prevented. However quantification of the virus level in this cultivar using Real-time PCR (Fig. 4) showed that BCTIV-Kaf replicates at a significantly lower level in Super Chief, a moderately resistant cultivar, as compared to the virus level in a susceptible cultivars such as Alindi 811, which explains production of mild and severe symptoms in Super Chief and Alindi 811, respectively.

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Supporting this result, symptom development and severity were also found to be positively correlated with viral DNA accumulation in Arabidopsis plants infected with BCTV (Lee *et al.*, 1994). In addition, a low level of ToLCV replication was also reported for tomato genotypes such as TY172 which has been classified as ToLCV and TYLCV-resistant genotypes (Bian *et al.*, 2007).

The coefficient of infection (CI) for resistant and moderately resistant plants was shown in a range of 0-10 and 10.1-30, respectively (Kanakala *et al.*, 2013). Based on the obtained CI, none of the tested tomato cultivars was found to be resistant to BCTIV-Kaf infection, while Super Chief cultivar was grouped into moderately resistant plants (Table 3). In addition, the obtained disease severity index for the tested cultivars was in a range of 30 to 75 present (Table 2) which also statistically ($P < 5\%$) confirmed the significant variations in response to the BCTIV-Kaf infection for tested tomato cultivars. In this indexing system (Kanakala *et al.*, 2013), disease severity scores less than 10 represents resistant phenotype. Therefore, based on this indexing system as well, no resistant phenotype was observed for the tested tomato cultivars. This finding suggested that there is no resistance source/s to BCTIV infection in the cultivated tomato plants. This result is in line with previous reports that almost all geminivirus resistance in tomato is derived from wild species (Bian *et al.*, 2007). Supporting our results, both tomato and pepper plants were also found to be moderately to highly susceptible to BCTV infection and no resistant phenotype was observed in these cultivated plants (Wang *et al.*, 1999).

The disease severity index has been developed for a large number of virus diseases and it has been used routinely in screening programmes (Akhtar *et al.*, 2004; Sabina *et al.*, 2010). Using this indexing system, the resistance source for breeding programs can be identified (Kanakala *et al.*, 2013). Interestingly, this scoring system was shown to positively correlate with the yield production in tomato plants infected with *Tomato yellow leaf curl virus* (TYLCV) (Lapidot *et al.*, 2006). Therefore, within a shorter time, 4 weeks after inoculation, tomato cultivars can be evaluated for their reaction to virus infection and yield lost. Our preliminary data also showed that the susceptible tomato plants produced lower yields of smaller and asymmetric fruits as compared to the healthy plants (data not shown).

It needs to be noted that, mechanical inoculation is not applicable for geminiviruses (Brown *et al.*, 2012). Therefore, we used the established agroinculation method (Elmer *et al.*, 1988;Stenger *et al.*, 1991) to deliver a similar level of virus to each plant. Using this system for inoculation rather than using an insect vector can reflect the pure reaction of each cultivar to the individual virus inoculation. In addition, it has been suggested that insect vectors may affect the rate and also the effects of viral infection (Jiang *et al.*, 2004). In addition, using this method we avoided the potential mixed infections. Finally, comparing disease severity index in sugar beet cultivars inoculated with *Beet severe curly top virus_Ir* using either agroinoculation method or the leafhopper vector showed a similar pattern (Fatahi, 2012). Therefore, both methods of inoculation are reliable for screening host plants for resistance to geminiviruses.

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چکیده: ویروس ایرانی پیچیدگی بوته چغندرقند عضو جدیدی از گروه جمعیتی ویروس‌ها می‌باشد که از عوامل محدودکننده کشت محصولات در مناطق مختلف ایران است. این ویروس یک پاتوژن مهم در چغندرقند *Beta vulgaris* L. و نیزمحصولات دیگر از قبیل گوجه‌فرنگی *Solanum lycopersicum* L. می‌باشد. گیاهان گوجه‌فرنگی آلوده به این ویروس علایم پیچیدگی و شکننده شدن برگ و نیز کوتولگی نشان می‌دهند. در این تحقیق واکنش ارقام مختلف گوجه‌فرنگی به ویروس ایرانی پیچیدگی بوته چغندرقند در شرایط گلخانه‌ای در دانشگاه زنجان در سال‌های ۱۳۹۲ و ۱۳۹۳ بررسی گردید. براساس طرح آماری کاملاً تصادفی واکنش دوازده رقم گوجه‌فرنگی به آلودگی به این ویروس بررسی گردید. تکثیر ویروس با انجام واکنش زنجیره‌ای پلی‌مراز و میزان بروز علایم در این گیاهان تعیین گردید. نتایج نشان داد براساس شاخص شدت بیماری هیچ‌کدام از ارقام مورد مطالعه به این ویروس مقاوم نبودند. اگرچه در رقم نسبتاً مقاوم سوپر چیف علایم پیچیدگی برگی مشاهده نشد و میزان تکثیر ویروس با روش واکنش زنجیره‌ای پلی‌مراز کمی (Quantitative PCR) در مقایسه با رقم حساس آلیندی ۸۱۱ به‌طور مشخصی کم‌تر بود. ارقام گروسی لیسی و ارلی اربانا نیز به این ویروس حساس بوده و سایر ارقام از قبیل سوپراستار نسبتاً حساس گروه‌بندی شدند. بنابراین کشت رقم سوپر چیف پس از انجام آزمایشات تکمیلی مزرعه‌ای می‌تواند سبب کاهش خسارت این محصول به‌واسطه ویروس ایرانی پیچیدگی بوته چغندرقند گردد. بررسی تعداد ارقام بیش‌تر و خصوصاً ارقام وحشی گوجه‌فرنگی جهت شناسایی ژن/های مقاوم به این ویروس نیازمند مطالعات بیش‌تر می‌باشد..

واژگان کلیدی: اگروواینفکشن، گوجه‌فرنگی، مقاومت

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